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Tesis doctoral

CPT1C and endocannabinoids as hypothalamic players in early stages of obesity development

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CPT1C AND ENDOCANNABINOIDS AS HYPOTHALAMIC PLAYERS IN EARLY STAGES OF OBESITY DEVELOPMENT

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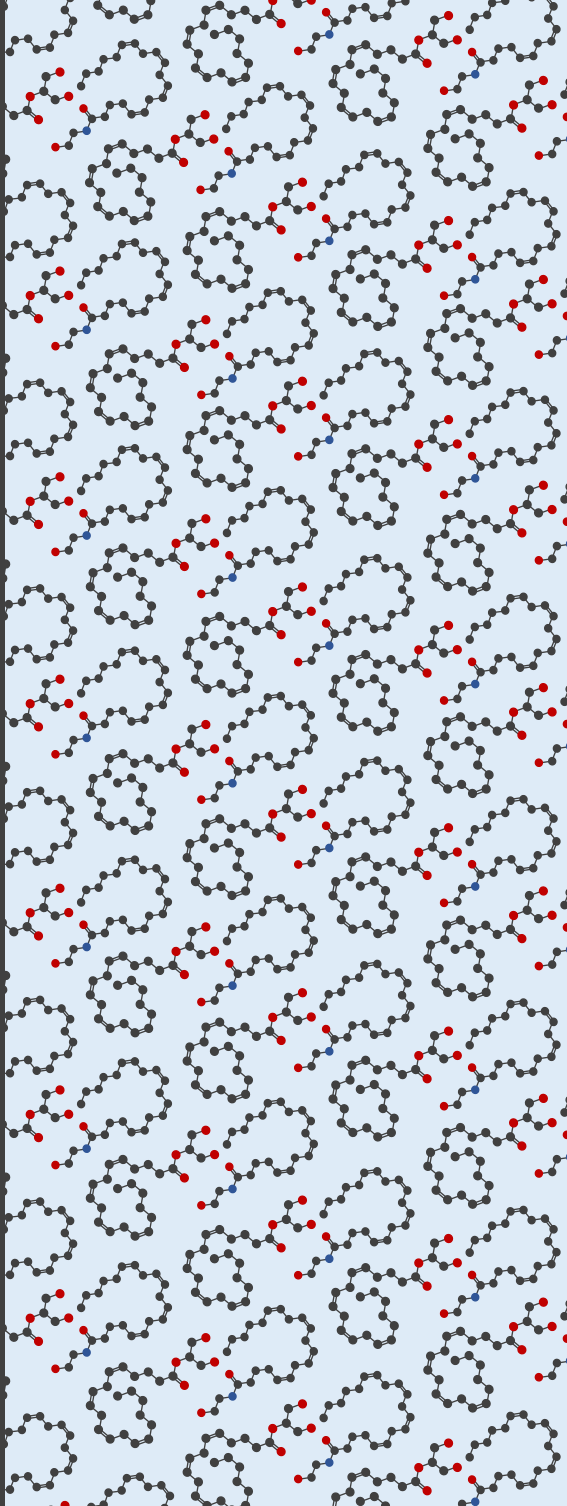

UNIVERSITAT INTERNACIONAL DE CATALUNYA
MEDICINE AND HEALTH SCIENCE FACULTY
BASIC SCIENCE DEPARTMENT

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Doctoral thesis manuscript presented by Cristina Miralpeix Monclús to opt for the degree of Doctor of Philosophy (PhD) awarded by the Universitat Internacional de Catalunya.

This thesis has been conducted in the Basic Science Department from the Medicine and Health Science Faculty of the Universitat Internacional de Catalunya, under the co-direction of Dr. Núria Casals Farré and Dr. Rosalía Rodríguez Rodríguez.

Sant Cugat del Vallès, 2019

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INTRODUCTION

1 OBESITY DEVELOPMENT

Obesity is defined as abnormal or excessive fat accumulation due to an imbalance between food intake and metabolic rate that presents a risk to health. The Wold Health Organization (WHO) has reported that in 2016, 13% of worldwide adult population were obese and 40% were overweight (**Figure 1**), and even more alarming is the increasing childhood obesity since 18% were overweight and 7% obese. It has been predicted that this situation will lead to obesity to 21% of global population and 34% of European citizens in 2030 (Krzysztoszek, Laudańska-Krzemińska, and Bronikowski 2019). Moreover, in the same study, it is forecasted that the increasing obesity prevalence will be greater in men than women in Europe. The increase evidence of obesity is a major public health problem as it is associated with the development of obesity-related-disorders such as type-2 diabetes and cardiovascular diseases, one of the major death cause (data from WHO). Nowadays, in most countries overweight and obesity kills more people than underweight (data from WHO). This situation is not only affecting live surveillance but it also increases the country expenditure in healthcare services (Cecchini 2018).

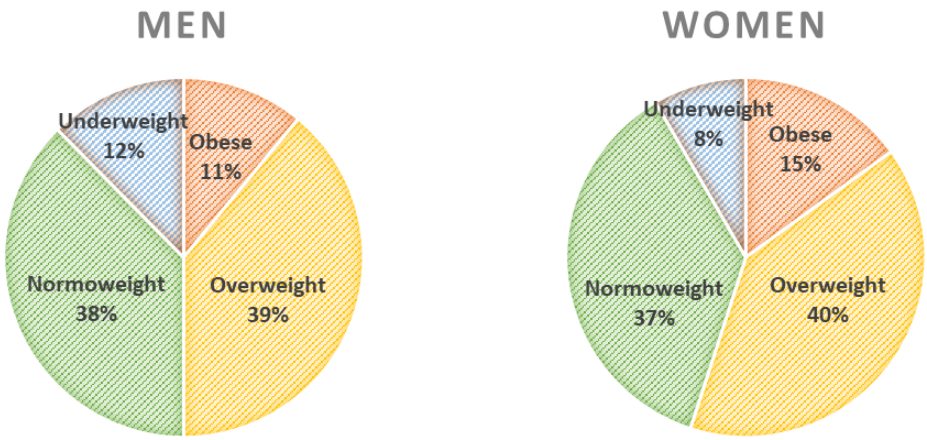


Figure 1. Whole Health Organization data from 2016 about worldwide adult obesity, overweight and underweight prevalence in men and women.

Considering this scenario, it is important to find new long-term therapeutic approaches to target obesity. One currently approach is bariatric surgery, which decreases food intake by reducing the size of the stomach, but also appears to alter metabolic pathways (Saltiel 2016). Moreover, although seven drugs are currently approved by the U.S. Food and Drug Administration for obesity, all of them have side effects (Dietrich and Horvath 2012; Saltiel 2016). Therefore, it is necessary to find new effective and safe therapeutic approaches to treat obese patients. One strategy is to target the central nervous system (CNS) to reduce appetite or to increase the feeling of satiety. The hypothalamus is the brain region involved in the control of energy intake. Unfortunately, satiety centres in the hypothalamus are linked to reward centres in the frontal cortex that govern hedonic behaviours, thus alteration of appetitive often leads to cognitive or psychiatric side effects (Dietrich and Horvath 2012). A promising strategy is to modulate peripheral mechanisms, such as increasing energy expenditure and thermogenesis, from the CNS (Whittle et al. 2011, 2013). In this thesis, we studied the obesity development process to better understand the hypothalamic mechanisms involve in the regulation of energy expenditure with the ultimate objective of developing new therapies against obesity.

In this introduction, I will focus on the obese animal models, the importance of the hypothalamus during obesity development and the thermogenesis activation as a new strategy against obesity. Finally, I will introduce two important systems in the hypothalamus involved in energy homeostasis: the CPT1 family and the endocannabinoids system (ECS), with a special attention to the α/β-hydrolase domain containing 6 (ABHD6).

1.1 Rodent models to study obesity

Obesity manifestation depends on individual genetic factors and the obesogenic environment characterized by sedentarily lifestyle and easy access to high palatable and energy–rich food (Saltiel 2016). During obesity research, several genetic and diet induced obese animal models have been produced, mostly small rodents (Kleinert et al. 2018). Monogenic rodent models have been largely used to study pathways controlling food intake and energy expenditure that are altered in obesity. For instance, the leptin-null *ob/ob* mouse has a spontaneous mutation in leptin gene that inhibits the secretion of bioactive leptin leading to a mild diabetic but severe obese mice. The *db/db* mouse has a spontaneous mutation in leptin receptor (LepR) that produces moderate obese but severe diabetic mice. Analogous to the *db/db* mouse model, there are rat models with spontaneous defects in leptin signal reception, the obese Zucker rat.

Monogenic animal models of metabolic diseases are valuable for understanding human-specific gene functions. However, these mouse models are based on a very specific mutation, while the patient populations cannot be reduced to a single mutation. For this reason, polygenic mouse models emerged. C57BL/6J is a polygenic obesity-prone mouse strain that develop pronounced weight gain, as well as hyperinsulinemia, when fed high caloric diets. The analogous model in rats is known as Sprague Dawley rats. Feeding rodents with high caloric foods enriched in fats (called high fat diet (HFD)) to mimic an obese phenotype is known as diet-induced obesity (DIO). The DIO model is considered the one that better resembles human obesity (Nilsson et al. 2012). The commonly used diets when inducing obesity are the 45% and 60% kcal energy from fat, where the fat source is soybean oil and lard, and the difference between the two diets is the lard content. HFD fed animals are compared to standard diet (SD) fed animals that contain 10% Kcal from fat.

1.2 Hypothalamus and obesity development

The brain is therefore playing a crucial role in counteracting the access of energy intake to maintain the energy homeostasis. Within the brain, there is an important area regulating these processes, the hypothalamus. The hypothalamus is the brain area with main concentration of nutrient-sensing nuclei and a major centre of convergence of multiple peripheral nutrient-related signals (Blouet and Schwartz 2010). The information is integrated to emit a response to regulate food intake, energy expenditure and nutrient partitioning to maintain the body energy homeostasis. In obesity, the energy homeostasis is lost leading to several consequences. For instance, white adipose tissue (WAT) is abnormally expanded leading to an accumulation of fat and an increase of hormonal signals, such as leptin, whereas the brown adipose tissue (BAT) activity, which is in charge of burning fats, is decreased (Trayhurn 2017). To carry out this regulatory function, the hypothalamus is composed of several neural populations distributed in different hypothalamic nuclei. Here, I briefly described the hypothalamic nuclei related to thermogenesis regulation and its principal neuronal populations (Figure 2):

- Arcuate nucleus of the hypothalamus (ARC): it is located close to a region of the blood brain barrier (BBB) that is permeable to allow access of peripheral hormonal and nutrient signals to the brain. The ARC main function is the regulation of feeding behaviour since it contains two functionally antagonistic neuronal populations: the orexigenic (appetite-stimulating) neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP), and the anorexigenic (appetite-suppressing)

expressing pro-opiomelanocortin (POMC) neuropeptides precursors. Thereby, the ARC integrates hormonal and nutritional metabolic signals from the peripheral circulation as well as peripheral and central neuronal inputs to generate a coordinated feedback response to regulate food intake and energy expenditure (Myers and Olson 2012).

- Ventromedial nucleus of the hypothalamus (VMH): it is a key hypothalamic nutrient-sensing region to control energy homeostasis including food intake, body weight and glucose homeostasis. The main role of this nucleus is to integrate peripheral signals to coordinate the thermogenic response, particularly the sympathetic tone to BAT and WAT. Many of the genes highly expressed in the VMH have been identified, among them, cannabinoid receptor type 1 (CB_1R), LepR, insulin receptors (IR) and brain-derived neurotrophic factor (BDNF). Although various genes are expressed in the VMH, the steroidogenic factor-1 (SF- 1) is the only gene that is exclusively expressed in the majority of VMH neurons and it mediates physiological processes particularly thermogenesis and energy expenditure (Choi et al. 2013).
- Dorsomedial nucleus of the hypothalamus (DMH): it is involved in the control of thermogenesis in brown and beige adipocytes and the febrile response through sympathetic transmission. It contains different type of neurons. On the one hand, the GABAergic neurons that continuingly inhibits the glutamatergic terminals. Some neurons express the LepR that contribute to the activation of fat depots depending on leptin signalling, and NPY-expressing neurons that are also able to regulate thermogenesis through sympathetic activation to BAT and WAT (W. Zhang, Cline, and Gilbert 2014).
- Paraventricular nucleus of the hypothalamus (PVH): it is composed by different neuronal population that integrates orexigenic and anorexigenic signals and project to other hypothalamic and extra-hypothalamic areas. PVH contains corticotropin (CRH), oxytocin (OXT) and vassopresin-releasing hormone (AVP) neurons associated to the control of feeding behaviour and neurons expressing the single-minded homolog 1 (Sim-1) necessary for the correct BAT thermogenesis activation. Moreover, the some PVH neurons express the melanocortin 4 receptor (Mc4R), a crucial controller of energy balance in the CNS (Sutton, Myers and Olson 2016).

- The lateral hypothalamic area (LHA): it is the third hypothalamic area where nutrient-sensing neurons have been identified. LHA has two specific groups of peptidergic neurons that play a significant role in energy balance: the neurons containing melanin-concentrating hormone (MCH) implicated in the regulation of feeding and sleep-wakefulness balance and orexin producing neurons. The LHA has also been implicated in the regulation of thermogenesis of BAT and WAT since orexins induce thermogenesis (Stuber and Wise 2016).

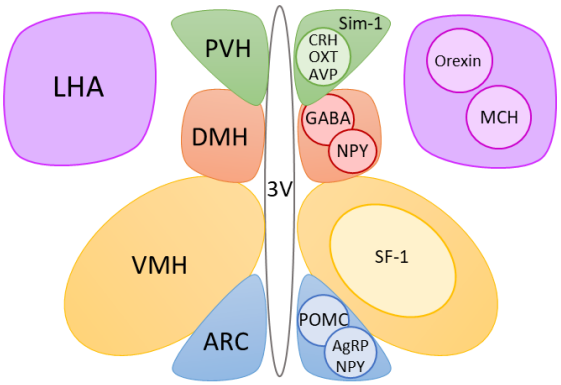


Figure 2. Hypothalamic nuclei and their principal neuronal populations. Arcuate nucleus (ARC) contains two neuronal populations, the neuropeptide Y (NPY) and agouti-related peptide (AgRP), and the pro-opiomelanocortin (POMC) to control food intake. The ventromedial hypothalamus (VMH) is composed by different type of neurons being those expressing the steroidogenic factor-1 (SF-1) to control energy homeostasis. The dorsomedial hypothalamus (DMH) is mainly composed by GABAergic neurons to control thermogenesis. The paraventricular nucleus (PVH) contains the single-minded 1 neurons (Sim-1) and the corticotropin (CRH), oxytocin (OXT) and vassopresin-releasing hormone (AVP). Finally, the lateral hypothalamus (LHA) contains melanin-concentrating hormone (MCH) and orexins controlling thermogenesis.

During obesity development, and once it is stablished, peripheral signals that go to these nuclei of the hypothalamus to regulate food intake and energy expenditure are altered. For instance, an increase in the production of leptin leads to hypothalamic resistance to this hormone (de Git and Adan 2015). Similarly, an imbalance in the ratio of fatty acids (FA) composition that arrives in the hypothalamus leads to an obesogenic phenotype (Moullé et al. 2014). Therefore, our interest is to reveal which mechanisms regulate the hypothalamic sensing of peripheral signals that controls energy expenditure and are dysregulated in obesity.

1.3 Hypothalamic leptin resistance

Leptin is a hormonal signal of energy surplus produced by the adipocytes that decrease food intake and to induce energy expenditure when acting on hypothalamic nuclei (Flak and Myers 2016). Within this context, leptin provides bidirectional communication between adipose tissue and hypothalamic regulation of food intake and energy expenditure (Flak and Myers 2016). Leptin release produces satiety effects and oxidation of fat depots that prevent further synthesis and release of leptin (Flak and Myers 2016). Although obese individuals have high circulating leptin levels, they do not respond to leptin signalling neither to reduce appetite nor to increase energy expenditure. This condition is known as "leptin resistance". Overnutrition, specifically consumption of a HFD, may induce leptin resistance when obesity is still not stablished by acting on neurons with LepR, and once obesity is developed, leptin resistance maintains overnutrition (de Git and Adan 2015). Different mechanisms have been proposed to mediate leptin resistance in DIO animal models, such as impaired leptin transport to BBB, blunted LepR trafficking and deficient leptin signalling (de Git and Adan 2015). Another factor enhancing leptin resistance in DIO rodents is the ER stress, since increased ER activity disturbs the balance between proteins folding and unfolding in the ER lumen. It has been showed that ER stress is induced in the hypothalamus after short and long-term exposure to HFD and that pharmacological inhibition of ER stress after exposure to HFD decreases body weight and increases leptin sensitivity (de Git and Adan 2015).

1.4 Hypothalamic lipid sensing

Hypothalamic regulation of energy homeostasis is not only depending on hormonal signalling but also by nutrients, such as glucose and FA. Particularly in the brain, FA represent 50% of brain dry weight. Hypothalamic FA sensing plays a key role in insulin secretion activity, hepatic glucose production, adipose deposition and food intake (Moullé et al. 2014). However, the molecular mechanism involved in the FA sensing is still unknown. Whether FA enter to the brain by crossing BBB or by specific transporter is in debate, but there are lipid-responsive hypothalamic areas (ARC and VMH) where FA can induce metabolic effects (Moullé et al. 2014).

The first evidences of FA sensing in the brain were done by direct intracerebroventricular (icv) infusion of the long chain fatty acid (LCFA) oleic acid that inhibited glucose production and food intake (Obici et al. 2002). However, in the same study, the short-chain fatty acid

(SCFA) octanoic acid failed to reproduce the metabolic effects of oleic acid, meaning that the effect of each FA depends on its nature. Another group demonstrated that icv administration of non-esterified fatty acids (NEFA) altered insulin secretion and reduced hepatic sensitivity to insulin, two typical phenotype of obesity and type 2 diabetes, through their effect on the CNS (Clément et al. 2002). Therefore, not only the CNS can sense FA but also their effects are different depending on their structure. Moreover, Intracellular FA metabolism leads to malonyl-CoA formation, an important sensor of energy levels in the hypothalamus (see section 2.2). When the supply of nutrients is increased, malonyl-CoA levels rise up and food intake is reduced. Therefore, malonyl-CoA is an indicator of the energy status of the neurons that regulates energy homeostasis (Wolfgang and Lane 2006).

Remarkably, another type of lipids that have impact on hypothalamic control of feeding are the endocannabinoids (eCBs). These are polyunsaturated fatty acids (PUFA)-derived lipid mediators, having key metabolic functions in energy homeostasis, such as BAT thermogenesis and WAT lipolysis (section 3 for further information) (Ruiz de Azua and Lutz 2019).

Therefore, given the physiological regulation of energy balance by neuronal FA sensing, impaired regulation of such sensing may then contribute to the development of metabolic diseases such as obesity and type 2 diabetes in predisposed subjects exposed to chronic lipid overload (Moullé et al. 2014).

1.5 Thermogenesis activation as a therapeutic target in obesity

There are three different types of adipose tissue: WAT, BAT and the “beige or brite” adipose tissue. WAT is the main energy storage depot within the body in the form of triglycerides. White adipocytes are large cells with a uni-locular lipid droplet (LD), few mitochondria and low oxidative rate. On the contrary, BAT is characterized by its capacity to underlie non-shivering thermogenesis, the ability to produce heat, to maintain body temperature. Brown adipocytes are specialized cells with multi-locular LD and a wide presence of mitochondria to perform uncoupled fatty acid oxidation (FAO). The third type of fat, the beige or brite fat is an intermediate between WAT and BAT. Beige adipocytes are specific localized white adipocytes that can undergo browning in concrete situations by expressing thermogenic markers (Bonet, Oliver, and Palou 2013). Initially, BAT was thought to exist only in small or hibernating mammals and newborn humans. However, in 2009 functional BAT depots were identified in adult humans through positron-emission

and computed tomographic scans (Cypess et al. 2009) (Figure 3). Moreover, they found that both BAT mass and activity are dependent of age and gender. In humans, BAT appears during gestation, increases until birth, and then, subsequently declines (Gilsanz, Hu, and Kajimura 2013). For instance, adolescent boys have bigger BAT mass than girls whereas adult women have higher BAT mass and activity than men (Gilsanz, Hu, and Kajimura 2013).

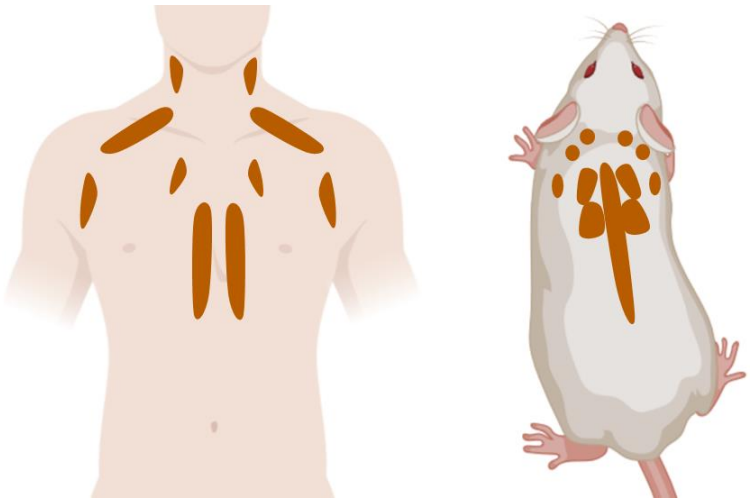


Figure 3. Schematic representation of BAT depots in humans and rodents. Humans BAT distribution is similar to found in rodents. Image modified from M. Carmen Soler-Vázquez, 2018.

Importantly, BAT activity is also susceptible to obesity and type 2 diabetes. Obesity is characterized by an expansion of WAT and a decrease in BAT thermogenesis (Trayhurn 2017). This is remarkably since the energy dissipating capacity of BAT is significant, it has the potential to increase daily energy expenditure by up to 20% in humans, suggesting its validity for the treatment of obesity (Cypess et al. 2009). In humans, there is a negative correlation between BAT activity and central obesity (visceral and subcutaneous adiposity) (Q. Wang et al. 2015). Several studies in experimental animals have shown that BAT activation reduces both obesity and diabetes (Y. Zhang et al. 2015). Thus, keeping BAT active and continuously combusting the excess of fat might be a potential new approach to treat obesity-induced disorders. For this purpose, several therapeutic approaches are in progress: i) studies focused on brown or beige cells transplantation in obese animals (Soler-Vázquez et al. 2018), ii) direct increase in the amount and activity of brown and beige adipocytes and iii) targeting central effectors of BAT thermogenesis (Whittle et al. 2013).

As mentioned above, adipocyte thermogenesis is regulated from the hypothalamus. BAT and WAT thermogenesis is typically activated by cold exposure; however, several peripheral signals relaying information about energy status act in the hypothalamus to regulate thermogenesis (Bonet, Oliver, and Palou 2013). The hypothalamic nuclei are in charge of processing this information and project it through the sympathetic nervous system (SNS), which in turn, innervates both WAT and BAT with the presence of postsynaptic β3-adrenoreceptors (β3-AR) on adipocytes. Induction of thermogenesis starts with the release of norepinephrine (NE) to the adipocytes, which binds the adrenergic receptors activating lipolysis in WAT and thermogenesis in brown and beige adipocytes (Contreras et al. 2017) . Then, the uncoupling protein 1 (UCP1) in the inner membrane of the mitochondria (IMM) is expressed. Activation of UCP1 enables oxidative phosphorylation without ATP production resulting in heat as the main product of this pathway.

Within the hypothalamic control of BAT thermogenesis, the VMH regulates thermogenesis in response to nutritional signals (Whittle et al. 2011). Moreover, peripheral signals, such as insulin, thyroid hormones (THs), estradiol, bone morphogenic protein 8B (BMP8B), leptin and glucagon-like-peptide-1 (GLP-1) analogs operate at the VMH to induce thermogenesis (Contreras et al. 2017). These signals are integrated in the VMH through an energy sensor, the AMP-activated protein kinase (AMPK). Generally, AMPK is activated in situations of low energy levels, leading to stop processes consuming ATP to activate catabolic processes to restore ATP levels. In the hypothalamus, fasting situations or ghrelin enhances AMPK to elicit feeding and body weight gain. On the contrary, during refeeding or leptin, AMPK is inhibited to induce hypophagia and weight lost (Minokoshi et al. 2004). In the VMH, AMPK plays a key role integrating hormonal signals to regulate the sympathetic firing and consequently modulating thermogenesis in BAT and browning in WAT (Figure 4) (López et al. 2016).

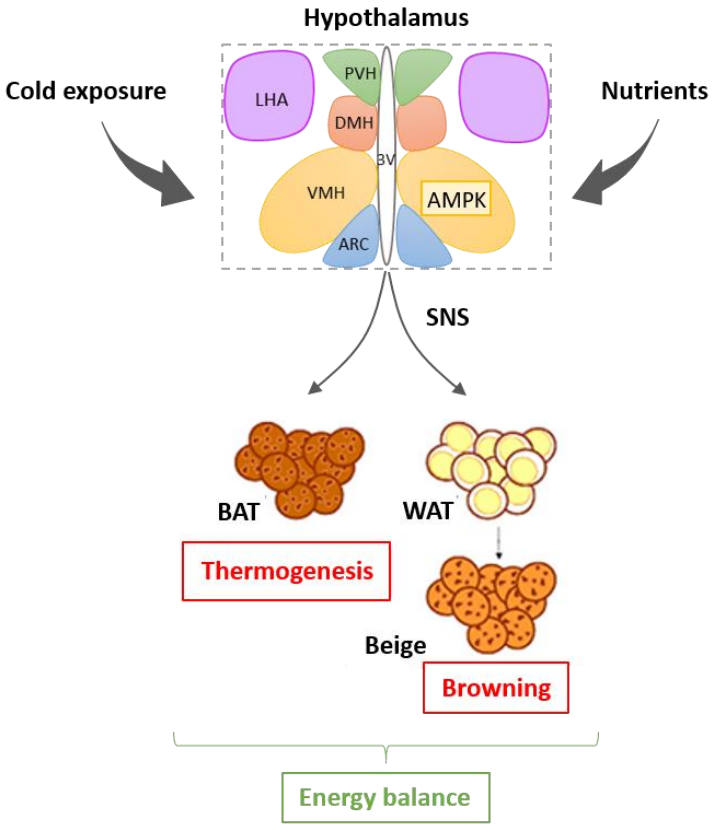


Figure 4. Hypothalamic control of thermogenesis upon cold exposure and nutrient sensing. VMH is an important nucleus that sense nutrients surplus through AMPK. The information travel from the hypothalamus by the SNS to BAT and WAT activating thermogenesis and browning of white adipocytes. This ultimately controls energy balance. Drug activation of thermogenesis could be a potential therapeutic approach to treat obesity. Modified from Lopez and Tena-Sempere 2017.

Besides, WAT and BAT are secretory organs. A number of WAT and BAT-secretory molecules (called adipokines and batokines, respectively) have been identified apart from leptin, although the secretory profile between tissues are quite different (Villarroya et al. 2017). Batokines can go from inflammatory factors, bone morphogenic proteins, growth factors and eCBs.

In conclusion, thermogenesis activation is a promising approach to treat obesity although we need to study more deeply which factors are activating thermogenesis from brain and periphery as well as which pathways are effected from this activation in both male and female mice.

2 CARNITINE PALMITOYLTRANSFERASE 1C (CPT1C)

CPT1C belongs to the family of proteins with carnitine acyltransferases activity. These proteins are crucial for the transport of FA to the mitochondria to undergo β-oxidation. There are different proteins with carnitine acyltransferase activity depending on the length of the FA that catalyse, for instance, the short-chain acyl-CoA-specific carnitine acetyltransferase (CrAT), the peroxisome medium chain-specific carnitine octanoyltransferase (COT) and the long-chain acyl-CoA carnitine palmitoyltransferases (CPTs). In the CPTs group, 4 isoforms have been identified, the CPT2, in the mitochondrial inner-membrane, the CPT1A and CPT1B, in the outer membrane of the mitochondrial (OMM), and the CPT1C, the latest to be discovered, in the ER (Figure 5). CPT1s are responsible of catalysing the transesterification of acyl-CoA esters and carnitine to form acylcarnitine esters and coenzyme A (CoA) to ultimately translocate FA to the mitochondria. Then, the acylcarnitines can be transported to the mitochondrial matrix by CPT2 that catalyse the reverse reaction transforming acyl-carnitines to acyl-CoA that now can be oxidised (Casals et al. 2016) (Figure 5).

CPT1A and CPT1B are transmembrane proteins found in the OMM but they are differentially expressed. CPT1A is ubiquitously expressed in liver, lungs, pancreas, gut, ovaries and brain (McGarry and Brown 1997), whereas CPT1B is mainly found in muscle, BAT and testis (Esser et al. 1996). CPT1A and CPT1B are the rate-limiting step of the FA oxidation and it is controlled by their physiological inhibitor, malonyl-CoA, an intermediate product of the FA synthesis (Figure 5). Although they show considerable sequence similarity, the sensitivity of CPT1A for malonyl-CoA is 2 times higher than CPT1B (Esser et al. 1996).

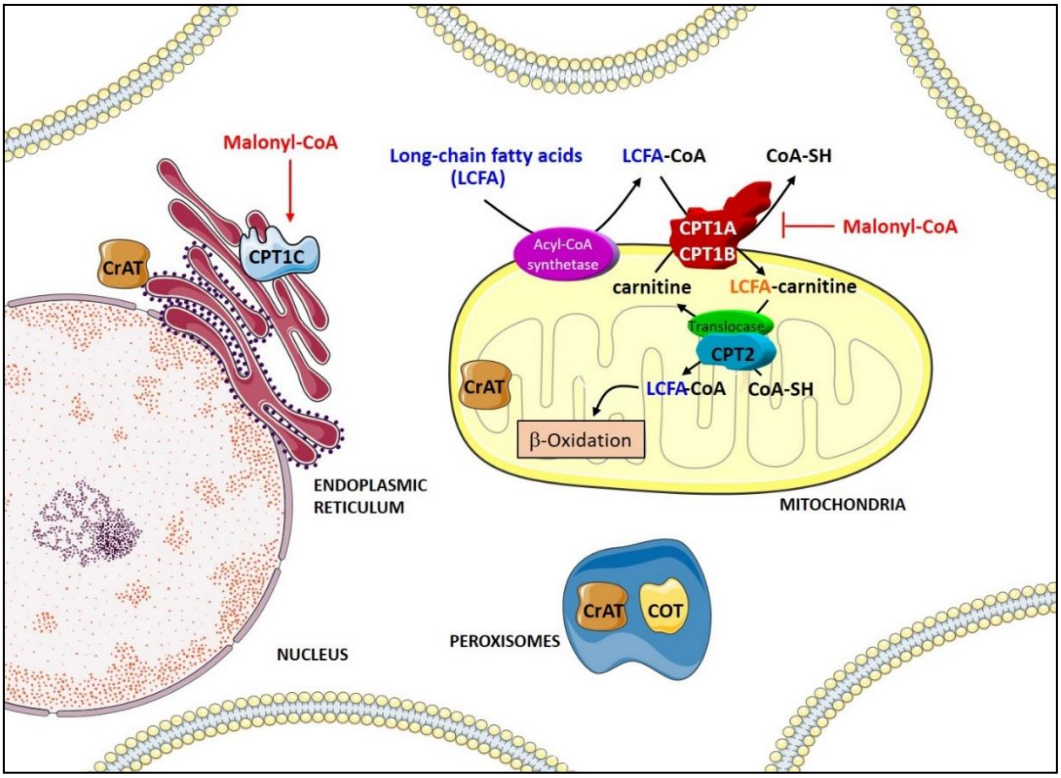


Figure 5. Carnitine palmitoyltransferase (CPT) enzymes and their localization. Long chain fatty acids (LCFA) are converted to LCFA-CoA by the acyl-CoA synthethase in the mitochondria. CPT1A and CPT1B enzymes are localized in the outer mitochondria membrane (OMM) to translocate LCFA-CoA into the mitochondria to undergo oxidation. Instead, CPT1C is localized in the endoplasmic reticulum (ER) and it does not have carnitine activity. CPT1 shared they ability to bind malonyl-CoA. Modified from Casals et al. 2016.

2.1 CPT1C general features

CPT1C was the latest CPT isoform to be discovered (Price et al. 2002) and remains the less characterized. Although CPT1C coding sequence shares 86% and 85% identity with CPT1A and CPT1B respectively, it is only expressed in mammalian neurons, testis (Price et al. 2002) and tumor cells (Zaugg et al. 2011). A deeper study of CPT1C localization showed widespread expression in the CNS with major signal in the hippocampus (involved in learning and cognition), hypothalamic nuclei and amygdala (that coordinates the autonomic and endocrine responses to emotional state) (Dai et al. 2007). Indeed, in the same study, the authors demonstrated that over-expression of CPT1C in the hypothalamus protected mice fed a HFD from DIO. Later, it was reported that CPT1C was also localized in the peripheral nervous system (PNS) (Rinaldi et al. 2015). The expression of CPT1C in the CNS and PNS is restricted to neurons since it was not found in glia or endothelial cells (Sierra et al. 2008). Another distinct feature of CPT1C, compared to the other CPT1s, is that it is

localized in the ER instead of the mitochondria. Price and colleagues evidenced that CPT1C was present in the microsomal fraction obtained from mouse brain and yeast (Price et al. 2002). These results were later confirmed using co-localization with specific markers of ER (Sierra et al. 2008). In the same study, they found that the N-terminal region of CPT1C was the responsible of its ER localization.

CPT1C preserves all the aminoacids motifs involved in the CPT activity as well as the binding pocket for acyl-CoAs. For this reason, several studies focused on determining the catalytic activity of this protein and they found that CPT1C had very low catalytic efficiency with carnitine and different acyl-CoA esters as substrate (Price et al. 2002; Wolfgang et al. 2006). Some years later, it was reported that CPT1C had high affinity to palmitoyl-CoA substrate albeit it showed 20-300 times lower catalytic efficiency than CPT1A (Sierra et al. 2008). In line with these results, Wolfgang MJ and collaborators evidenced that CPT1C was not involved in FAO in COS-1 cells and that primary neurons culture from CPT1C knock out (KO) animals had the same FAO rate than WT neurons. All together, these data demonstrated that CPT1C has minimal catalytic activity and does not participate in neuronal FAO. Some authors have proposed that one reason for this lack of activity could be due to the conformational structure of the N-terminal domain (a peptide of 50 residues) (Samanta, Situ, and Ulmer 2014). Nuclear magnetic resonance spectroscopy studies found that the N-terminal domain of CPT1A and CPT1C can adopt two different conformations, Nα and Nβ, able to switch between them depending on the physiological conditions (Rao et al. 2011; Samanta, Situ, and Ulmer 2014). Mutagenesis studies suggested that CPT1A would adopt the Nα conformation in the presence of malonyl-CoA, and the Nβ conformation under malonyl-CoA depletion (Rao et al. 2011). In the case of CPT1C, since the Nβ conformation showed a disordered 3D structure, Samanta S. and collaborators proposed that CPT1C would be permanently in the Nα conformation, the inactive state (Samanta, Situ, and Ulmer 2014).

2.2 CPT1C and malonyl-CoA sensing

Although CPT1C is not catalytically active, it binds malonyl-CoA, an intermediate in the FA biosynthetic pathway that is able to bind CPT1A and CPT1B and inhibit their activities, thus downregulating FAO (Foster 2012). Malonyl-CoA is synthetized from acetyl-CoA in a reaction carried by the acetyl-CoA carboxylase (ACC) and deactivated by the malonyl-CoA decarboxylase (MCD). Malonyl-CoA acts as a substrate for fatty acid synthase (FAS) to produce FA. Malonyl-CoA synthesis is regulated by AMPK, a nutrient-sensitive kinase that

inhibits ACC. When nutrient availability is reduced, AMPK is activated leading to an inhibition of ACC and lowered malonyl-CoA levels (**Figure 6**). Indeed, malonyl-CoA levels in different brain regions (hippocampus, cortex and hypothalamus among others) increase during feeding and decrease in fasted situation (Tokutake et al. 2010). Moreover, acute inhibition of FAS in the hypothalamus increased malonyl-CoA levels that led to a reduction in food intake and body weight whereas genetic overexpression of MCD in the VMH decreased malonyl-CoA levels leading to an increase in food intake and body weight (**Figure 6**) (Hu et al. 2005). Therefore, in the hypothalamus, malonyl-CoA fluctuations have been proposed as a crucial indicator of the energy status that regulates food intake and energy homeostasis and CPT1C its downstream target (Wolfgang and Lane 2011). Malonyl-CoA binding affinity assays performed in yeast and HEK-293T cells demonstrated that CPT1C binds malonyl-CoA with the same affinity as CPT1A and its K_d in HEK-293T cells for Malonyl-CoA is 0.3 μM, which is within the dynamic range of neuronal malonyl-CoA levels. Hence, CPT1C has been suggested to act as a malonyl-CoA sensor that can regulate other proteins depending on the energy status of the cell (Casals et al. 2016). Specifically, we postulate that CPT1C changes its conformation (Nα or Nβ) depending on malonyl-CoA to regulate the function of other interacting proteins.

2.3 Metabolic functions of CPT1C

2.3.1 *CPT1C and the control of food intake*

Following CPT1C discovery, many researches were focused on its control of food intake since it is highly expressed in appetite regulatory nuclei, such as the ARC, the PVN and the VMH (Price et al. 2002), and it binds malonyl-CoA. The CPT1C-KO mouse was developed in 2006 to study the role of CPT1C in food intake and energy homeostasis (Wolfgang et al. 2006). Studies with this transgenic model showed that, CPT1C is crucial in the response to lipid hormonal signals that regulate food intake, such as ghrelin and leptin. Particularly, icv administration of ghrelin induced food intake and food-seeking behaviour in satiated control mice, whereas these effects were blunted in CPT1C-KO mice (Ramírez et al. 2013). The authors also evidenced that the orexigenic effect of ghrelin was mediated by CPT1C-dependent increase of ceramides in the ARC. Related to the opposite hormone, leptin, it was proved that CPT1C had an important role in leptin's ARC anorectic signalling pathways (S. Gao et al. 2011). The authors suggested that CPT1C mediated malonyl-CoA's signalling aspect in leptin feeding actions by regulating ceramide de novo synthesis. Recently, it has been reported that CPT1C is involved in the diet preference selection after fasting in CRH

neurons of the PVH (Okamoto et al. 2018). In food deprivation, AMPK activation was necessary and sufficient for selection of high-carbohydrate diet (HCD) over HFD during refeeding. Thus, CPT1C is not only modulating appetite but also food preference (Figure 6).

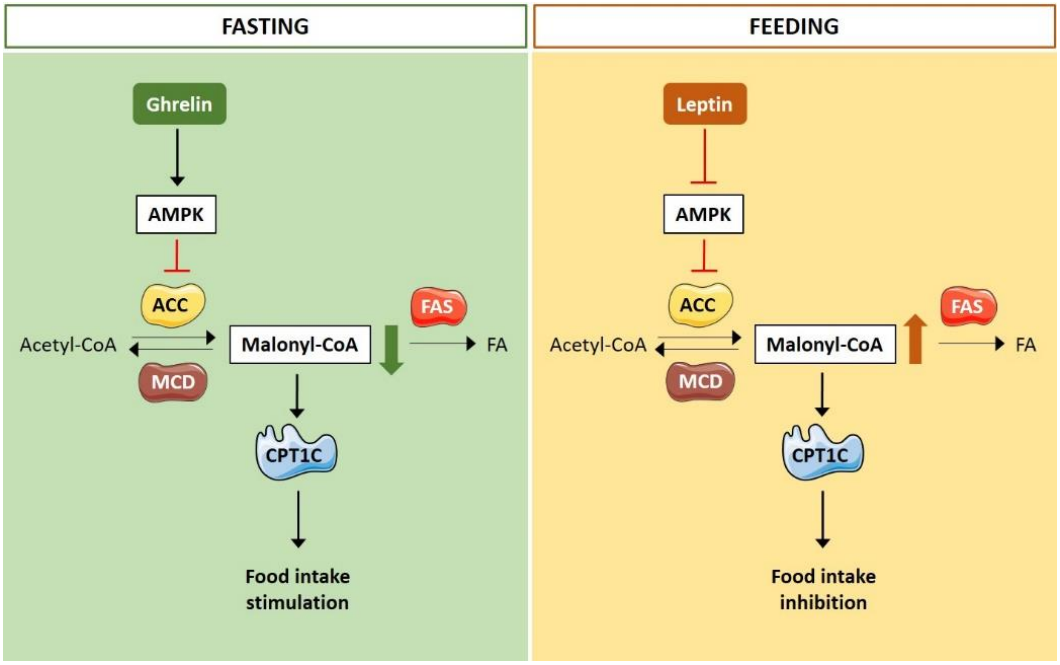


Figure 6. AMPK – Malonyl-CoA – CPT1C axis on food intake control. A situation of fasting where ghrelin levels increase, AMPK inhibits the acetyl-CoA carboxylase (ACC) to decrease malonyl-CoA levels. CPT1C, as a sensor of malonyl-CoA, in the hypothalamus decreases food intake. On the contrary, during feeding or after a leptin increase, this hormone inhibits AMPK that leads to an increase of malonyl-CoA levels and ultimately a decrease on food intake mediated by CPT1C.

2.3.2 *Regulation of peripheral lipid metabolism*

CPT1C deficient mice under normal conditions displayed a normal phenotype, however, when these mice were fed a HFD, they were more prone to obesity since they gained more body weight than wild type (WT) animals (Figure 7) (Wolfgang et al. 2008; Wolfgang et al. 2006). Surprisingly, these animals ate nearly the same amount as their WT littermates suggesting that CPT1C-KO mice had supressed the peripheral energy expenditure, which led to an obese phenotype. The authors suggested that CPT1C is able to integrate lipid sensing and respond to the nutritional environment to maintain energy homeostasis (Wolfgang et al. 2008; Wolfgang et al. 2006). Moreover, CPT1C-KO mice had increased susceptibility to HFD-induced insulin resistance due to elevated hepatic gluconeogenesis and decreased skeletal muscle glucose uptake (Figure 7) (X. F. Gao et al. 2009). The insulin resistance phenotype led to a decrease of liver and muscle FAO leading to an increase in

triglycerides content in these tissues. In the opposite situation, fasting of CPT1C-KO animals attenuated adiposity and body weight lost (Pozo et al. 2017). Those mice presented defective induction of FAO in liver and muscle accompanied with enhanced gluconeogenesis and glycolysis, respectively (Figure 7). CPT1C expression in the mediobasal hypothalamus was able to reverse KO animal's phenotype meaning that CPT1C in the hypothalamus is necessary for sensing the negative energy balance and regulate fuel partitioning in liver and muscle. In line with these results, ectopic overexpression of CPT1C in the ARC and VMH was sufficient to attenuate body weight gain in SD and HFD mice, respectively, thus indicating a protective role of hypothalamic CPT1C against DIO (Dai et al. 2007; S. Gao et al. 2011). These results suggest that CPT1C is involved in the hypothalamic-peripheral crosstalk to regulate liver and muscle metabolism in response to nutritional challenging situations (Figure 7). Whether hypothalamic CPT1C is regulating peripheral energy expenditure through the activation of BAT thermogenesis was unknown at the moment of starting my PhD thesis project.

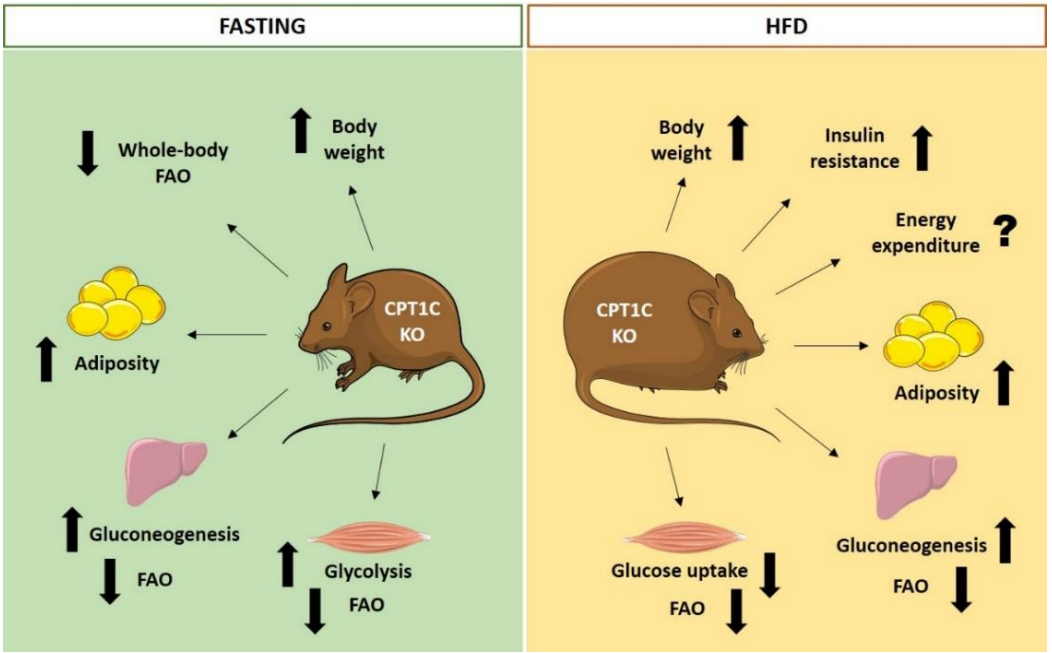


Figure 7. CPT1C-KO mice are unable to properly adapt to metabolic challenging situations. Under fasting conditions, CPT1C-KO animals show less body weight lost, increased adiposity, hepatic gluconeogenesis and muscle glycolysis, decreased its global fatty acid oxidation (FAO) as well as in liver and muscle. In contrast, under high fat diet (HFD), mice are more prone to obesity since they have increased body weight, adiposity and insulin resistance and increased glucose uptake in muscle and gluconeogenesis in liver. What is not known is whether this obesogenic phenotype is accompanied by changes in energy expenditure.

2.4 Non-metabolic functions of CPT1C

As noted above, in addition to the hypothalamus, CPT1C is expressed in brain areas related to cognition, such as cortex and hippocampus. Learning and memory tests were performed to CPT1C-KO mice demonstrating that they learned more slowly than the WT mice during the knowledge acquisition period (Carrasco et al. 2012). This learning impairment was associated with poor maturation of neuronal dendritic spines in CPT1C-KO mice. It is in these dendritic spines were α-amino-3-hydrozyl-5-methyl-4-isoxazole-propionate-type glutamate receptor (AMPAR) are recruited to mediate excitatory synaptic transmission. In relation to this, a high-resolution proteomics study identified CPT1C as part of the AMPAR complex in rodent brain (Brechet et al. 2017; Schwenk et al. 2012). Our group reported that CPT1C regulates the synthesis (translation efficiency) of the most abundant subunit of AMPAR, GluA1, and its trafficking to the postsynaptic membrane (**Figure 8**) (Fadó et al. 2015; Gratacacós-Batlle et al. 2015).

Moreover, CPT1C deficiency has been associated to motor dysfunction and hypoactivity. Different neurological tests showed that the lack of CPT1C causes impaired coordination, severe muscle weakness and reduced daily motor activity; and these motor deficiencies progressively increased with age (Carrasco et al. 2013). In line with these evidences, it was described the first disease-causing *Cpt1c* mutation in humans, which is associated with hereditary spastic paraplegia (HSP) (Rinaldi et al. 2015). HSP are a group of neurological disorders characterized by impaired function of corticospinal motor neurons resulting in higher-extremity spasticity and weakness (Fink 2013). In the case of CPT1C, the mutation Arg37Cys was found to decrease LD synthesis, the main organelle for storing FA, after the challenging with oleate (Rinaldi et al. 2015). However, more research is needed to understand the pathogenic mechanisms involved (**Figure 8**).

Finally, CPT1C is involved in tumor cells survival. Zaugg and collaborators evidenced that CPT1C confers higher capacity of survival and promotes tumor growth in stress metabolic conditions (Zaugg et al. 2011). They observed that overexpression of CPT1C in tumor cell lines increased FAO, ATP production and higher resistance to glucose and oxygen deprivation whereas deletion of CPT1C produced the opposite effects. At the same time, the expression of CPT1C was induced by glucose deprivation and hypoxia, two common situations in tumors environment. For these reasons, the inhibition or deletion of CPT1C is a potential target for cancer therapy (**Figure 8**). Recently, it has been reported that CPT1C is also expressed in human mesenchymal stem cells (hMSCs) and it also protects them

against glucose starvation and oxygen deprivation (Roa-Mansergas et al. 2018). In contrast to tumor cells, CPT1C overexpression in hMSCs did not increase FAO capacity, but its protective role in hMSCs was found to be the result of autophagy enhancement, leading to higher number of LD and increased intracellular ATP levels (Figure 8).

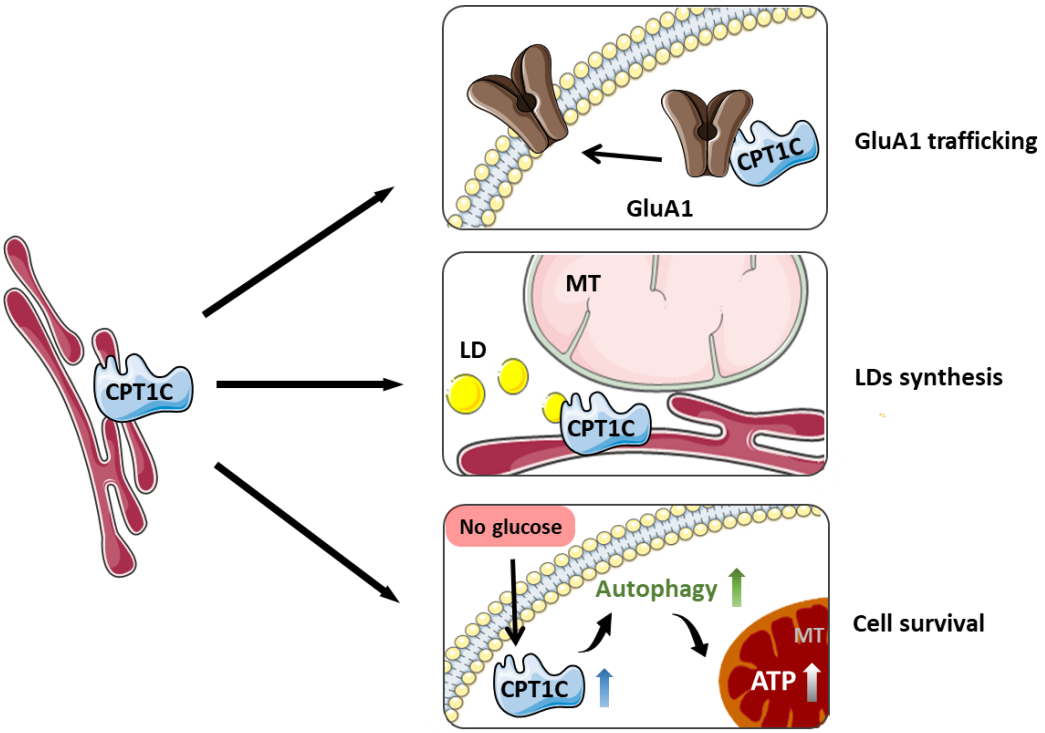


Figure 8. Non-metabolic functions of CPT1C. CPT1C is involved in learning and cognition since regulates the synthesis and trafficking of GluA1 subunit of AMPAR. CPT1C dysfunction in lipid droplet (LD) synthesis is associated to hereditary spastic paraplegia (HSP) disorder. CPT1C increases tumor and hMSCs survival increasing autophagy and the availability of fuel for mitochondria (MT). Modified from Casals et al. 2016.

3 ENDOCANNABINOID SYSTEM IN OBESITY

In the context of obesity, the ECS has been involved in the regulation of energy balance in central and peripheral tissues. In fact, obesity has been associated with an increase of the main eCBs tone (Matias and Di Marzo 2007; Naughton et al. 2013) but whether this increase is a cause or a consequence of obesity is not well stablished. Treatment with Rimonabant, an inverse agonist of the cannabinoids receptors (CBR), demonstrated to have powerful effects on body weight and fat mass decrease, as well as improving metabolic impairments of obese mice (Herling et al. 2008a; 2008b) and humans (Van Gaal et al. 2005). However, after chronic treatment with Rimonabant, patients suffered several neuropsychiatric side effects leading to the withdrawal from the European market in 2008 as an anti-obesity drug. For this reason, it is important to further study the role of the ECS in the CNS to elucidate whether their receptors can be a suitable target to treat obesity.

3.1 General description of the ECS

The cannabinoid signalling system started to be important in the scientific community with the isolation and identification of Δ^9-tetrahyrocannabinol (THC, the major psychoactive component of *Cannabis Sativa*) back in the 60s (Gaoni and Mechoulam 1964). In the late 80s, it was found that cannabinoids acted through a receptor in the brain (Devane et al. 1988) and nowadays a whole ECS has been identified. The ECS consists of cannabinoid receptors, the endogenous ligands of CBRs, the eCBs, and their synthesis and degradation enzymes.

There are two main CBRs, the cannabinoid type 1 receptor (CB_1R) and the cannabinoid type 2 receptor (CB_2R). Both are G-protein coupled receptors (GPCRs) expressed practically in all tissues of the body. CB_1R is widely distributed in peripheral tissues and in the brain, mainly in GABA and glutamatergic neurons, but also in other neuronal subtypes such as serotonergic, noradrenergic, cholinergic and in glial cells (Busquets-Garcia, Bains, and Marsicano 2018). Neuronal CB_1R is preferentially expressed at presynaptic sites but it was also found in the IMM, where it regulates cellular respiration and energy production (Busquets-Garcia, Bains, and Marsicano 2018). Regarding the periphery, CB_1R was found in adipose tissue, liver, skeletal muscle, pancreas, kidney, and gastrointestinal tract (Piazza, Cota, and Marsicano 2017; Silvestri and Di Marzo 2013). On the other hand, CB_2R is mainly expressed in immune cells, but also in adipocytes, liver, neurons and astrocytes (Cassano et al. 2017). Activation of CB_1R and CB_2R receptors leads to Gi/o-mediated inhibition on

adenylyl cyclase (AC) modulating cyclic adenosine monophosphate (cAMP) levels and the activation on mitogen-activated protein kinase (MAPK) pathway (Turu and Hunyady 2010).

Arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the two best-characterized endocannabinoids (**Figure 9**). They both are PUFAs derived from membrane phospholipids precursors and arachidonic acid (ARA). The major pathway of AEA synthesis starts by phophatidyletanolamine and ARA being transformed into N-arachidonoylphosphatidylethanolamine (NAPE) by the enzyme N-acyltransferase (NAT). Then, NAPE is hydrolysed by the NAPE selective phospholipase D (NAPE–PLD) producing AEA and phosphatidic acid (**Figure 10**) (Simon and Cota 2017). Although the NAPE-PLD pathway is the best characterized, there are two additional pathways, one that involves the serine α/β-domain containing 4 (ABHD4) and the second one that regards the protein tyrosine phosphatase PTPN22 (Simon and Cota 2017). From these synthesis pathways not only AEA is formed but also palmitoyl-ethanolamine (PEA) and oleoyl ethanolamide (OEA), both lipids being important in the regulation of food intake and body weight (Mattace Raso et al. 2014). Regarding 2-AG production, the precursor phosphatidylinositol (PI) is converted in 1,2 diacylglycerol (1,2-DAG) by the phospholipase C (PLC) and then in 2-AG by the diacylglycerol lipase (DAGL) (**Figure 10**). An alternative pathway for 2-AG synthesis exists in which PI is transformed to 2-arachidonoyl-lysophospholipid (lyso-PI) by the phospholipase A1. Lyso-PI is then hydrolysed by the lyso-phospolipase C (lyso-PLC) into 2-AG (Simon and Cota 2017).

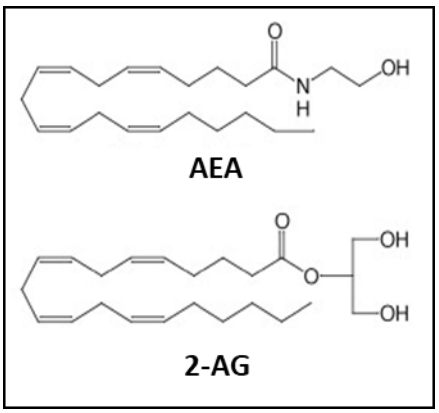


Figure 9. The two main eCBs, the anandamide (AEA) and 2-arachidonoylglycerol (2-AG) structure.

In the CNS, eCBs are synthesized on demand at the postsynaptic site from where they diffuse to act on the presynaptic CB_1R to inhibit neurotransmitter release. This is considered a retrograde negative feedback mechanism. The stimulus triggering eCBs synthesis from the postsynaptic membrane is an elevation of intracellular Ca^{2+} due to metabotropic or ionotropic receptor activation (Castillo et al. 2012). In non-neuronal cells, it has been described that eCBs can act in a paracrine or autocrine manner, being synthesized in a different or in the same cell type expressing CB_1R (Busquets-Garcia, Bains,

and Marsicano 2018). However, it is still unclear how these ligands move through the extracellular space to reach their targets in a paracrine manner. In both neuronal and non-neuronal cells, activation of CBR results in inhibition of different voltage-gated Ca^{2+} channels and AC, leading to the activation of K^+ channels and reduced cAMP levels along with elevated activity of MAPK and phospholipases (Busquets-Garcia, Bains, and Marsicano 2018).

In addition to the synthetizing machinery, eCBs are tightly regulated by their degradation enzymes. Following the re-uptake of eCBs after CBR activation, AEA is destroyed principally by the fatty acid amide hydrolase (FAAH) into ethanolamide and ARA (**Figure 10**). N-acylethanolamide-hydrolyzing acid amidase (NAAA) has been also described as a degradation enzyme of AEA (Tsuboi et al. 2005). On the contrary, 2-AG is manly inactivated by the monoacylglycerol lipase (MAGL) and secondarily by ABHD6 and ABHD12 (Blankman, Simon, and Cravatt 2007) into glycerol and ARA (**Figure 10**). Furthermore, the degradation of 2-AG and AEA results into ARA, which is a pro-inflammatory molecule. ARA is converted to prostacyclins, thromboxanes and prostaglandins esters (Kozak et al. 2002). Moreover, cyclooxygenase 1 and 2 (COX-1/2) are also responsible of 2-AG and AEA transformation to prostaglandin H_2 that subsequently is transformed to different prostaglandins glycerol esters (PGE) (**Figure 10**) (Alhouayek, Masquelier, and Muccioli 2014).

Despite CBR are the canonical pathway signalling for eCBs, there are alternative receptors less studied (**Figure 10**) (Di Marzo 2018). For instance, AEA and 2-AG activate the cation channel transient receptor potential vanilloid 1 (TRPV1) at an intracellular site expressed in peripheral sensory fibres and in several nuclei of the CNS (Zygmunt et al. 2013). Moreover, both eCBs can act on the peroxisome proliferator-activated receptors (PPARs) subtype α and γ for the regulation of lipid and glucose metabolism and inflammatory responses (Bouaboula et al. 2005; Sullivan and Kendall 2010).

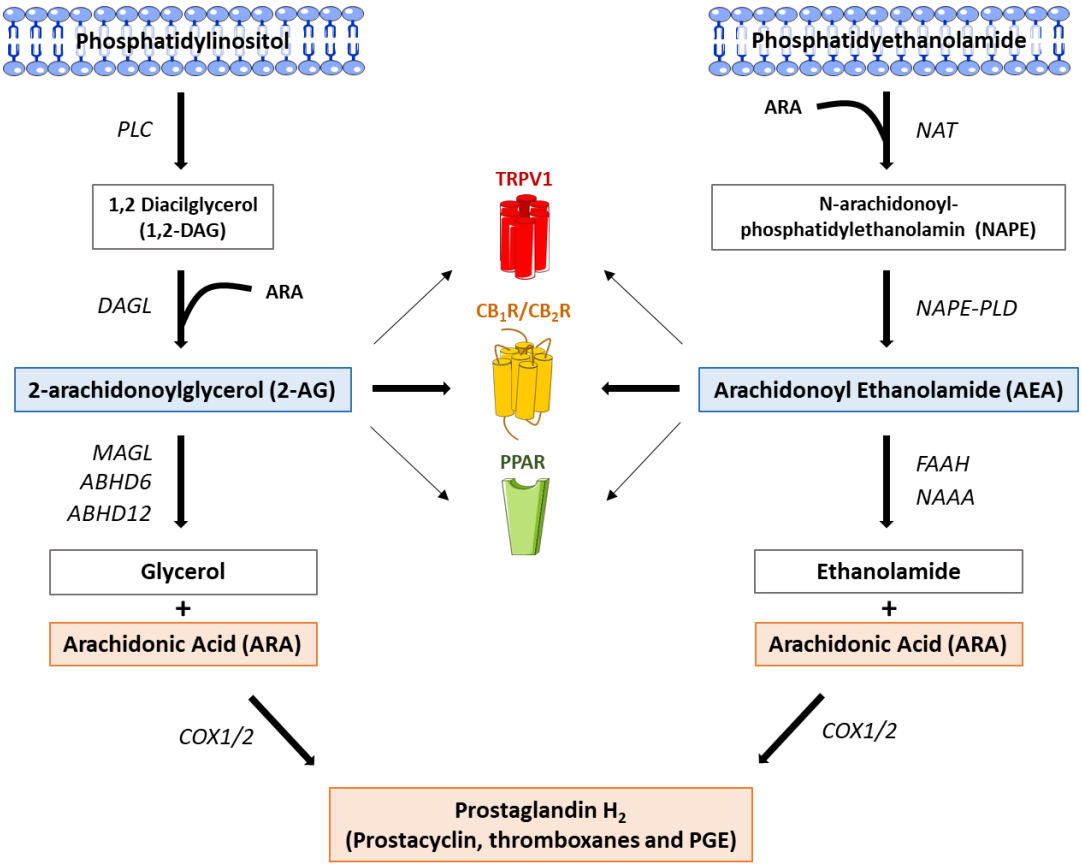


Figure 10. Principal synthesis and degradation pathways of 2-AG and AEA and their target receptors. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), phospholipase C (PLC), N-arachidonoylphosphatidylethanolamine (NAPE), N-acyltransferase (NAT), NAPE selective phospholipase D (NAPE–PLD), 1,2 diacylglycerol (1,2-DAG), diacylglycerol lipase (DAGL), fatty acid amide hydrolase (FAAH), N-acylethanolamide-hydrolyzing acid amidase (NAAA), monoacylglycerol lipase (MAGL), α/β-hydrolase domain 6 and 12 (ABHD6 and ABHD12), cyclooxygenase 1 and 2 (COX-1/2), prostaglandins glycerol esters (PGE), arachidonic acid (ARA), cannabinoid type 1 receptor (CB_1R), cannabinoids type 2 receptor (CB_2R), cation channel transient receptor potential vanilloid 1 (TRPV1) and proliferator-activated receptors (PPAR). Modified from Inigo Ruiz de Azua, 2019.

3.2 Hypothalamic ECS in the control of food intake

Several studies have described the role of hypothalamic ECS in the control of food intake under physiological conditions and in obesity (DiPatrizio and Piomelli 2012).

In the early 2000s, Kirkham and collaborators observed that food deprived rats had increased 2-AG hypothalamic levels whereas under satiation, 2-AG levels were decreased (Kirkham et al. 2002). Moreover, different studies demonstrated that acute administration of CB_1R agonists in different nuclei of the hypothalamus (Jamshidi and Taylor 2001; Koch et al. 2015; Verty, McGregor, and Mallet 2005) or in the nucleus accumbens (Kirkham et al. 2002) induced hyperphagia whereas CB_1R antagonist reduced food intake (Verty, McGregor, and Mallet 2005) (**Figure 11**). In line with these results, intraperitoneal (ip.) administration of a DAGLα inhibitor induced a decrease of hypothalamic 2-AG levels leading to a reduction of food intake and body weight in mice fed a HFD (**Figure 11**) (Bisogno et al. 2013). Therefore, an activation of hypothalamic CB_1R induce an increase in food intake whereas inactivation of this receptor produce the contrary effect. In fact, CB_1R-KO mice had altered expression of hypothalamic neuropeptide genes, indicating a role of CB_1R in food intake regulation (Cota et al. 2003). However, in an elegant study of Luigi Bellochio and collaborators reported that CB_1R activation exert a bimodal control of food intake. Low doses of THC induced hyperphagia depending on CB_1R activation in glutamatergic terminals whereas high doses of THC activated CB_1R on GABAergic neurons that leads to hypophagia (Bellocchio et al. 2010). These results indicate that this modulation of the food intake is dependent on neuronal type and CB_1R agonist dosage (**Figure 11**).

3.3 Hypothalamic ECS in the control of energy expenditure

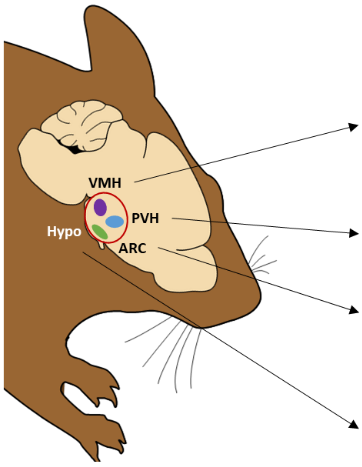
The ECS in the hypothalamus not only regulates food intake but also energy expenditure in normal fed conditions and in HFD feeding (DiPatrizio and Piomelli 2012).

Different genetic models have been used to study the role of the ECS in distinct hypothalamic nuclei. Genetic mice model of CB_1R deletion in forebrain and sympathetic neurons (CaMK-CB_1R-KO) (Quarta et al. 2010) or in the hypothalamus (CB_1R-KO) (Cardinal et al. 2012) showed decrease body weight gain due to an increase in energy expenditure providing to those mice resistance to DIO (**Figure 11**). Similarly, the group of Daniela Cota studied the role of CB_1R on Sim-1 neurons (Sim1-CB_1R-KO), which account for the majority of glutamatergic PVH neurons, in the DIO model and they reported that Sim1-CB_1R-KO mice

had decreased adiposity and increased BAT thermogenesis (**Figure 11**) (Cardinal et al. 2015). Therefore, these results evidenced that blunting of CB_1R in the hypothalamus proportionate resistance to obesity development. Surprisingly, mice deficient of CB_1R on SF-1 neurons (SF1-CB_1R-KO), an exclusive neuron-type of the VMH, challenged with a HFD, displayed increased body weight gain and adiposity linked to a reduction in WAT lipolysis (**Figure 11**) (Cardinal et al. 2014). The authors concluded that CB_1R in VMH neurons is necessary for a proper metabolic flexibility, meaning the ability of an animal to adjust to different diets.

The metabolic role of ECS by modulating the eCBs synthesis and degradation enzymes has also been evaluated. *In vivo* studies using MAGL-overexpressing mice in the forebrain, leading to a decrease of 2-AG levels in the hypothalamus, showed that MGL-mice were resistant to DIO due to an enhanced thermogenic response (**Figure 11**) (Jung et al. 2012). In contrast, deletion of the 2-AG degrading enzyme ABHD6 on the VMH, resulting in 2-AG increase in the hypothalamus, favoured DIO and impaired energy expenditure (**Figure 11**) (Fisette et al. 2016). In accordance with previous studies in the ECS of the VMH, they suggested that ABHD6 in the VMH is necessary for metabolic flexibility.

In conclusion, the hypothalamic ECS has a key role in the adaptation to challenging energetic situation such as a HFD. Generally, hypothalamic deletion of CB_1R or decrease of 2-AG improves energy expenditure counteracting DIO development; however, ECS functions might be nuclei-specific.



Treatment	Food intake	Body weight	Energy expenditure
AEA administration	↑	n.d.	n.d.
SF1-CB_1R-KO	=	=	↑
VMH-ABHD6-KO	↓	↑	↓
THC administration	↑	n.d.	n.d.
Sim1-CB_1R-KO	=	↓	↑
ACEA administration	↑	n.d.	n.d.
CaMK-CB_1R-KO	↓	↓	↑
CB_1R-KO	=	↓	↑
DAGLα inhibition	↓	↓	n.d.
MAGL overexpression	↑	↓	↑

Figure 11. ECS regulation of food intake, body weight and energy expenditure depending on the hypothalamic area. Arcuate nucleus (ARC), paraventricular nucleus (PVH) and ventromedial nucleus (VMH) of the hypothalamus have been the target of agonist CB_1R administration and ECS genetic modification to study its implication on food intake, body weight and energy expenditure. = means no changes, ↑ increase, ↓ decrease and n.d. not determined.

3.4 Hypothalamic eCBs levels in obesity

As mentioned above, CB_1R stimulation or inhibition regulates food intake and energy expenditure. However, other studies have described how levels of hypothalamic eCBs change in obesity depending on hormonal signalling, diet composition and gender.

3.4.1 *Hypothalamic eCBs levels in genetic vs DIO models*

To study the role of the eCBs in the control of obesity, both DIO and obese genetic models has been used.

Hypothalamic eCBs levels have been quantified in genetic models of obese mice induced by disrupted leptin signalling (*ob/ob* and *db/db*) and in obese metabolic syndrome rats (*fa/fa* and Zucker). The first evidence came up from the Di Marzo's group in 2001 when they demonstrated that *ob/ob and db/db* mice as well as Zucker rats had 2-AG and AEA hypothalamic levels increased (**Table 1**) (Balsevich et al. 2018; Di Marzo et al. 2001). Later, it was reported that old *ob/ob* mice had increased 2-AG but not AEA levels in the LHA (**Table 1**) (Cristino et al. 2013).

Regarding studies using DIO model, most of them have been performed after long-term exposure to HFD when obesity is already stablished. Of note, hypothalamic changes at initial stages of obesity are important for preventing the development of obesity and for understanding the timeline of the pathology. However, scarce studies have described the eCBs changes at short-term HFD exposure and the results are controversial. For instance, it was reported that mice fed HFD for 10 days had increased hypothalamic 2-AG levels compared to SD animals (**Table 2**) (Bisogno et al. 2013). Moreover, Higuchi and collaborators assessed hypothalamic 2-AG levels after 3, 7, 14, 28 and 42 days demonstrating that 14 days of HFD feeding increases hypothalamic 2-AG levels lasting for 42 days (**Table 2**) (Higuchi et al. 2011). Concerning experiments done after long-term HFD exposure, two different groups had reported that after 16 or 24 weeks of HFD feeding, 2-AG hypothalamic levels were increased whereas AEA remained unchanged (**Table 2**) (Gamelin et al. 2016; Cristino et al. 2013). However, a recent study showed that mice fed a HFD for 19 weeks showed no changes on hypothalamic 2-AG and AEA levels (**Table 2**) (Balsevich et al. 2018).

In conclusion, genetic models of leptin deficient signalling (*ob/ob, db/db* and Zucker) have clearly increased 2-AG levels in the hypothalamus demonstrating a link between leptin system and endocannabinoids signalling. However, concerning DIO models, results on the hypothalamic eCBs dynamics are more controversial and how they behave in the early stages of obesity is poorly known.

Hypothalamic eCBs levels in genetic models

Genetic model	2-AG	AEA	Reference
ob/ob mice	↑	=	Di Marzo *et al.*, 2001
	↑	↑	Balsevich *et al.*, 2018
	↑	=	Cristino *et al.*, 2013
db/db mice	↑	↑	Di Marzo *et al.*, 2001
Zucker rats	↑	=	Di Marzo *et al.*, 2001

Table 1. Hypothalamic eCB levels in obese genetic models of rats and mice. ↑ means increase, ↓ decrease, = no changes and n.d. not determined.

Hypothalamic eCBs levels in DIO model

Genetic background	Treatment		2-AG	AEA	Reference
ICR mice	60% fat	3-7 d	=	n.d.	Higuchi *et al.*, 2012
C57BL/6N	45% fat	10 d	↑	n.d.	Bisogno *et al.*, 2013
ICR mice	60% fat	14-42 d	↑	n.d.	Higuchi *et al.*, 2012
C57BL/6J	49% fat	16 w	↑	=	Cristino *et al.*, 2013
C57BL/6	60% fat	19 w	=	=	Balsevich *et al.*, 2018
Wistar rats	60% fat	24 w	↑	=	Gamelin *et al.*, 2017

Table 2. Hypothalamic eCB levels of DIO in rats and mice depending on the type of diet and the treatment period. d means days, w weeks, ↑ increase, ↓ decrease, = no changes and n.d. not determined.

3.4.2 *Effect of leptin and ghrelin in hypothalamic eCBs levels*

Leptin and ghrelin act at both peripheral and hypothalamic sites to modulate appetite and energy homeostasis.

The relationship between leptin and the ECS has been extensively investigated in the past 18 years. Acute leptin administration in normal rats, obese Zucker rats and *ob/ob* mice decreased hypothalamic eCBs levels indicating that leptin is a negative regulator of the CB_1R signalling (Balsevich et al. 2018; Di Marzo et al. 2001). Recently, it has been showed that normal mice treated with leptin had decreased AEA levels, but not 2-AG levels, and when mice were fed a HFD for 19 weeks, they developed resistance to the effects of leptin (Balsevich et al. 2018). Moreover, some studies reported that leptin, by reducing eCBs levels, affected CB_1R signalling that in turn modulates hypothalamic neurotransmission to induce satiety (Jo et al. 2005; Malcher-Lopes et al. 2006). Using genetic models, it was demonstrated that CB_1R-KO mice were insensitive to the anorectic action of leptin (Cardinal et al. 2012). However, the same group a couple of years later showed that SF-1-CB_1R-KO ameliorate periphery metabolic effects of leptin (Cardinal et al. 2014). Surprisingly, when SF1-CB_1R-KO animals were challenged with a HFD, they did not respond properly to leptin signalling leading to an increase in body weight and adiposity, thus suggesting that CB_1R on VMH may actually protect from the development of HFD-induced leptin resistance (Cardinal et al. 2014).

In close relationship with leptin, the gastric hormone that stimulates food intake and body weight gain, ghrelin, is also linked with the ECS (Edwards and Abizaid 2016). For instance, administration of ghrelin in WT mice affected AMPK activity and increased hypothalamic eCBs levels, which acting through CB_1R, stimulated appetite (Kola et al. 2005). Disruption of CB_1R signalling or infusion of Rimonabant in the PVH turned into the blocking of central ghrelin action on food intake, concluding that intact ECS signalling was necessary for its orexigenic effect (Kola et al. 2005; Tucci et al. 2004). On the other way around, functional ghrelin system is required to mediate the orexigenic effect of cannabinoids since a CB_1R agonist tended to increase food intake in WT mice but not in ghrelin receptor deficient mice (Lim et al. 2013).

In conclusion, leptin and ghrelin directly modify the eCBs levels in the hypothalamus to induce or supress appetite. This system gets dysregulated in obesity due to resistance to both hormones.

3.4.3 *Effect of diet and gender in hypothalamic eCBs levels*

The influence of HFD and the effect of dietary FA in eCBs have been widely studied (Bisogno and Maccarrone 2014). However, most of the investigations are focused on changes in peripheral tissues but not in brain areas, such as the hypothalamus, and have been only studied in male rodents.

Firstly, dietary fats are the unique source of PUFA responsible of the eCBs biosynthesis. There are two major PUFA families: the n-3 that includes eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the n-6 FA where it belongs the eCBs precursor ARA. The amount and the ratio of n-3 and n-6 PUFA intake is essential for the eCBs synthesis and, subsequently, their levels in obesity (Bisogno and Maccarrone 2014; Naughton et al. 2013) (**Figure 12**). Different studies demonstrated that diets supplemented with n-3 PUFA (EPA and DHA) decreased 2-AG levels in the whole brain (Alvheim et al. 2012; Di Marzo et al. 2010; Wood et al. 2010; Watanabe, Doshi, and Hamazaki 2003). In contrast, in experiments using diets with high n-6 or with low n-3 PUFA composition resulted in an increase of 2-AG in the whole brain and in the hypothalamus (Alvheim et al. 2012; Watanabe, Doshi, and Hamazaki 2003).

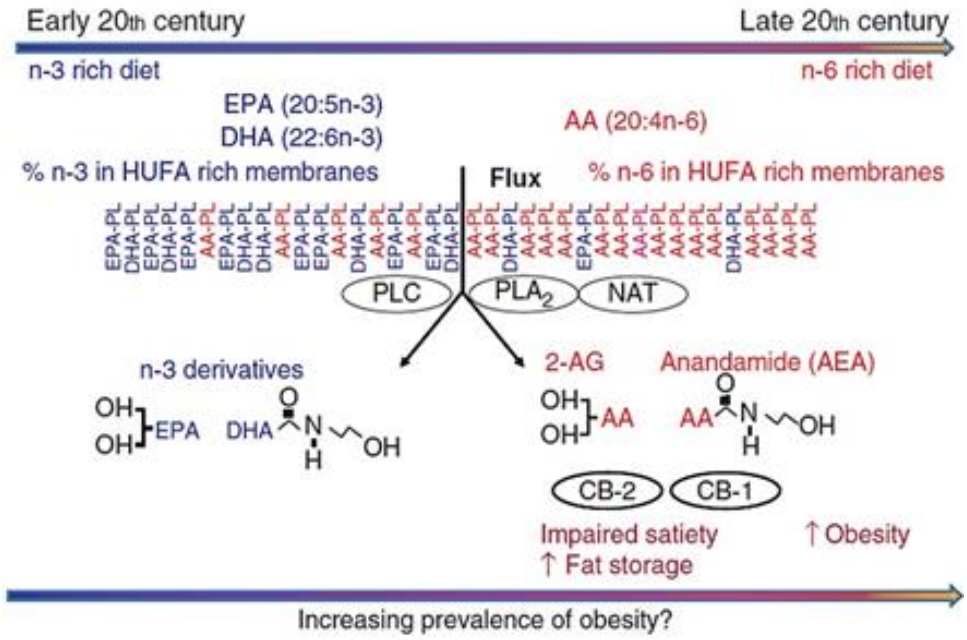


Figure 12. Impact of dietary FA on eCBs synthesis and obesity prevalence. The 2-AG and AEA are synthesized on demand from the essential fatty acid arachidonic acid (AA in the picture) in membrane phospholipids (AA-PL). AA can be elevated by dietary n-6 rich diets or diminished consumption of omega-3 fatty acids notably eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). High proportion of AA-PL will derive in higher eCBs levels that will act on CB-1 and CB-2 receptors favouring obesity. HUFA, highly unsaturated fatty acids; NAT, N-acetyltransferase; PLA2, phospholipase A2; PLC, phospholipase C. Modified from Anita R. Alvheim, 2012.

Secondly, in humans, it is known that females are more sensitive to cannabinoid effects than males, which may be related to different pharmacokinetic responses (Craft, Marusich, and Wiley 2013). However, studies targeting sex-dependent changes in the ECS are scarce. In rodents, Michael Walker and colleagues have evaluated the effect of sex and female hormonal rat cycle on different brain areas, within the hypothalamus (Bradshaw et al. 2006). They found that the female had more hypothalamic 2-AG than male rats. Then, they demonstrated that 2-AG and AEA levels production was subjected to the estrus cycle of females (Bradshaw et al. 2006). Some years later, a different group corroborated that female mice had higher 2-AG and AEA brain levels than male mice (Martin et al. 2016, 2017). Moreover, the group of Edward J. Wagner have extensively studied sex differences in the cannabinoids regulation of energy homeostasis focusing on the hypothalamic feeding circuits (Wagner 2016). They reported that hypothalamic POMC neurons were modulated by sex hormones since estradiol in POMC impaired CB_1R signalling at glutamatergic inputs leading to a reduction in food intake whereas testosterone did the contrary effect (Borgquist, Meza, and Wagner 2014; Mela et al. 2016). More recently, the

same group observed that in POMC neurons, estradiol reduction of the ECS tone proportionated resistance to DIO development in female mice (Qiu et al. 2018) while HFD administration enhanced testosterone activation of the ECS tone (Fabelo et al. 2018).

To summarise, on the one hand, the ratio of eCBs precursors present in diet are crucial for brain eCBs levels and, on the other hand, brain eCBs are sexually dimorphic. Therefore, it is important to stablish the eCBs levels in a concrete DIO model in both male and female animals.

3.5 Circulating eCBs levels in obesity

Not only central eCBs are modulated in obesity but also circulating levels of 2-AG and AEA are altered in animals and obese patients (Hillard 2018; Matias, Gatta-Cherifi, and Cota 2012). Moreover, some researchers are starting to identify from where these circulating eCBs come from.

Concerning studies on circulating eCBs in DIO model, recently, Eline N. Kuipers and collaborators have revealed that 2-AG circulating levels were increased after 4 weeks of HFD feeding, and AEA after just 1 day of HFD exposure, and both were maintained elevated until 18 week of HFD feeding in male mice. Moreover, eCBs levels positively correlated with body weight gain (Kuipers et al. 2019). A study carried out with female mice fed a HFD observed that 2-AG circulating levels were elevated after 6 weeks of treatment whereas AEA levels increased only after 36 weeks of HFD (Pati et al. 2018). In addition, mice fed a HFD for 12 weeks showed increase 2-AG in female plasma but no alteration in male mice (Martin et al. 2017). Hence, these studies evidenced that circulating 2-AG levels increase after HFD exposure, but the exact time-profile of 2-AG and AEA levels and the sexual dimorphism is still controversial

In patients, it was found that obese individuals had increased plasmatic 2-AG, but not AEA, levels, which positively correlated with visceral fat area (Blüher et al. 2006). Moreover, plasma AEA levels were increased in female compared to male subjects (Blüher et al. 2006). Later, it was demonstrated that 2-AG and AEA concentration in plasma positively correlated with body mass index (BMI) of normal weight and obese patients (Gatta-Cherifi et al. 2012; Weis et al. 2010). However, another study reported that this correlation depends on the fat-mass distribution (Côté et al. 2007). In patients with access in intra-abdominal adiposity increased 2-AG and AEA circulating levels positively correlated with insulin, abdominal fat and BMI; whereas obese subjects with low intra-abdominal adiposity

or high subcutaneous adipose tissue had circulating eCBs levels comparable to those with normal weight (Côté et al. 2007). Therefore, 2-AG circulating levels in obese patients increase depending on the fat accumulation distribution.

Taken together, although it is clear that 2-AG and AEA circulating levels are increased in obese patients, the dynamic of these levels in the DIO model is less clear (**Figure 13**).

In addition, the source of these circulating eCBs and which tissue are directed to is still an unresolved matter (Hillard 2018). One option is that the adipose tissue behaves as a producer and secretory eCBs organ. A recent study has demonstrated a positive correlation between increased circulating AEA levels after HFD with the expression of AEA synthesis enzymes in BAT (Kuipers et al. 2019). In addition, acute treatment with a β3-AR agonist induced BAT thermogenesis activation and WAT browning as well as an increase in BAT and WAT eCBs, accompanied by higher mRNA expression of 2-AG synthesis enzymes (Krott et al. 2016).

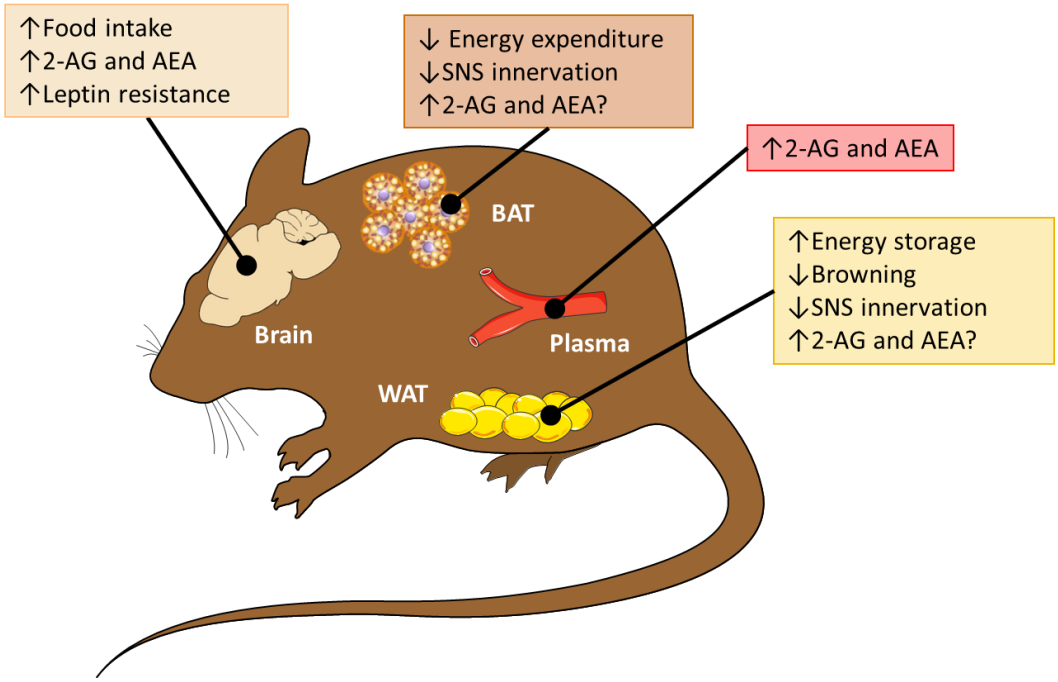


Figure 13. General overview of eCBs-mediated responses in obesity. eCBs are involved in the regulation of metabolism from different organs. Here we highlighted the brain control of food intake, the control of peripheral energy expenditure and fat storage. Moreover, circulating eCBs in plasma are increased during obesity. Modified from Inigo Ruiz de Azua, 2019.

4 α/β-HYDROLASE CONTAINING 6 (ABHD6)

ABHD6 is a relatively new serine hydrolase of the ECS that breaks specific pools of 2-AG generating ARA and glycerol (Cao, Kaplan, and Stella 2019). The canonical ABHD6 mechanism of action is the hydrolysis of 2-AG levels that reduce CB_1R activation leading to a decrease in its downstream signalling: MAPK inhibition, cAMP production and modulation of ion channels (Cao, Kaplan, and Stella 2019). ABHD6 is highly expressed in the brain, BAT, small intestine and immune system (Thomas et al. 2013) but it is also expressed in pancreatic islets, liver, kidney and testis (Poursharifi, Madiraju, and Prentki 2017). In the brain, 2-AG is mainly hydrolysed by MAGL at pre-synaptic membranes, representing an ∼85% of the total 2-AG deactivation in mouse (Blankman, Simon, and Cravatt 2007). ABHD6, from post-synaptic neurons, is responsible of the ∼4% hydrolysis of 2-AG in the brain and ∼20% in cells lacking MAGL. Despite MAGL is the major player in 2-AG deactivation, it has been demonstrated that ABHD6 controls a distinct sub-cellular pool of 2-AG compared to MAGL suggesting a differentiate role of ABHD6 from MAGL (Marrs et al. 2010).

ABHD6 has been involved in numerous functions overall related to peripheral and central control of metabolism and energy homeostasis. It has also been described to play a role in cognition processes, in late endosomes/lysosomes machinery, in epilepsies and in the inflammatory response. However, how is ABHD6 regulating these functions remains unknown. For instance, some of ABHD6 functions are not controlled by CBR, thus we might think on non-canonical receptors of 2-AG that ABHD6 could perform its signalling. Up to date, ABHD6 has been linked to GABA receptors in the control of seizures (Naydenov et al. 2014). Moreover, no partners of ABHD6 to controls its activity has been found.

4.1 Obesity related functions of ABHD6

ABHD6 has important roles on metabolic functions in both the periphery and the CNS.

In the periphery, ABHD6 has been postulated to be a negative regulator of glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells (**Figure 14**) (Zhao et al. 2014, 2015). Whole body and β-cell specific ABHD6 deficient mice or ABHD6 inhibition in diabetic mice resulted in an enhanced GSIS and increased MAG in pancreatic islets (Zhao et al. 2014, 2015, 2016). Moreover, they found that ABHD6 inhibition of GSIS was not regulated by $CB_{1/2}R$. MAG directly modulated the mammalian Uc13-1 protein (Munc13-1) involved in the priming of secretory vesicles (Zhao et al. 2014, 2015).

The same author observed that global suppression of ABHD6 prevented DIO by enhancing energy expenditure, elevated adipose tissue browning, BAT thermogenesis and insulin sensitivity. Furthermore, they evidenced that in ABHD6-KO mice 1-AG directly bounded to PPARα and PPARγ to mediate adipose tissue browning independently of CBRs (Zhao et al. 2016). In accordance with these data, selective knock down of ABHD6 in peripheral tissues protected mice from DIO, insulin resistance and hepatic steatosis (**Figure 14**) (Thomas et al. 2013).

In addition to 2-AG degradation, the Zimmerman laboratory demonstrated that ABHD6 hydrolyse the bis(monoacylglycero)phosphate (BMP) in brain and liver (**Figure 14**) (Pribasnig et al. 2015). BMP is a phospholipid enriched in intraluminal vesicles (ILVs) of late endosomes favouring the ILV formation and playing a critical role in stimulating degradation and sorting of lipids (Schulze and Sandhoff 2011). In their first study, they found that peripheral knockdown of ABHD6 in mice resulted in elevated hepatic BMP, which was further increased when exposed to a HFD (Pribasnig et al. 2015). In the second study, they observed that global ABHD6-KO mice, patients with liver cirrhosis and a patient with ABHD6 mutation showed increased levels of circulating BMP (Grabner et al. 2019). The authors concluded that ABHD6 regulation of BMP content and composition in mice and humans might be important for common metabolic diseases.

Concerning ABHD6 functions in the CNS, it was demonstrated that ABHD6 in the VMH regulates energy metabolism flexibility (Fisette et al. 2016). ABHD6-VMH-KO mice showed increased levels of 2-AG and failed to adapt to metabolic challenges such as fasting, cold exposure and HFD. Indeed, these animals exhibited blunted fasting-induced feeding and reduced food intake, energy expenditure, and adaptive thermogenesis in response to cold and HFD feeding (**Figure 14**).

Therefore, ABHD6, although is not the principal 2-AG hydrolytic enzyme, plays a key role in regulating metabolic flexibility from the periphery as well as from the CNS.

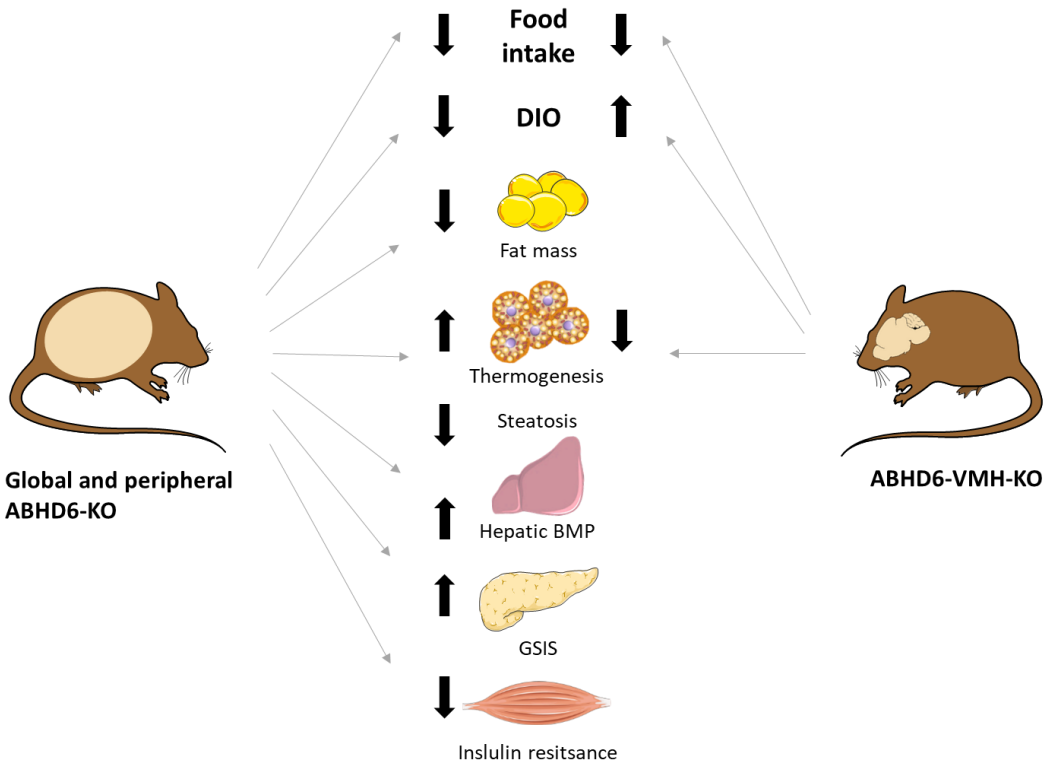


Figure 14. Metabolic ABHD6 functions. ABHD6 is a key regulator of metabolic flexibility when animals face to HFD or fasting conditions. This enzyme exert different effects depending on its localization, peripheral organs or the brain.

4.2 Non-obesity related functions of ABHD6

ABHD6 is also an important regulator of non-obesity related functions. In 2010, Marrs and collaborators demonstrated that ABHD6 blockade was able to increase 2-AG signalling at CB_1R thus regulating synaptic long-term depression in the prefrontal cortex (**Figure 15**) (Marrs et al. 2010). Moreover, high-resolution proteomics revealed that ABHD6 was part of the AMPAR complex (Brechet et al. 2017; Schwenk et al. 2012). Indeed, ABHD6 negatively regulated trafficking and synaptic function of AMPARs, independently of its catalytic activity. The authors found that overexpression of ABHD6 in neurons reduced both the surface GluA1, GluA2 and GluA3 subunits of AMPARs and the excitatory neurotransmission mediated by AMPARs, but not the mutated ABHD6 catalytically inactive (**Figure 15**) (Wei et al. 2016, 2017).

In addition, ABHD6 was implicated in the development of epilepsies by direct 2-AG control of $GABA_AR$ activity, positioning ABHD6 as a target for new antiepileptic treatments (**Figure 15**) (Naydenov et al. 2014; Sigel et al. 2011).

ABHD6 has also been studied as an anti-inflammatory target (**Figure 15**). In the mouse model of traumatic brain injury, 2-AG exhibited a neuroprotective role via CB_1R after brain injury (Panikashvili et al. 2001). Inhibition of ABHD6 increased 2-AG levels that exerted anti-inflammatory and regenerative effects after the brain injury (Tchantchou and Zhang 2013). In the experimental model of autoimmune encephalomyelitis, the mouse model of multiple sclerosis and in macrophages, inhibition of ABHD6 showed anti-inflammatory effects and ameliorated the clinical symptoms via CB_2R signalling. (Wen et al. 2015; Alhouayek, Masquelier, and Muccioli 2014; Bottemanne et al. 2019). However, recent studies revisiting the therapeutic effects of ABHD6 in multiple sclerosis evidenced low efficacy of ABHD6 inhibitors in autoimmune-mediated diseases (Manterola, Bernal-Chico, Cipriani, Canedo-Antelo, et al. 2018; Manterola, Bernal-Chico, Cipriani, Ruiz, et al. 2018). Besides, it was demonstrated that the suppression of inflammatory processes of multiple sclerosis was independent of ABHD6 mechanisms (Tanaka et al. 2017). Therefore, ABHD6 as a target for anti-inflammatory treatments is still controversial.

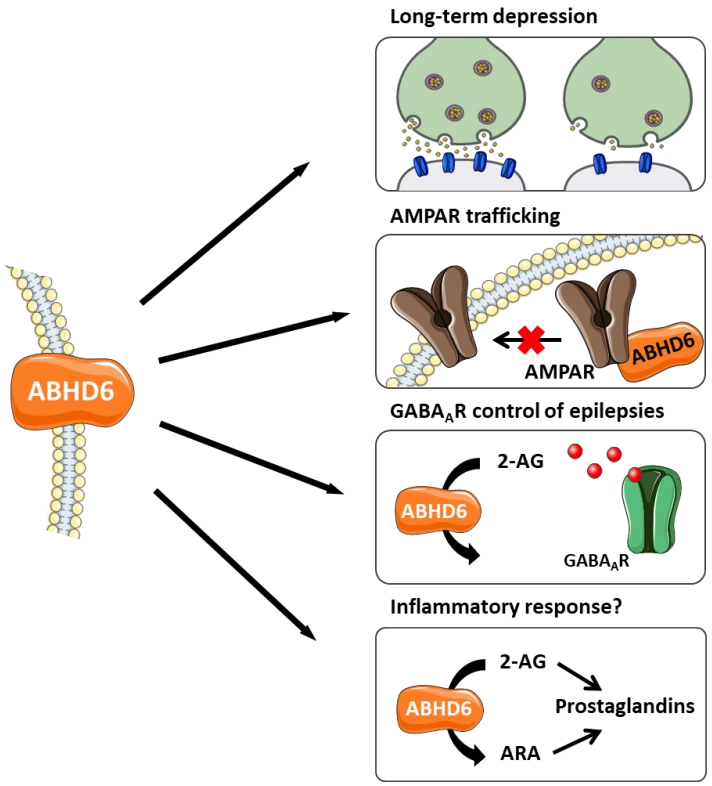


Figure 15. Non-metabolic functions of ABHD6. ABHD6 is involved in the regulation of inflammatory responses by controlling the prostaglandins precursors availability. ABHD6 inhibitions increases 2-AG levels that can activate GABA receptors to inhibit seizures. ABHD6 is negatively regulating the AMPA receptors to the membrane. Inhibition of ABHD6 increases CB_1R signalling leading to long-term depression of synapsis.

4.3 Techniques to study eCBs hydrolases

In the last decades several non-radiolabeled activity assays have been developed to allow directly assay of ECS enzymes activity and to test different substrates and inhibitors. A well-known technique is the activity-base protein profiling (ABPP) that consist on the labelling of probes to the active site of a given enzyme facilitating a wide screening of inhibitors at the same time (Cravatt, Wright, and Kozarich 2008). The ABPP technique can be coupled to a liquid chromatography-mass spectrometry (LC/MS) to identify the peptides bound to the probes. Using LC/MS the activity of ECS enzymes can be detected by quantification of ARA releases from 2-AG and AEA breakdown (Cravatt, Wright, and Kozarich 2008). The newest technique used is the fluorescent activity assay. Yuren Wang and collaborators in 2008 were the first to develop a fluorescent-based MAGL activity assay using the 7-hydroxycoumarinyl-arachidonate (7-HCA). 7-HCA is a fluorogenic substrate that, when hydrolysed by MAGL, generates ARA and the highly fluorescent 7-hydroxylcoumarin (7-HC) (Table 3) (Y. Wang et al. 2008). The following year, the group of Jarmo T. Laitinen improved the 7-HCA assay and characterized a wide range of MAGL inhibitors (Table 3) (Savinainen et al. 2010).

I would like to emphasize ABHD6 since big efforts have been done to characterize new hydrolases. With the purpose of studying substrate preference and the catalytic residues of hABHD6, hABHD12 and hMAGL, a glycerol assay was developed, which, following subsequent enzyme reactions, glycerol was transformed to resorufin, a fluorescent compound (Navia-Paldanius, Savinainen, and Laitinen 2012). The glycerol assay allowed the use of natural substrates against ABHD6, ABHD12 and MAGL and the authors for the first time could study the substrate profile of these enzymes (Table 3) (Navia-Paldanius, Savinainen, and Laitinen 2012). The glycerol assay was adapted to study DAGLα based on the production of 2-AG from 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) hydrolysis by DAGLα coupled to the glycerol production by MAGL (Table 3) (van der Wel et al. 2015). Recently, another fluorogenic compound based on 7-HCA reporter came up for studying ABHD6 activity: the arachidonoyl 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE) and it has been used to study both the catalytic site of ABHD6 and its inhibitors WWL70 and AM6701 (Table 3) (Shields et al. 2019). The AHMMCE reporter was previously used to study MAGL (Table 3) (Zvonok et al. 2008).

In conclusion, different activity assays have been developed to assess ECS enzymes. However, the kinetics parameters obtained vary between the assays. Thus, there is a need to develop new highly specific strategies to study these enzymes.

Enzyme	Assay	Km ± SE (μM)	Vmax ± SE (nmol/mg/min)	Reference
ABHD6	Glycerol	159 ± 26	45 ± 5	Navia *et al.*, 2012
		200 ± 38	0.07 ± 0.006	Shields *et al.*, 2019
	AHMMCE	26 ± 1.2	0.008 ± 0.0002	Shields *et al.*, 2019
	HPLC	144 ± 10	0.08 ± 0.002	Shields *et al.*, 2019
	1(3)-MO	1900	7976	Thomas *et al.*, 2013
ABHD12	Glycerol	117 ± 14	42 ± 3	Navia *et al.*, 2012
MAGL	Glycerol	110 ± 15	120 ± 10	Navia *et al.*, 2012
	Glycerol	9.7 ± 3.6	0.6 *	Savinainen *et al.*, 2014
	7-HCA	9.8	1700	Wang *et al.*, 2008
	AHMMCE	8-9	n.d.	Kvonok *et al.*, 2008
DAGL	Glycerol	150 ± 25	11 ± 07	Wel *et al.*, 2015

Table 3. Activity assays and kinetics parameters of 2-AG synthesis and degradation enzymes. Standard error (SE). *Data extracted from the graph.

HYPOTHESIS

CPT1C and endocannabinoids are key factors of hypothalamic regulation of brown fat thermogenesis at early stages of diet-induced obesity. One of the molecular mechanism underlying the effects of CPT1C on energy homeostasis is through the interaction with the hydrolase of endocannabinoids, ABHD6.

OBJECTIVES

1. To investigate whether CPT1C in the ventromedial nucleus of the hypothalamus is a key modulator of brown fat thermogenesis.

2. To study the profile of hypothalamic endocannabinoids during diet-induced obesity development and their correlation with brown fat thermogenesis.

3. To elucidate whether CPT1C interacts with ABHD6 and regulates its activity.

RESULTS

Chapter I

CPT1C in the ventromedial nucleus of the hypothalamus is necessary for brown fat thermogenesis activation in obesity. *Molecular Metabolism,* 2019.

ARTICLE SUMMARY

In this chapter, we aimed to investigate whether CPT1C is controlling BAT thermogenesis during obesity development and whether it is a downstream factor of AMPK in the VMH.

A brief reminder, the brain-specific carnitine CPT1C is necessary for the correct adaptation to metabolic challenges in the hypothalamus. However, it has not been studied whether CPT1C is important in the control of diet-induced thermogenesis. The BAT is the principal thermogenic organ and it is regulated from the VMH, among other nuclei. In this nucleus, it has been described that AMPK acts as an energy sensor able to regulate BAT thermogenesis.

We exposured WT and CPT1C-KO mice to HFD for 7 and 14 days or to icv leptin administration and we studied BAT thermogenesis activation, body weight, adiposity, food intake and leptinemia (**Experimental procedure, Figure 16A**). Moreover, we stereotaxically injected virus carrying CPT1C or an inactive form of AMPK in the VMH of CPT1C-KO mice and BAT thermogenesis was monitored (**Experimental procedure, Figure 16B**).

Results showed that 7 days of HFD and central leptin administration induced an activation of BAT thermogenesis that was attenuated in CPT1C-KO mice. Moreover, CPT1C-KO animals evidenced higher body weight, adiposity, hyperleptinemia, ER stress, and disrupted hypothalamic leptin signalling after HFD feeding. Expression of CPT1C in the VMH of CPT1C-KO mice was sufficient to restore normal body weight gain, leptin plasma levels and BAT thermogenesis activation after 7 days of HFD. Inactivation of endogenous AMPK within the VMH induced body weight loss and impaired BAT thermogenesis activation in WT animals whereas these effects were blunted in CPT1C-KO animals.

Altogether, we concluded that CPT1C is a key factor for the activation of BAT thermogenesis induced by HFD or leptin probably being an AMPK downstream signalling in the VMH (**Results summary, Figure 17**).

EXPERIMENTAL PROCEDURE

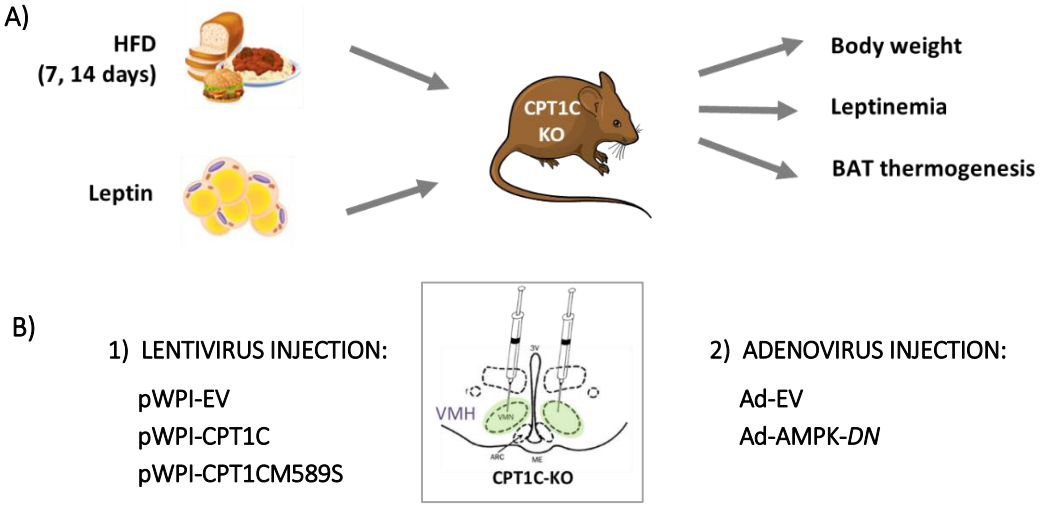


Figure 16. A) WT and CPT1C-KO animals were fed with SD or HFD for 7 and 14 days and body weight, leptinemia and BAT thermogenesis were assessed. B) CPT1C-KO mice were stereotaxically injected with (1) lentivirus encoding for CPT1C and CPT1CM589S, a mutated form insensitive to malonyl-CoA, and (2) adenovirus encoding for a dominant negative form of AMPK (AMPK-*DN*).

RESULTS SUMMARY

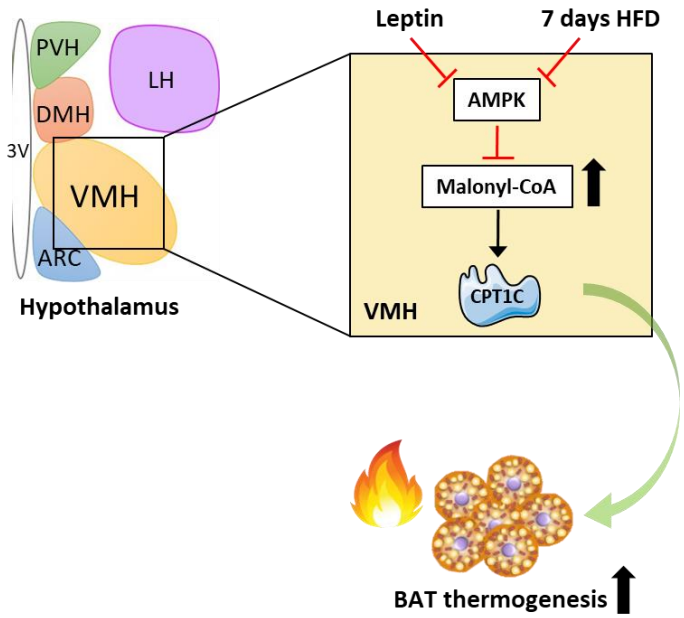


Figure 17. CPT1C in the VMH is necessary for leptin and diet-induced BAT thermogenesis since is likely an AMPK downstream signalling.

CPT1C IN THE VENTROMEDIAL NUCLEUS OF THE HYPOTHALAMUS IS NECESSARY FOR BROWN FAT THERMOGENESIS ACTIVATION IN OBESITY

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ABSTRACT

Objective: Carnitine palmitoyltransferase 1C (CPT1C) is implicated in central regulation of energy homeostasis. Our aim was to investigate whether CPT1C in the ventromedial nucleus of the hypothalamus (VMH) is involved in the activation of brown adipose tissue (BAT) thermogenesis in the early stages of diet-induced obesity.

Methods: CPT1C KO and wild type (WT) mice were exposed to short-term high-fat (HF) feeding or to intracerebroventricular leptin administration and BAT thermogenesis activation was evaluated. Body weight, adiposity, food intake and leptinemia were also assayed.

Results: Under 7 days of HF diet, WT mice showed a maximum activation peak of BAT thermogenesis that counteracted obesity development whereas this activation was impaired in CPT1C KO mice. KO animals evidenced higher body weight, adiposity, hyperleptinemia, ER stress, and disrupted hypothalamic leptin signaling. Leptin-induced BAT thermogenesis was abolished in KO mice. These results indicate an earlier onset leptin resistance in CPT1C KO mice. Since AMPK in the VMH is crucial in the regulation of BAT thermogenesis, we analyzed if CPT1C was a downstream factor of this pathway. Genetic inactivation of AMPK within the VMH was unable to induce BAT thermogenesis and body weight loss in KO mice, indicating that CPT1C is likely downstream AMPK in the central mechanism modulating thermogenesis within the VMH. Quite opposite, the expression of CPT1C in the VMH restored the phenotype.

Conclusion: CPT1C is necessary for the activation of BAT thermogenesis driven by leptin, HF diet exposure and AMPK inhibition within the VMH. This study underscores the importance of CPT1C in the activation of BAT thermogenesis to counteract diet-induced obesity.

1 INTRODUCTION

Obesity is ultimately the result of a sustained imbalance between energy intake and energy expenditure. A key mechanism to maintain body weight homeostasis against an overload of energy is diet-induced thermogenesis [1,2]. Brown adipose tissue (BAT) is considered a major site for the regulation of diet-induced thermogenesis through the sympathetic nervous system (SNS), and it is precisely orchestrated by the hypothalamus [3,4]. In fact, an intact hypothalamic function will ensure a fine-tune activation of BAT thermogenesis in response to short-term high fat (HF) diet or leptin to counteract excessive body weight gain [2,5,6]. Despite this evidence, to date, little is known about the exact molecular hypothalamic pathways regulating thermogenesis under conditions of nutrient surplus [7]. In light of the current obesity epidemic, identification of the hypothalamic pathways and potential targets mediating short-term activation of thermogenesis in response to nutritional status would provide valuable information about obesity development and progression [2,7–9].

Recent findings have demonstrated that hypothalamic AMPK is a major regulator of BAT thermogenesis through its modulation of the SNS [3,10]. Particularly, it has revealed AMPK activity in the ventromedial nucleus of the hypothalamus (VMH) on thermogenic response. Remarkably, selective inactivation of AMPK within the VMH increased ventral hypothalamic malonyl-CoA levels, BAT activity and promoted weight loss, in a feeding-independent manner [10,11]. Although this pathway constitutes a canonical circuit that mediates the effect of several thermogenic molecules (*e.g.* T3 or leptin) [3,10,12], further studies are necessary to explore the sub-cellular mechanisms and neuronal networks involved in the AMPK(VMH)-SNS-BAT axis. In this regard, recent data have demonstrated that selective ablation of the isoform AMPKα1 in steroidogenic factor 1 (SF1) neurons of the VMH promotes BAT activation and subsequently a leaner, feeding-independent and obese-resistant phenotype [12,13].

The acetyl-CoA (ACC) / malonyl-CoA pathway is one of the most important signaling pathways downstream AMPK [14]. Within the hypothalamus, malonyl-CoA levels fluctuate in response to the nutritional status, acting as a canonical signal of energy surplus [15,16]. Malonyl-CoA is the physiological inhibitor of carnitine palmitoyltransferase 1 (CPT1) enzymes, which catalyze the transport of long chain fatty acids into the mitochondria [16]. Among CPT1s, the neuron-specific CPT1C isoform is the most puzzling carnitine acyltransferase [17,18]. In contrast to the canonical isoforms (CPT1A and CPT1B), CPT1C is located in the endoplasmic reticulum (ER) of neurons, instead of the mitochondrial

membrane, and has insignificant CPT1 activity [19]. Nevertheless, it is still able to bind malonyl-CoA with similar affinity than CPT1A [20], suggesting that CPT1C could act as a sensor of this lipid intermediary in the hypothalamus [16].

The expression of CPT1C in the brain has been found particularly high in neurons of hypothalamic areas involved in the regulation of feeding and energy expenditure including arcuate nucleus (ARC), paraventricular hypothalamus (PVH) and VMH [17,21]. Studies from our group and others have demonstrated that CPT1C within these areas plays a major role in the modulation of energy balance. For example, hypothalamic CPT1C mediates that central effects of leptin and ghrelin on feeding behavior [22,23]. Hypothalamic CPT1C is also determining fuel selection and food preference during fasting [24,25]. Moreover, CPT1C KO mice are more prone to become obese when chronically fed a HF diet with a reduced peripheral fatty acid oxidation [20,21,26,27]. In these studies, the expression of CPT1C especially in the mediobasal hypothalamus (MBH) was found to be crucial in mediating the effects in energy homeostasis [21,24]. However, the possible role of CPT1C in the hypothalamic regulation of BAT thermogenesis is totally unknown.

Here, we show that the obese phenotype and metabolic inflexibility that characterizes to CPT1C KO mice is related to an impaired BAT thermogenesis following a short-term HF diet exposure and central leptin injection. We also demonstrate that the lack of CPT1C disrupts the canonical pathway of AMPK(VMH)-SNS-BAT-mediated thermogenesis. Our data thus uncover CPT1C as a key downstream factor of the hypothalamic AMPK/ACC pathway in the control of brown fat thermogenesis.

2 MATERIALS AND METHODS

2.1 Animals

Male (8-10 week old) CPT1C KO mice and their wild-type (WT) littermates with the same genetic background (C57BL/6J) were used for the experiments [24]. All animals were housed on a 12 h/12 h light/dark cycle (light on at 8 am, light off at 8 pm) in a temperature- and humidity-controlled room. The animals were allowed free access to water and standard laboratory chow, unless otherwise specified. For HF diet studies, animals were placed on an HF diet (60 % kcal from fat, D12492) or standard diet (SD) (10 % kcal from fat, D12450B, Research Diets, New Brunswick, USA) for 3, 7 or 14 days. At the end of the studies, animals were sacrificed, and tissues collected for further molecular and biochemical analysis as further detailed. All animal procedures were performed in

agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Local Ethical Committee (Procedure ref. 9606 from the Generalitat de Catalunya).

2.2 Intracerebroventricular administration of leptin

Chronic cannulae were stereotaxically implanted into the lateral cerebral ventricle under ketamine/xylazine intraperitoneal anesthesia (ketamine 75 mg/kg body weight plus xylazine 10 mg/kg body weight). The coordinates were 0.58 mm posterior to Bregma, 1 mm lateral to the midsagittal suture and to a depth of 2.2 mm. Mice were individually caged and allowed to recover for 5 days before the experiment. Prior to the experiment, cannula placement was verified by a positive dipsogenic response to angiotensin II (1 nmol in 1 ml; Sigma-Aldrich). On experimental day, WT and CPT1C KO mice received an intracerebroventricular (ICV) administration of 2 µl of either leptin (0.1 µg/µl) (PeproTech, London, UK) or vehicle (aqueous buffer containing 0.1% BSA), three hours after lights-on. 200 min after the injection, mice were sacrificed by cervical dislocation and MBH and BAT were collected for further analysis.

2.3 Stereotaxic microinjection and viral vectors

The lentiviral vectors, pWPI-IRES-GFP and pWPI-CPT1C-IRES-GFP, were produced and titrated as previously described [24]. In addition, a lentiviral vector with a mutated isoform of CPT1C insensitive to malonyl-CoA, pWPI-CPT1CM589S-IRES-GFP, was produced. Mouse malonyl-CoA CPT1C sensitive site was identified by sequence homology with CPT1A. The homologous mutation in CPT1A (M593S) abolishes malonyl-CoA sensitivity while maintaining CPT1 activity [28]. CPT1C mutant M589S was constructed using the Q5 Site-Directed mutagenesis procedure (New England BioLabs) with the pWPI-IRES-CPT1C plasmid as template. The primers were obtained from the online design software NEBaseChanger and designed with 5' ends phosphorylated and annealing back-to-back: forward 5'-GAGTCAGCCAGTACCCGACTGTTC-3' and reverse 5'-ATAAGTCAGGCAGAATTGAC-3' (the mutated nucleotide were underlined). The appropriate substitutions as well as the absence of unwanted mutations were confirmed by sequencing the inserts in both directions.

Adenoviral vectors (GFP and AMPKα1-dominant negative + AMPKα2-dominant negative, AMPK-*DN*; Viraquest; North Liberty, IA, USA) were kindly provided by Dr. Miguel Lopez [12]. Stereotaxic surgery to target the VMH was performed in mice under ketamine/xylazine

anesthesia. Purified lentivirus (1 x 10^9 pfu ml^{-1}) or adenovirus (1 x 10^{12} pfu ml^{-1}) in artificial cerebrospinal fluid were injected bilaterally in the VMH over 10 min through a 33-gauge injector connected to a Hamilton Syringe and an infusion pump (0.5 µl per injection site) [24]. The injections were directed to the following stereotaxic coordinates: 1.6 mm posterior from Bregma, ± 0.4 mm lateral to midline and 5.6 mm deep. Mice underwent 6 days (adenovirus) or 7 days (lentivirus) of recovery before other experiments were performed. Correct bilateral infection was confirmed by western blot and histologically by GFP fluorescence in brain slices.

2.4 BAT temperature measurements

Skin temperature surrounding BAT was visualized using a high-resolution infrared camera (FLIR Systems) and analyzed with a specific software package (FLIR-Tools-Software, FLIR; Kent, UK), as previously described [12]. For ICV administration of leptin, images were recorded and analyzed every 10 minutes during 220 min. For the rest of experiments, thermal images were acquired the day of sacrifice.

2.5 Sample collection and processing

Mice were killed by cervical dislocation. From each animal, either the whole brain (for histology) or the MBH, as well as blood (for plasmatic determinations), liver, interscapular BAT, visceral and subcutaneous WAT were weighted, collected and stored at -80°C until further processing. To dissect the MBH, brains were placed in a coronal brain matrix and sectioned from Bregma (-1 to -2.5 mm) and the MBH was obtained using a tissue collector measuring 1 mm in diameter.

2.6 Plasma analysis

Plasma was obtained after blood centrifugation (2000 g, 15 min). Plasmatic levels of leptin were determined by mouse ELISA kit (Crystal Chem, Zaandam, Netherlands), following the manufacturer's instructions.

2.7 Tissue morphology

Interscapular BAT, visceral and subcutaneous WAT were fixed overnight in 10% PBS-buffered formalin. Histological samples were paraffin-embedded and stained with hematoxylin and eosin (H&E), as previously described [29]. Tissue sections were captured by light microscopy (Olympus, Hamburg, Germany) at 20X magnification and using NIS-Elements software (Nikon, Japan).

2.8 Liver triglycerides (TG) quantification

Liver samples were homogenized and lipids were extracted as previously described [30]. TG were measured in the lipid extract using a commercial kit (Sigma, Madrid, Spain), following the manufacturer's instructions.

2.9 RNA preparation and quantitative RT-PCR

Total RNA was extracted from tissues using Trizol Reagent (Fisher Scientific, Madrid, Spain). Retrotranscription and quantitative RT-PCR (qPCR) was performed as previously described [24]. Proprietary SYBR Green or Taqman Gene Expression assay primers used (IDT DNA Technologies, Leuven, Belgium) are detailed in supplementary material (Table S.I.). Relative mRNA levels were measured using the CFX96 Real-time System, C1000 Thermal Cycler (BioRad).

2.10 Western blotting

Western blot was performed as previously described [24]. Briefly, tissue was homogenized in RIPA buffer (Sigma-Aldrich, Madrid, Spain) containing protease and phosphatase inhibitor cocktails. Protein extracts were separated on SDS-PAGE, transferred into Immobilion-PVDF membranes (Merck Millipore, Madrid, Spain) and probed with antibodies against: ACC, AMPKα, pACC (Ser79), pAMPKα (Thr172), pSTAT3 (Tyr705) (Cell Signaling; Danvers, MA, USA); GAPDH, UCP1 (Abcam, Cambridge, UK) β-actin (Fisher Scientific, Madrid, Spain) and tubulin (Sigma, Madrid, Spain). Each membrane was then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody, anti-mouse or anti-rabbit (DAKO, Glostrup, Denmark), and developed using LuminataForte Western HRP substrate (Merck Millipore). Images were collected by GeneTools software (Syngene, Cambridge, UK) and quantified by densitometry using ImageJ-1.33 software (NIH, Bethesda, MD, USA). GAPDH or β-actin was used as an endogenous control to normalize protein expression levels. In all the figures showing images of gels, all the bands for each picture come from the same gel, although they may be spliced for clarification.

2.11 Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was conducted using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Statistical analysis was determined by ANOVA (more than 2 groups were compared) followed of post hoc two-

tailed Bonferroni test. $P < 0.05$ was considered significant. The number of animals used in each experiment is specified in each figure legend.

3 RESULTS

3.1 CPT1C KO mice show impaired diet-induced thermogenesis

The induction of thermogenesis in the interscapular BAT of mice was first analyzed after short-term exposure to HF diet, compared to SD. To identify the maximum activation peak of diet-induced thermogenesis over time, experiments were performed in response to 3, 7 and 14 days HF diet feeding (**Fig. 1 and Fig. S.1**). The peak in the thermogenesis was reached in interscapular temperature (**Fig. 1A**), gene expression of thermogenic markers (**Fig. 1B-D, Fig. S.1A**) and UCP1 protein expression (**Fig. 1E**) in the BAT of WT mice after 7 days of HF diet when compared to other timings and SD. Of note, HF feeding over 7 days resulted in significantly ameliorated responses in CPT1C KO mice, when compared to WT mice (**Fig. 1A-E**).

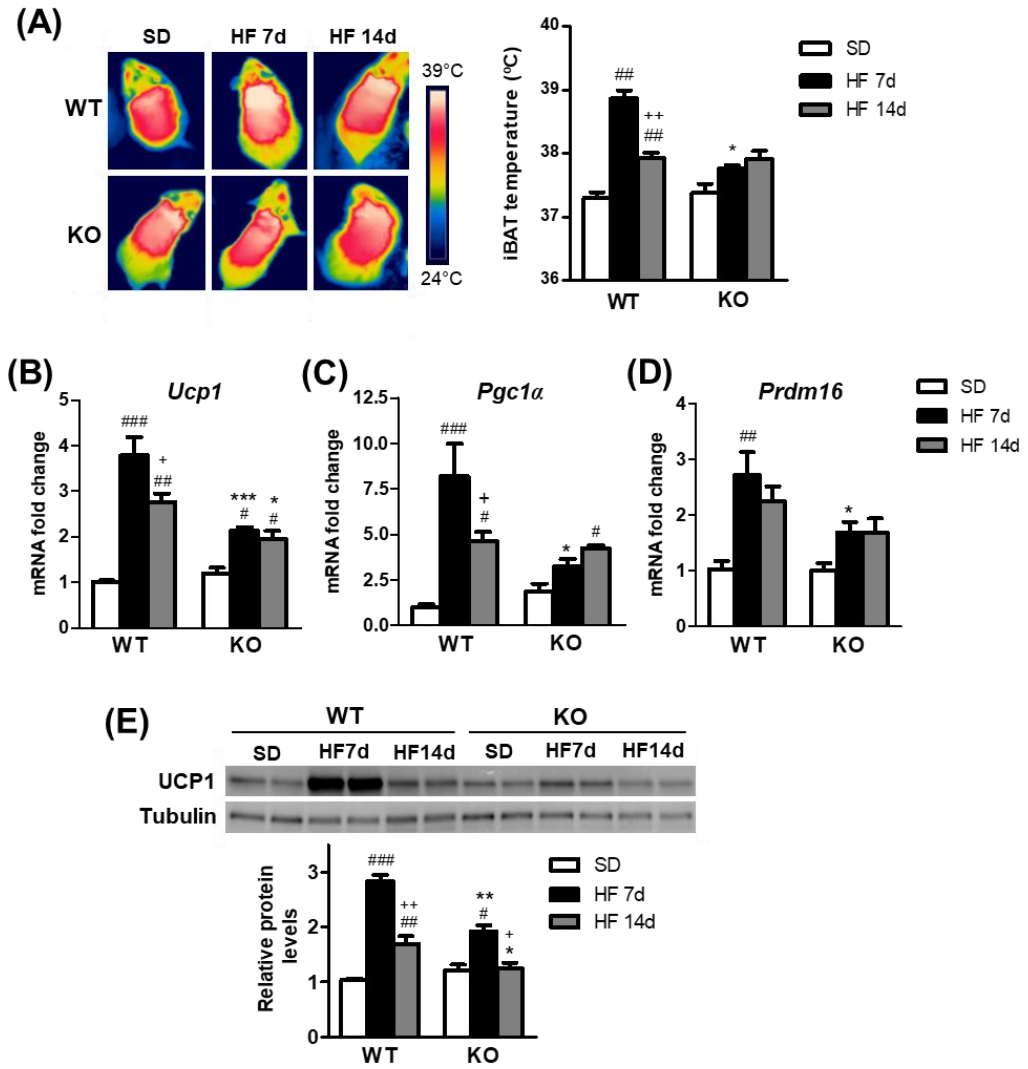

Figure 1. Impaired diet-induced thermogenesis in CPT1C KO mice. (A) Representative infrared thermal images and quantification of interscapular temperature adjacent to the BAT depot of WT and CPT1C KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 and 14 days. (B-D) Relative mRNA expression of the thermogenic markers UCP1 (B), PGC1α (C) and PRDM16 (D) in BAT of WT and KO mice fed SD or HF diet. (E) Protein levels of UCP1 in BAT of WT and KO fed SD or HF diet for 7 and 14 days. Data are expressed as mean ± SEM (n=5-9). **P*<0.05, ***P*<0.01, ****P*<0.001 *versus* WT with the same diet; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *versus* SD within the same genotype; +*P*<0.05, ++*P*<0.01 *versus* HF 7d within the same genotype.

In WT mice, a significant increase in body weight gain was not appreciated until 14 days of administration of a HF diet (Fig. 2A). In contrast, CPT1C KO mice already revealed higher body and visceral WAT weight over 7 days of HF diet compared to SD (Fig. 2A and B; Fig. S.2A).The induction of thermogenesis and body weight gain of WT and CPT1 KO mice were associated with a reduction in the size of unilocular lipid droplets observed after 7 days of HF diet feeding in histological sections of BAT (Fig. 2C). As illustrated in Fig. 2D, HF feeding over 7 days resulted in a substantial increase of leptin levels in plasma of CPT1C KO mice, whereas these levels remained unaltered in WT mice. Altogether, these data indicate that CPT1C KO mice show an earlier obesogenic phenotype in response to acute HF diet administration, likely due to an impaired activation of BAT thermogenesis, compared to WT. This is also supported by the fact that food intake, measured during 7 and 14 days of HF feeding, was comparable in the WT and KO mice (Fig. S.2B), indicating that the obesogenic phenotype observed in CPT1C KO mice is not due to alterations in food intake.

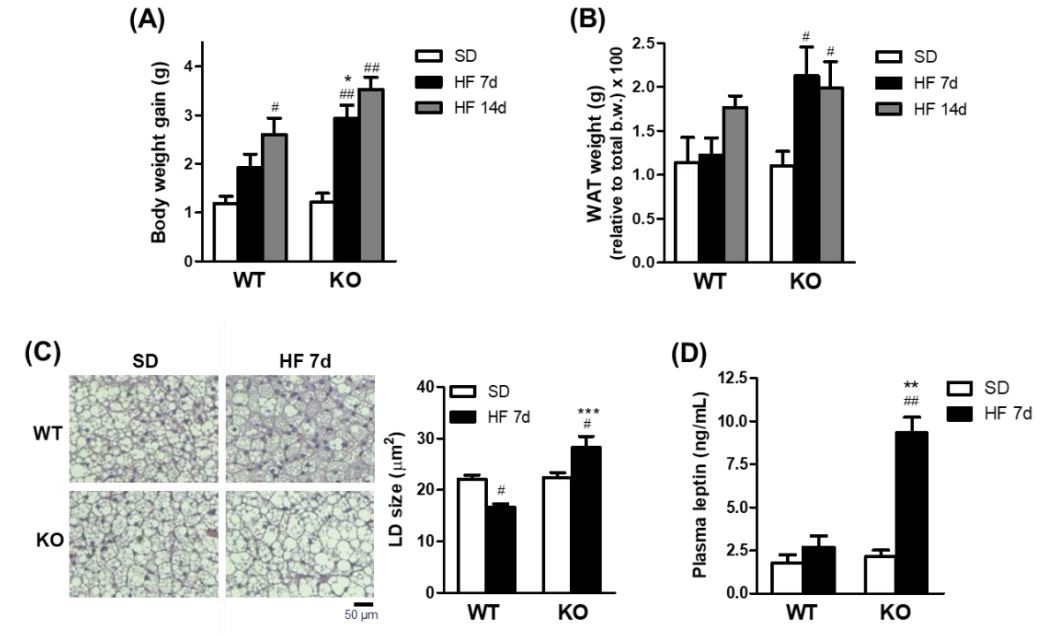

Figure 2. CPT1C KO mice show an earlier obesogenic phenotype compared to WT. (A and B) Body weight gain (A) and visceral WAT weight (B) of WT and KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 and 14 days. (C) Representative histological H&E staining and quantification of the unilocular lipid droplets (LD) size of interscapular BAT of WT and KO mice fed a SD or a HF diet for 7 days. (D) Plasma leptin levels of WT and KO mice fat a SD or HF diet for 7 days. Data are expressed as mean ± SEM (n=5-7). **P*<0.05, ***P*<0.01, ****P*<0.001 *versus* WT with the same diet; #*P*<0.05, ##*P*<0.01 *versus* SD within the same genotype.

3.2 The thermogenic response to central leptin is impaired in CPT1C KO mice

In another set of experiments, activation of BAT thermogenesis was also evaluated after central administration of leptin (**Fig. 3**). We found an increase of BAT interscapular temperature by central leptin that was maintained for at least 3 h in WT mice (**Fig. 3A and C**). This effect was confirmed by an increase in gene expression of thermogenic markers in BAT of WT (**Fig. 3D**). However, these acute leptin-induced responses were significantly attenuated in CPT1C KO mice (**Fig. 3B-D**).

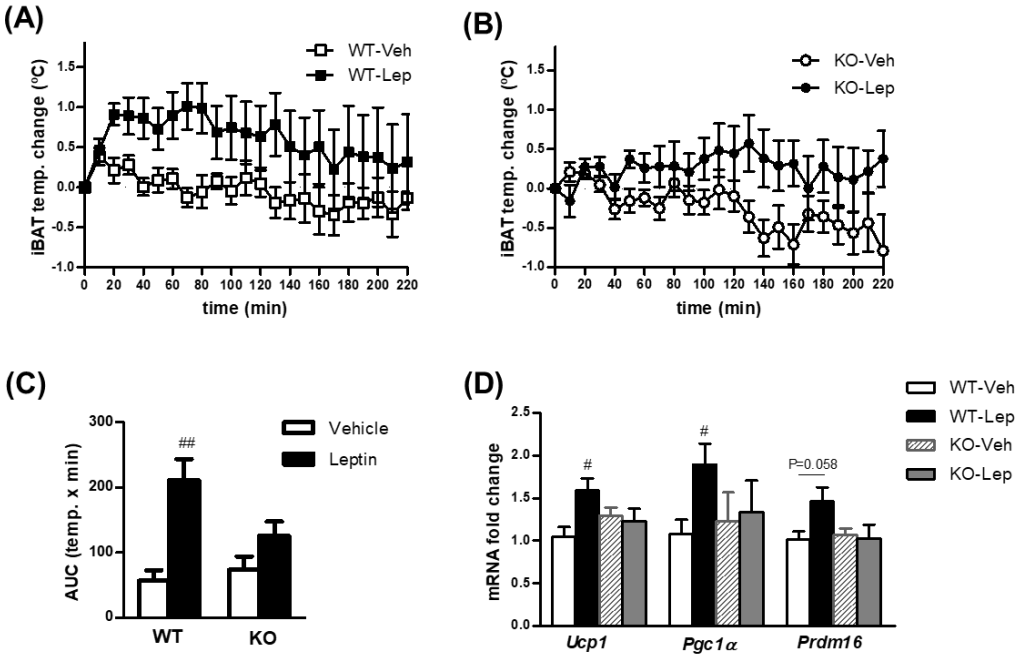

Figure 3. Impaired leptin-induced thermogenesis in CPT1C KO mice. (A-C) Quantification of interscapular temperature changes adjacent to the BAT depot (iBAT) after ICV leptin treatment in WT (A) and CPT1C KO mice (B) compared with ICV vehicle. (C) Area under the curve (AUC) of iBAT temperature during 220 minutes. (D) Gene expression analysis of thermogenic markers in BAT of WT and KO mice after ICV leptin. Data are expressed as mean ± SEM (n=5-8). #*P*<0.05, ##*P*<0.01 *versus* Vehicle within the same genotype.

3.3 CPT1C KO mice display an altered expression of hypothalamic leptin signaling markers and ER stress after short-term HF diet feeding

The hyperleptinemia after 7 days of HF diet administration and the impaired central leptin-induced thermogenesis observed in CPT1C KO mice suggest an earlier onset in the disruption of leptin signaling in these mice compared to WT. Therefore, the expression of proteins involved in leptin signaling in the MBH was evaluated. First, expression levels of pSTAT3 and SOCS3, important transcription factors in leptin signaling, were analyzed in MBH of WT and KO mice fed a HF diet for 7 days. MBH of WT mice showed a reduced phosphorylation of STAT3 (**Fig. 4A**) and increased mRNA levels of SOCS3 (**Fig. 4B**) with HF diet compared to SD. Conversely, CPT1C KO mice fed a HF diet exhibited a substantial increase in pSTAT3 without changes in SOCS3 (**Fig. 4A and B**).

Next, we evaluated the impact of HF and/or CPT1C ablation on the AMPK signaling in the MBH. No significant alterations in the expression of pAMPK and pACC were detected in either genotype after 7 days of HF diet exposure (**Fig. 4C**). Remarkably, the leptin-induced inhibition of pAMPK in the MBH was suppressed in CPT1C KO mice, indicating that a normal CPT1C function is required for a normal leptin hypothalamic signaling (**Fig. 4D**).

Finally, since CPT1C is located in the ER, and hypothalamic ER stress has been strongly related to leptin signaling disruption and obesity, as well as on the central control of thermogenesis [31–33], ER stress markers were analyzed in MBH. Whereas no changes in mRNA expression levels for ER stress markers were appreciated in WT mice-fed HF diet for 7 days, significant increases were shown in MBH of CPT1C KO mice (**Fig. 4E-G**), in keeping with their impaired thermogenic responses, leptin signaling and more prone obese phenotype.

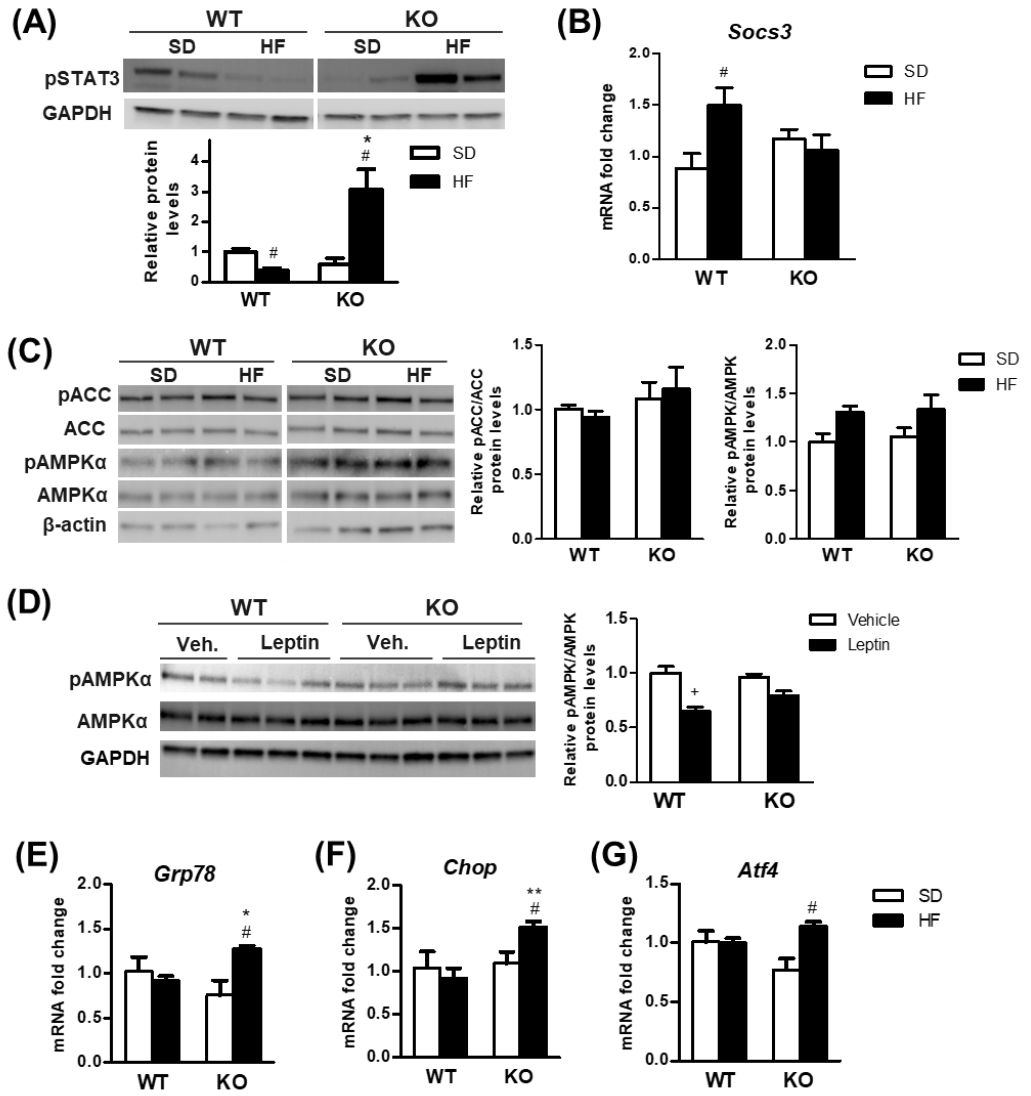

Figure 4. CPT1C KO mice show an altered expression of markers of leptin signalling and ER stress in the mediobasal hypothalamus after short-term administration of a HF diet. (A-C) Protein levels of pSTAT3 (A), mRNA levels of SOCS3 (B) and protein expression of pAMPKα, pACC, AMPK and ACC (C) in the mediobasal hypothalamus of WT and CPT1C KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 days. (D) Protein levels of pAMPKα and AMPK in the mediobasal hypothalamus of WT and CPT1C KO mice after ICV administration of leptin or vehicle. (E) mRNA levels of ER stress markers in the mediobasal hypothalamus of WT and CPT1C KO mice fed a SD or a HF diet for 7 days. Data are expressed as mean ± SEM (n=5-7). **P*<0.05 *versus* WT with the same diet; #*P*<0.05 *versus* SD within the same genotype; +*P*<0.01 *versus* vehicle within the same genotype.

3.4 Expression of CPT1C in the VMH is enough to restore short-term diet-induced response in CPT1C KO mice

Due to the importance of CPT1C in the MBH region (which includes the VMH) in the regulation of energy homeostasis [21,24], and considering the crucial role of the VMH in the control of BAT thermogenesis [10], firstly we evaluated if the expression of CPT1C in this hypothalamic area was able to restore the phenotype observed after short-term HF feeding in KO mice. Lentiviral vectors expressing CPT1C-GFP or empty vector (EV)-GFP were microinjected in the VMH of WT and CPT1C KO mice and, after 7 days, mice were fed SD or HF diet for 7 days (see experimental protocol illustrated in **Fig. 5A**). Injection site was confirmed by direct fluorescence of GFP in brain sections or by CPT1C expression analysis by western blot in the MBH (**Fig. 5B**). The expression of CPT1C in the VMH was enough to reverse the body weight gain (**Fig. 5C**), the hyperleptinemia (**Fig. 5D**) and the expression of gene thermogenic markers in BAT (**Fig. 5E**) of KO mice fed a HF diet for 7 days. Stereotaxic injection of adeno-associated viruses expressing the EV-GFP or CPT1C in the VMH of CPT1C KO mice (see Supplementary Methods) also revealed a significant restoration of iBAT temperature and body weight gain in response to 7 days HF diet feeding (**Fig. S.3**).

These data confirm a key role of CPT1C in this hypothalamic area during diet-induced thermogenesis.

We also investigated the role of malonyl-CoA in the impaired hypothalamic function of CPT1C null mice. For this purpose, lentiviral vectors expressing a mutant CPT1C insensitive to malonyl-CoA (CPT1CM589S, see Methods), were used and compared to vectors expressing EV and CPT1C. Expression of the mutated isoform of CPT1CM589S in VMH of KO mice was not able to fully restore body weight gain (**Fig. 5C**), leptinemia (**Fig. 5D**) and expression of gene thermogenic markers in BAT (**Fig. 5E**) in response to HF diet. These data indicate that malonyl-CoA sensing by CPT1C is relevant to regulate short-term diet-induced responses.

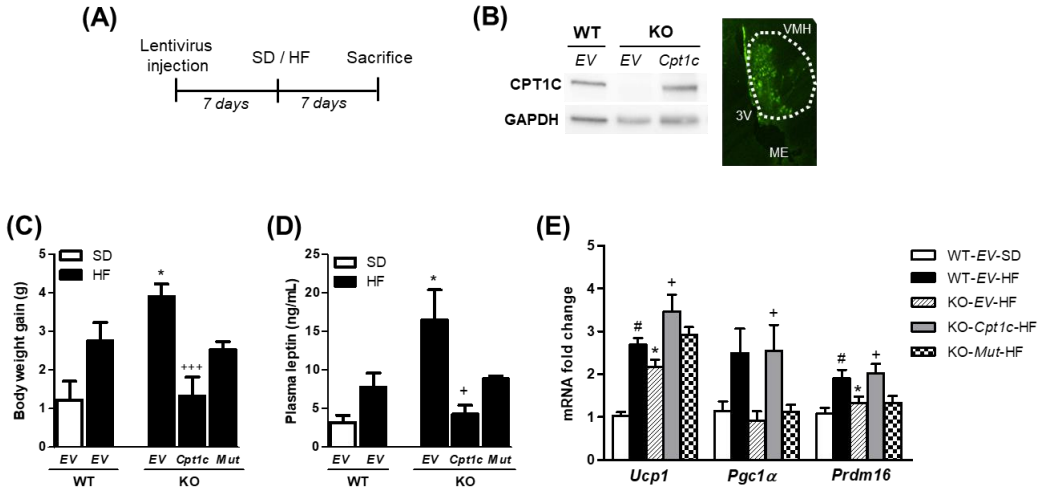

Figure 5. Expression of CPT1C in the VMH restores short-term diet-induced response in CPT1C KO mice. (A) GFP (empty vector, *EV*) or CPT1C-GFP (*Cpt1c*)-expressing lentiviruses were microinjected in the VMH of WT and CPT1C KO mice and after 1 week, mice were fed a standard diet (SD) or a high fat (HF) diet for 7 days. (B) Injection site was confirmed by direct fluorescence of GFP in brain sections or by CPT1C expression analysis by western blot in the ventral hypothalamus. (C-E) Body weight gain (C), plasma leptin (D) and gene expression analysis of thermogenic markers in BAT of WT-*EV*, KO-*EV*, and KO expressing CPT1C (KO-*Cpt1c*) or CPT1CM589S (KO-*Mut*) fed SD or HF diet for 7 days. Data are expressed as mean ± SEM (n=6-8). **P*<0.05 *versus* WT-*EV*-HF; #*P*<0.05 *versus* WT-*EV*-SD; +*P*<0.05, +++*P*<0.001 *versus* KO-*EV*-HF.

3.5 Selective inactivation of AMPKα in the VMH was not able to induce BAT thermogenesis and body weight loss in CPT1C KO mice

To assess whether CPT1C is a downstream factor in the AMPKα-mediated regulation of energy balance, we selectively inactivated AMPK in VMH of WT and KO mice by stereotaxic delivery of a dominant-negative AMPKα1+α2 isoforms (AMPK-*DN*) [11–13,34]. This inactivation was confirmed by reduced hypothalamic protein levels of pACC (**Fig. S.4**) [34]. Previous data demonstrated that AMPK-*DN* delivery into the VMH increased malonyl-CoA concentrations in the ventral hypothalamus, inducing weight loss and increased expression of BAT thermogenic markers, without altering food intake [11,12]. As illustrated in **Fig. 6**, selective inactivation of AMPK in the VMH of WT mice involved a substantial reduction of body weight gain (**Fig. 6A**) with a significant increase in interscapular temperature adjacent to the BAT depot (**Fig. 6B**), elevated UCP1 protein expression levels (**Fig. 6C**) and increased gene expression of thermogenic markers in BAT (**Fig. 6D**). Notably, CPT1C KO mice showed a significant attenuation in all these parameters compared to WT mice (**Fig. 6A-D**).

Recent data from our group have shown that the inhibition of AMPK in the VMH promotes decreased hepatic AMPK signaling through the vagus nerve and subsequently increased

lipogenesis [12]. Our data showed that while in WT recapitulated that response, it was totally blunted in CPT1C KO mice (Fig. 6E-F). Altogether, AMPK-*DN*-mediated effects within the VMH in body weight change, BAT and liver were impaired in mice lacking CPT1C. This is of importance because, it has been recently demonstrated that increased lipogenesis after VMH inhibition of AMPK is demanding for BAT thermogenesis. Therefore, CPT1C KO mice, which show impaired BAT function, also display altered associated liver responses.

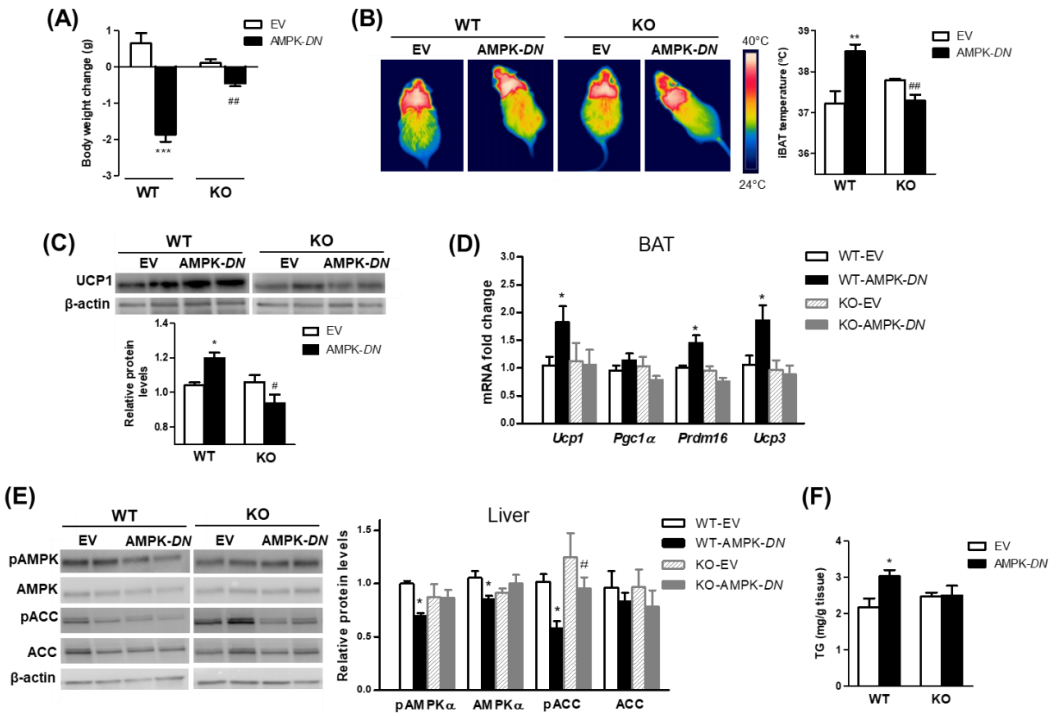

Figure 6. CPT1C KO mice show impaired AMPK-mediated effects within the VMH on body weight change, BAT thermogenesis and liver. (A) Body weight change of WT and CPT1C KO mice treated with adenoviruses encoding GFP (Empty vector, EV) or AMPK-*DN* in the VMH. (B-D) Representative infrared thermal images and quantification of interscapular temperature adjacent to the BAT depot (B), protein levels of UCP1 in BAT (C) and gene expression analysis of thermogenic markers in BAT of WT and KO mice treated with EV or AMPK-*DN* in the VMH (D). (E and F) Protein levels of the AMPK pathway (E) and TG levels in the liver of mice treated with EV or AMPK-*DN* in the VMH (G). Data are expressed as mean ± SEM (n=6-7). **P*<0.05, ***P*<0.01, ****P*<0.001 *versus* WT-EV; #*P*<0.05, ##*P*<0.01 *versus* WT-AMPK-*DN*.

4 DISCUSSION

Development and progression of obesity are mediated by short-term neurological changes in response to nutritional status that progressively impair hypothalamic neuronal functions and therefore body weight regulation. In the last few years, several investigations have been directed towards the identification of proteins involved in the temporal dysregulation of neuronal functions to control aspects of energy balance beyond food intake, during the development of diet-induced obesity [2,7–9].

The present research demonstrates that the neuron-specific CPT1 isoform, CPT1C, plays a critical role in hypothalamic regulation of BAT thermogenesis, particularly in response to metabolic challenges activating BAT, such as short-term diet and central leptin. Considering the importance of the canonical pathway dependent on AMPK in the VMH to regulate BAT thermogenesis during the development of diet-induced obesity [10,12,13], our study also reveals that CPT1C might be a crucial factor in this canonical pathway.

Although CPT1C is still the most unknown CPT1 and its neuronal function is uncertain, our group and others have demonstrated its critical role in energy homeostasis [18], and has been suggested to be a key indicator of the energetic status of neurons by sensing malonyl-CoA, a canonical signal of energy surplus [16,35]. The present study reveals that the obesogenic phenotype and acute alterations in metabolic flexibility already described in CPT1C KO mice [20,22–24] are related to impaired hypothalamic regulation of BAT thermogenesis, as exposed in response to short-term diet or central leptin administration. These metabolic challenges imply an increase in hypothalamic levels of malonyl-CoA [16,35,36] that need to be sensed by CPT1C. We show that, under short-term HF diet feeding (7 days), a robust activation peak of BAT thermogenesis was appreciated in WT mice, which helps mice to maintain normal body weight, adiposity and leptinemia, thus counteracting obesity development. Previous studies analyzing initial hypothalamic events during development of diet-induced obesity in mice [7–9,37] have also demonstrated that C57BL/6J mice do show hypothalamic compensatory changes at early time points in response to HF diet (from 2 days to 7 days), but they may not be able to maintain them (from 14 days onwards). This could contribute to their obese phenotype after a prolonged period of HF diet. Our study describes a pronounce activation of BAT thermogenesis after 7 days of feeding a HF diet, which could be directly related to the short-term hypothalamic compensatory changes that have been previously described to counteract obesity. In contrast, HF feeding over 7 days resulted in a diminish activation of BAT thermogenesis, higher body weight gain, hyperleptinemia and adiposity in CPT1C KO mice. This indicates

that the lack of neuronal CPT1C determines an early obesogenic phenotype in response to fat-rich diets. In relation to this result, acute activation of BAT thermogenesis in response to central leptin administration was also attenuated in mice lacking CPT1C. These data could correlate with the fact that CPT1C KO mice are resistant to the satiety effect of central leptin (Fig. S.5 and [23]).

Development of obesity has been linked to increased plasmatic levels of leptin that positively correlates to high adiposity and body weight gain, and an altered hypothalamic leptin signaling [38]. Evaluation of molecular mediators of leptin signaling during initial exposure to HF diets in MBH revealed that WT mice showed transient reduced levels of pSTAT3 with increased levels of SOCS3 levels after 7 days of HF diet. These results are in line with previous findings analyzing hypothalamic responses after short-term administration of fat-rich diets [8,9]. The authors reported decreased mRNA levels of hypothalamic SOCS3 after 2 days and 1 week of HF diet administration, whereas these levels were reverted after 2 weeks of HF feeding [8]. SOCS3 expression is stimulated by leptin and provides an *in vivo* indicator of direct leptin stimulation of neurons, acting as a negative feedback regulator of leptin receptor activation [38] and being particularly important in response to short-term changes of nutritional status [7]. Therefore, transient increased levels of SOCS3 could be related to the reduced expression in pSTAT3 of WT mice fed HF diet during 7 days, whereas these levels were restored after 14 days of HF diet administration. These temporal hypothalamic changes observed after short-term HF feeding have been suggested as a compensatory response to the positive energy surplus and to maintain a stable body weight during initial stages of fat-rich administration [7,8]. CPT1C KO mice fed a HF diet for 7 days presented, in contrast to WT mice, increased hypothalamic pSTAT3 levels and unchanged expression of SOCS3. Although these transitory hypothalamic changes in KO mice are not specifically indicating leptin resistance, this response to short-term HF diet is the opposite of that observed in WT mice. The lack of physiological response in the hypothalamus of KO mice during initial stages of diet-induced obesity could indicate the lack of compensatory changes at early time points of HF diet feeding and therefore an earlier obesogenic phenotype.

In addition to these findings, MBH of mice deficient in CPT1C fed a HF diet showed significant increases in ER stress markers. The hypothesis that hypothalamic ER stress is causally linked with leptin resistance and obesity has gained substantial support in the recent years [39]. Although the exact mechanisms by which HF diet feeding can directly perturb hypothalamic neuronal function remain unclear, a number of investigations

associate hypothalamic lipotoxicity derived from exposure to HF diet with ER stress as a possible explanation for the onset of obesity [31–33,40,41]. CPT1C is suggested to act as a sensor of hypothalamic malonyl-CoA levels fluctuations and also as a main regulator of the metabolism of complex lipids such as ceramides in neurons [18,20,42]. In addition, hypothalamic ER stress induced by lipotoxicity has been shown to impair the BAT thermogenic process [31–33]. A plausible hypothesis would be that the lack of CPT1C is determining an inaccurate lipid sensing, leading to hypothalamic lipotoxicity and subsequently ER stress, an idea that will require data to be confirmed.

It is known that the AMPK pathway is dysregulated in hypothalamus in obese states resulting from chronic HF feeding and that lack of dynamic responsiveness of this pathway is crucial in the pathophysiology of leptin resistance during diet-induced obesity [37]. In our study, administration of a HF diet during 7 days did not induce significant changes in pAMPK and pACC in MBH of WT or KO mice. This result agrees with previously reported data, showing that short-term administration of a HF diet (1-3 weeks) to rats did not modify hypothalamic AMPK phosphorylation [43,44]. Longer periods of HF feeding (from 3 weeks onwards) induced increased levels of the active phosphorylated form of AMPK in the hypothalamus of rats [43] and mice [37], mediating the interplay between hypothalamic and peripheral response to diet. When analyzing central administration of leptin, we found a significant attenuation of pAMPK expression levels in MBH of WT mice after leptin injection whereas this attenuation was not evidenced in CPT1C KO mice. Considering the findings that leptin has a role in SNS-mediated activation of BAT thermogenesis [45], and that inhibition of hypothalamic AMPK activity by leptin implies sympathetic activation to BAT and WAT [6], we could suggest that the lack of changes in pAMPK in the MBH of CPT1C KO mice could be related to the impaired leptin-induced thermogenesis in these animals.

To further demonstrate if CPT1C, particularly in the VMH, is a factor involved in the AMPK-SNS-BAT axis, specific strategies were achieved in this study. Firstly, considering the importance of the VMH in the control of BAT thermogenesis [6,10], we showed that the lentiviral expression of CPT1C in the VMH was enough to restore the phenotype observed after short-term HF feeding in KO mice. In addition, the phenotype was not fully restored when expressing the mutated isoform of CPT1C insensitive to malonyl-CoA in the VMH. This result suggests that sensing malonyl-CoA by CPT1C is relevant to regulate short-term diet-induced responses in this hypothalamic area. Secondly, our virogenetic approaches showed that BAT thermogenesis and body weight of CPT1C KO mice did not respond to selective inactivation of AMPK in the VMH, indicating that CPT1C is a crucial factor in the

AMPKα(VMH)-mediated regulation of BAT thermogenesis. Our data are in line with recent investigations proposing CPT1C as a downstream factor of AMPK in different hypothalamic nuclei to regulate feeding. A study from our group demonstrated the existence of a downstream pathway to SIRT1/p53/pAMPK axis in response to ghrelin, involving CPT1C, triggering acute changes in ceramide levels to regulate food intake by the modulation of NPY/AgRP expression in the ARC [22]. Interestingly, a recent investigation from Minokoshi's group [25] found that activation of an AMPK-CPT1C pathway in a subset of CRH-positive neurons in the PVH mediates the fasting-induced increase in high-carbohydrate diet selection. Our current investigation shows for the first time a role of CPT1C in the AMPK-brown fat axis to regulate thermogenic program in the VMH. These data suggest CPT1C as a downstream factor of hypothalamic AMPK to maintain energy homeostasis. Despite of these results, using a whole-body CPT1C KO mouse is a main limitation in our study, and therefore the importance of other hypothalamic nuclei (e.g. PVH or ARC) in these thermogenic responses could not be excluded. Further work, will be necessary to determine the specific neuronal VMH population mediating these effects. An interesting candidate could be SF1 neurons, as we have recently demonstrated that the specific ablation of AMPKα1 at these levels promotes a lean feeding-independent, but thermogenic-dependent phenotype that protects against HF-induced obesity [12,13].

Overall, the present investigation demonstrates that CPT1C in the VMH is necessary for the activation of BAT thermogenesis in response to central leptin and short-term HF diet administration. Also, we demonstrate that the role of CPT1C in adaptive thermogenesis is throughout the canonical pathway dependent on AMPK in the VMH. This study underscores the importance of CPT1C to provide metabolic adaptation during short-term consumption of fat-rich diets and during obesity development.

5 CONCLUSIONS

A better understanding of the neuronal pathways mediating short-term hypothalamic changes in response to nutritional status would provide valuable information about obesity development and progression. Therefore, identification of potential targets involved in these hypothalamic pathways to control aspects of energy balance beyond food intake, such as the BAT thermogenic activity, has gained relevance in the last few years.

The neuron-specific CPT1C, the most enigmatic CPT1 isoform, seems to play a key role in central regulation of energy homeostasis, mostly in terms of fuel selection and food preference during fasting or in response to ghrelin by AMPK-dependent mechanisms. The

present investigation reveals that mice lacking CPT1C show an impaired activation of BAT thermogenesis in response to short-term HF feeding and central leptin administration. In this phenotype, expression of CPT1C, by sensing malonyl-CoA, in the VMH is enough to restore diet-induced thermogenesis and counteract body weight gain. Considering the importance of the canonical pathway dependent on AMPK in the VMH to regulate BAT thermogenesis during the development of diet-induced obesity, our study also demonstrates for the first time that CPT1C is a crucial factor in this canonical pathway. The link between hypothalamic CPT1C and adaptive thermogenesis by the AMPK-brown fat axis could explain the obesogenic phenotype characteristic of CPT1C KO mice and also emphasize the role of CPT1C in the VMH to provide metabolic adaptation during short-term consumption of fat-rich diets. Altogether, this study underscores the importance of CPT1C in the development and progression of obesity and could add insight into the understanding of the mechanisms underlying diet-induced obesity.

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SUPPLEMENTARY INFORMATION

Supplementary Figures

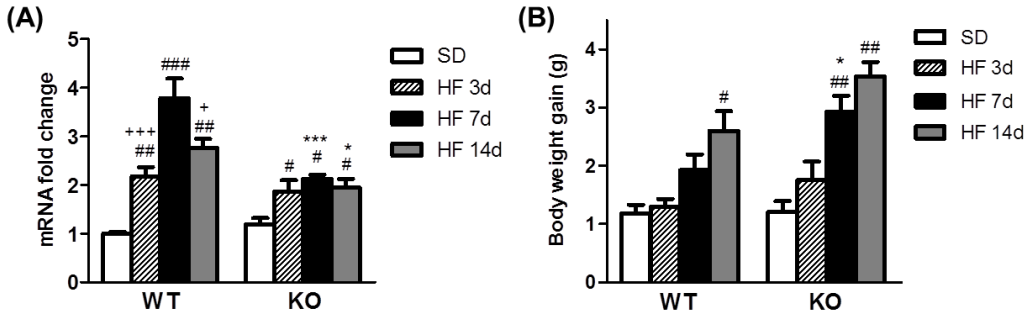

Figure S.1. Expression of mRNA levels of UCP1 in BAT (A) and body weight gain (B) of WT and CPT1C KO mice fed a standard diet (SD) or a high fat (HF) diet for 3, 7 or 14 days. Data are expressed as mean ± SEM (n=5-7). **P*<0.05, ****P*<0.001 *versus* WT with the same diet; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *versus* SD within the same genotype; +*P*<0.05, +++*P*<0.001 *versus* HF 7d within the same genotype.

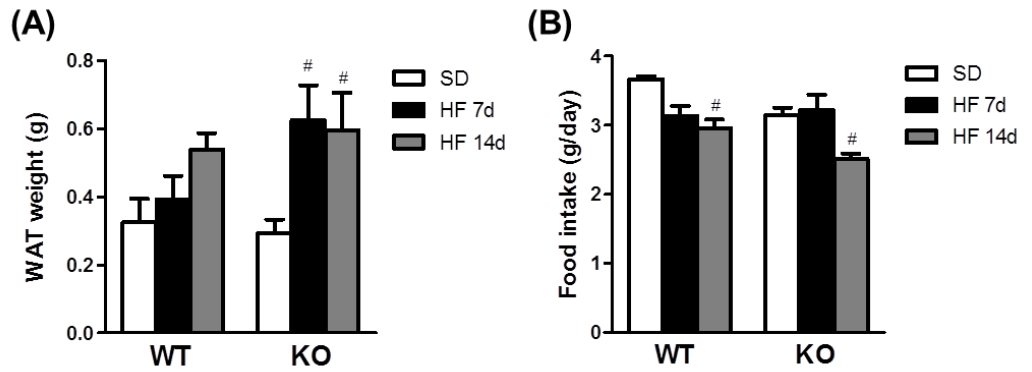

Figure S.2. Visceral WAT weight (A) and food intake (B) of WT and CPT1C KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 and 14 days. Data are expressed as mean ± SEM (n=5-8). #*P*<0.05 *versus* SD within the same genotype.

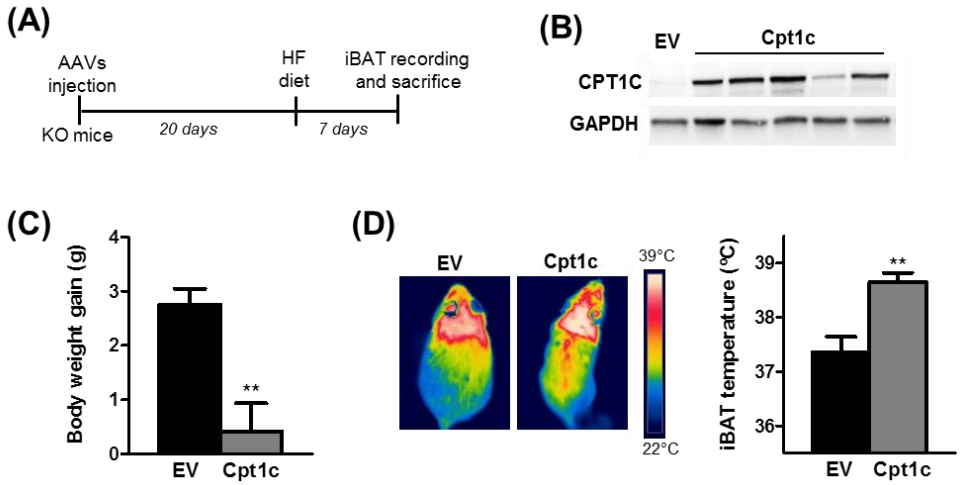

Figure S.3. Expression of CPT1C in the VMH restores short-term diet-induced body weight gain and iBAT temperature in CPT1C KO mice. (A) GFP (empty vector, *EV*) or CPT1C (*Cpt1c*) expressing adeno-associated viruses (AAVs) were microinjected in the VMH of CPT1C KO mice and after 20 days, mice were fed a high fat (HF) diet for 7 days. (B) Injection site was confirmed by CPT1C expression analysis by western blot in the hypothalamus. (C-D) Body weight gain (C), representative infrared thermal images and quantification of interescapular temperature adjacent to the BAT depot (D) of KO-*EV* and KO expressing CPT1C (KO-*Cpt1c*) fed HF diet for 7 days. Data are expressed as mean ± SEM (n=5). ***P*<0.01 *versus* KO-*EV*.

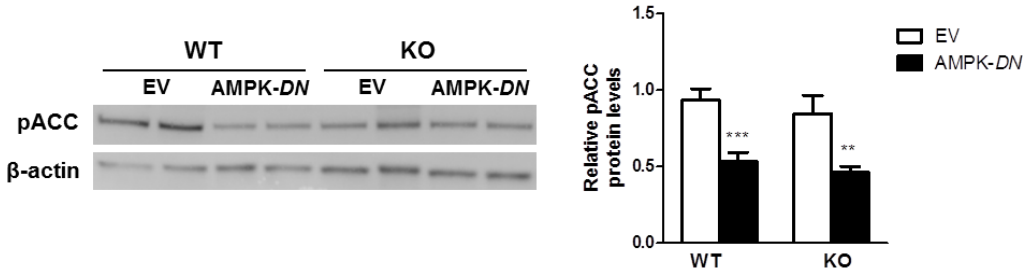

Figure S.4. Western blot representative images (left panel) and ventral hypothalamic protein levels of pACC (right panel) of WT and CPT1C KO mice stereotaxically treated with adenoviruses encoding GFP and AMPK-*DN* in the VMH. Data are expressed as mean ± SEM (n=5). ***P*<0.01, ****P*<0.001 *versus* EV within the same genotype.

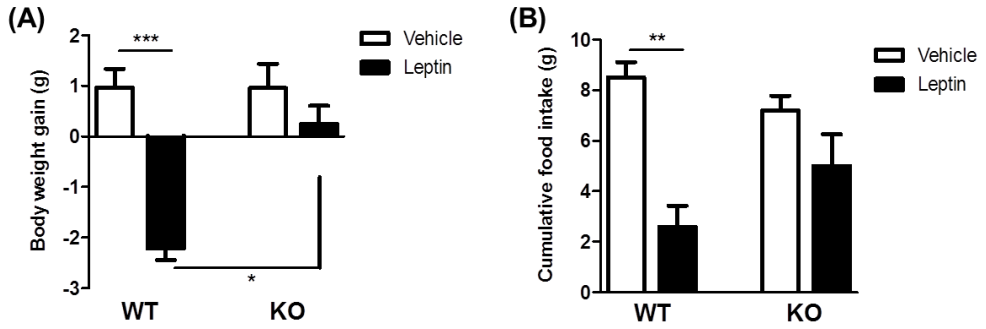

Figure S.5. Leptin sensitivity test. For leptin sensitivity test, mice were acclimatized by subjecting them to handling and sham injections. Ten-week-old WT and CPT1C KO mice were i.p. injected with either 1.5 mg/g of mouse leptin or vehicle twice a day (1 hr before lights out (7 p.m.) and at 8.00 a.m.) for 3 consecutive days, as previously described [1]. Food intake and body weight were recorded daily. Body weight gain (A) and cumulative food intake (B) of WT and CPT1C KO mice after 3 days leptin injection. Data are expressed as mean ± SEM (n=5). **P*<0.05, ***P*<0.01, ****P*<0.001.

Table S.I. Quantitative real-time PCR primers (SyBr or Taqman).

Gene (SyBr)	Sequence
Atf4	Fw: 5'-ATGATGGCTTGGCCAGTG Rv: 5'-CCATTTTCTCCAACATCCAATC
Chop	Fw: 5'-GGAGAAGGAGCAGGAGAATGA Rv: 5'-AGACAGACAGGAGGTGATGC
Gapdh	Fw: 5'-TCCACTTTGCCACTGCA Rv: 5'-GAGACGGCCGCATCTTCTT
Grp78	Fw: 5'-GCCTCATCGGACGCACTT Rv: 5'-AACCACCTTGAATGGCAAGAA
Pgc1α	Fw: 5'- GAAAGGGCCAAACAGAGAGA Rv: 5'- GTAAATCACACGGCGCTCTT
Prdm16	Fw: 5'-CCTAAGGTGTGCCCAGCA Rv: 5'-CACCTTCCGCTTTTCTACCC
Socs3	Fw: 5'- CCTTCAGCTCCAAAAGCGAG Rv: 5'- GCTCTCCTGCAGCTTGCG
ß-actin	Fw: 5'-CGCCACCAGTTCGCCATGGA Rv: 5'-TACAGCCCGGGGAGCATCGT
Gene (Taqman)	
Ucp1	Fw: 5'-CACACCTCCAGTCATTAAGCC Rv:5'-CAAATCAGCTTTGCCTCACTC Assay name: Mm.PT.58.7088262
Ucp3	Fw: 5'-GTCACCATCTCAGCACAGTT Rv: 5'-ATGCCTACAGAACCATCGC Assay name: Mm.PT.56a.9090376

Supplementary Methods

Stereotaxic injection of adeno-associated viruses in the VMH of CPT1C KO mice and evaluation of body weight gain and iBAT temperature in response to short-term HF diet feeding

Adeno-associated virus (AAV) encoding CPT1C (AAV-EIF1α-CPT1C) or the control virus (AAV-EIF1α-GFP), were generated at the Viral Vector production Unit of the Universitat Autonoma de Barcelona. Stereotaxic surgery was performed in CPT1C KO mice under ketamine/xylazine anesthesia. AAVs were bilaterally injected into the VMH over 10 min through a 33-gauge injector connected to a Hamilton Syringe and an infusion pump (0.5 µl per injection site), followed by 20 days post-surgery recovery. Then, KO mice were fed a HF diet during 7 days (**Fig. S.3**). During these days, body weight and food intake was daily recorded. At the end of the experiment, iBAT temperature was monitored using a high-resolution infrared camera (FLIR Systems) and analyzed with a specific software package (FLIR-Tools-Software, FLIR; Kent, UK).

Supplementary References

[1] Schneeberger, M., Dietrich, M.O., Sebastián, D., Imbernón, M., Castaño, C., Garcia, A., et al., 2013. Mitofusin 2 in POMC Neurons Connects ER Stress with Leptin Resistance and Energy Imbalance. Cell 155(1): 172–87, Doi: 10.1016/j.cell.2013.09.003.

Chapter II

Hypothalamic endocannabinoids inversely correlate with the development of diet-induced obesity in male and female mice. *Journal of Lipid Research,* 2019.

ARTICLE SUMMARY

In this chapter, we aimed to describe the exact time-profile of hypothalamic eCBs and their correlation with obesity in male and female mice.

A brief reminder that the ECS has been related to the control of energy homeostasis and it is known to be dysregulated in obese rodents and patients. Numerous studies have investigated the role of the CBR and the enzymatic eCBs machinery in obesity; however, scarce investigations have studied the dynamic of 2-AG and AEA in the hypothalamus during obesity development, overall at the onset of the pathology.

C57BL/6J male and female mice were fed with SD or HFD for 7, 14, 28, 60 and 90 days and the body weight, food intake and leptinemia were assessed. Hypothalamic and plasmatic 2-AG and AEA levels were measured by HPLC-MS/MS during obesity development and after acute peripheral activation of thermogenesis (**Experimental procedure, Figure 18**). BAT thermogenesis activation was measured after HFD feeding, central leptin and eCBs administration (**Experimental procedure, Figure 18**).

Results showed that 7 days of HFD induced a remarkable increase of hypothalamic eCBs that gradually decrease to basal levels or below to HFD exposure. The dynamic of hypothalamic eCBs were sexual dimorphic since female had higher eCBs at the beginning of HFD feeding. Moreover, hypothalamic eCBs profile positively correlates with BAT thermogenesis activation and inversely correlates with body weight, leptinemia and plasmatic eCBs. The peak of hypothalamic eCBs at 7 days of HFD was accompanied by an increased expression of 2-AG and AEA synthesis enzymes. Finally, administration of thermogenic activators, such as central leptin and peripheral b3-AR agonist, increased hypothalamic eCBs whereas direct injection of 2-AG and AEA did not induce BAT thermogenesis activation.

These results indicate that early stages of obesity development is characterized by an increase of hypothalamic eCBs levels probably triggered by peripheral thermogenesis activation (**Results summary, Figure 19**).

EXPERIMENTAL PROCEDURE

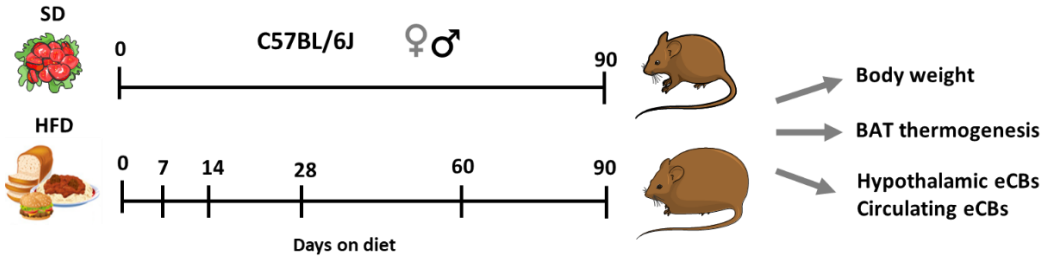

Figure 18. C57BL/6J mice were fed for 7, 24, 28, 60 and 90 days with SD or HFD and body weight, BAT thermogenesis and eCBs from the hypothalamus and plasma were monitored.

RESULTS SUMMARY

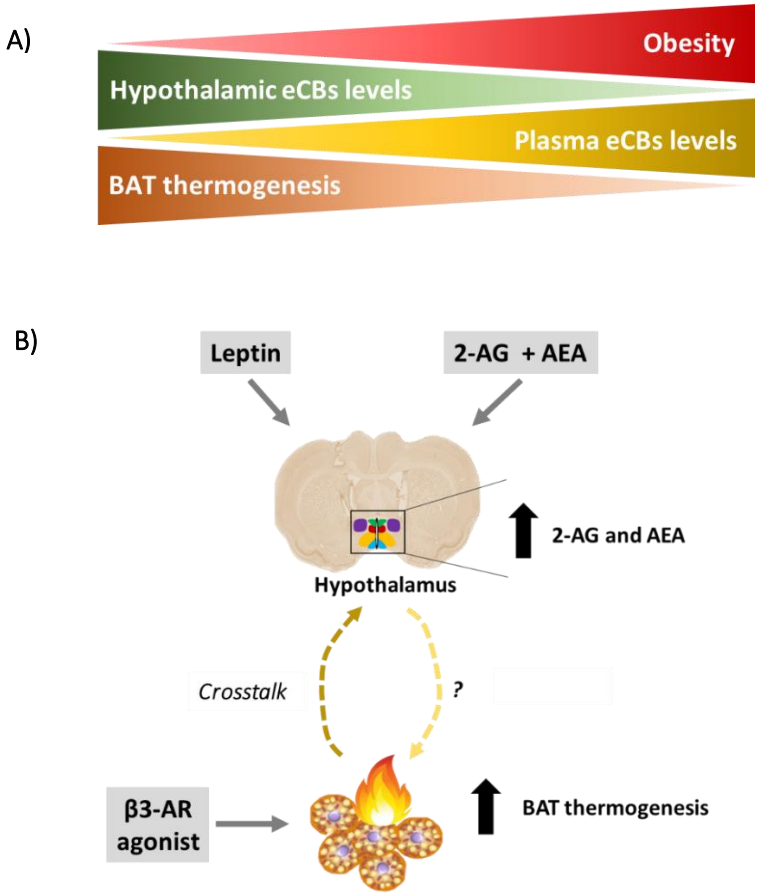

Figure 19. A) Obesity development positively correlates with plasma eCBs whereas inversely correlates with hypothalamic eCBs levels and BAT thermogenesis activation, being the correlation more evidence in female mice. B) Peripheral and central activation of thermogenesis is signalling hypothalamic ECS.

HYPOTHALAMIC ENDOCANNABINOIDS INVERSELY CORRELATE WITH THE DEVELOPMENT OF DIET-INDUCED OBESITY IN MALE AND FEMALE MICE

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ABSTRACT

The endocannabinoid (eCB) system regulates energy homeostasis and is linked to obesity development. However, the exact dynamic and regulation of eCBs in the hypothalamus during obesity progression remains incompletely described and understood. Our study shows the time-course of the hypothalamic eCBs 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (AEA) in male and female mice during diet-induced obesity, and its association to changes in brown adipose tissue (BAT) thermogenesis and body weight. High-fat diet (HFD) feeding induced a transient increase (with a substantial rise at 7 days) in hypothalamic eCBs followed by a progressive decrease to basal levels under longer HFD feeding. The eCB dynamic was sexually dimorphic: hypothalamic eCBs were higher in females and became obese at later time-points than male mice. The hypothalamic eCBs time-course positively correlated to thermogenesis activation whereas negatively matched to body weight, leptinemia and circulating eCBs. Increased expression of eCB synthetizing enzymes accompanied the transient hypothalamic eCB elevation. Intracerebroventricular injection of 2-AG+AEA did not promote BAT thermogenesis, while administration of thermogenic molecules such as central leptin or a peripheral β3-adrenoreceptor agonist induced a significant increase in hypothalamic eCBs, suggesting a directional link from BAT thermogenesis to hypothalamic eCBs. The transient rise in hypothalamic eCBs at early stages of obesity is understood as a physiological compensatory response to BAT thermogenesis activation triggered by diet surplus. This study could add insight into the understanding of hypothalamic regulation of obesity.

1 INTRODUCTION

Endocannabinoid (eCB) system is a highly conserved lipid-derived signaling system that plays a critical role in the control of energy homeostasis and body weight (1). The most well-known eCBs are 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide, AEA). eCBs are synthetized on demand in brain and peripheral tissues where they can act in autocrine or paracrine manner, or be secreted to bloodstream (2). In peripheral tissues such as liver, fat, pancreas and muscle, eCBs exert a wide range of metabolic effects including the modulation of food digestion, energy expenditure, lipid storage and glucose homeostasis (3, 4). The overall action of eCBs in the periphery favors energy intake and storage, promoting obesity development (1, 5). In humans, evidences demonstrate that circulating 2-AG and/or AEA levels are increased in people with obesity, and these levels specifically correlate with visceral fat mass, either in females or males patients (6, 7).

The eCB signaling is particularly critical in the brain, where it modulates neurotransmitter release and provides neuroprotection (8, 9). The eCB system is widely expressed in brain areas associated with the regulation of energy homeostasis, like the hypothalamus, the brain-steam and the cortico-limbic system (8). In the hypothalamus, 2-AG levels are increased in different genetic models of obesity: Zucker rats, *db*/*db* mice or *ob*/*ob* mice (10). In line with these evidences, specific deletion of the main eCB receptor (CB1) in the hypothalamus resulted in increased energy expenditure and brown adipose tissue (BAT) thermogenesis, leading to a reduction in body weight, while food intake remained unchanged (11). These studies, together with others on genetic animal models (12–14) suggest that the activation of the eCB system in the hypothalamus leads to reduced energy expenditure and promotes obesity (1).

Despite of these findings in genetic models, the exact dynamic of eCBs levels and their modulation in the hypothalamus during diet-induced obesity (DIO), the model that best resembles human obesity, has been poorly explored, and the scarce results in response to a high fat diet (HFD) are contradictory. For instance, hypothalamic 2-AG levels were increased in rats after long-term exposure to a HFD (24 weeks) (15), while they were not changed in another study performed with mice fed a HFD for 19 weeks (16). In both studies, AEA levels remained unchanged. Moreover, the time-course fluctuations and regulation of the eCB profile in the hypothalamus during obesity development remains incompletely described and understood.

In addition to this unsolved issue, recent evidences showed some differences in brain eCBs levels between male and female mice after long-term administration of a HFD (17), in line with the sexually dimorphic brain response in obesity (18–20). However, eCB dynamics in the hypothalamus between male and female animals during DIO development have not been explored.

In the present study, we have analyzed hypothalamic 2-AG and AEA levels in male and female mice at different stages of DIO development. Our results demonstrate that 2-AG and AEA levels transitorily increase in both genders with maximum levels at 7 days of HFD administration, followed by a gradual decline to levels similar to those observed in control groups. These changes positively correlate with BAT thermogenesis and inversely correlate with body weight gain. Acute activation of BAT thermogenesis under different stimuli also increased eCB levels in the hypothalamus, indicating early rises in hypothalamic eCBs as a compensatory response to the increased thermogenesis. This is the first study revealing the exact dynamic of hypothalamic 2-AG and AEA during diet-induced obesity development, and its potential link to BAT activation in male and female mice.

2 METHODS

2.1 Animals, diets and sample collection

Male and female C57BL/6J mice (8 week old) were used for the experiments. All animals were housed on a 12h/12h light/dark cycle in a temperature- and humidity-controlled room, and allowed free access to water and standard laboratory chow. Animals were placed on a HFD (60 % kcal from fat, D12492) or standard diet (SD) (10 % kcal from fat, D12450B, Research Diets, New Brunswick, NJ, USA) for 7, 14, 28, 60 or 90 days. Diets were administered in two different sets of animals: 1) mice fed SD or HFD for 7, 14 or 28 days; 2) mice fed SD or HFD for 60 or 90 days. At the end of the studies, animals were fasted for one hour, killed by cervical dislocation and tissues collected for further molecular and biochemical analysis. For each animal, the hypothalamus and interscapular BAT were quickly removed, weighed and stored at -80ºC. Plasma was obtained after blood centrifugation. Tissue processing and analysis from both sets of animals was simultaneously performed. All animal procedures were performed in agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Local Ethical Committee (Procedure ref. 9659, Generalitat de Catalunya).

2.2 Extraction and analysis of eCBs

Hypothalamic and plasma eCBs from both sets of animals were simultaneously extracted and analyzed as previously described by Gong et al. (21). Hypothalamus (6-8 mg wet tissue) was dounce-homogenized in 200 µL ice-cooled deionized water containing a final concentration of 0.362 µM of N-Oleylethanolamine-d2 (OEA-d2) (Cayman Chemicals, Ann Arbor, MI, USA) as internal standard for 2-AG and AEA calibration, 100 µM of PMSF and 0.01 % of BHT (Sigma-Aldrich, Madrid, Spain) followed by a brief sonication. After that, half of the homogenized sample was kept at -20ºC for protein quantification and the other half was mixed with 400 µL ethyl acetate/n-hexane (9:1, v/v) and vortexed for 5 min. After centrifugation (14,000 x g, 4ºC, 5 min), the upper layer was collected and evaporated using a nitrogen evaporator.

Plasma (25 µL) was mixed with 0.362 µM of OEA-d2, 100 µM of PMSF and 0.01 % of BHT per sample. Lipid extraction was made with 250 µL of ethyl acetate/n-hexane (9:1, v/v) following the same protocol as for hypothalamus extraction.

eCB levels were analyzed by LC-MS/MS, following the protocol described by Gong et al (21). Briefly, 2-AG, AEA and OEA-d2 (Cayman Chemicals, Ann Arbor, MI, USA) where used for the calibration curve in a Acquity ultra-high performance liquid chromatography (UPLC) (Waters, Singapore) system connected to a Xevo-TQS triple-quadropole Detector (Waters, Ireland) and controlled with Waters/Micromass MassLynx software. Chromatographic separation was performed on an Acquity UPLC BEH C_{18} column (1.7 µm particle size, 100 mm x 2.1 mm, Waters, Ireland) with an isocratic mobile phase of formic acid 0.1% in water-acetonitrile (30:70, v/v). The flow rate was 0.3 mL/min. Detection was performed with an electrospray interface operating in the positive ion mode. The capillary voltage was set to 3.1 kV, the source temperature was 150 °C and the desolvation temperature was 500°C, acquiring the following selected reaction monitoring transitions: OAE-d2, 328.2–62.2 Da, cone voltage 50V, collision energy 10 eV; AEA, 348.2–62.2 Da, cone voltage 50V, collision energy 10 eV; and 2-AG 379.2–287.1 Da, cone voltage 50V, collision energy 10 eV. eCB levels from each experimental group were normalized to its corresponding control group (SD).

2.3 Analysis of plasma leptin levels

Plasma levels of leptin were determined by mouse ELISA Kit (Crystal Chem, Zaandam, Netherlands), following the manufacturer's instructions.

2.4 RNA preparation and quantitative RT-PCR

Total RNA was extracted from tissues using Trizol Reagent (Fisher Scientific, Madrid, Spain). Retrotranscription and quantitative RT-PCR (qPCR) was performed as previously described (22). SYBR Green or Taqman Gene Expression assay primers used (IDT DNA Technologies, Leuven, Belgium) (Table S.1). Relative mRNA levels were measured using the CFX96 Real-time System, C1000 Thermal Cycler (BioRad, Madrid, Spain).

2.5 BAT temperature measurements

Skin temperature surrounding BAT was visualized using a high-resolution infrared camera (FLIR Systems) and analyzed with a specific software package (FLIR-Tools-Software, FLIR; Kent, UK), as described (23, 24). Thermal images were acquired the day of sacrifice.

2.6 Intracerebroventricular (ICV) administration of leptin and 2-AG + AEA combination

Two different experiments were performed: i) ICV leptin administration followed by evaluation of BAT thermogenesis and hypothalamic eCBs levels; ii) ICV administration of a mixture of the eCBs, 2-AG + AEA in different dosages to evaluate BAT thermogenesis. For both experiments, cannulae were stereotaxically implanted into the lateral cerebral ventricle under ketamine/xylazine intraperitoneal anesthesia, as previously described (24). Mice were individually caged and allowed to recover for 5 days before the experiment.

For leptin injection experiment: On experimental day, lean male mice received an ICV administration of 2 µl of either vehicle (aqueous buffer containing 0.1 % BSA) or leptin (0.1 µg/µl) (PeproTech, London, UK), three hours after lights-on (24). 4 hours after the injection, mice were sacrificed by cervical dislocation and hypothalamus and BAT were collected for further analysis.

For endocannabinoids injection experiment: On experimental day, lean male mice received an ICV administration of 2 µl of either vehicle (saline buffer containing 5 % DMSO) or the eCBs combination, 2-AG + AEA (Chayman Chemical, Ann Arbor, MI, USA), in two different dosages: Dose 1 (0.5 µg 2-AG + 0.005 µg AEA) and Dose 2 (2 µg 2-AG + 0.02 µg AEA). The doses of 2-AG and AEA were selected based on previous publications using ICV or intrahypothalamic administration of these eCBs separately (25, 26), and also considering the different range of concentration found in the hypothalamic region under standard conditions (x50-100 higher concentrations of 2-AG compared to AEA).

2.7 β3-adrenergic agonist-induced thermogenesis activation

Hypothalamic eCB levels were determined after adrenergic stimulation of BAT thermogenesis with the selective β3-adrenergic agonist CL 316243 (Tocris Bioscience, Bristol, UK), as previously described (27, 28). 4 hours after intraperitoneal injection of either CL 316243 (10 mg/kg) or vehicle (aqueous buffer) (27), mice were sacrificed by cervical dislocation and hypothalamus and BAT were collected for further analysis.

2.8 Statistical analysis

All results are expressed as mean ± SEM (n=8-12). Analysis was conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Statistical analysis was determined by One-Way ANOVA when different diets groups within the same gender where compared and Two-Way ANOVA when groups between different genders where compared. In both cases, ANOVA was followed by post hoc two-tailed Bonferroni test. For the analysis of relation between variables, parameters were mathematically Log transformed to improve symmetry and correlation was analyzed by Pearson's test and lineal regression. $P < 0.05$ was considered significant.

3 RESULTS

3.1 Progression of diet-induced obesity was delayed in female compared to male mice

Age-matched male and female mice were fed a SD or a HFD for 90 days. During this period, progression of body weight gain, plasmatic levels of leptin, food and caloric intake were evaluated (**Fig. 1, Fig. S.1, Fig. S.2**). Males fed a HFD for a period of administration equal or longer than 28 days gained significantly more weight than controls (**Fig. 1A, Fig. S.1**). In female mice, a significant increase in body weight gain was not appreciated until 60 days of administration of a HFD compared to SD (**Fig. 1A, Fig. S.1**). In line with these results, plasmatic levels of leptin were significantly increased in male mice after 28 days of HFD feeding compared to control diet, whereas female mice did not show hyperleptinemia until 60 days of HFD administration, and leptin levels were considerably lower at this point than those observed in male mice (**Fig. 1B**). These data show that male mice became obese and hyperleptinemic at earlier time points of HFD feeding than female and the final body weights remained higher in comparison to female mice (**Fig. 1A and 1B, Fig. S.1**).

As expected, total caloric intake in male and female mice was increased when the animals were fed a HFD, whereas food intake was decreased in HFD animals of both sexes

compared to SD (Fig. S.2). In addition, both caloric and food intake was significantly lower in female mice fed SD or HFD in comparison to diet-matched male animals (Fig. S.2).

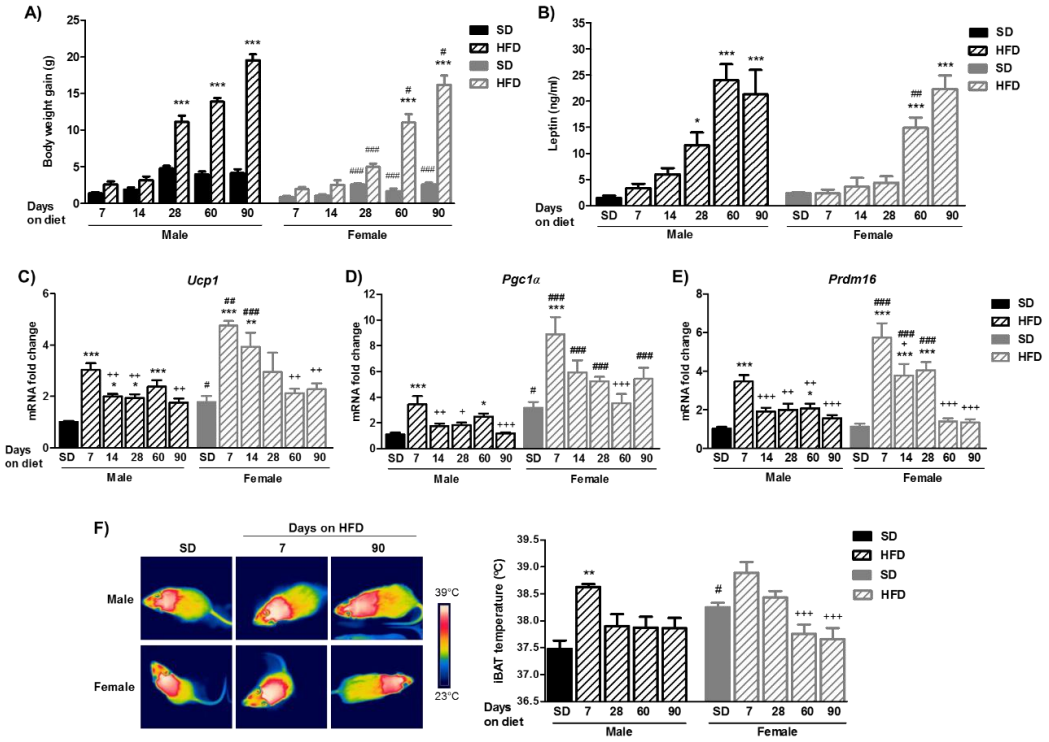

Figure. 1. Diet-induced obesity development of male and female mice fed a standard diet (SD) or a high fat diet (HFD) for 90 days. (A) Body weight gain, (B) plasmatic levels of leptin, (C-E) relative mRNA expression of the thermogenic markers UCP1 (C), PGC1α (D) and PRDM16 (E) in BAT of male and female mice fed SD or HFD for 90 days (n=8-10). (F) Representative infrared thermal images and quantification of interscapular temperature adjacent to the BAT depot of male and female mice fed SD or HFD for 7, 28, 60 and 90 days (n=5). Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *versus* its corresponding SD; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *versus* male under the same diet conditions; ^{+}P < 0.05, ^{++}P < 0.01, ^{+++}P < 0.001 *versus* same gender fed HFD for 7 days.

3.2 Short-term administration of a HFD induced a transient increase of both thermogenesis activation and hypothalamic eCB levels in male and female mice

The induction of thermogenesis in the interscapular BAT of male and female mice was analyzed at different time points during 90 days of HFD feeding, compared to SD (**Fig. 1C-F**). A substantial activation peak was reached in gene expression of thermogenic markers (**Fig. 1C-E**) and interscapular temperature (**Fig. 1F**) in the BAT of male and female mice after 7-14 days of HFD when compared to SD. Longer administration periods of HFD (from 28 days onwards) were not able to induce such a considerable activation of BAT thermogenesis (**Fig. 1C-F**). The activation of gene expression of thermogenic markers was higher in BAT of female compared to male mice, particularly after 7, 14 and 28 days of HFD (**Fig. 1C-E**). In addition, an increased basal expression of specific thermogenic genes (**Fig. 1C and D**) and basal interscapular BAT temperature (**Fig. 1F**) was appreciated in female mice in comparison to male mice, as described previously in the literature (29, 30).

Analysis of hypothalamic eCB levels also revealed a pronounced transitory increase after short-term administration of HFD (**Fig. 2**). Hypothalamic 2-AG levels were significantly increased after 7 days HFD feeding in both male and female mice, and these values were progressively attenuated reaching basal levels in a time-dependent manner (**Fig. 2A**). AEA levels in the hypothalamus of male and female mice remained elevated during 7 to 28 days of HFD feeding whereas they were significantly reduced after longer exposure to HFD (**Fig. 2B**). Interestingly, transitory hypothalamic increases in 2-AG and AEA were substantially higher in female than in male mice, and decreases were more pronounced in female than in male mice (**Fig. 2**). Hypothalamic concentrations of 2-AG and AEA (ng eCB / mg tissue) derived from the two different sets of animals during dietary administration are shown on supplementary information (**Table S.2**).

In summary, short-term exposure to a HFD induced a transitory activation of BAT thermogenesis and a transient increase in hypothalamic 2-AG and AEA in mice, and these responses were sexually dimorphic (**Fig. 1 and 2**).

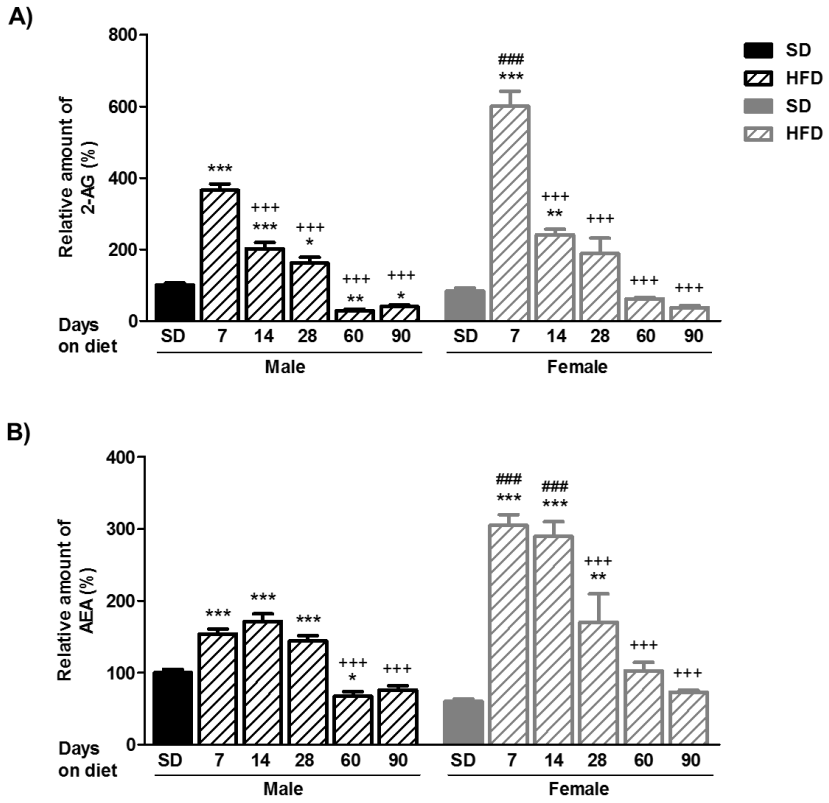

Figure. 2. Time-profile of hypothalamic endocannabinoid levels during the development of diet-induced obesity in male and female mice. (A) Relative amount of 2-arachidonoylgycerol (2-AG) to male SD. (B) Relative amount of anandamide (AEA) to male SD. Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM (n=8-10). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* its corresponding SD; $^{\#\#\#}P < 0.001$ *versus* male under the same diet conditions; $^{++}P < 0.01$, $^{+++}P < 0.001$ *versus* same gender fed HFD for 7 days.

3.3 Hypothalamic eCBS levels correlated with body weight gain, leptinemia and brown fat thermogenesis

Firstly, we analyzed the relationship between hypothalamic eCB levels and body weight gain or plasma leptin levels in the animals fed the experimental diets. 2-AG and AEA levels in the hypothalamus showed a negative correlation with both body weight gain (**Fig. 3A-D**) and leptinemia (**Fig. S.3**) in male and female mice. Then, evaluation of the relationship between eCBs in the hypothalamus and the mRNA expression levels of BAT thermogenesis activation revealed that hypothalamic 2-AG and AEA levels positively correlated with mRNA levels of Ucp1, Pgc1α and Prdm16 in BAT of female (**Fig. 3F and H; Fig. S.4**). In male mice, this correlation was only significantly appreciated when analyzing 2-AG levels (**Fig. 3E and G; Fig. S.4**).

These results indicate a negative association between eCB levels in the hypothalamus and obesity progression in male and female mice, but a positive association of hypothalamic eCBs with thermogenic activation in response to HFD, particularly evidenced in female mice.

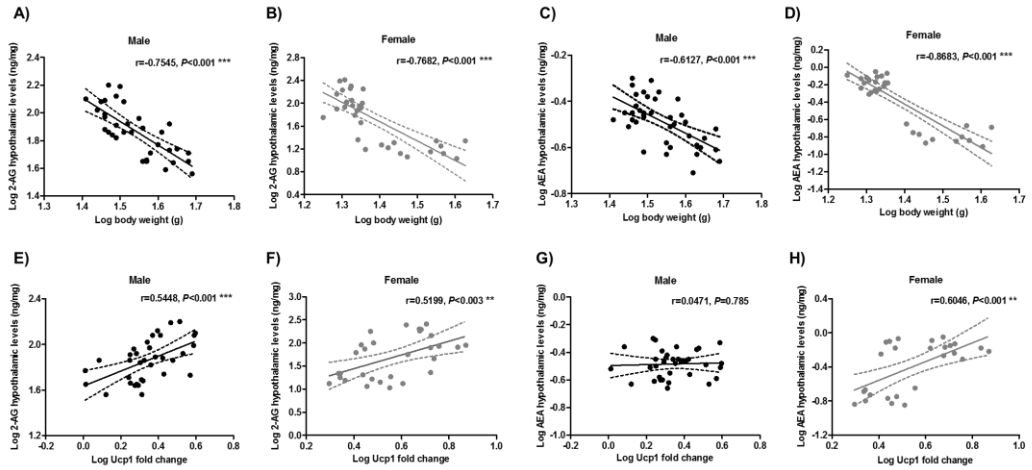

Figure. 3. Correlation between hypothalamic endocannabinoids and body weight or UCP1 mRNA expression in BAT. (A-B) Hypothalamic 2-AG levels negatively correlates with body weight at time of sacrifice in both male (A) and female (B) mice. (C-D) Hypothalamic AEA levels negatively correlates with body weight at time of sacrifice in both male (C) and female (D) mice. (E-F) Hypothalamic 2-AG levels positively correlate with UCP1 mRNA expression in BAT of male (E) and female mice (F). (G-H) Hypothalamic AEA levels do not correlate with UCP1 mRNA expression in BAT of male mice (G) but they positively correlate in BAT of female mice (H). Statistical significance and correlation was determined by Pearson correlation coefficients (XY values=30-40).

3.4 Plasmatic eCBs increased after longer administration periods of a HFD and showed a negative correlation to hypothalamic eCB levels

In contrast to the hypothalamus, plasmatic levels of 2-AG and AEA remained unchanged after short-term administration of HFD compared to SD in both male and female mice (**Fig. 4A and 4B**). However, exposure to HFD for 60 or 90 days revealed an increase in plasmatic 2-AG in both male and female mice (**Fig. 4A**), and a substantial increase in plasmatic AEA, particularly observed in female mice (**Fig. 4B**). In addition, 2-AG and AEA levels in plasma evidenced a positive correlation with body weight gain in both male and female mice (**Fig. 4C-F**). Analysis of the relationship between plasmatic and hypothalamic eCB levels revealed that plasmatic eCBs negatively correlated with those levels in the hypothalamus of male and female mice fed a HFD (**Fig. S.5**). Plasmatic concentrations of 2-AG and AEA (ng eCB/mL plasma) derived from the two different sets of animals during dietary administration are shown on supplementary information (**Table S.3**).

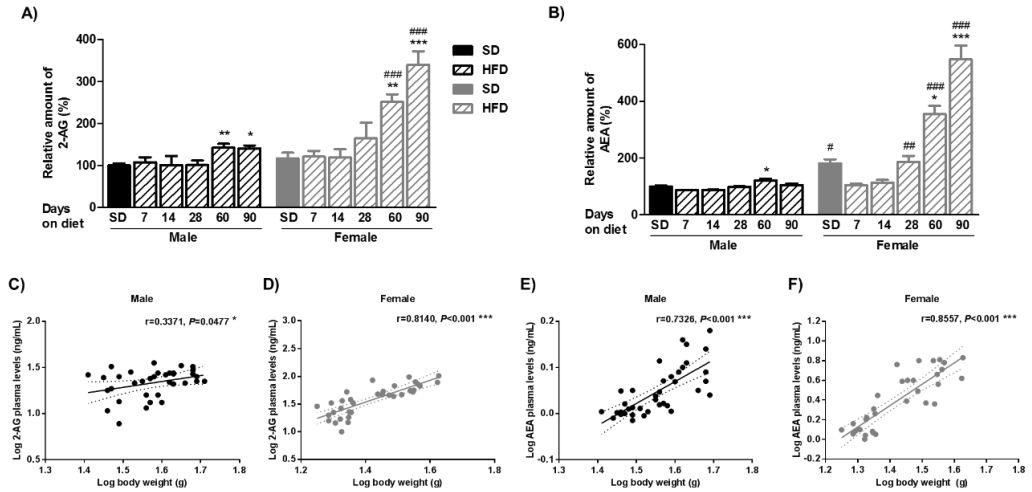

Figure. 4. Time-profile of plasmatic endocannabinoids levels during the development of diet-induced obesity and correlation to body weight in male and female mice. (A) Relative amount of 2-arachidonoylgycerol (2-AG) compared to values found in male SD. (B) Relative amount of anandamide (AEA) compared to values found in male SD. (C-D) Plasmatic 2-AG levels positively correlate with body weight at time of sacrifice in both male (C) and female (D) mice. (E-F) Plasmatic AEA levels positively correlate with body weight at time of sacrifice in both male (E) and female (F) mice. Statistical significance was determined by ANOVA and Bonferroni post-test and correlation was determined by Pearson correlation coefficients (XY values=33-39). Error bars represent SEM (n=8-10). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* its corresponding SD; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* male under the same diet conditions.

3.5 Short-term HFD feeding increased gene expression of eCB synthesis enzymes in the hypothalamus

We analyzed gene expression of the enzymes responsible for the synthesis (*Daglα*, *Daglβ* and *Nape*) and degradation (*Mgll*, *Abhd6* and *Faah*) of 2-AG and AEA in hypothalamus of mice fed SD or HFD for 7 days. This analysis revealed a significant increase in the expression of the hypothalamic enzymes synthetizing 2-AG and AEA after short-term HFD administration, whereas this increase was not evidenced in the expression of degradation enzymes (Fig. 5). Overall, this result shows that the transient increase in hypothalamic 2-AG and AEA over 7 days HFD feeding concurs with an enhanced expression of synthesis enzymes in the hypothalamus.

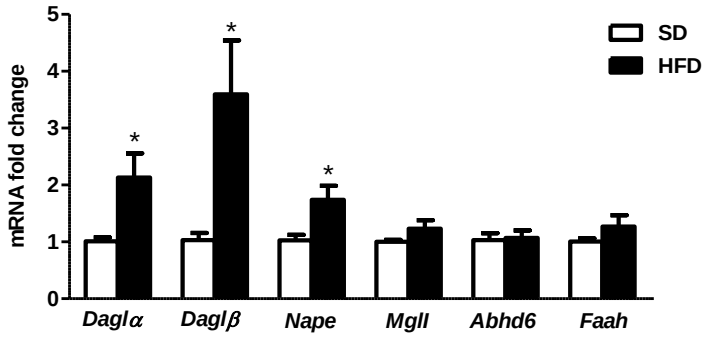

Figure. 5. Relative mRNA expression of 2-AG and AEA synthesis and degradation enzymes in hypothalamus of male mice fed standard diet (SD) or high fat diet (HFD) for 7 days. Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM (n=5-8). **P* < 0.05 *versus* SD.

3.6 Acute leptin and a β3-adrenergic agonist both induced a thermogenic response with an increase in hypothalamic eCB levels

We decided to measure the levels of hypothalamic eCBs in another conditions well-known to activate BAT thermogenesis, such as central leptin administration and intraperitoneal administration of a β3-adrenergic agonist. In agreement with our previous results (24), 4h-leptin ICV increased gene expression of thermogenic markers in BAT (Fig. 6A). Under these experimental conditions, hypothalamus of the same animals evidenced a significant increase in both 2-AG and AEA concentrations (Fig. 6B).

To compare the effects of central leptin *versus* peripheral adrenergic activation of BAT, mice received intraperitoneal injection of the potent and selective β3-adrenergic agonist CL 316243, an agonist that has demonstrated minimal access to the brain after peripheral

injection (31). In line with leptin experiment, CL 316243 induced adrenergic activation of BAT thermogenesis marker genes (Fig. 6C) with a substantial increase in both 2-AG and AEA in the hypothalamus (Fig. 6D), revealing an association between hypothalamic eCBs and thermogenesis activation.

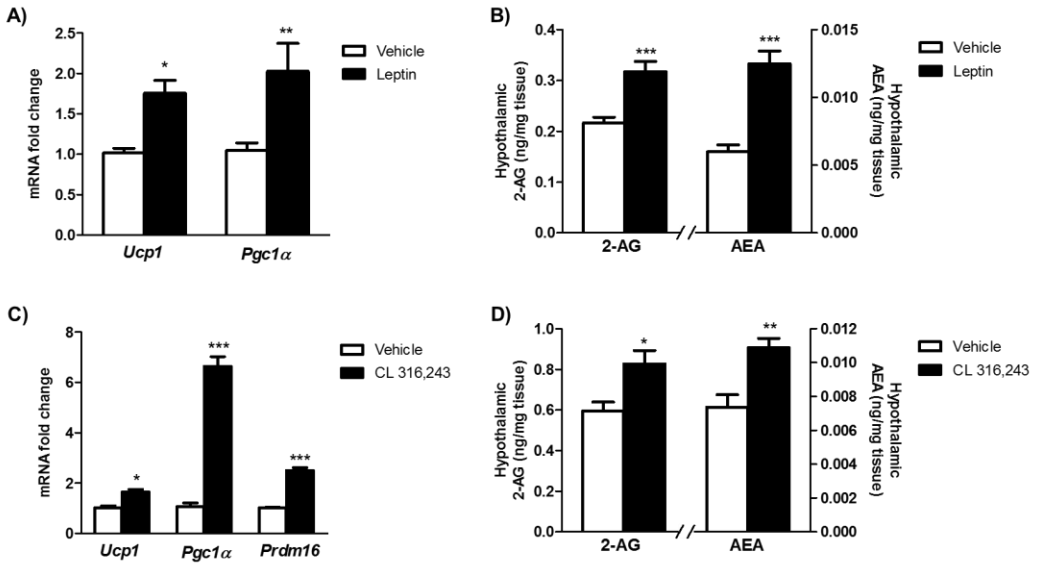

Figure. 6. Hypothalamic endocannabinoid levels and BAT thermogenesis in response to acute ICV leptin (A-B) or to intraperitoneal (i.p.) injection of the β3-adrenoreceptor agonist, CL 316,243 (C-D). Relative mRNA expression of the thermogenic markers in BAT samples after 4h of ICV leptin (A) or i.p. CL 316,243 administration (C). Concentration of 2-arachidonoylgycerol (2-AG) and anandamide (AEA) in the hypothalamus of mice after 4h of ICV leptin (B) or i.p. CL 316,243 administration (D). Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM (n=8-12). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *versus* vehicle.

3.7 Acute central administration of 2-AG + AEA did not induce BAT thermogenesis

ICV administration of the mixture of the eCBs, 2-AG + AEA, in two different doses for 4 hours, were not able to induce a significant alteration of either interscapular BAT temperature (**Fig. 7A**) or gene expression levels of thermogenic markers in BAT (**Fig. 7B**). Food intake was not significantly altered in response to ICV injection of the doses of these eCBs (*data not shown*), in agreement with a previous publication (25).

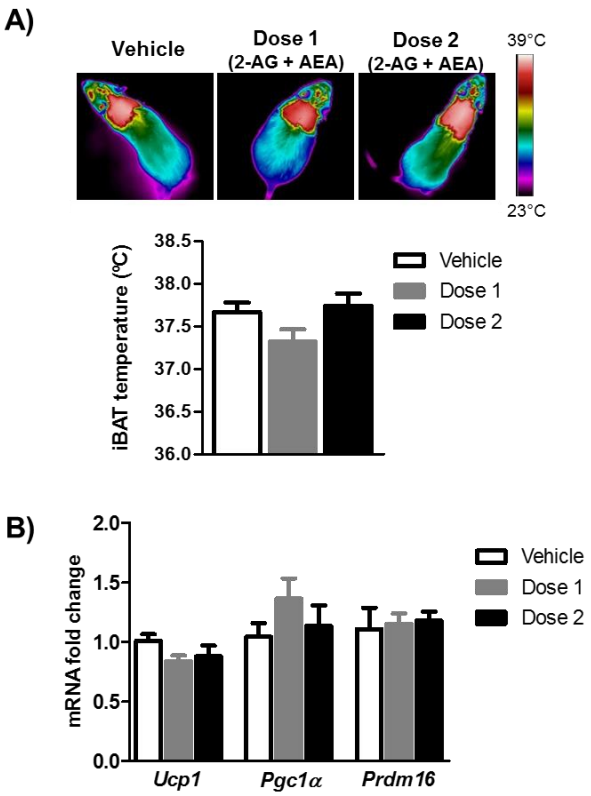

Figure. 7. BAT thermogenesis in response to acute ICV injection of the endocannabinoids 2-AG and AEA in two different dosages: Dose 1 (0.5 µg 2-AG + 0.005 µg AEA) and Dose 2 (2 µg 2-AG + 0.02 µg AEA). (A) Representative infrared thermal images and the corresponding quantification of interscapular temperature adjacent to the BAT depot (iBAT), and (B) relative mRNA expression of the thermogenic marker UCP1 in BAT samples of mice after 4h of ICV injection of 2-AG + AEA in two different dosages. Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM (n=8-10).

4 DISCUSSION

Despite the well-established function of the eCB system on energy homeostasis, our knowledge on their exact dynamic and regulation under dietary conditions leading to obesity and associated complications are still limited. Our current study presents for the first time the temporal profile of hypothalamic eCBs changes during the development of DIO and its association with BAT thermogenesis activation and leptin response in male and female mice. This is an intriguing finding, considering the very few and contradictory evidences that exist in the literature on hypothalamic eCBs in response to a HFD, its relation to BAT activation and the potential contribution of this association to sexual dimorphism in obesity.

One of the most remarkable results in our study was the transitory and substantial increase on hypothalamic eCBs after short-term administration of a HFD in both male and female, while these levels were progressively attenuated under long-term exposure to this diet. The transient increase was particularly pronounced on 2-AG levels after 7 days of HFD feeding (4-6 times higher than the basal levels). At this time-point, the early rise on eCB levels was sustained by a significant increase in the hypothalamic expression of the enzymes responsible for the synthesis of 2-AG and AEA, without significant alterations in degrading enzymes expression.

It has been reported that eCB levels become deregulated in the hypothalamus during obesity (8, 10). Di Marzo et al. (10) were the first detecting elevated levels of 2-AG in genetically obese Zucker rats and *ob*/*ob* mice, and of both 2-AG and AEA in the hypothalamus of *db*/*db* mice, compared to lean controls. Since those genetic models are either leptin-deficient or express mutated forms of the leptin receptor, the increased eCB levels in the hypothalamus were suggested as an additional component of leptin-sensitive regulatory mechanisms (10). Studies on DIO animals, the model that best resembles human obesity, are scarce and controverted in terms of hypothalamic eCB changes. Moreover, some of these investigations are limited to their analysis after long-term HFD administration (19 weeks onwards) and thereby when obesity has been already established (15, 16). Our current data show that hypothalamic 2-AG and AEA levels are not increased when DIO is established (at 60 or 90 days of HFD), but they are at earlier stages (7-28 days) of high-fat feeding, in contrast to that observed in plasma. These data indicate that the eCBs dynamic in the hypothalamus is different in genetic animal models of obesity compared to diet-induced obese models. Furthermore, this is the first time that a negative

association between hypothalamic eCBs and body weight during DIO development is demonstrated.

Our group has recently reported that the robust activation peak of BAT thermogenesis observed under 7 days of HFD feeding matches with a transient preservation of normal body weight, adiposity and leptinemia in the initial phases of DIO (24). The fact that the transitory increase in hypothalamic eCBs after short-term HFD administration found in the present investigation correlated with the activation peak of BAT thermogenesis, lead us to propose this early rise on hypothalamic eCBs as a physiological compensatory response (found in the hypothalamus, but not in plasma) to BAT thermogenesis activation triggered by diet surplus. These data also suggest the existence of a crosstalk between BAT and hypothalamic eCBs in the initial stages of obesity.

The link between the increase in hypothalamic 2-AG and AEA levels and BAT thermogenesis activation was further evaluated by measuring hypothalamic eCBs following acute thermogenic activation by different stimuli. Acute administration of either central leptin or intraperitoneal injection of CL 316,243, a selective and peripherally restricted β3-adrenergic agonist, induced BAT thermogenesis activation with a substantial increase in hypothalamic eCB levels. This is the first study revealing that acute stimulation of BAT, enhances eCB levels in the hypothalamus. In relation to these findings, it was recently demonstrated that *in vivo* stimulation of β3-adrenoreceptors in brown adipocytes by CL 316,243 (acutely or chronically injected), elevated the levels of the eCBs, 2-AG and AEA, in BAT and were suggested to act as local autocrine negative feedback regulators (28). Our assumption that the hypothalamic eCB elevation is a compensatory response, and not a cause, to BAT thermogenesis activation was also supported by the absence of stimulation of thermogenesis under ICV administration of different doses of the combination of 2-AG + AEA. The lack of BAT activation by central eCBs injection, at least with the selected dosages, suggests the unidirectional link from BAT to hypothalamic eCBs. However, further research is needed to unravel the molecular mechanisms by which BAT is possibly signaling the hypothalamic eCB system, particularly in early stages of DIO development.

Therefore, the present investigation evidences that, in early stages of obesity, when BAT thermogenesis is more active, there is a higher increase in hypothalamic eCBs, as a physiological compensatory response to BAT activation. However, when diet-induced obesity is already established, the decline of thermogenesis activation is accompanied by

a decrease in hypothalamic eCB levels. Further investigation will be needed to study the specific role of hypothalamic eCBs in response to BAT activation.

Interestingly, we can find in the literature other examples of short-term changes in the hypothalamus in response to nutritional surplus suggested to be compensatory mechanisms in obesity (32–34). These changes have also been described as processes preceding insulin/leptin sensitivity disruption and inflammation in peripheral tissues and therefore promoting positive energy balance (35, 36). Therefore, we might also understand the transitory increase in hypothalamic eCBs as an early indicator to precede leptin resistance and peripheral obesity.

Despite the discrepancy on central eCBs during DIO, the dysregulation of circulating eCBS levels in metabolic diseases have been widely investigated (1, 3, 37). To date, these data are more conclusive and the results on plasmatic eCBs in rodents are similar to those observed in humans (1, 38–41). Circulating eCBs positively correlate with markers of obesity and metabolic disorders, such as body mass index, waist circumference, visceral fat mass and insulin resistance (6, 7, 37, 40, 42, 43). Our data on circulating eCBs agree with that previously reported, that is, plasmatic 2-AG and AEA levels were increased, particularly in female mice, after long-term exposure to a HFD, but not at early stages of DIO. Interestingly, these results negatively correlated with those concentrations in the hypothalamus. In line with these evidences, Kuipers and colleagues have recently demonstrated that there is a link between eCBs metabolism in adipose tissues and plasmatic eCBs during DIO development (44). They showed that long-term HFD feeding increases circulating eCBs accompanied by increased synthesis enzymes in adipose tissue (particularly BAT) of DIO mice (44). The authors also suggest that adipose tissues are likely important organs that release 2-AG and AEA levels in HFD-induced obesity (44).

An important finding in the current investigation was the difference on eCB levels observed depending on the gender. Relevant differences were appreciated between male and female mice, particularly when analyzing the transient increase of hypothalamic eCB and the peak of activation of thermogenesis after short-term HFD, which were substantially higher in females. In addition, obesity progression was delayed and less severe in female, suggesting a sexual dimorphism in hypothalamic eCB system that could determine obesity progression. The relationship of central eCB system and sexual dimorphism in obesity has been poorly explored (17). Recent findings on hypothalamic dimorphism in fatty acid concentration, chain length and saturation, in response to HFD feeding were associated to

protection to obesity and cardiovascular diseases in female compared to male mice (18, 20). Our data are the first comparing the dynamic of hypothalamic eCBs between male and female mice during diet-induced obesity development. These results are therefore contributing to elucidate the relevance of central lipid metabolism in the sexual dimorphism in obesity.

Overall, this is the first study revealing the exact dynamic of hypothalamic eCBs during the development of obesity in DIO models, and these temporal changes correlated positively to BAT thermogenesis, and negatively to circulating eCB, leptin and body weight. Our data evidence a transitory elevation in hypothalamic eCBs after short-term HFD feeding accompanied by increased expression of 2-AG and AEA synthesis enzymes, understood as a physiological compensatory response to BAT thermogenesis activation triggered by diet surplus. The link between hypothalamic eCBs and BAT thermogenesis activation was also supported by a substantial upregulation of eCB in the hypothalamus following acute thermogenic activation by central leptin or peripheral β3-adrenergic stimulation. Our findings could add significant insight into the understanding of the hypothalamic mechanisms regulating obesity progression and its relationship to BAT function.

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SUPPLEMENTARY INFORMATION

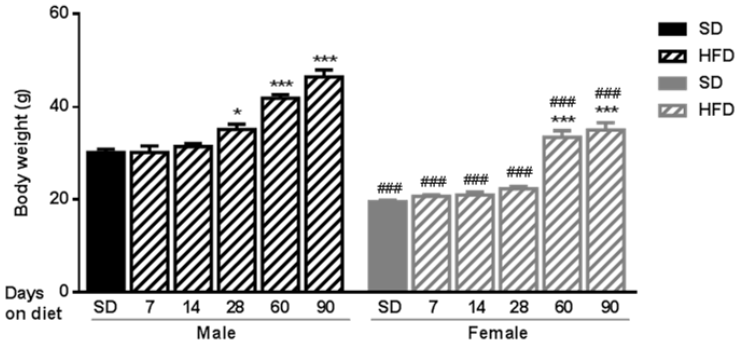

Figure. S.1. Body weight at time of sacrifice. Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM (n=8-10). **P*<0.05, ****P*<0.001 *versus* its corresponding SD; ###*P* <0.001 *versus* male under the same diet conditions.

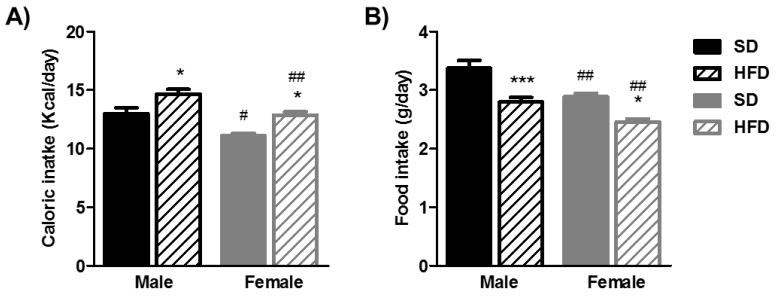

Figure. S.2. Total caloric (A) and food intake (B) of male and female mice fed a standard diet (SD) or a high fat diet (HFD) for 90 days. Statistical significance was determined by ANOVA and Bonferroni post-test. Error bars represent SEM (n=8-10). **P* < 0.05, ****P* < 0.001 versus its corresponding SD; #*P* < 0.05, ##*P* < 0.01 versus male under the same diet conditions.

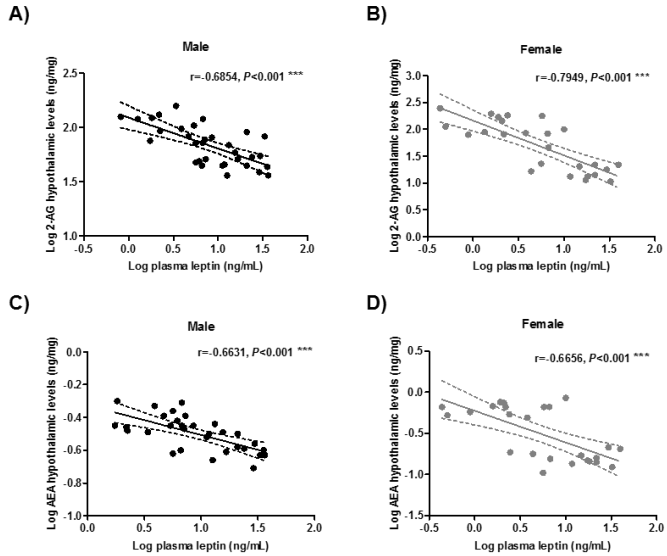

Figure. S.3. Correlation between hypothalamic endocannabinoids and plasmatic leptin levels. (A-B) Hypothalamic 2-AG levels negatively correlates with leptinemia in both male (A) and female (B) mice. (C-D) Hypothalamic AEA levels negatively correlates with leptinemia in both male (C) and female (D) mice. Statistical significance and correlation was determined by Pearson correlation coefficients (XY values=25-33).

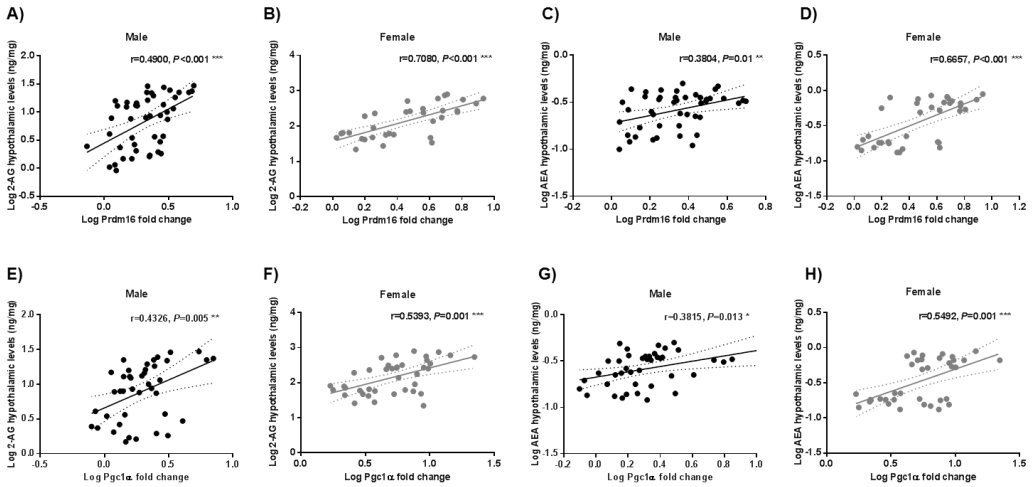

Figure. S.4. Correlation between hypothalamic endocannabinoids and Prdm16 or Pgc1α mRNA expression in BAT. (A-B) Hypothalamic 2-AG levels positively correlate with Prdm16 mRNA expression in BAT in both male (A) and female (B) mice. (C-D) Hypothalamic AEA levels positively correlate with Prdm16 mRNA expression in BAT in both male (C) and female (D) mice. (E-F) Hypothalamic 2-AG levels correlate with Pgc1α mRNA expression in BAT in both male (E) and female mice (F). (G-H) Hypothalamic AEA levels correlate with Pgc1α mRNA expression in BAT in both male (G) and female mice (H). Statistical significance and correlation was determined by Pearson correlation coefficient (XY values=30-36).

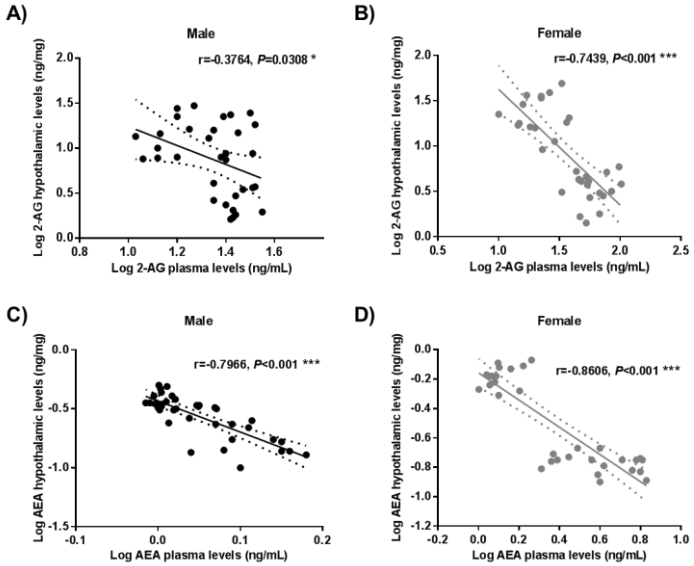

Figure. S.5. Correlation between hypothalamic and plasmatic endocannabinoids in male and female mice. (A-B) Hypothalamic 2-AG levels negatively correlate with plasmatic 2-AG levels in both male (A) and female (B) mice. (C-D) Hypothalamic AEA levels negatively correlate with plasmatic AEA levels in both male (C) and female (D) mice. Statistical significance and correlation was determined by Pearson correlation coefficients (XY values=26-36).

Table S.1. Quantitative real-time PCR primers (SyBr or Taqman).

Gene (SyBr)	Sequence
Abhd6	Fw: 5'-AGACCAGGTGCTTGATGT Rv: 5'-CTCTCCATCACTACCGAAT
Daglα	Fw: 5'-TATCTTCCTCTTCCTGCT Rv: 5'-CCATTTCGGCAATCATAC
Daglβ	Fw: 5'-GGGTCTTTTGAGCTGTTC Rv: 5'-AAGGAGGACTATCAGGTA
Faah	Fw: 5'-CAGCTACAAGGGCCATGCT Rv: 5'-TTCCACGGGTTCATGGTCTG
Gapdh	Fw: 5'-TCCACTTTGCCACTGCA Rv: 5'-GAGACGGCCGCATCTTCTT
Mgll	Fw: 5'-CCCAGTGGCACACCCAAG Rv: 5'-TAACGGCCACAGTGTTCCC
Nape	Fw: 5'-AAAACATCTCCATCCCGAA Rv: 5'-CGTCCATTTCCACCATCA
Pgc1α	Fw: 5'-GAAAGGGCCAAACAGAGAGA Rv: 5'- GTAAATCACACGGCGCTCTT
Prdm16	Fw: 5'-CCTAAGGTGTGCCCAGCA Rv: 5'-CACCTTCCGCTTTTCTACCC
Gene (Taqman)	**Sequence**
Ucp1	Fw: 5'-CACACCTCCAGTCATTAAGCC Rv:5'-CAAATCAGCTTTGCCTCACTC Assay name: Mm.PT.58.7088262

Table S.2. Hypothalamic endocannabinoids levels (ng/mg tissue).

		2-AG		AEA	
Days on diet		*Male*	*Female*	*Male*	*Female*
SD		2.7 ± 0.3	1.6 ± 0.5	0.013 ± 0.001	0.008 ± 0.001
HFD	7	8.6 ± 1.4 [a]	11.5 ± 1.4 [a, b]	0.023 ± 0.005 [a]	0.040 ± 0.008 [a, b]
	14	4.1 ± 0.7 [a]	4.6 ± 0.6 [a]	0.022 ± 0.003 [a]	0.039 ± 0.007 [a, b]
	28	3.5 ± 1.1	3.3 ± 1.8	0.019 ± 0.003 [a]	0.022 ± 0.016 [a]
SD		1.1 ± 0.2	0.9 ± 0.4	0.012 ± 0.002	0.010 ± 0.002
HFD	60	0.4 ± 0.2 [a]	0.6 ± 0.2 [a]	0.009 ± 0.001 [a]	0.013 ± 0.003 [a]
	90	0.5 ± 0.1 [a]	0.5 ± 0.1 [a]	0.011 ± 0.002	0.012 ± 0.002

Data are mean ± SD (n=8-10). Statistical significance was determined by ANOVA and Bonferroni post-test. [a]*P*<0.05 *versus* its corresponding SD; [b]*P*<0.05 *versus* male under the same diet conditions.

Table S.3. Plasmatic endocannabinoids levels (ng/mL plasma).

		2-AG		AEA	
Days on diet		*Male*	*Female*	*Male*	*Female*
SD		18.9 ± 2.3	22.0 ± 6.3	1.15 ± 0.09	2.0 ± 0.4 [b]
HFD	7	19 ± 5.8	22.9 ± 6.3	1.01 ± 0.02	1.2 ± 0.2
	14	19 ± 10	24.0 ± 9.6	1.01 ± 0.03	1.3 ± 0.3
	28	22.2 ± 6.4	36.0 ± 12.6	1.13 ± 0.08	2.2 ± 0.6 [b]
SD		6.3 ± 3.4	3.9 ± 0.9	1.9 ± 0.1	1.4 ± 0.6
HFD	60	10.8 ± 2.0 [a]	9.8 ± 2.3 [a]	2.2 ± 0.4	2.7 ± 0.6 [a]
	90	10.7 ± 1.5 [a]	13.2 ± 3.6 [a]	2.0 ± 0.3	4.0 ± 1.0 [a, b]

Data are mean ± SD (n=8-10). Statistical significance was determined by ANOVA and Bonferroni post-test. [a]*P*<0.05 *versus* its corresponding SD; [b]*P*<0.05 *versus* male under the same diet conditions.

Chapter III

CPT1C as the first negative regulator of the endocannabinoids hydrolase ABHD6.

In preparation.

ARTICLE SUMMARY

In this chapter, we aimed to confirm and characterized CPT1C and ABHD6 interaction and to study whether CPT1C could regulate ABHD6 activity.

A brief reminder, CPT1C is a multifunctional enzyme involved in metabolic and non-metabolic functions. However, CPT1C is not catalytically active and is not known how it is mediating these functions. For this reason, we looked for proteins that might interact with CPT1C. Using high-resolution proteomic, it was evidenced that CPT1C was interacting with ABHD6, a hydrolase of 2-AG eCB, in the AMPA glutamate receptors.

HEK-293T cells were transiently transfected with ABHD6 in combination with CPT1C and CPT1CM589S, a CPT1C form insensitive to malonyl-CoA and their interaction was studied by co-immunoprecipitation and FRET assay (**Experimental procedure, Figure 20A**). Moreover, we optimized a new activity assay to study CPT1C regulation of ABHD6 activity in HEK-293T cells and brain homogenates. Finally, eCBs levels had been measured in both systems (**Experimental procedure, Figure 20B**).

The results have demonstrated that CPT1C is interacting with ABHD6 and this interaction is crucial for CPT1C inhibition of ABHD6 activity. In addition, CPT1C is able to modulate 2-AG availability to CB_1R affecting its downstream signalling. Interestingly, CPT1C regulates ABHD6 activity depending on malonyl-CoA sensing.

In conclusion, these results describe for the first time a link between CPT1C and the ECS and provide additional information about molecular mechanisms underlying the effects of CPT1C. Moreover, we have identified CPT1C as the first regulator of ABHD6 able to inhibits its 2-AG hydrolase activity (**Results summary, Figure 21**). These finding might be relevant since blocking of ABHD6 activity could be beneficial for different pathologies, such as epilepsies, traumatic brain injury, multiple sclerosis and metabolic disorders.

EXPERIMENTAL PROCEDURE

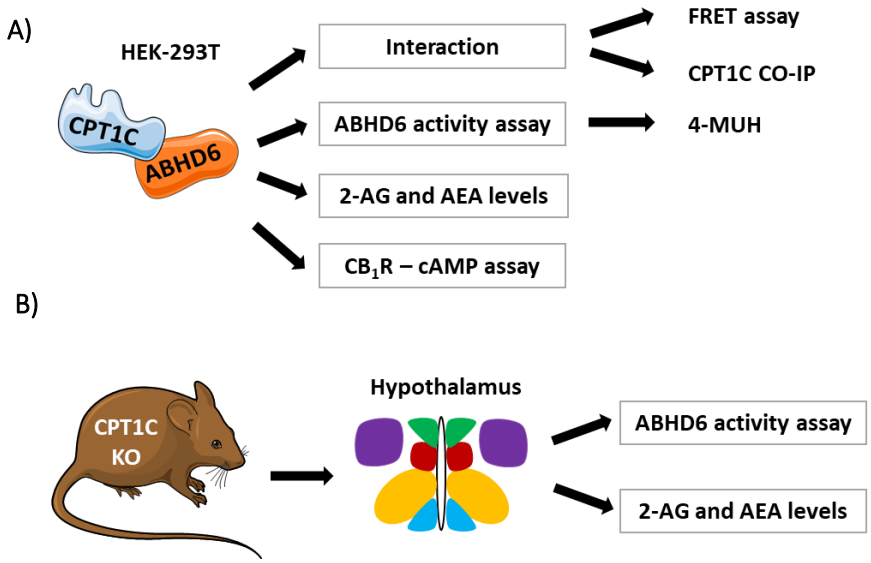

Figure 20. A) CPT1C and ABHD6 were transiently transfected in HEK-293T cells to study their interaction, ABHD6 activity, endogenous 2-AG and AEA levels and CB_1R response. B) Wild type (WT) and CPT1C-Knock out (KO) mice were used to assess hypothalamic ABHD6 activity and 2-AG and AEA levels.

RESULTS SUMMARY

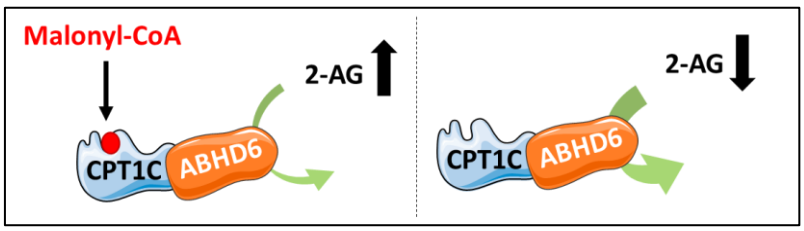

Figure 21. CPT1C inhibits ABHD6 activity depending on its ability to sense malonyl-CoA.

CPT1C AS THE FIRST NEGATIVE REGULATOR OF THE ENDOCANNABINOIDS HYDROLASE ABHD6

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In preparation

ABSTRACT

Brain-specific carnitine palmitoyltransferase 1 (CPT1) C has minimal CPT1 catalytic activity but it is able to bind malonyl-CoA, a major mediator of fatty acid synthesis, whose levels fluctuate depending on the energy status of the cell. CPT1C has been proposed to be a sensor of malonyl-CoA in neurons. However, the mechanism by which CPT1C exerts its functions is poorly understood. Using high-resolution proteomics, it was found that CPT1C interact with α/β-hydrolase domain containing 6 (ABHD6), as part of the AMPA glutamate receptor macromolecular complex. ABHD6 is a serine hydrolase of the endocannabinoid system (ECS) that catabolizes the main endocannabinoid, 2-arachydonoyl glycerol (2-AG). Although multiple functions have been related to ABHD6, it is unknown how the ABHD6 activity is regulated. Therefore, the main goal of this study was to characterize the CPT1C/ABHD6 interaction and to evaluate whether CPT1C regulates the hydrolase activity of ABHD6. For this purpose, we confirmed the interaction by both co-immunoprecipitation and FRET analysis, and we optimized a new and high sensitive fluorescent assay for measuring ABHD6 activity. We found that this interaction was crucial for the regulation of ABHD6, since CPT1C inhibited more than 50% of ABHD6 activity, and this regulation was dependent on CPT1C sensing of malonyl-CoA. Consequently, CPT1C was also able to modulate the CB_1R signalling. Altogether, we have identified for the first time a connection between CPT1C and the endocannabinoids systems, being CPT1C the first negative regulator of ABHD6 activity. These results brings new insights into the characterization of these two proteins.

1 INTRODUCTION

The family of carnitine palmitoyltransferase 1 (CPT1) enzymes catalyse the transport of long chain fatty acids into the mitochondria to undergo β-oxidation (Wolfgang and Lane 2011). Among CPT1s, CPT1C expression is mainly restricted to neurons and its subcellular location is the endoplasmic reticulum (ER) membrane (Sierra et al. 2008). In addition, contrary to the other CPT1 isoforms (CPT1A and CPT1B), CPT1C has minimal catalytic activity, although it maintains the ability to bind malonyl-CoA (Price et al. 2002; Wolfgang et al. 2006). Malonyl-CoA is an intermediate in fatty acid biosynthesis that acts as a nutritional-sensing mechanism in the hypothalamus (Wolfgang and Lane 2011). In a negative energy state, such as under fasting conditions, hypothalamic malonyl-CoA levels decreases stimulating food intake. In contrast, a positive energy state, such as after refeeding, implies an increases of malonyl-CoA levels leading to a satiated status (Hu et al. 2005; Wolfgang et al. 2007). In line with these results, our group has demonstrated that CPT1C within the hypothalamus plays a critical role in fuel selection and food preference during fasting (Pozo et al. 2017) as well as in the modulation of energy expenditure depending on malonyl-CoA levels (Rodríguez-Rodríguez et al. 2019). Besides, CPT1C is also largely expressed in the hippocampal area, where it directly interact with α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor (AMPAR) thus regulating synaptic plasticity and learning (Carrasco et al. 2012; Fadó et al. 2015; Gratacacós-Batlle et al. 2015).

However, the mechanisms by which CPT1C is achieving its neuronal functions are still poorly understood. The group of Bernd Fakler was the first to evidence, by high-resolution proteomic, that CPT1C was part of the AMPAR complex, and in this context CPT1C interacts with the α/β-hydrolase domain containing 6 and 12 (ABHD6 and ABHD12, respectively) among other proteins (Brechet et al. 2017; Schwenk et al. 2012). ABHD6 is a relatively new serine hydrolase of the endocannabinoids system (ECS) that hydrolyses specific pools of the endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG) generating arachidonic acid (ARA) and glycerol (Cao, Kaplan, and Stella 2019). 2-AG is the main eCB, biosynthesized from membrane phospholipid precursors “on demand”, whose biological actions are mainly mediated via the G-coupled protein receptors, cannabinoid type 1 receptor (CB_1R) and cannabinoid type 2 receptor (CB_2R). ABHD6 is highly expressed in the brain, brown adipose tissue, small intestine and immune system (Thomas et al. 2013). In the brain, 2-AG is mainly hydrolysed by the monoacylglycerol lipase (MAGL) at pre-synaptic membranes,

representing an ~85% of the total 2-AG hydrolysis in mouse (Blankman, Simon, and Cravatt 2007). ABHD6, from post-synaptic neurons, is responsible of the ~4% hydrolysis of 2-AG in the brain and ~20% in cells lacking MAGL. Despite MAGL is the major player in 2-AG deactivation, it has been found that ABHD6 controls a distinct sub-cellular pool of 2-AG compared to MAGL suggesting a differentiate role of ABHD6 from MAGL (Marrs et al. 2010).

ABHD6 has been related to different functions in the central nervous system (CNS), some of them being related to the hydrolysis of 2-AG and the subsequent regulation of CB_1R signalling and some others being ECS-independent. For instance, ABHD6 controls long-term synaptic (Marrs et al. 2010), the recovery of traumatic brain injury (Tchantchou and Zhang 2013) and the progression of multiple sclerosis (Manterola et al. 2018; Wen et al. 2015) by regulating the availability of 2-AG in the synapsis. Moreover, ABHD6 is also regulating the metabolic flexibility in the ventromedial nucleus of the hypothalamus (VMH) through controlling 2-AG levels in this nucleus (Fisette et al. 2016). In contrast, other ABHD6 functions have been reported to be independent on CBR signalling, such as the development of epilepsies that, in fact, are regulated by direct 2-AG control of $GABA_AR$ activity (Naydenov et al. 2014; Sigel et al. 2011). Furthermore, ABHD6 is physically associated with AMPAR in the CNS (Erlenhardt et al. 2016) to negatively regulate the surface delivery and synaptic functions of these receptors, in a way independent of its hydrolase activity (Wei et al. 2016, 2017). Finally, ABHD6 is able to hydrolyse non-cannabinoids lipids such as the bis(monoacylglycero)phosphate (BMP) in brain and liver (Pribasnig et al. 2015). BMP is enriched in late endosomal/lysosomes suggesting its participation in the lipid sorting machinery (Grabner et al. 2019; Pribasnig et al. 2015).

Although multiple functions have been related to ABHD6, there is no evidence of the molecular mechanism that regulates ABHD6 activity. In this study, we show that CPT1C is a new partner of ABHD6 that negatively regulates its activity. In addition, ABHD6 activity regulation by CPT1C depends on malonyl-CoA binding, a nutritional-sensing molecule. These results suggest a novel molecular interaction between CPT1C and ABHD6 that might link changes in energy metabolism and eCBs signalling.

2 MATERIAL AND METHODS

2.1 Cell culture and expressing vectors

HEK-293T cells were maintained in High Glucose DMEM (Sigma-Aldrich, Madrid, Spain) containing 10% FBS (Sigma-Aldrich, Madrid, Spain), 1% antibiotics (penicillin/streptomycin) and 1% glutamine at 37ºC in a humidified atmosphere of 5% CO_2/ 95% air. Plasmids used for HEK-293T transfection were cloned at BamHI in pmTurquoise-N1 containing cyan florescent protein (CFP; Addgene, Teddington, UK, ref. 60559) or in psYFP-C1 containing yellow fluorescent protein (YFP; Addgene, Teddington, UK, ref. 22878). For engineering of different plasmids the primers and vectors used are specified on Table 4. psYFP-C1-CPT1C plasmid was kindly given by Dr. Dolors Serra. We assessed the role of both malonyl-CoA using a mutated CPT1C insensitive to malonyl-CoA, mCPT1CM589S, referred as M589S from now on (Rodríguez-Rodríguez et al. 2019), and the CPT1C C-terminal using a mCPT1C form that substitutes its last 39 aminoacids (772-810) for the FLAG epitope (DYKDDDDK), referred as ΔCter in this paper. pmTurquoise-ER-5 (Addgene, Teddington, UK, ref. 55550) was used to express the Turquoise protein fused to KDEL (lysine (K), aspartic acid (D), glutamic acid (E) and leucine (L)), a ER targeting sequence. cDNA for the human version of CB_1R was cloned in pRluc-N1 plasmid as previously described (Reyes-Resina et al. 2018) obtaining CB_1R-Rluc. These constructs were used for the fluorescence resonance energy transfer (FRET) assay, co-immunoprecipitation, ABHD6 activity assay and cyclic adenosine monophosphate (cAMP) determination.

Plasmid	*Primers*
GluA1-CFP	F: 5’ GCGACCGGTGGATCCAACAATCCTGTGGCTCCCAAGGGCATCC 3’ R: 5’ CGCGGGCCCGGGATCCATGCCGTACATCTTTGCCTTTTTCTGC 3’
ABHD6-YFP	F: 5’ CGCGGGCCCGGGATCCATGGATCTCGATGTGGTTAACATGT 3’ R: 5’ GCGACCGGTGGATCCAAGTTCAGCTTCTTGTTGTCTGTGTT 3’
ABHD6-CFP	F: 5’ CGCGGGCCCGGGATCCATGGATCTCGATGTGGTTAACATGT 3’ R: 5’ GCGACCGGTGGATCCAAGTTCAGCTTCTTGTTGTCTGTGTT 3’
M589S-YFP	F: 5’ CGCGGGCCCGGGATCCATGGCTGAGGCACACCAGGCCTCGA 3’ R: 5’ GCGACCGGTGGATCCAACAAGTTGGTGGAGGATGTAGGGGT 3’
ΔCter-YFP	F: 5’ CGCGGCCCGGGATCCATGGCTGAGGCACACCAGGC 3’ R: 5’ GCGACCGGTGGATCCAAAACCCGGAACAGGGAGGCTACATCC 3’

Table 4. Primers obtained for recombination of plasmids.

2.2 Animals and sample collection

Male C57BL/6J and wild type (WT) and CPT1C-Knock out (KO) mice (8 week old) were used for the experiments. All animals were housed on a 12h/12h light/dark cycle in a temperature- and humidity-controlled room, and allowed free access to water and standard laboratory chow. Animals were killed by cervical dislocation and the hypothalamus and hippocampus were quickly removed, weighed and stored at -80ºC. All animal procedures were performed in agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Local Ethical Committee (Procedure ref. 9659 from the Generalitat de Catalunya).

2.3 Co-immunoprecipitation

HEK-293T cells were transfected by calcium-phosphate standard protocol with 4.5 µg/plate of cDNAs encoding CPT1C and ABHD6 or M589S and ABHD6 for 48h. Then, cells were washed twice with ice-cold PBS, scrapped using 500 µL for 60-mm plates of ComplexioLyte-47 buffer (CL-47, Logopharm, Freiburg, Germany) and homogenates were kept for 30 min at 4 ºC in an orbital shaker for solubilisation of membrane proteins. CL-47 was diluted ¼ with its buffer solution, sonicated for 5 minutes and supplemented with protease and phosphatase inhibitor cocktails. Samples were processed as previously described (Miñano-Molina et al. 2011). CPT1C antibody (Sigma, Madrid, Spain) was coupled to the sepharose beads (G Sephadex, GE Healthcare Life Science, Barcelona, Spain) for 1 h at 4 ºC in an orbital shaker. ABHD6 fraction bound to CPT1C was co-immunoprecipitated.

2.4 Western Blotting

Western blot was performed as previously described (Pozo et al. 2017). For the co-immunoprecipitation, precipitated complexes were washed in solubilisation buffer three times and eluted with 2x SDS/DTT sample buffer. Whole cell lysates were eluted with 1x SDS sample buffer. Protein extracts were heated 10 min at 95 ^{0}C, separated on 10% SDS-PAGE and transferred into PVDF membranes. Proteins were probed with antibodies against: CPT1C (Sigma-Aldrich, Madrid, Spain), ABHD6 (Abcam, Cambridge, UK) and GAPDH (Abcam, Cambridge, UK). Each membrane was then incubated with the corresponding secondary antibody and developed using Luminata Forte Western HRP substrate (Merck Millipore, Madrid, Spain). Images were collected by Gene Tools software (Syngene, Cambridge, UK) and quantified by densitometry using ImageJ-1.33 software (NIH, Bethesda, MD, USA).

2.5 Fluorescence resonance energy transfer (FRET) assay

HEK-293T cells were cultured in 24-well plate on coverslips pre-coated with poly-L-Lysine for 1h at 37 ºC. The day after seeding, cells were transiently transfected using calcium-phosphate standard protocol with 0.5 µg/well of donor plasmids containing CFP or 0.75 µg/well of acceptor plasmids containing YFP. cDNAs encoding for empty vector (EV), KDEL-CFP, GluA1-CFP, CPT1C-YFP, M589S-CYFP, ΔCter-YFP and ABHD6-CFP were used. 24 h after transfection, cells were fixed with 4% paraformaldehyde. Coverslips were mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Madrid, Spain). All FRET imaging experiments were performed on a Leica TCS SP2 microscope. A 63x objective with immersion oil and argon lasers with 458 and 514 lines (for donor and acceptor, respectively) were used for all image acquisition. The pinhole size was set to 2 (airy units, UA) and the ROI to 5 x 5 µm. 458 laser power was set up to 23-60% and 514 laser up to 5-10% so that no pixel saturation occurred. The number of bleaches were between 3 and 4, all at 40%. For the calculation of FRET efficiency from acceptor to donor, we used that operated at pixel basis,

$E = I_{fret}/I_d = (I_a - I_{dcrossem} - I_{crossex})/I_d$, where I_{fret} refers to FRET intensity, I_d refers to donor intensity in the absence of acceptor, I_a is the acceptor intensity in the presence of donor, $I_{dcross\ em}$ is the fraction of donor emission cross-emitted into the acceptor channel and $I_{cross\ ex}$ is the fraction of acceptor intensity cross-excited by donor excitation.

2.6 4-MUH-based activity assay

This protocol was based on the one kindly provided by Stephen Alexander (013P, 8th European Workshop on Cannabinoids Research, London, UK). The ABHD6 assay is based on its natural capacity to hydrolyse 4-methylumbelliferyl-heptanoate (4-MUH; Sigma, Madrid, Spain), a fluorogenic substrate whose hydrolysis produces the fluorescent compound 4-methylumbelliferone (4-MU; Sigma, Madrid, Spain) (λ_{ex} = 355; λ_{em} = 460nm). 4-MUH was dissolved in ethanol at 20x and kept at -20 ºC. HEK-293T were seeded in 6-well plate and transiently transfected with 1.2 µg/well of cDNAs encoding EV-YFP, ABHD6-YFP, CPT1C-YFP or M589S-YFP alone or in combination using FuGene HD (Promega, Madrid, Spain). To maintain the same ratio of DNA in co-transfections, we used EV-YFP to equilibrate the amount of total cDNA transfected. After 48 h, cells were washed twice with ice-cold PBS, scrapped and centrifuged to collect them. Pellets were frozen-thawed once and then homogenized with Tris-EDTA 50:1 mM buffer at pH 7.4 and briefly sonicated. The

assay was performed in black 96-well plates using 1 µg/well of protein in a total volume of 100 µL/well. Cell homogenates were incubated with 5 µL of the ABHD6 inhibitor, WWL70 (from 1 nM to 100 µM), or 2-AG (from 25 to 100 µM) (both from Cayman Chemicals, Ann Arbor, MI, USA) for 20 min at 37ºC. Subsequently, 5 µL of 4-MUH was added (50 µM final concentration) and fluorescence was immediately measured in 5 min intervals for 60 min or 120 min on a Synergy HT Reader (BioTek, Bad Friedrichshall, Germany). 4-MU was used to conduct a standard curve to express ABHD6 activity as nmol/mg.

2.7 cAMP determination

HEK-293T cells were seeded in 6-well plates and transfected for 48 h using polyethylenimine (Sigma-Aldrich, Madrid, Spain) as previously described (Carriba et al. 2008) with 1.5 µg/well of plasmids encoding CB_1R, ABHD6, CPT1C and M589S alone or in combination. To maintain the same ratio of DNA in co-transfections, we used EV-YFP to equilibrate the amount of total cDNA transfected. Two hours before adding reagents, HEK-293T cells were placed in serum-free medium. Then, cells were detached by suspension in medium containing 50 µM of zardaverine (Sigma, Madrid, Spain). Cells were placed in white 384-well plates (2500 cells/well), predated for 15 min with 1 µM of Rimonabant (Sigma, Madrid, Spain), a CB_1R antagonist, or 10 µM of WWL70, and stimulated with 100 nM of arachidonyl-2'-chloroethylamide (ACEA), a highly selective CB_1R agonist (Sigma, Madrid, Spain) or increasing concentrations of 2-AG (from 100 nM to 1 µM) for 15 min. Then 0.5 µM forskolin (FK) was added to increase intracellular cAMP levels for 15 min at conditions expressing CB_1R alone. Homogeneous time-resolved fluorescence energy transfer (HTRF) measures were performed, as previously described (Reyes-Resina et al. 2018), using the Lancer Ultra cAMP Kit (PerkinElmer, Waltham, MA, USA) after 1h of plate incubation at room temperature protected from light. Fluorescence at 665nm was analysed on a PHERAstar Flagship plate reader equipped with HTRF optical module (BMG Lab technologies, Offenburg, Germany).

2.8 Extraction and analysis of endocannabinoids

Hypothalamic eCBs were extracted and measured by HPLC-MS/MS exactly as previously described (Miralpeix et al. 2019). The same extraction protocol was used for HEK-293T cells, with some modifications. Briefly, cells were seeded on a 6-well plate and transfected with EV, ABHD6, CPT1C and M589S using FuGene HD (Promega Biotech Iberica, Madrid, Spain). 48 h after transfection, cells were washed twice with ice-cold PBS and centrifuged

(10000 x g, 5 min, 4ºC). Immediately, cells were suspended in 200 µL ice-cooled deionized water containing a final concentration of 0.362 µM of N-Oleylethanolamine-d_2 (OEA-d_2; Cayman Chemicals, Ann Arbor, MI, USA) as internal standard, 100 µM of phenylmethylsulfonyl fluoride (PMSF) and 0.01 % of butylated hydroxytoluene (BHT; Sigma-Aldrich, Madrid, Spain) and homogenised by a brief sonication. Then, 50 µL of the homogenized sample were kept at -20ºC for protein quantification and the other 150 µL were mixed with 600 µL ethyl acetate/n-hexane (9:1, v/v) and vortexed for 5 min. After centrifugation (14000 x g, 5 min, 4ºC), the upper layer was collected, evaporated in a nitrogen stream and analysed by HPLC-MS/MS as described before.

2.9 Statistical analysis

All results are expressed as mean ± S.D. Analysis of kinetics parameters was conducted using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA) using nonlinear regression with variable slope and Michaelis-Menten analysis. Statistical significance was obtained by t-test and one or two-way ANOVA followed by post hoc two-tailed Bonferroni test. $P < 0.05$ was considered significant.

3 RESULTS

3.1 CPT1C interacts with ABHD6 independently of malonyl-CoA sensing and CPT1C C-terminal

The group of Bern Fackler described that CPT1C interacts with ABHD6 since both proteins are constituents of the AMPAR proteome. We aimed to demonstrate that CPT1C and ABHD6 interacted outside of the AMPAR context and to investigate whether malonyl-CoA was important for this interaction. We also explored the role of the C-terminal region of CPT1C since this region is not present in the other CPT1 isoforms (CPT1A and CPT1B). For these purposes, we used HEK-293T cells to express ABHD6 in combination with CPT1C, the mutated form M589S, which is insensitive to malonyl-CoA (Rodríguez-Rodríguez et al. 2019), or the truncated form ΔCter, which the last 39 aminoacids have been replaced by the FLAG tag. First, we performed a co-immunoprecipitation assay using CPT1C antibodies and we observed that ABHD6 interacted with CPT1C, and this interaction was still maintained with the mutated M589S form (**Figure 1A and 1B**). These results indicate that ABHD6 interacts with CPT1C and that this interaction is independent of CPT1C malonyl-CoA sensing. Secondly, we performed FRET assays in HEK-293T cells (**Figure 1C and D**).

Plasmids encoding for the mTurquoise-ER (KDEL) and calnexin were used as a control of negative interaction whereas GluA1, a subunit of AMPAR that has been already proved to interact with CPT1C (Fadó et al. 2015), was considered as a positive control of interaction. Results showed that CPT1C and GluA1 FRET efficiency was significantly different from that evidenced by CPT1C with KDEL and calnexin (**Figure 1D**). Moreover, CPT1C, M589S and ΔCter exhibited similar FRET efficiency for ABHD6 compared to CPT1C-GluA1 (**Figure 1C and 1D**). In conclusion, both approaches evidence that CPT1C interacts with ABHD6. Neither CPT1C malonyl-CoA sensing nor CPT1C C-terminus are crucial for this interaction.

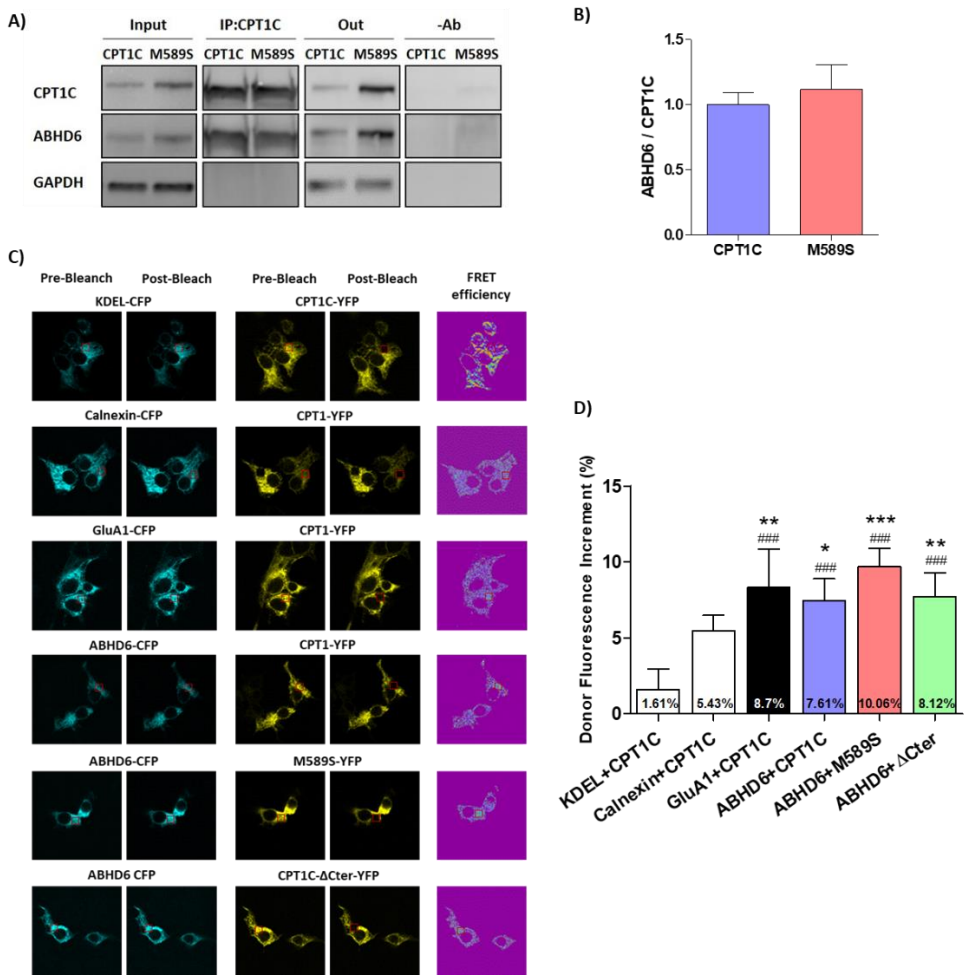


Figure 1. ABHD6 interacts with CPT1C. (A) Co-immunoprecipitation of ABHD6 using CPT1C antibody in HEK-293T expressing CPT1C or CPT1CM589S. Proteins were detected in whole lysate (Input), immunoprecipitated samples (IP), unbound proteins (out) and in samples incubated with protein G without CPT1C antibody (-Ab). (B) Quantification of ABHD6 co-immunoprecipitated by CPT1C (n=2, in duplicats). (C) Representative pictures of pre- and post-bleach and FRET efficiency. (D) FRET efficiency of ABHD6 and CPT1C interaction (n=3, 10 cells each). KDEL+CPT1C represents a negative FRET interaction and GluA1+CPT1C a positive FRET interaction. Error bars represent SD. ###P<0.001 *versus* KDEL+CPT1C and *P<0.05, ** P<0.01, ***P<0.001 *versus* Calnexin+CPT1C.

3.2 Development of 4-MUH-based ABHD6 activity assay

The CPT1C-ABHD6 interaction raised the question of whether CPT1C was regulating ABHD6 activity. Therefore, in this study, we optimized a rapid, direct and high sensitive fluorescent activity assay for ABHD6, based on the hydrolysis of a 4-MUH fluorogenic substrate (**Figure 2A**). HEK-293T cells lysates expressing ABHD6 showed higher accumulation of fluorescence over time than cells expressing EV when adding 50 µM of MUH as substrate (**Figure 2B**). The fluorescent progression curve displayed a lineal kinetics from 5 to 20 min. After 20 min, the fluorescence signal reached a plateau, whereas the background signal continued to increase (**Figure 2B**). We selected the 10 min point within the lineal fluorescence increase for further experiments otherwise specified.

Furthermore, ABHD6 activity depending on 4-MUH concentration showed that up to 50 µM of 4-MUH, the formation of 4-MU was constant, whereas at higher 4-MUH concentrations, a plateau was reached (**Figure 2C**). Nonlinear regression analysis was used to calculate an apparent K_m of 24.1 ± 7 µM, and a V_{max} of 324.8 ± 25.9 nmol/mg/min (**Figure 2C**). These parameters were similar to those previously described using the fluorogenic reporter 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE) (Shields et al. 2019). To validate the ABHD6 activity assay, we tested the effectiveness of an inhibitor of ABHD6, WWL70, in a dose-inhibition experiment (**Figure 2D**). We observed that WWL70 potently inhibited ABHD6 activity with an IC_{50} of 184.7 ± 0.07 nM. Moreover, to corroborate the specificity of 4-MUH as a substrate for ABHD6, we performed a competitive substrate assay by adding increasing concentrations of 2-AG (**Figure 2E**). We found that 2-AG, at a concentration of 100 µM, was able to evoke 55 % of 4-MUH hydrolysis compared to control conditions (**Figure 2E**).

Previous ABHD6 activity assays were unable to detect its activity in tissue homogenates (van der Wel et al. 2015). Here, we optimized the 4-MUH assay for hypothalamus and hippocampus homogenates (**Figure 2F and G**, respectively). The fluorescent progression curve displayed a lineal kinetics up to 80 min in hypothalamus and 60 min in hippocampal homogenates (**Figure 2F**). Dose-response inhibition of ABHD6 activity by WWL70 showed that up to 40-50% of brain homogenates 4-MUH hydrolysis was achieved by ABHD6 (**Figure 2G**). In summary, 4-MUH-based activity assay is a suitable and reliable method to study ABHD6 activity in both heterologous and endogenous expressing systems.

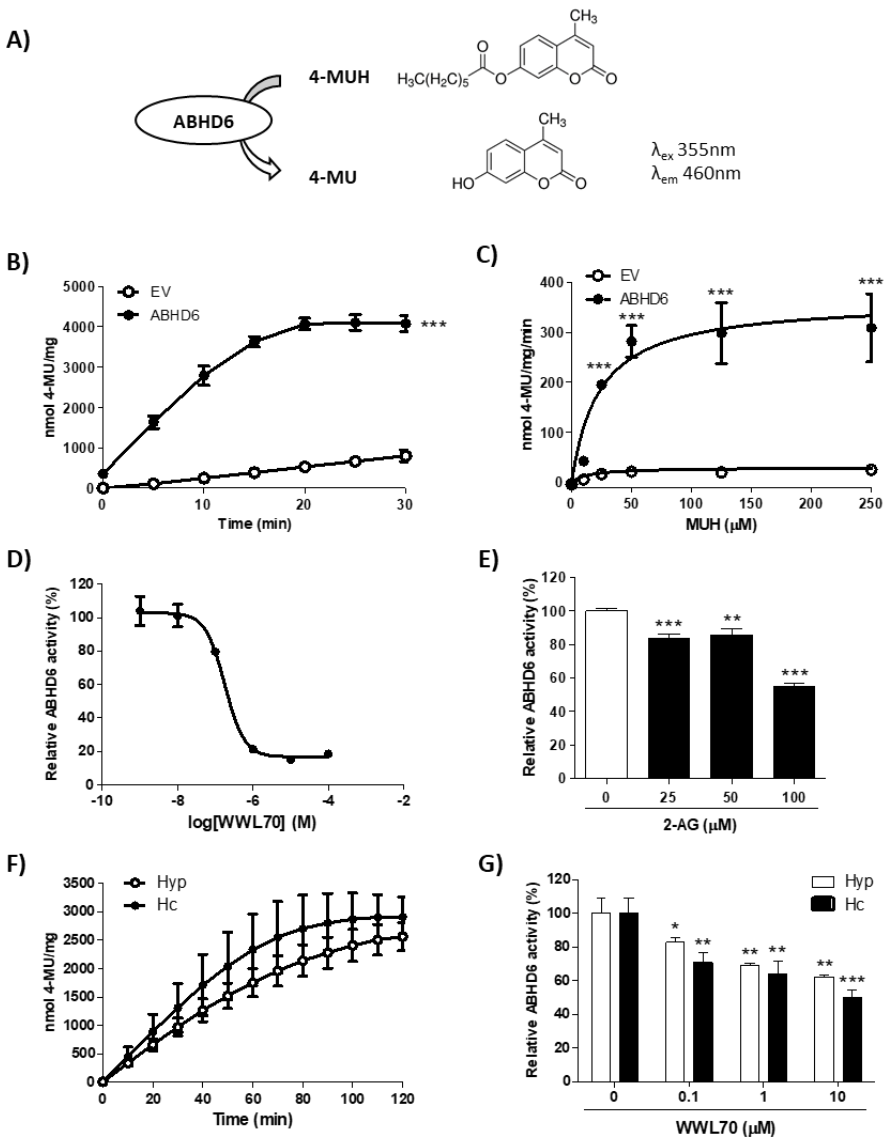
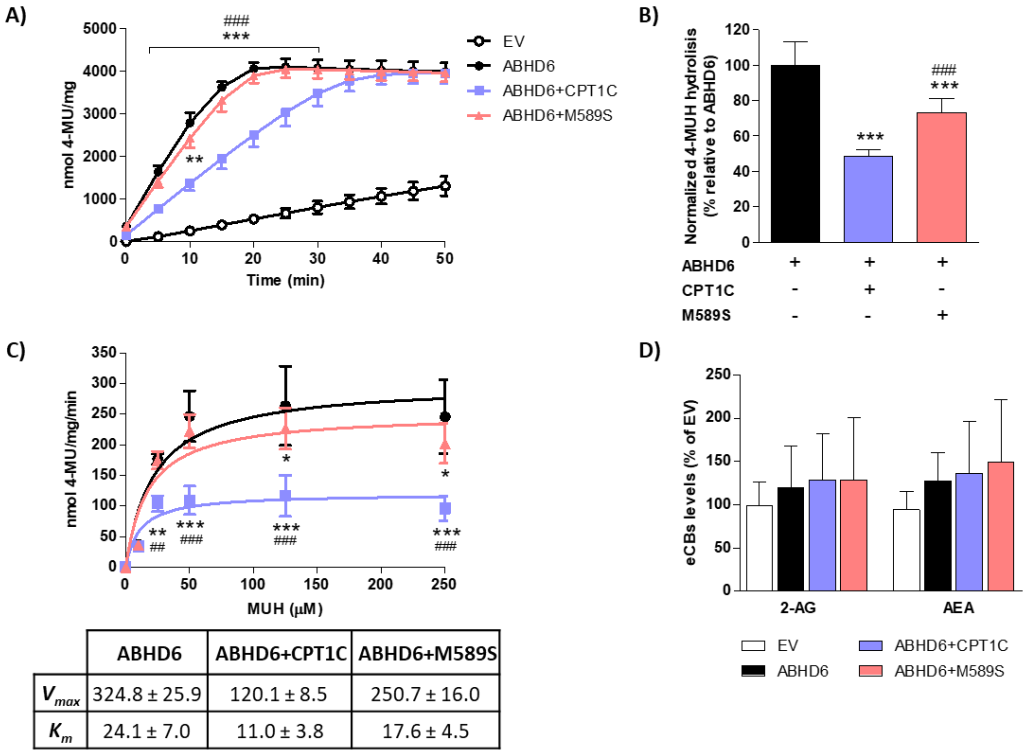


Figure 2. ABHD6 assay based on 4-MUH hydrolysis in HEK-293T cells. (A) ABHD6 hydrolyzes 4-MUH producing 4-MU, a highly florescent compound (excitation = 355nm; emission =460nm). (B) 4-MUH hydrolysis by cells expressing EV or ABHD6 (n=3, in triplicates). (C) ABHD6 activity dependent of 4-MUH concentration at t=10 min (n=3,in triplicates). (D) ABHD6 activity inhibition by WWL70 at t=10 min (n=3, in triplicates). (E) 2-AG competition with 4-MUH in cells expressing ABHD6 (n=2, in triplicates). (F) 4-MUH hydrolysis in hypothalamus (Hyp) and hippocampus (Hc) homogenates (n=5 mice). (G) ABHD6 activity inhibition by WWL70 in hypothalamus and hippocampus homogenates at t=120 min (n=2 mice). Errors bars represent SD. **P*<0.05, ***P*<0.01, ****P*<0.001 *versus* vehicle.

3.3 CPT1C regulates the activity of ABHD6 depending on malonyl-CoA sensing

Once we set up the ABHD6 activity assay, we were interested on evaluating whether CPT1C was able to regulate ABHD6 activity. HEK-293T cells were transfected with ABHD6 in combination with CPT1C or M589S and then ABHD6 activity was assessed. We observed that CPT1C downregulated the ABHD6 activity kinetic curve (**Figure 3A**). When ABHD6 was expressed alone, the activity curve displayed a lineal kinetics from 5 to 20 min and after that a plateau was reached (**Figure 3A**). In contrast, when CPT1C was present, ABHD6 activity reached the plateau after 40 min of reaction (**Figure 3A**). Normalization of ABHD6 activity by its protein expressing levels (**Figure S.1A and B**) showed that CPT1C reduced 50% of the ABHD6 activity (**Figure 3B**). Besides, nonlinear regression analysis of ABHD6 activity as a function of 4-MUH concentration demonstrated that CPT1C decreased the reaction rate of ABHD6 (**Figure 3C**). However, cells transfected with ABHD6 and M589S only evidenced a reduction of 33% of ABHD6 activity (**Figure 3B**) and its kinetics was partially restored (**Figure 3C**). Moreover, endogenous 2-AG and AEA levels were measured in HEK-293T co-expressing ABHD6 with CPT1C or M589S. Nevertheless, no changes on 2-AG and AEA levels were found (**Figure 3D**), suggesting that ABHD6 activity is not enough to modulate endogenous 2-AG in HEK-293T cells. Finally, cells non-expressing ABHD6, transfected with CPT1C or M598S alone, showed similar fluorescent accumulation than empty vector expressing cells, demonstrating that CPT1C and its mutated form M589S had no 4-MUH hydrolysis activity (**Figure S.1C**). These results indicate that CPT1C acts as a negative regulator of ABHD6 hydrolysis activity depending on malonyl-CoA sensing.



	ABHD6	ABHD6+CPT1C	ABHD6+M589S
V_{max}	324.8 ± 25.9	120.1 ± 8.5	250.7 ± 16.0
K_m	24.1 ± 7.0	11.0 ± 3.8	17.6 ± 4.5

Figure 3. CPT1C regulates ABHD6 activity depending on malonyl-CoA sensing. (A) Time course of 4-MUH hydrolysis by cells overexpressing EV and ABHD6 alone or in combination with CPT1C and M589S. (B) Normalization of ABHD6 activity by ABHD6 protein expression at t=10 min. (C) ABHD6 activity dependent of 4-MUH concentration at t=10 min. V_{max} (nmol/mg/min) and K_m (µM) values of ABHD6 obtained by Michelis-Menten analysis. (D) HEK-293T endogenous 2-AG and AEA levels. These assays were performed three times in triplicates. Error bars represents SD. **P*<0.05, ***P*<0.01, ****P*<0.001 *versus* ABHD6 and ##*P*<0.01, ###*P*<0.001 *versus* ABHD6+CPT1C.

3.4 CPT1C regulation of ABHD6 is able to modulate CB_1R signalling

It is known that 2-AG is the main ligand of CB_1R, a GPCR that inhibits the adenylyl cyclase (AC) and thus modulates the cAMP signalling pathway, among others (Busquets-Garcia, Bains, and Marsicano 2018). To elucidate whether CPT1C regulation of ABHD6 activity could modify the CB_1R downstream signalling, we studied cAMP levels in HEK-293T cells transiently expressing CB_1R, ABHD6, CPT1C and M589S alone or in combination. cAMP detection was set up using ACEA, a CB_1R agonist that decreases cAMP levels, and Rimonabant, a CB_1R (**Figure S.2A**). Dose-response assay with increasing concentrations of 2-AG (from 100 nM to 1 µM) in cells expressing CB_1R diminished intracellular cAMP levels as expected (**Figure 4A**). However, when CB_1R and ABHD6 were co-expressed, 2-AG significantly increased cAMP over basal levels in all concentrations (**Figure 4A**). Moreover, CPT1C expressed in combination with CB_1R and ABHD6 decrease cAMP to basal levels and were significantly different from CB_1R + ABHD6 condition (**Figure 4A**).

When M589S was expressed with CB_1R and ABHD6, cAMP levels were restored to those levels found under CB_1R+ABHD6 condition (**Figure 4A**). These results are in accordance with those obtained in the ABHD6 activity assay. To corroborate that CPT1C and M589S alone do not activate CB_1R downstream signalling, we expressed them in combination with CB_1R and observed that CPT1C and M589S effected cAMP levels similarly than CB_1R expressed alone when is stimulated with 2-AG (**Figure S.2B**).

The fact that cAMP levels in cells expressing ABHD6 were higher than those observed under control conditions was unexpected since basal cAMP levels were determined after incubation with FK, an activator of the AC. We postulated that cAMP levels could be increased due to an accumulation of the 2-AG hydrolysis products, ARA and glycerol. Therefore, increasing concentrations of both products were added to HEK-293T cells observing that neither 2-AG not glycerol were able to modify cAMP levels, albeit ARA induced significant increases in cAMP levels (**Figure 4B**).

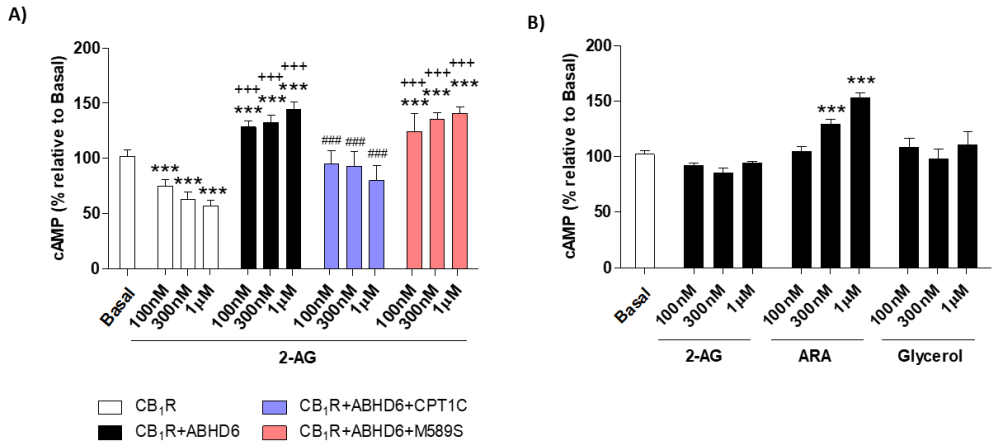


Figure 4. Effect of ABHD6 and CPT1C on CB_1R cAMP signalling pathway in HEK-293T cells. (A) cAMP intracellular levels in cells expressing CB_1R alone or in combination with ABHD6 and CPT1C or M589S and treated with increasing concentrations of 2-AG. (B) Effect of 2-AG, arachidonic acid (ARA) and glycerol in cAMP levels of HEK-293T without CB_1R. This assay was performed three times in triplicates. Error bars represent SD. ***P<0.001 *versus* basal; ###P<0.001 *versus* corresponding treatment in CB_1R+ABHD6 and CB_1R+ABHD6+M589S; +++P<0.001 *versus* corresponding treatment in CB_1R.

3.5 CPT1C-KO mice show altered ABHD6 activity and endocannabinoids levels in the hypothalamus.

To study the ability of CPT1C on regulating ABHD6 activity in a physiological system we first measured 4-MUH hydrolysis in hypothalamic homogenates of WT and CPT1C-KO mice. CPT1C deficient mice showed higher hydrolase activity than WT mice in hypothalamus (**Figure 5A**), in accordance with the results found in HEK-293T cells. Surprisingly, hypothalamic 2-AG levels were 4 times higher in CPT1C-KO mice compared to WT mice (**Figure 5B**). Moreover, AEA level were also increased in CPT1C-KO animals.

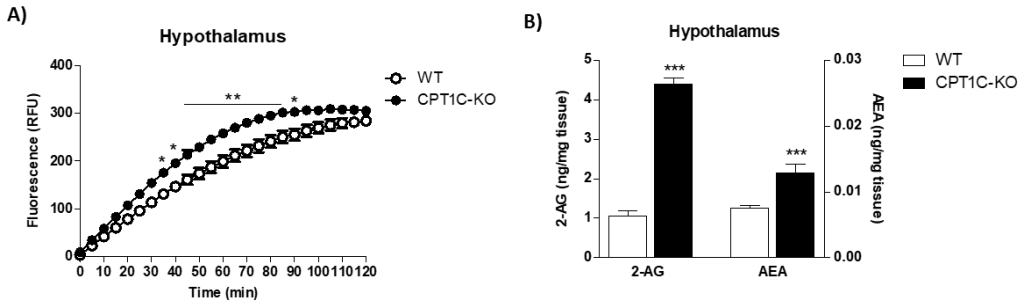


Figure 5. CPT1C-KO brain hypothalamus have increased 4-MUH hydrolysis and eCBs levels. (A) 4-MUH hydrolysis by WT and CPT1C-KO hypothalamic homogenate (n=3 mice). (B) Hypothalamic 2-AG and AEA levels of WT and KO mice (n=6-10 mice). Errors bars represents SEM. *P<0.05, **P<0.01, ***P<0.001 *versus* WT.

4 DISCUSSION

CPT1C is crucial in the regulation of multiple functions such as obesity, cognition and motor impairment, however, little is known about the molecular mechanisms underlying its functions since it lacks catalytic activity. Here, we have identified CPT1C as the first negative regulator of ABHD6 activity, one of the newest 2-AG hydrolases of the ECS. These results are relevant since blocking ABHD6 activity has emerged as an important therapeutic target (Cao, Kaplan, and Stella 2019) and helps to understands how these two proteins interact mechanistically.

First, we have reported that CPT1C directly interacts with ABHD6 in transfected HEK-293T cells. These results are in accordance with previous studies that identified both CPT1C and ABHD6 as components of AMPAR complexes (Brechet et al. 2017; Schwenk et al. 2012). On the one hand, our group demonstrated that CPT1C was interacting with GluA1 subunit of AMPAR to regulate the synthesis and trafficking of these receptors to the synaptic area (Fadó et al. 2015; Gratacacós-Batlle et al. 2015). On the other hand, it was shown that ABHD6 was able to interact with GluA1-3 and to negatively regulate its surface delivery (Wei et al. 2016, 2017). Further work will be necessary to elucidate the role that CPT1C/ABHD6 interaction plays in AMPAR trafficking. In addition, we have reported that CPT1C-ABHD6 interaction does not depends on malonyl-CoA sensing or on the presence of the CPT1C C-terminal sequence. Regarding the latter, it is important to note that the CPT1C C-terminal region is critical for CPT1C-GluA1 interaction (non-published data of our group). However, CPT1C C-terminal domain is not crucial for ABHD6-CPT1C interaction suggesting that, even being part of the same AMPAR complex, CPT1C interacts in a different manner with ABHD6 and GluA1. Therefore, our results evidence that CPT1C and ABHD6 interact outside of the AMPAR atmosphere and that this interaction is not regulated by either malonyl-CoA or the CPT1C C-terminal region.

Secondly, we have optimized an enzymatic activity assay for ABHD6. The activity of the ECS enzymes has been commonly studied using radiolabelled substrates (Dinh et al. 2002; Gopapaju et al. 2003; Karlsson, Tornqvist, and Holm 2000; Sakurada and Noma 1981; Somma-Delpéro et al. 1995; Tornqvist, Krabisch, and Belfrage 1974) or mass-spectrometry-based (Blankman, Simon, and Cravatt 2007) and HPLC detection of ARA (Saario et al. 2005), all laborious or expensive assays. Additionally, assays using colorigenic (Imamura and Kitaura 2000) and fluorogenic (Wang et al. 2008; Zvonok et al. 2008) substrates have been developed for the study of different hydrolases, including ABHD6 (Navia-Paldanius,

Savinainen, and Laitinen 2012; Shields et al. 2019). However, these assays required multiple-enzymatic steps or long incubation times. Hence, we have optimized an easy, rapid, cheap and high sensitive ABHD6 activity assay using 4-MUH as a substrate.

4-MUH has been used to study lipases activity for decades since it is well solubilized in aqueous mediums, it is not easily hydrolysed by non-lipolytic esterase and its hydrolysis produces a highly fluorescent compound, 4-MU (Dolinsky et al. 2004; Gilham et al. 2005). The ABHD6 kinetic parameters obtained (K_m = 24.1 ± 7.0 µM and V_{max} = 324.8 ± 25.9 nmol/mg/min) compared to previous parameters using 2-AG (K_m = 160 µM and V_{max} = 45 nmol/mg/min; Navia-Paldanius, Savinainen, and Laitinen 2012) or AHMMCE (K_m = 26 µM and V_{max} = 67 pmol/mg/min; Shields et al. 2019), as substrates demonstrate that 4-MUH is a high affinity substrate that is rapidly hydrolysed by ABHD6. K_m and V_{max} values have to be considered as apparent values since they are subjected to the assay protocol used and type of sample. For validation of this method we inhibited ABHD6 activity using WWL70 (IC_{50} = 180 nM) and performed a substrate competition with 2-AG evoking 55% of 4-MUH hydrolysis. Previous assays using activity-based protein profiling (ABPP) (Li, Blankman, and Cravatt 2007) or 2-AG as a substrate (Navia-Paldanius, Savinainen, and Laitinen 2012) obtained lower IC_{50} values (IC_{50} = 70nM and IC_{50}=85nM, respectively) although an assay using fluorogenic substrate obtained higher inhibition constant (IC_{50} = 285nM; Shields et al. 2019). These differences on WWL70 inhibition constant might be due to the affinity of ABHD6 for the probe or substrate used. Furthermore, 4-MUH activity-based assay is suitable to detect endogenous ABHD6 activity since WWL70 reduced 40-50% of lipase activity in brain homogenates. In conclusion, we have adapted the fluorogenic 4-MUH substrate to use it as a high sensitive ABHD6 activity assay in both heterologous and endogenous expression systems.

Using this enzymatic assay, we wondered whether CPT1C could regulate the hydrolase activity of ABHD6. We have evidenced that CPT1C negatively regulates ABHD6 activity by reducing up to 50% of ABHD6 hydrolysis velocity (ABHD6+CPT1C: V_{max} = 120.1 ± 8.5 and K_m = 11.0 ± 3.8). Since CPT1C has no catalytic activity, it has been postulated to act as a malonyl-CoA sensor (Rodríguez-Rodríguez et al. 2019; Wolfgang and Lane 2011) and therefore to regulate the function of other interacting proteins in response to changes in malonyl-CoA levels. Our results show that CPT1C sensing of malonyl-CoA is necessary for the regulation of ABHD6 activity, being inhibited only when CPT1C is detecting malonyl-CoA. These results are relevant since malonyl-CoA acts as a nutritional-sensing mechanism

in the hypothalamus and is crucial for CPT1C regulation of energy expenditure (Rodríguez-Rodríguez et al. 2019; Wolfgang and Lane 2011). Hence, this finding report suggests that ABHD6 activity could be under the control of malonyl-CoA levels, but more studies have to be done to elucidate whether the cell energy status can regulate ABHD6 activity through malonyl-CoA/CPT1C axis. However, when we measured endogenous eCBs levels in cells expressing ABHD6+CPT1C or CPT1CM598S we could not observe changes in 2-AG levels. These results are in accordance with the fact that ABHD6 only hydrolyses 2-AG upon "on demand" production (Marrs et al. 2010). Thus, we would need to perform studies measuring 2-AG and AEA levels in HEK-293T cells expressing ABHD6 upon 2-AG production stimulation.

Since we were unable to observe changes in endogenous levels of 2-AG in HEK-293T cells, we decided to study the modulation of CB_1R signalling pathway as a more sensitive method. The canonical 2-AG activated CB_1R downstream signalling leads to AC inhibition and, consequently, to the decrease of intracellular cAMP levels (Busquets-Garcia, Bains, and Marsicano 2018). Results showed that ABHD6 elevated cAMP intracellular levels in response to increasing 2-AG concentrations whereas CPT1C abolished that increase. Moreover, the effect of CPT1C was depending on malonyl-CoA sensing. Therefore, CPT1C control of ABHD6 is able to modify the 2-AG-mediated CB_1R response. Of note, these findings agree with ABHD6 activity assays results.

CPT1C regulation of ABHD6 activity might be important in some ABHD6 functions of the CNS. For instance, ABHD6 in the VMH is necessary to regulate food intake, body weight and energy expenditure in response to metabolic challenges such as fasting or high fat diet (HFD) conditions (Fisette et al. 2016). Since, CPT1C has also been described to be necessary for the regulation of energy homeostasis under a HFD in the VMH (Rodríguez-Rodríguez et al. 2019), CPT1C modulation of ABHD6 activity could be important to regulate metabolic flexibility in the VMH to counteract obesity development. In addition, ABHD6 hydrolyse BMP in the brain, a MAG related to lysosomal storage disorders (Pribasnig et al. 2015). The amount and composition of this lipid changed when animals were treated with a HFD (Grabner et al. 2019). Then, it might be interesting to study the role of CPT1C - ABHD6 complex in the regulation of BMP levels in response to metabolic challenges, such as HFD. Lastly, blocking ABHD6 activity stopped the development of seizures by activating $GABA_AR$ (Naydenov et al. 2014; Sigel et al. 2011). For a long time, patients with seizures have been treated with special diets, decreasing the amount of sugar or increasing FA composition

(Martin et al. 2016). CPT1C in this context could also play an important role in the regulation of ABHD6 activity. Overall, CPT1C regulates ABHD6 activity and consequently modulates CB_1R-cAMP response depending on malonyl-CoA levels. However, in which physiological and pathological conditions the interaction CPT1C - ABHD6 plays a crucial role needs to be further elucidated.

Finally, the activity of ABHD6 in brain homogenates was assessed in CPT1C-KO mice. Results demonstrated that hypothalamus of CPT1C deficient mice have more 4-MUH hydrolysis activity than WT mice, in accordance with the results obtained in heterologous cells. Interestingly, a metabolomics profile study comparing brain of WT and CPT1C-KO (Lee and Wolfgang 2012) found that 2-oleoylglycerol, an eCBs also hydrolysed by ABHD6 (Navia-Paldanius, Savinainen, and Laitinen 2012), were decreased in CPT1C-KO mice. We measured hypothalamic 2-AG and AEA levels, in WT and CPT1C-KO mice. Surprisingly, hypothalamic 2-AG and AEA were higher in CPT1C-KO than WT mice. These results were the opposite to those expected from the ABHD6 activity assay. We have to consider that ABHD6 is responsible for the hydrolysis of concrete pools of 2-AG upon activity-stimulation of its production (Marrs et al. 2010) and we suggested that, it is in this situation when fine regulation of ABHD6 activity by CPT1C might be crucial. Moreover, AEA levels (an eCB not hydrolysed by ABHD6) were also increased. For these reasons, we conclude that CPT1C regulation of ABHD6 activity is not the main cause of the eCBs levels detected in the hypothalamus of CPT1C-KO mice. From where the increase of eCBs levels in the hypothalamus of deficient CPT1C mice comes from needs to be explore.

Altogether, this study describes for the first time a link between CPT1C and the ECS proportionating valuable information for the characterization of CPT1C. Moreover, we have described CPT1C as the first physiological inhibitor of ABHD6. These results are remarkable since blocking of ABHD6 activity has been showed to have therapeutic benefit in epilepsies, traumatic brain injury, multiple sclerosis and obesity (Cao, Kaplan and Stella 2019). Therefore, CPT1C as a negative regulator of ABHD6 might be an interesting target to these pathologies.

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SUPPLEMENTARY INFROMATION

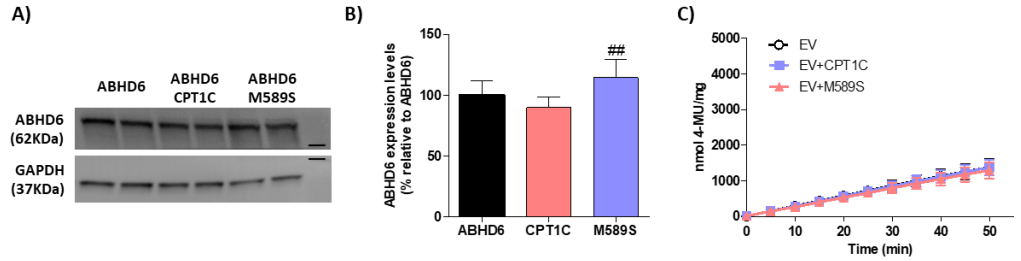

Figure S.1. ABHD6 activity assay based on 4-MUH. Representative western blot (A) and quantification (B) of ABHD6 protein expression in HEK-293T after 48h of transfection. (C) 4-MUH hydrolysis of HEK-293T cells expressing EV, CPT1C and M589S. This assay was performed three times in triplicates. Errors bars represents SD. $^{\#\#}P<0.01$ versus ABHD6+CPT1C.

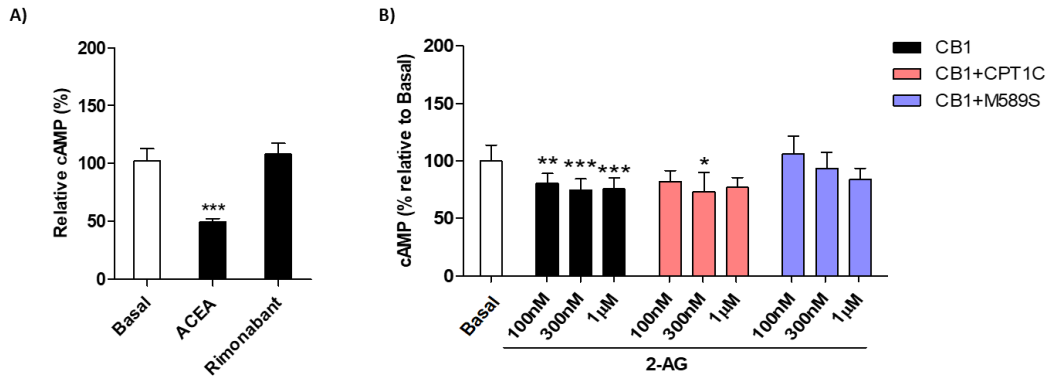

Figure S.2. Intracellular cAMP assay was set up in HEK-293T. (A) cAMP levels of HEK-293T expressing CB1R incubated with an agonist of CB1R, ACEA, and an antagonist, Rimonabant. (B) CPT1C and its mutated form M589S expressed in combination with CB1R and incubated with 2-AG are not able to modify cAMP levels. This assay was performed three times in triplicates. Errors bars represents SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus basal.

DISCUSSION

Obesity prevalence in worldwide population is increasing every day due to an imbalance between the energy intake and the energy expenditure. Western societies offer highly palatable and easily accessible rich-caloric food that in combination with a sedentary lifestyle makes difficult to maintain energy homeostasis and leads to obesity development. Up to now, finding competent treatment for obesity has failed due in part to a lack of knowledge on the molecular mechanisms underlying obesity development. In the last few years, the central mechanisms leading to the activation of BAT thermogenesis have emerged as a promising therapeutic approach against obesity (Whittle et al. 2011, 2013).

In this thesis, for the first time, we have evidenced that the brain specific isoform of the CPT1s, the CPT1C, is a crucial factor in the hypothalamus for the activation of BAT thermogenesis upon fat intake. Previous studies demonstrated that CPT1C-KO mice were more prone to obesity since they failure to adapt to HFD (Dai et al. 2007; Wolfgang et al. 2008, 2006) and now we know that, in part, this is due to a failure to activate BAT thermogenesis from the VMH. However, the molecular mechanisms underlying CPT1C in the control of energy homeostasis are not known.

The ECS is another critical system controlling energy homeostasis and metabolic flexibility from the hypothalamus. In obesity, the ECS gets dysregulated leading to an increased adiposity, leptin resistance and decreased BAT thermogenesis (Ruiz de Azua, 2019). Although the role of the ECS in obesity has been extensively studied, it is not known how hypothalamic eCBs behave in the development of the pathology and whether they are related to BAT thermogenesis activation. For this reason, here we have addressed this topic in DIO male and female mice and we have described a crosstalk from peripheral thermogenesis activation to hypothalamic 2-AG and AEA levels.

Finally, we found a link between the CPT1 and ECS, the ABHD6, which is one of the newest hydrolases of 2-AG (Marrs 2010). CPT1C, by direct interaction with ABHD6, negatively regulates its activity. Therefore, we have identified the first negative regulator of ABHD6. This results are remarkable since blocking ABHD6 activity provides therapeutic benefits in epilepsies, traumatic brain injury, multiple sclerosis and obesity (Cao, Kaplan, Stella 2019), highlighting the importance of CPT1C role on ABHD6 activity.

1 AMPK-MALONYL-COA-CPT1C AXIS IS NECESSARY FOR BAT THERMOGENESIS ACTIVATION IN THE VMH

One of the newest results of this thesis (Chapter I) is the demonstration that CPT1C regulates BAT thermogenesis activation under short-term fat intake. CPT1C-KO mice presented an attenuated BAT thermogenesis in response to 7 days of HFD feeding or after central leptin administration. Previous results had linked CPT1C deficiency with body weight gain, but the pathological mechanisms involved were poorly known. For instance, under HFD feeding, CPT1C-KO mice showed altered hepatic and muscle glucose metabolism suggesting a role for CPT1C in the control of whole-body glucose homeostasis (Dai et al. 2007). More recently, it was reported that during fasting (Pozo et al. 2017), CPT1C in the MBH acted as a regulator of lipid metabolism in liver and muscle. Here we show that CPT1C at the VMH, is necessary for a proper activation of BAT thermogenesis to counteract body weight gain during HFD feeding. Even though we have used a global KO model, we can assure that CPT1C effects are exerted at the VMH because re-expression of CPT1C in the VMH of CPT1C-KO mice using genetic approaches was sufficient to restore the phenotype induced by 7 days of HFD feeding.

Secondly, we have observed that malonyl-CoA sensing through CPT1C in the VMH is necessary for diet-induced BAT thermogenesis activation. For this purpose, I developed a CPT1C with a mutation in the methionine 589 for a serine (M589S) in the malonyl-CoA binding pocket. This mutation was previously described for CPT1A, making it constitutively active but unable to bind malonyl-CoA (Morillas et al. 2003). Expression of M589S in the VMH of CPT1C-KO mice was neither able to decrease body weight gain nor to restore BAT thermogenesis activation. Hence, this is the first time demonstrating that CPT1C sensing of malonyl-CoA is necessary for a correct thermogenic response to a HFD feeding.

Thirdly, considering the importance of the canonical pathway dependent on AMPK in the VMH to regulate BAT thermogenesis (Contreras et al. 2017); we have demonstrated that CPT1C is necessary for BAT thermogenesis activation via AMPK in the VMH. Using genetic approaches we overexpressed a dominant negative form of AMPK into the VMH that induced weight loss and BAT thermogenesis activation in WT mice whereas CPT1C-KO mice did not respond. Therefore, we suggest that CPT1C is downstream the AMPK signalling in the VMH, although more experiments are needed to confirm these preliminary results. For instance, silencing CPT1C at the VMH using genetic approaches and study the AMPK response in the DIO model would be a suitable assay. Nonetheless, we are not the first

group proposing that CPT1C is positioned under the hypothalamic AMPK control. Last year, Minokoshi's group observed that activation of the AMPK-CPT1C pathway in CRH neurons of the PVH mediates the fasting-induced HCD preference selection (Okamoto et al. 2018). They demonstrated that fasting increased AMPK activity resulting in activation of CPT1C, which promoted intracellular calcium release and increased synaptic activity of CRH neurons and HCD selection. Moreover, they observed that AMPK activation increases CPT1C mRNA expression in the PVH (Okamoto et al. 2018). Thus, AMPK might be able to regulate CPT1C by both regulating its expression levels and modifying malonyl-CoA levels. Altogether, hypothalamic CPT1C, under the control of AMPK, is a key regulator of BAT thermogenesis and diet-preference selection in a situation of energy surplus. Taking into account that AMPK pathway at the VMH regulates BAT thermogenesis in response to hormones and other effectors, such as estrogens, thyroid, or nicotine (López et al. 2016), it would be interesting to explore in the future the role of CPT1C under these conditions.

In conclusion, the study described in chapter I positions CPT1C as a new target to regulate BAT thermogenesis in the development of obesity, but it also opens new questions that need to be addressed. For instance, CPT1C maintains the structure to detect LCFA as substrates. Dietary fatty acids such as linoleic or oleic acid have been demonstrated to regulate body weight and food intake from the hypothalamus (Moullé et al. 2014). Thus, we still do not know whether hypothalamic regulation of BAT activation is triggered by both CPT1C lipid sensing that comes from HFD and/or by malonyl-CoA sensing. The study of which fatty acids are able to activate BAT thermogenesis and whether CPT1C is involved in their sensing in the VMH would proportionate valuable information about the effect of dietary fatty acids composition in obesity development and would provide valuable knowledge about CPT1C as a target to treat obesity.

2 CPT1C-KO MICE HAVE DYSREGULATED ENDOCANNABINOIDS LEVELS IN THE HYPOTHALAMUS AND PLASMA

The fact that CPT1C is not catalytically active made us to hypothesise that it would exert its functions by interacting with other proteins. Moreover, the ability of CPT1C to sense malonyl-CoA would proportionate a regulation of these other proteins depending on the energetic status of the cell. Dr. Bern Fakler and colleagues carried out a proteomic study comparing brains of WT and CPT1C-KO mice to find proteins possibly interacting with CPT1C. One of the proteins that more abundantly co-purified with CPT1C was the ABHD6

(data not shown in this thesis), a hydrolase of the main eCB, the 2-AG. This finding was particularly interesting since the ECS is involved in the regulation of energy homeostasis from the hypothalamus and is dysregulated in obesity. Increased eCBs tone in the hypothalamus generally leads to an increase of body weight and to a decrease in energy expenditure (Ruiz de Azua and Lutz 2019).

ABHD6 is one of the newest enzymes of the ECS and has been already described to be involved in multiple functions, even though no partners of ABHD6 have been characterized yet (Cao, Kaplan, and Stella 2019). ABHD6 has also been described to play a role in metabolic flexibility in the VMH nuclei of the hypothalamus since disruption of ABHD6 in this nucleus diminished energy expenditure and enhanced DIO (Fisette et al. 2016). Considering that, the molecular mechanism by which CPT1C controls energy homeostasis is not clear we hypothesized that CPT1C could regulate its metabolic functions through modifying the eCBs levels in the hypothalamus via the interaction with ABHD6. For this reason, we first studied 2-AG and AEA levels in the hypothalamus of WT and CPT1C-KO mice under normal conditions. We found that CPT1C-KO mice have increased both eCBs in the hypothalamus compared to WT mice. Moreover, we assessed the eCBs levels after short-term HFD administration, when CPT1C is crucial in the regulation of BAT thermogenesis activation. After 7 days of HFD feeding, hypothalamic eCBs of both WT and CPT1C-KO mice were increased comparing to their respective SD fed animals, and CPT1C-KO mice showed higher eCBs levels compared to WT mice (Appendix I, Figure 23A and B).

The diet and CPT1C-dependent increase of eCBs were restricted to the hypothalamic area since hippocampal eCBs levels were not altered in any of the conditions assessed (Appendix I, Figure 23C and D). These results highlight the important role of CPT1C in the regulation of the ECS in the hypothalamus. In line with this result, a recent publication reported that the effect of leptin on eCBs signalling was restricted to the hypothalamus, without affecting the hippocampus. The authors suggested that the ECS is only sensitive to leptin in brain functional domains related to feeding (Balsevich et al. 2018). In contrast to the hypothalamus, the eCBs circulating levels in CPT1C-KO mice were diminished compared to WT mice in both SD and HFD (Appendix I, Figure 23E and F). In line with this result, some studies found that circulating eCBs inversely correlates with central ones in both rodent and healthy patients (Martin et al. 2017; Jumpertz et al. 2011).

The eCBs phenotype of CPT1C-KO mice led us to question whether the increase in hypothalamic eCBs levels could be related to the impaired diet- and leptin-induced BAT

thermogenesis in CPT1C-KO mice and whether CPT1C was able to regulate the eCBs levels through ABHD6. However, the eCBs dynamic in the hypothalamus of DIO model and its relationship to BAT thermogenesis activation in early stages of obesity had been poorly studied, therefore we had difficulties to interpret our results correctly. In addition, we realised that all the studies about hypothalamic eCBs in obesity were done in male but not in obese female rodents, so we decided to address these topics. Hence, at this point, we concurrently studied i) the eCBs dynamic in the hypothalamus during obesity development in the DIO model and whether they correlate with BAT thermogenesis in male and female mice (Chapter II), and ii) whether CPT1C interacts and regulates ABHD6 activity depending on malonyl-CoA sensing (Chapter III).

3 BAT THERMOGENESIS ACTIVATION CORRELATES WITH HYPOTHALAMIC ENDOCANNABINOID LEVELS IN EARLY STAGES OF OBESITY DEVELOPMENT IN MALE AND FEMALE MICE

Concerning hypothalamic eCBs during DIO development (Chapter II), we have described for the first time the time-profile of hypothalamic eCBs from early stages of obesity development (7 days of HFD) to obesity establishment (90 days of HFD) in male and female mice. Our results have demonstrated that at short-term HFD feeding hypothalamic 2-AG and AEA substantially increase followed by a progressive decrease until basal levels or even below at 90 days of HFD. Furthermore, the hypothalamic 2-AG and AEA profile negatively correlates with body weight gain and plasma leptin levels, indicating that hypothalamic eCBs dynamic behave inversely to obesity development.

Studies investigating hypothalamic eCBs levels at short-term HFD feeding are scarce. Sei Higuchi and collaborators found that 14 days of HFD administration increased 2-AG levels lasting for 42 days whereas AEA levels were not studied (Higuchi et al. 2012). When these animals were challenged with a preference test to HFD, the 2-AG levels increases at 3 and 7 days of feeding (Higuchi et al. 2011, 2012). Thus, an early increase of eCBs levels in the hypothalamus are related to both the HFD feeding and the hedonic component of fat.

Concerning studies about hypothalamic eCBs levels after long-term administration of a HFD, the results are controversial. The general and most accepted idea is that eCBs levels in the hypothalamus are elevated in obesity (Quarta et al. 2011). This conception started with the investigation of hypothalamic eCBs in genetic models of obesity such as *ob/ob* and *db/db* mice or Zucker rats (Balsevich et al. 2018; Cristino et al. 2013; Di Marzo et al. 2001).

Obesity in these genetic models is led by a disruption on leptin signalling (Kleinert et al. 2018). Since the ECS is in close relationship with leptin, eCBs levels on genetic obese models are probably a consequence of blunted leptin signalling (Di Marzo et al. 2001). However, the same experiments but instead using DIO mice model, found that 2-AG levels were either slightly increased (Cristino et al. 2013) or did not change (Balsevich et al. 2018) whereas AEA levels did not increase in any of the studies. In another study using DIO rats, hypothalamic 2-AG levels were elevated whereas AEA levels were not modified (Gamelin et al. 2016). Taking into consideration the discrepancy between hypothalamic eCBs levels in studies using DIO models, our results contribute with a well-defined 2-AG and AEA time profile in the hypothalamus of male and female DIO mice.

The time profile of circulating eCBs was also assessed. 2-AG and AEA levels were low at early stages of HFD feeding and increased after 90 days of HFD feeding. Therefore, plasmatic eCBs positively correlated with obesity development and negatively correlated with hypothalamic eCBs. According to our results, several studies in obese and diabetic patients have positively correlated the adiposity and the BMI with the circulating eCBs (Hillard 2018; Matias, Gatta-Cherifi, and Cota 2012).

Since the hypothalamic eCBs levels in female mice during DIO development were not studied before, we monitored the hypothalamic and plasmatic eCBs levels in both genders finding a consistent but slight sexual dimorphism. Both the hypothalamic and circulating eCBs levels were higher in female than male mice. Previous studies had demonstrated that hypothalamic 2-AG levels under standard diet conditions were higher in female than male mice (Bradshaw et al. 2006) and that estradiol in POMC neurons proportionate resistance to DIO by decreasing ECS tone and increasing energy expenditure (Fabelo et al. 2018). Thus, it seems that hormonal control of hypothalamic and plasmatic eCBs could explain the gender dimorphism. Therefore, the use of both genders for studying the ECS is advisable for future research.

We also monitored BAT thermogenesis activation in these animals and its correlation with obesity progression. In accordance with results obtained in Chapter I, 7 days of HFD feeding produced a high increase in BAT thermogenesis that decreased progressively during obesity. The BAT thermogenesis profile correlated with the hypothalamic eCBs levels, during obesity development. Interestingly, at 7 days of HFD, the rise of eCBs levels was accompanied with an increase in the expression of the eCBs synthesis enzymes in the hypothalamus whereas the degrading enzymes were not altered. The fact that

hypothalamic eCBs correlated with BAT thermogenesis activation were surprising since it is generally accepted that an increase in the ECS tone in the hypothalamus activates CB_1R, which inhibits SNS transmission leading to a decrease in BAT thermogenesis (Quarta et al. 2011). However, for the first time, we observed that diet-induced activation of BAT thermogenesis correlates with higher eCBs levels in the hypothalamus associated to an increase of their synthesis.

To better understand the crosstalk between BAT thermogenesis activation and hypothalamic eCBs levels, we acutely activated BAT thermogenesis by central leptin injection or peripheral restricted β3-AR agonist administration. Surprisingly, both treatments led to an increase of eCBs levels in the hypothalamus. In contrast, acute administration of central 2-AG and AEA did not alter BAT thermogenesis. Collectively, these data suggest that the peripheral and central stimulation of thermogenesis should signal the hypothalamic ECS to increase the eCBs levels. This is not the first time identifying a crosstalk between periphery organs and central ECS. The study curried out by Martin GG and collaborators evidenced that a deficiency in liver fatty acid binding protein (FABP1), which facilitate hepatic clearance of fatty acids, had lowered AEA and 2-AG levels in the brain compare to WT mice (Martin et al. 2017).

In the near future, we would like to assess which factors are involved in signalling the thermogenesis activation to hypothalamic ECS. One possibility would be that BAT upon thermogenesis activation secrete batokines (Villarroya et al. 2017) that could induce eCB synthesis in the hypothalamus. For instance, the bone morphogenetic protein 8b (BMP8b) is produced by mature brown adipocytes and its expression increases in response to β3-AR stimulation and HFD (Whittle et al. 2013). Moreover, the hypothalamus is able to respond to BMP8b since its central and inta-VMH administration is able to activate BAT thermogenesis and reduces body weight gain through the SNS (Martins et al. 2016). Another batokine released in fasting and HFD conditions is the fibroblast growth factor 21 (FGF21), which can act in autocrine or endocrine manners since it is released to the circulatory system and can reach the CNS. FGF21 has been extensively studied because it enhances BAT thermogenesis and prevents obesity (Giralt, Gavaldà-Navarro, and Villarroya 2015). However, it is unknown whether BMP8b nor FGF21 trigger the synthesis of eCBs when reach the hypothalamus. For these reasons, studying circulating levels of batokines and whether their signalling in the hypothalamus is related to the ECS in response to BAT thermogenesis activation would add valuable information about this crosstalk mechanism.

Although we have focused our study on the crass-talk between BAT-hypothalamus, we should take into account that the β3-AR, besides being expressed in BAT, is also functional in WAT, liver, gut, blood vessels and cardiovascular system (Ursino et al. 2009), thus other organs beyond BAT could be mediating the effect on the eCBs levels in the hypothalamus that we found. The evaluation of which organs are implicated in this crosstalk would be interesting.

Another consideration is that BAT and WAT would act as eCBs secretory organs to the circulating system, which would reach the hypothalamus to increase eCBs levels. A recent study has pointed out BAT as an eCBs producer after short- and long-term exposure to HFD determining the circulating eCBs (Kuipers et al. 2019). In a different study, the authors demonstrated that acute activation of thermogenesis increases BAT and WAT eCBs levels (Krott et al. 2016). Although we have not analysed the eCBs levels in BAT or WAT, our results demonstrated that increased BAT thermogenesis at 7 days of HFD correlated with lower eCBs circulating levels and higher hypothalamic eCBs. Therefore, we consider that the increase in eCBs in the hypothalamus do not come from the circulating ones.

Altogether, this study has proportionated a time-profile of the hypothalamic and circulating eCBs during obesity development in male and female mice. Furthermore, we suggest a crosstalk between stimuli leading to BAT thermogenesis activation and the hypothalamic ECS system.

4 CPT1C IS A NEW NEGATIVE REGULATOR OF ABHD6 ACTIVITY

Regarding the study of CPT1C and ABHD6 (Chapter III), the first result obtained has been the confirmation of CPT1C and ABHD6 interaction. CPT1C and ABHD6 interaction was previously described in AMPAR complexes by using high-resolution proteomics (Brechet et al. 2017; Schwenk et al. 2012). In these studies, they observed that CPT1C and ABHD6 form a macromolecular complex with AMPAR. Moreover, recent investigations report that ABHD6 interacts with GluA1, GluA2 and GluA3 subunits of the AMPAR and regulates GluA1 trafficking (Wei et al. 2016, 2017). Here we have demonstrated that CPT1C and ABHD6 are able to interact without being part of the AMPAR complex (in HEK293T cells that do not express AMPAR), and consequently this interaction might be also involved in other cellular functions beyond AMPAR. Besides, this interaction was independent of CPT1C malonyl-CoA sensing. Moreover, although the last aminoacids of the CPT1C C-terminal region are critical for CPT1C-GluA1 interaction (data not published of our group), this domain is not crucial

for the CPT1C-ABHD6 interaction suggesting that, even being part of the same AMPAR complex, CPT1C interacts in a different manner with ABHD6 and GluA1.

Next, we have demonstrated that CPT1C acts as a negative regulator of ABHD6 activity. I would like to highlight this result since no partners of ABHD6 have been described until today. For this purpose, I set up a new, easy and rapid fluorimetric assay able to measure ABHD6 activity in transiently transfected cells and, for the first time, in brain homogenates. In contrast to the interaction, CPT1C regulates ABHD6 activity depending on malonyl-CoA sensing. The fact that CPT1C inhibits ABHD6 activity depending on malonyl-CoA positioned CPT1C as an energy sensor for ABHD6 indicating when to hydrolyse 2-AG or other monoacylglycerols. We are now investigating whether the C-terminal region of CPT1C is also regulating ABHD6 activity.

Malonyl-CoA binding of CPT1C is controlled by the N-terminal regulatory domain of CPT1C. The N-terminal adopts two different conformations, the Nα – in the presence of malonyl-CoA - or the Nβ – in the absence of it (Samanta, Situ, and Ulmer 2014). Depending on malonyl-CoA binding, CPT1C can change its conformation, although this change might not be crucial for ABHD6 interaction. We postulate that this conformational change does not affect the interaction between the two proteins, but could be the cause of ABHD6 inhibition. However, more studies need to be addressed using N-terminal mutated forms of CPT1C to elucidate whether ABHD6 activity regulation by CPT1C is N-terminal dependent.

Since CPT1C modifies ABHD6 activity, we decided to measure CB_1R activation by detecting cAMP intracellular levels. When CB_1R is activated by elevated 2-AG levels, intracellular cAMP levels decrease (Turu and Hunyady 2010). Thus, cells expressing CB_1R and ABHD6 treated with 2-AG had increased cAMP levels whereas expression of CPT1C decreased intracellular cAMP. Its levels were also regulated by malonyl-CoA. ABHD6 not only controls the availability of 2-AG that reaches the pre-synaptic CB_1R but also 2-AG levels in the post-synaptic terminal, which deactivate $GABA_AR$, involved in the control of epilepsies. Moreover, ABHD6 is able to hydrolyse other monoacylglycerols, such as the BMP in the late endosomes/lysosomes (Cao, Kaplan, and Stella 2019). These results suggest that CPT1C could act as a sensor of malonyl-CoA able to modify ABHD6 physiological responses.

In the cAMP assays, not only CB_1R response regulated cAMP levels, but also the ARA produced in the hydrolysis of 2-AG. ARA is transformed to prostaglandins by COX-1/2 and

they act on G-coupled receptors, whose signalling is through cAMP pathway (Hata and Breyer 2004). Thus, ARA could be modulating cAMP by CB_1R-independent pathways.

Considering these results, we wondered whether CPT1C negative regulation of ABHD6 activity was the cause of dysregulated hypothalamic eCBs levels in CPT1C-KO mice and, consequently, impaired BAT thermogenesis. We showed that hypothalamic homogenates of CPT1C-KO mice had increased hydrolase activity and, consequently, we expected to find a decrease in eCBs level. However, what we found was that CPT1C-KO mice had markedly increased 2-AG levels (4 times higher than WT mice) and that AEA levels were also increased. Considering the fact that ABHD6 is not the principal 2-AG hydrolase in the brain (Marrs et al. 2010), increased 2-AG and AEA levels in the hypothalamus of CPT1C-KO mice could be consequence of the activity of other enzymes of the ECS. The study of the gene expression pattern of eCBs synthesis and degrading enzymes in CPT1C-KO mice would add valuable information here. One possibility is that the CPT1C/ABHD6 complex can be modulating specific pools of 2-AG in the post-synaptic terminal upon a concrete stimulus. Then, the total hypothalamic eCBs levels might not be representative of CPT1C regulation of ABHD6. In addition, the CPT1C-KO mice phenotype is closer to a genetic obese model, such as *ob/ob* or *db/db* (Balsevich et al. 2018; Di Marzo et al. 2001) than to a DIO mice. Obese genetic models have increased hypothalamic eCBs levels, mainly due to an impaired leptin signalling, and CPT1C-KO mice under HFD feeding presents disrupted leptin signalling. Hence, we conclude that the increase in hypothalamic eCBs levels of CPT1C-KO mice appears not to be the result of disrupted CPT1C control of ABHD6 activity.

Our next question was, what functions of AHBD6 might be regulated by CPT1C and malonyl-CoA? In 2016 it was demonstrated that ABHD6 in the VMH is important for a correct metabolic adaptation in challenging situations such as cold, fasting or HFD (Fisette et al. 2016). Conditional ABHD6-VMH-KO mice showed decreased cold-induced BAT thermogenesis and HFD-induced energy expenditure resulting in an increased body weight. Our results from chapter I, demonstrate that CPT1C also plays a key role in the regulation of BAT thermogenesis and body weight in the VMH and it is dependent on AMPK-malonyl-CoA pathway. Moreover, previous results of our group demonstrated that CPT1C in the MBH was necessary for a proper adaptation to fasting (Pozo et al. 2017). These findings lead us to suggest that CPT1C sensing of malonyl-CoA would be necessary for the regulation of ABHD6 response to metabolic challenges, such as fasting and HFD, and consequently to the modulation of 2-AG levels. Thus, AMPK-CPT1C-ABHD6 axis within

the VMH might not be discarded as a regulator of metabolic flexibility although more experiments are needed to prove this hypothesis.

Then, we wondered which other functions of ABHD6 could be regulated by CPT1C and different possibilities should be considered. Firstly, both CPT1C (Fadó et al. 2015; Gratacacós-Batlle et al. 2015) and ABHD6 (Wei et al. 2016) regulate the trafficking of the subunit GluA1 of the AMPAR. However, GluA1 regulation is independent of ABHD6 activity. This suggests that, even though CPT1C and ABHD6 are part of the AMPAR complex, both partners might be controlling GluA1 trafficking in an independent manner (Wei et al. 2016). Secondly, the group of Robert Zimmerman has reported that ABHD6 is also a hydrolase of the BMP in brain and liver, accounting for 50 and 90% of BMP hydrolysis, respectively (Pribasnig et al. 2015). BMP is enriched in late endosomes/lysosomes where it plays a key role in the formation of ILVs and endosomes cargo delivery. BMP accumulation leads to lysosomal lipid disorders and therefore its levels needs to be tightly regulated (Schulze and Sandhoff 2011). Recently, the same group has demonstrated that both BMP levels and its lipid content are altered after a HFD administration in liver and plasma (Grabner et al. 2019). In addition, data not published of our group indicate a role for CPT1C in late endosomal trafficking dependent on malonyl-CoA sensing. Hence, the CPT1C control of ABHD6 activity could regulate BMP levels in late endosomes, by indicating to the cell when is energetically necessary to deliver the late endosomes cargo. Finally, blocking ABHD6 activity have emerged as a therapeutic target to treat epilepsies (Naydenov et al. 2014; Sigel et al. 2011). ABHD6 is able to control 2-AG availability to $GABA_AR$, which inhibits seizures development. Therefore, it would be interesting to study the role of CPT1C as a natural ABHD6 inhibitor in seizures.

Altogether, this thesis has defined for the first time a link between CPT1C and the ECS. Both systems at the hypothalamus are crucial regulators of BAT thermogenesis and DIO. We have demonstrated that CPT1C in the VMH is a key sensor of energy status necessary for the regulation of BAT thermogenesis and body weight gain (**Figure 22, Chapter I**). On the other hand, we have described a crosstalk between BAT thermogenesis and endocannabinoids in the hypothalamus at early stages of DIO in male and female mice (**Figure 22, Chapter II**). Finally, we have characterized CPT1C as the first ABHD6 physiological inhibitor (**Figure 22, Chapter III**). Overall, our results have contributed to a better understanding of CPT1C functions and the molecular mechanisms underlying energy homeostasis.

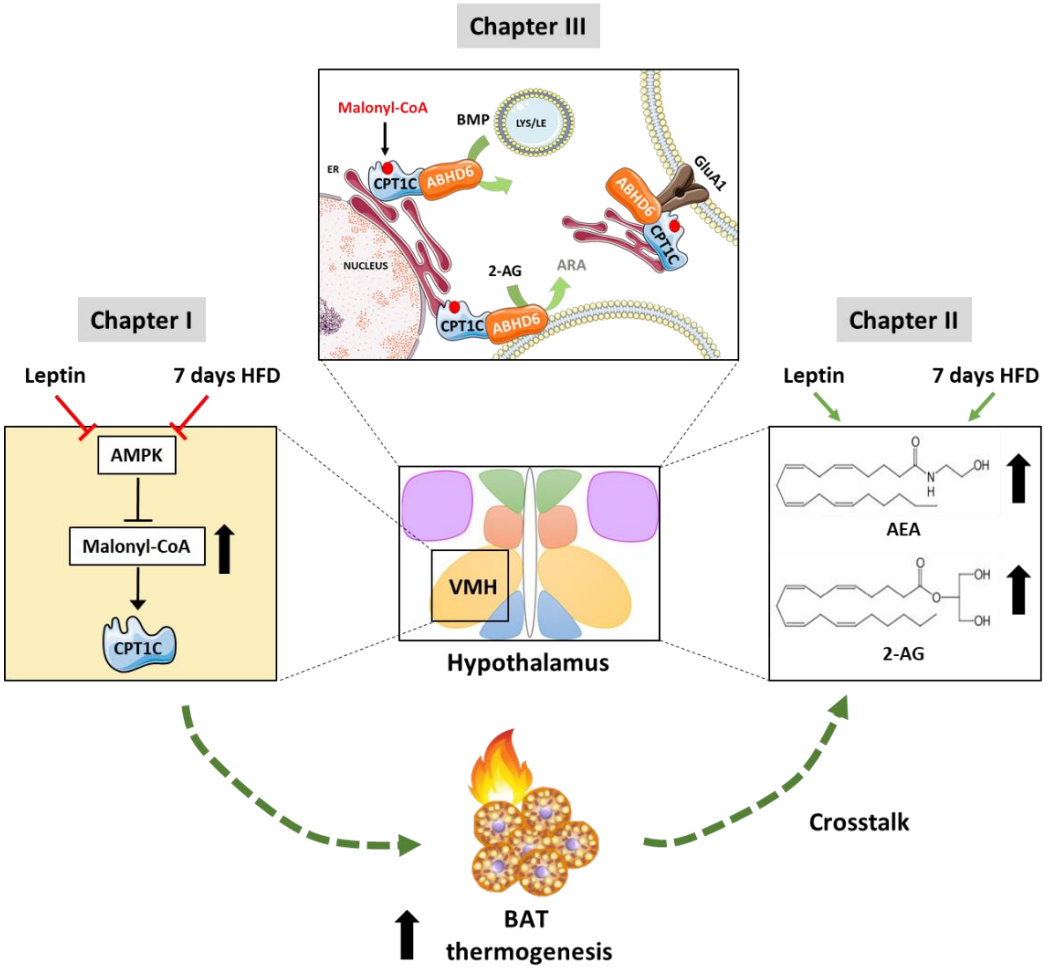

Figure 22. Chapter I demonstrated that CPT1C in the VMH nucleus of the hypothalamus is a key regulator of BAT thermogenesis when mice were challenged with leptin or HFD administration. CPT1C is able to sense malonyl-CoA levels regulated by the upstream AMPK signalling. Chapter II demonstrated that short-term HFD, acute leptin administration and BAT thermogenesis activation increases eCBs levels at the hypothalamus. Moreover, it describes a crosstalk from BAT thermogenesis to hypothalamic ECS. Finally, Chapter III defined CPT1C as the first negative regulator of ABHD6 activity with putative physiological roles in the ECS, late endosome function, and GluA1 trafficking and cognition. ER = endoplasmic reticulum and LYS/LE = Lysosome/Late endosome.

CONCLUSIONS

1. CPT1C-KO mice present an attenuated brown fat thermogenesis activation after short-term high-fat diet feeding or central leptin administration.

2. CPT1C in the VMH is necessary for metabolic adaptation to short-term consumption of a high-fat diet.

3. CPT1C is a crucial factor in the canonical AMPK pathway in the VMH to regulate brown fat thermogenesis.

4. CPT1C-KO mice have dysregulated endocannabinoids levels in the hypothalamus and plasma.

5. Hypothalamic endocannabinoids positively correlate with brown fat thermogenesis activation, and negatively correlate with body weight, plasma leptin levels and circulating endocannabinoids.

6. Female mice have higher increase of endocannabinoids levels and brown fat thermogenesis than male mice after short-term high fat diet feeding.

7. Short-term administration of a high-fat diet induces a substantial increase of 2-AG and AEA in the hypothalamus accompanied by an upregulation on the expression of their synthesis enzymes.

8. Two different thermogenic activators, central leptin administration and peripheral stimulation of the β3-Adrenoreceptor, increase hypothalamic endocannabinoids, whereas central 2-AG and AEA administration does not stimulate BAT thermogenesis.

9. CPT1C is linked to the ECS by direct interaction with ABHD6 hydrolase, and this interaction is not dependent on malonyl-CoA binding or the C-terminus of CPT1C.

10. CPT1C negatively regulates ABHD6 activity depending on malonyl-CoA sensing in HEK-293T cells.

11. CPT1C is able to modulate CB_1R response in HEK-293T cells depending on malonyl-CoA sensing.

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ABBREVIATIONS

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1,2-DAG	1,2 diacylglycerol
2-AG	2-arachidonoylglycerol
4-MUH	4-methylumbelliferyl heptanoate
4-MU	4-methylumbelliferone
7-HC	7-hydroxylcoumarin
7-HCA	7-hydroxycoumarinyl-arachidonate
β3-AR	β3-adrenoreceptors

A

ABHD4	α/β-hydrolase domain containing 4
ABHD6	α/β-hydrolase domain containing 6
ABHD12	α/β-hydrolase domain containing 12
ABPP	Activity base protein profiling
AC	Adenylyl cyclase
ACC	Acetyl-CoA carboxylase
ACEA	Arachidonoyl-2'-chloroethylamide
AEA	Anandamide or arachidonoylethanolamide
AgRP	Agouti-related peptide
AHMMCE	Arachidonoyl 7-hydroxy-6-methoxy-4-methylcoumarin ester
AMPAR	α-amino-3-hydrozyl-5-methyl-4-isoxazole-propionate-type glutamate receptors
AMPK	AMP-activated protein kinase
ARA	Arachidonic acid
ARC	Arcuate nucleus of the hypothalamus
AVP	Vassopresin-releasing hormone

B

BAT	Brown adipose tissue
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
BMI	Body mass index
BMP	Bis(monoacylglycero)phosphate
BMP8B	Bone morphogenic protein 8B
BDNF	Brain-derived neurotrophic factor

C

cAMP	Cyclic adenosine monophosphate
CB_1R	Cannabinoid receptor type 1
CB_2R	Cannabinoids reveptor type 2
CNS	Central nervous system
CoA	Coenzyme A

COT	Carnitine octanoyltransferase
COX-1/2	Cyclooxygenase 1 and 2
CPT	Carnitine palmitoyltransferase
CrAT	Carnitine acetyltransferase
CRH	Corticotropin-releasing hormone
D	
DAGL	Diacylglycerol lipase
DHA	Docosahexaenoic acid
DIO	Diet-induced obesity
DMH	Dorsomedial nucleus of the hypothalamus
E	
eCB	Endocannabinoid
ECS	Endocannabinoid System
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
EV	Empty vector
F	
FA	Fatty acids
FAAH	Fatty acid amide hydrolase
FABP1	Fatty acid binding protein 1
FAO	Fatty acids oxidation
FAS	Fatty acid
FK	Forskolin
FRET	Fluorescence resonance energy transfer
G	
GLP-1	Glucagon-like-peptide-1 AMP-activated protein kinase
GPCR	G-protein coupled receptor
GSIS	Glucose-stimulated insulin secretion
H	
HCD	High carbohydrate diet
HFD	High fat diet
hMSC	Human mesenchymal stem cells
HSP	Hereditary spastic paraplegia
HTRF	Homogenous time-resolved fluorescence
I	
icv	Intracerebroventricular

ILV	Intraluminal vesicles
IMM	Mitochondrial inner-membrane
ip	Intraperitoneal
IR	Insulin receptor
K	
KO	Knock-out
L	
LD	Lipid droplets
LepR	Leptin receptor
LHA	Lateral hypothalamic area
LCFA	Long chain fatty acid
LC/MS	Liquid chromatography-mass spectrometry
M	
MAG	Monoacylglycerol
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
Mc4R	Melanocortin 4 receptor
MCD	Malonyl-CoA decarboxylase
MCH	Melanin-concentrating hormone
Munc13-1	Mammalian Uc13-1 protein
N	
NAAA	N-acylethanolamide-hydrolyzing acid amidase
NAPE	N-arachidonoylphosphatidylethanolamine
NAT	N-acyltransferase
NE	Norepinephrine
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
O	
OEA	Oleoyl ethanolamide
OMM	Outer membrane of the mitochondria
OXT	*Oxytocin-releasing hormone*
P	
PEA	Palmitoyl-ethanolamine
PGE	Prostaglandin glycerol ester
PI	Phosphatidylinositol
PLC	Phospholipase C

PLD	Phospholipase D
PNS	Peripheral Nervous System
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptors
PUFA	Polyunsaturated fatty acids
PVH	Paraventricular nucleus of the hypothalamus
S	
SAG	1-stearoyl-2-arachidonoyl-sn-glycerol
SD	Standard diet
SCFA	Short-chain fatty acid
SF-1	Steroidogenic factor-1
Sim-1	Single-minded homolog 1
SNS	Sympathetic nervous system
T	
TH	Thyroid hormone
THC	Δ^9-tetrahyrocannabinol
TRPV1	Transient receptor potential vanilloid 1
U	
UCP1	Uncoupling protein 1
V	
VMH	Ventromedial nucleus of the hypothalamus
W	
WAT	White adipose tissue
WHO	Wold Health Organization
WT	Wild type

APPENDIX I

Supplementary figure

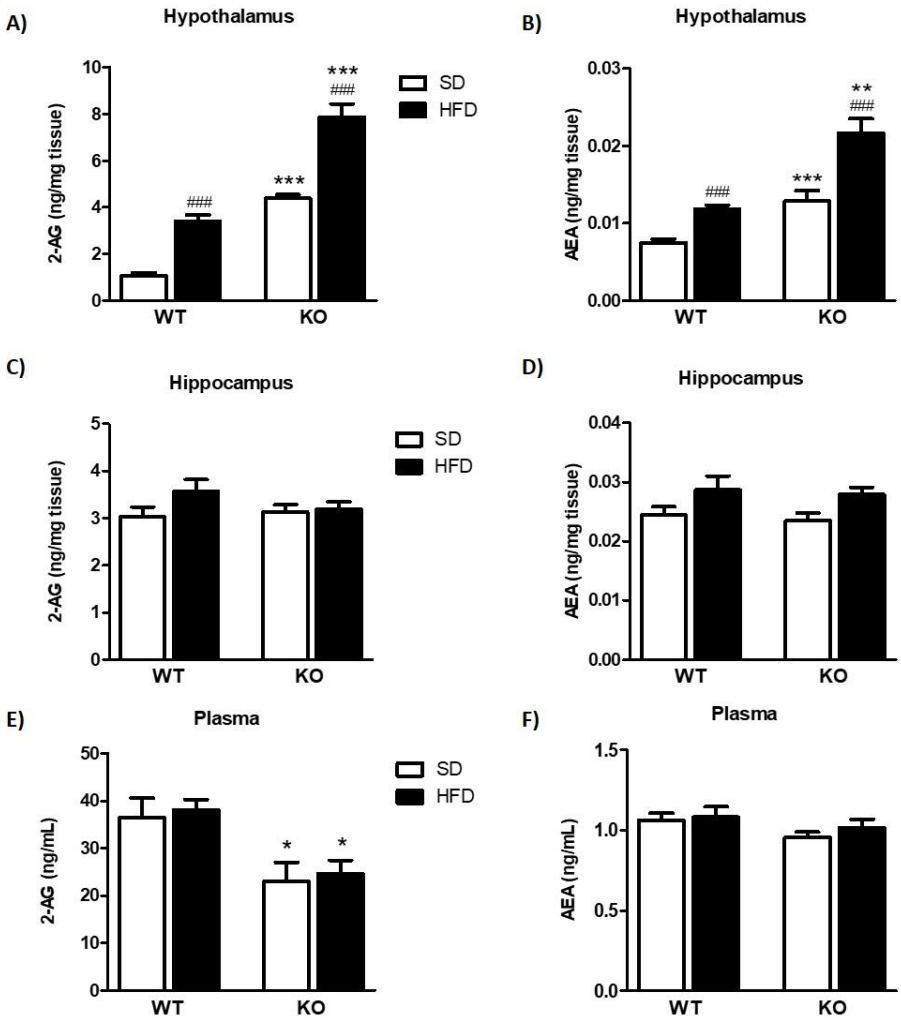

Figure 23. eCBs levels in hypothalamus, hippocampus and plasma of male WT and CPT1C-KO mice fed a SD or HFD for 7 days. CPT1C-KO mice had increased 2-AG (A) and AEA (B) levels in the hypothalamus when fed a SD. This 2-AG (A) and AEA (B) increase is more dramatic when fed a HFD in both WT and CPT1C-KO mice. Hippocampal 2-AG (C) and AEA (D) were not affected by neither CPT1C deficiency nor diet treatment. Plasma 2-AG (E) is decreased in CPT1C-KO mice whereas AEA (F) in not affected. HFD during 7 days of feeding did not altered 2-AG (E) or AEA (F) levels in plasma. Error bars represent SEM (n=6-10). Statistical analysis carried out by two-way ANOVA were *p<0.05, **p<0.01, ***p<0.001 versus WT and ###p<0.001 versus SD.

APPENDIX II

Publications

Original Article

MOLECULAR METABOLISM

CPT1C in the ventromedial nucleus of the hypothalamus is necessary for brown fat thermogenesis activation in obesity

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ABSTRACT

Objective: Carnitine palmitoyltransferase 1C (CPT1C) is implicated in central regulation of energy homeostasis. Our aim was to investigate whether CPT1C in the ventromedial nucleus of the hypothalamus (VMH) is involved in the activation of brown adipose tissue (BAT) thermogenesis in the early stages of diet-induced obesity.

Methods: CPT1C KO and wild type (WT) mice were exposed to short-term high-fat (HF) diet feeding or to intracerebroventricular leptin administration and BAT thermogenesis activation was evaluated. Body weight, adiposity, food intake, and leptinemia were also assayed.

Results: Under 7 days of HF diet, WT mice showed a maximum activation peak of BAT thermogenesis that counteracted obesity development, whereas this activation was impaired in CPT1C KO mice. KO animals evidenced higher body weight, adiposity, hyperleptinemia, ER stress, and disrupted hypothalamic leptin signaling. Leptin-induced BAT thermogenesis was abolished in KO mice. These results indicate an earlier onset leptin resistance in CPT1C KO mice. Since AMPK in the VMH is crucial in the regulation of BAT thermogenesis, we analyzed if CPT1C was a downstream factor of this pathway. Genetic inactivation of AMPK within the VMH was unable to induce BAT thermogenesis and body weight loss in KO mice, indicating that CPT1C is likely downstream AMPK in the central mechanism modulating thermogenesis within the VMH. Quite opposite, the expression of CPT1C in the VMH restored the phenotype.

Conclusion: CPT1C is necessary for the activation of BAT thermogenesis driven by leptin, HF diet exposure, and AMPK inhibition within the VMH. This study underscores the importance of CPT1C in the activation of BAT thermogenesis to counteract diet-induced obesity.

Keywords CPT1C; Hypothalamus; Thermogenesis; Brown adipose tissue; Diet-induced obesity

1. INTRODUCTION

Obesity is ultimately the result of a sustained imbalance between energy intake and energy expenditure. A key mechanism to maintain body weight homeostasis against an overload of energy is diet-induced thermogenesis [1,2]. Brown adipose tissue (BAT) is considered a major site for the regulation of diet-induced thermogenesis through the sympathetic nervous system (SNS), and it is precisely orchestrated by the hypothalamus [3,4]. In fact, an intact hypothalamic function will ensure a fine-tune activation of BAT thermogenesis in response to short-term high fat (HF) diet or leptin to counteract excessive body weight gain [2,5,6]. Despite this evidence, to date, little is known about the exact molecular hypothalamic pathways regulating thermogenesis under conditions of nutrient surplus [7]. In light of the current obesity epidemic, identification of the hypothalamic pathways and potential targets mediating short-term activation of thermogenesis in response to nutritional status would provide valuable information about obesity development and progression [2,7–9].

Recent findings have demonstrated that hypothalamic AMPK is a major regulator of BAT thermogenesis through its modulation of the SNS

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[3,10]. Particularly, it has revealed AMPK activity in the ventromedial nucleus of the hypothalamus (VMH) on thermogenic response. Remarkably, selective inactivation of AMPK within the VMH increased ventral hypothalamic malonyl-CoA levels and BAT activity and promoted weight loss, in a feeding-independent manner [10,11]. Although this pathway constitutes a canonical circuit that mediates the effect of several thermogenic molecules (*e.g.* T3 or leptin) [3,10,12], further studies are necessary to explore the sub-cellular mechanisms and neuronal networks involved in the AMPK(VMH)-SNS-BAT axis. In this regard, recent data have demonstrated that selective ablation of the isoform AMPKα1 in steroidogenic factor 1 (SF1) neurons of the VMH promotes BAT activation and subsequently a leaner, feeding-independent and obese-resistant phenotype [12,13].

The acetyl-CoA (ACC)/malonyl-CoA pathway is one of the most important signaling pathways downstream AMPK [14]. Within the hypothalamus, malonyl-CoA levels fluctuate in response to the nutritional status, acting as a canonical signal of energy surplus [15,16]. Malonyl-CoA is the physiological inhibitor of carnitine palmitoyltransferase 1 (CPT1) enzymes, which catalyze the transport of long chain fatty acids into the mitochondria [16]. Among CPT1s, the neuron-specific CPT1C isoform is the most puzzling carnitine acyltransferase [17,18]. In contrast to the canonical isoforms (CPT1A and CPT1B), CPT1C is located in the endoplasmic reticulum (ER) of neurons, instead of the mitochondrial membrane, and has insignificant CPT1 activity [19]. Nevertheless, it is still able to bind malonyl-CoA with similar affinity than CPT1A [20], suggesting that CPT1C could act as a sensor of this lipid intermediary in the hypothalamus [16].

The expression of CPT1C in the brain has been found particularly high in neurons of hypothalamic areas involved in the regulation of feeding and energy expenditure including arcuate nucleus (ARC), paraventricular hypothalamus (PVH) and VMH [17,21]. Studies from our group and others have demonstrated that CPT1C within these areas plays a major role in the modulation of energy balance. For example, hypothalamic CPT1C mediates that central effects of leptin and ghrelin on feeding behavior [22,23]. Hypothalamic CPT1C also determines fuel selection and food preference during fasting [24,25]. Moreover, CPT1C KO mice are more prone to become obese when chronically fed a HF diet with a reduced peripheral fatty acid oxidation [20,21,26,27]. In these studies, the expression of CPT1C, especially in the mediobasal hypothalamus (MBH) was found to be crucial in mediating the effects in energy homeostasis [21,24]. However, the possible role of CPT1C in the hypothalamic regulation of BAT thermogenesis is totally unknown. Here, we show that the obese phenotype and metabolic inflexibility that characterizes to CPT1C KO mice is related to an impaired BAT thermogenesis following a short-term HF diet exposure and central leptin injection. We also demonstrate that the lack of CPT1C disrupts the canonical pathway of AMPK(VMH)-SNS-BAT-mediated thermogenesis. Our data thus uncover CPT1C as a key downstream factor of the hypothalamic AMPK/ACC pathway in the control of brown fat thermogenesis.

2. MATERIALS AND METHODS

2.1. Animals

Male (8–10 week old) CPT1C KO mice and their wild-type (WT) littermates with the same genetic background (C57BL/6J) were used for the experiments [24]. All animals were housed on a 12 h/12 h light/dark cycle (light on at 8 am, light off at 8 pm) in a temperature- and humidity-controlled room. The animals were allowed free access to water and standard laboratory chow, unless otherwise specified. For HF diet studies, animals were placed on an HF diet (60% kcal from fat, D12492) or standard diet (SD) (10% kcal from fat, D12450B, Research Diets, New Brunswick, USA) for 3, 7, or 14 days. At the end of the studies, animals were sacrificed and tissues collected for further molecular and biochemical analysis as further detailed. All animal procedures were performed in agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Local Ethical Committee (Procedure ref. 9606 from the Generalitat de Catalunya).

2.2. Intracerebroventricular administration of leptin

Chronic cannulae were stereotaxically implanted into the lateral cerebral ventricle under ketamine/xylazine intraperitoneal anesthesia (ketamine 75 mg/kg body weight plus xylazine 10 mg/kg body weight). The coordinates were 0.58 mm posterior to Bregma, 1 mm lateral to the midsagittal suture, and 2.2 mm deep. Mice were individually caged and allowed to recover for 5 days before the experiment. Prior to the experiment, cannula placement was verified by a positive dipsogenic response to angiotensin II (1 nmol in 1 ml; Sigma–Aldrich). On experimental day, WT and CPT1C KO mice received an intracerebroventricular (ICV) administration of 2 μl of either leptin (0.1 μg/μl) (PeproTech, London, UK) or vehicle (aqueous buffer containing 0.1% BSA), 3 h after lights-on. 200 min after the injection, mice were sacrificed by cervical dislocation and MBH and BAT were collected for further analysis.

2.3. Stereotaxic microinjection and viral vectors

The lentiviral vectors pWPI-IRES-GFP, and pWPI-CPT1C-IRES-GFP were produced and titrated as previously described [24]. In addition, a lentiviral vector with a mutated isoform of CPT1C insensitive to malonyl-CoA, pWPI-CPT1CM589S-IRES-GFP, was produced. Mouse malonyl-CoA CPT1C sensitive site was identified by sequence homology with CPT1A. The homologous mutation in CPT1A (M593S) abolishes malonyl-CoA sensitivity while maintaining CPT1 activity [28]. CPT1C mutant M589S was constructed using the Q5 Site-Directed mutagenesis procedure (New England BioLabs) with the pWPI-IRES-CPT1C plasmid as template. The primers were obtained from the online design software NEBaseChanger and designed with 5′ ends phosphorylated and annealing back-to-back: forward 5′-GAGTCAGCCAGTACCCGACTGTTC-3′ and reverse 5′-ATAAGTCAGGCAGAATTGAC-3′ (the mutated nucleotide were underlined). The appropriate substitutions as well as the absence of unwanted mutations were confirmed by sequencing the inserts in both directions.

Adenoviral vectors (GFP and AMPKα1-dominant negative + AMPKα2-dominant negative, AMPK-*DN*; Viraquest; North Liberty, IA, USA) were kindly provided by Dr. Miguel Lopez [12]. Stereotaxic surgery to target the VMH was performed in mice under ketamine/xylazine anesthesia. Purified lentivirus (1×10^9 pfu ml^{-1}) or adenovirus (1×10^{12} pfu ml^{-1}) in artificial cerebrospinal fluid were injected bilaterally in the VMH over 10 min through a 33-gauge injector connected to a Hamilton Syringe and an infusion pump (0.5 μl per injection site) [24]. The injections were directed to the following stereotaxic coordinates: 1.6 mm posterior from Bregma, $\pm$0.4 mm lateral to midline, and 5.6 mm deep. Mice underwent 6 days (adenovirus) or 7 days (lentivirus) of recovery before other experiments were performed. Correct bilateral infection was confirmed by western blot and histologically by GFP fluorescence in brain slices.

2.4. BAT temperature measurements

Skin temperature surrounding BAT was visualized using a high-resolution infrared camera (FLIR Systems) and analyzed with a specific software package (FLIR-Tools-Software, FLIR; Kent, UK), as previously described [12]. For ICV administration of leptin, images were

recorded and analyzed every 10 min during 220 min. For the rest of experiments, thermal images were acquired the day of sacrifice.

2.5. Sample collection and processing

Mice were killed by cervical dislocation. For each animal, either the whole brain (for histology) or the MBH, as well as blood (for plasmatic determinations), liver, interscapular BAT, visceral and subcutaneous WAT were collected, weighed, and stored at −80 °C until further processing. To dissect the MBH, brains were placed in a coronal brain matrix and sectioned from Bregma (−1 to −2.5 mm) and the MBH was obtained using a tissue collector measuring 1 mm in diameter.

2.6. Plasma analysis

Plasma was obtained after blood centrifugation (2000 g, 15 min). Plasma levels of leptin were determined by mouse ELISA kit (Crystal Chem, Zaandam, Netherlands), following the manufacturer's instructions.

2.7. Tissue morphology

Interscapular BAT and visceral and subcutaneous WAT were fixed overnight in 10% PBS-buffered formalin. Histological samples were paraffin-embedded and stained with hematoxylin and eosin (H&E), as previously described [29]. Tissue sections were captured by light microscopy (Olympus, Hamburg, Germany) at 20X magnification and using NIS-Elements software (Nikon, Japan).

2.8. Liver triglycerides (TG) quantification

Liver samples were homogenized, and lipids were extracted as previously described [30]. TG were measured in the lipid extract using a commercial kit (Sigma, Madrid, Spain), following the manufacturer's instructions.

2.9. RNA preparation and quantitative RT-PCR

Total RNA was extracted from tissues using Trizol Reagent (Fisher Scientific, Madrid, Spain). Retrotranscription and quantitative RT-PCR (qPCR) was performed as previously described [24]. Proprietary SYBR Green or Taqman Gene Expression assay primers used (IDT DNA Technologies, Leuven, Belgium) are detailed in the supplementary material (Table S.I). Relative mRNA levels were measured using the CFX96 Real-time System, C1000 Thermal Cycler (BioRad).

2.10. Western blotting

Western blot was performed as previously described [24]. Briefly, tissue was homogenized in RIPA buffer (Sigma−Aldrich, Madrid, Spain) containing protease and phosphatase inhibitor cocktails. Protein extracts were separated on SDS-PAGE, transferred into Immobilion-PVDF membranes (Merck Millipore, Madrid, Spain) and probed with antibodies against: ACC, AMPKα, pACC (Ser79), pAMPKα (Thr172), pSTAT3 (Tyr705) (Cell Signaling; Danvers, MA, USA); GAPDH, UCP1 (Abcam, Cambridge, UK) β-actin (Fisher Scientific, Madrid, Spain) and α-tubulin (Sigma, Madrid, Spain). Each membrane was then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody, anti-mouse or anti-rabbit (DAKO, Glostrup, Denmark), and developed using Luminata-Forte Western HRP substrate (Merck Millipore). Images were collected by GeneTools software (Syngene, Cambridge, UK) and quantified by densitometry using ImageJ-1.33 software (NIH, Bethesda, MD, USA). GAPDH or β-actin was used as an endogenous control to normalize protein expression levels. In all the figures showing images of gels, all the bands for each picture come from the same gel, although they may be spliced for clarification.

2.11. Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was conducted using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Statistical analysis was determined by ANOVA (more than 2 groups were compared) followed of post hoc two-tailed Bonferroni test. $P < 0.05$ was considered significant. The number of animals used in each experiment is specified in each figure legend.

3. RESULTS

3.1. CPT1C KO mice show impaired diet-induced thermogenesis

The induction of thermogenesis in the interscapular BAT of mice was first analyzed after short-term exposure to HF diet, compared to SD. To identify the maximum activation peak of diet-induced thermogenesis over time, experiments were performed in response to 3, 7, and 14 days HF diet feeding (Figure 1 and Fig. S.1). The peak in the thermogenesis was reached in interscapular temperature (Figure 1A), gene expression of thermogenic markers (Figure 1B−D, Fig. S.1A) and UCP1 protein expression (Figure 1E) in the BAT of WT mice after 7 days of HF diet when compared to other timings and SD. Of note, HF feeding over 7 days resulted in significantly ameliorated responses in CPT1C KO mice, when compared to WT mice (Figure 1A−E).

In WT mice, a significant increase in body weight gain was not appreciated until 14 days of administration of a HF diet (Figure 2A). In contrast, CPT1C KO mice already revealed higher body and visceral WAT weight over 7 days of HF diet compared to SD (Figure 2A,B; Fig. S.2A). The induction of thermogenesis and body weight gain of WT and CPT1 KO mice were associated with a reduction in the size of unilocular lipid droplets observed after 7 days of HF diet feeding in histological sections of BAT (Figure 2C). As illustrated in Figure 2D, HF feeding over 7 days resulted in a substantial increase of leptin levels in plasma of CPT1C KO mice, whereas these levels remained unaltered in WT mice. Altogether, these data indicate that CPT1C KO mice show an earlier obesogenic phenotype in response to acute HF diet administration, likely due to an impaired activation of BAT thermogenesis, compared to WT. This is also supported by the fact that food intake, measured during 7 and 14 days of HF feeding, was comparable in the WT and KO mice (Fig. S.2B), indicating that the obesogenic phenotype observed in CPT1C KO mice is not due to alterations in food intake.

3.2. The thermogenic response to central leptin is impaired in CPT1C KO mice

In another set of experiments, activation of BAT thermogenesis was also evaluated after central administration of leptin (Figure 3). We found an increase of BAT interscapular temperature by central leptin that was maintained for at least 3 h in WT mice (Figure 3A,C). This effect was confirmed by an increase in gene expression of thermogenic markers in BAT of WT (Figure 3D). However, these acute leptin-induced responses were significantly attenuated in CPT1C KO mice (Figure 3B−D).

3.3. CPT1C KO mice display an altered expression of hypothalamic leptin signaling markers and ER stress after short-term HF diet feeding

The hyperleptinemia after 7 days of HF diet administration and the impaired central leptin-induced thermogenesis observed in CPT1C KO mice suggest an earlier onset in the disruption of leptin signaling in these mice compared to WT. Therefore, the expression of proteins involved in leptin signaling in the MBH was evaluated. First, expression levels of pSTAT3 and SOCS3, important transcription factors in leptin signaling, were analyzed in MBH of WT and KO mice fed a HF diet for 7

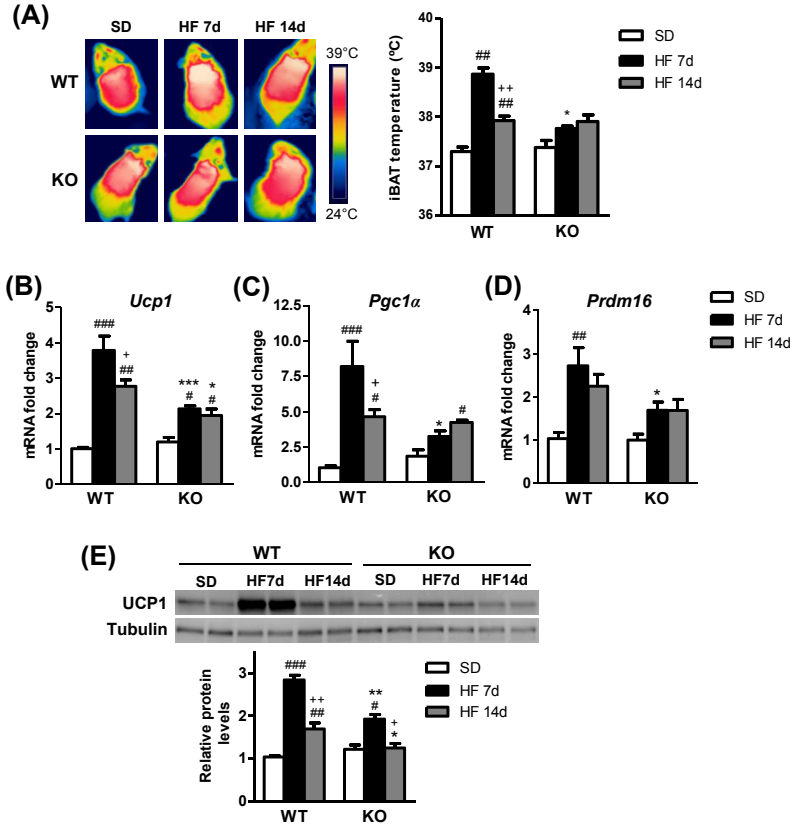

Figure 1: Impaired diet-induced thermogenesis in CPT1C KO mice. (A) Representative infrared thermal images and quantification of interscapular temperature adjacent to the BAT depot of WT and CPT1C KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 and 14 days. (B–D) Relative mRNA expression of the thermogenic markers UCP1 (B), PGC1α (C) and PRDM16 (D) in BAT of WT and KO mice fed SD or HF diet. (E) Protein levels of UCP1 in BAT of WT and KO fed SD or HF diet for 7 and 14 days. Data are expressed as mean ± SEM (n = 5–9). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* WT with the same diet; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* SD within the same genotype; $^{+}P < 0.05$, $^{++}P < 0.01$ *versus* HF 7d within the same genotype.

days. MBH of WT mice showed a reduced phosphorylation of STAT3 (Figure 4A) and increased mRNA levels of SOCS3 (Figure 4B) with HF diet compared to SD. Conversely, CPT1C KO mice fed a HF diet exhibited a substantial increase in pSTAT3 without changes in SOCS3 (Figure 4A,B).

Next, we evaluated the impact of HF and/or CPT1C ablation on the AMPK signaling in the MBH. No significant alterations in the expression of pAMPK and pACC were detected in either genotype after 7 days of HF diet exposure (Figure 4C). Remarkably, the leptin-induced inhibition of pAMPK in the MBH was suppressed in CPT1C KO mice, indicating that a normal CPT1C function is required for a normal leptin hypothalamic signaling (Figure 4D).

Finally, since CPT1C is located in the ER, and hypothalamic ER stress has been strongly related to leptin signaling disruption and obesity, as well as on the central control of thermogenesis [31–33], ER stress markers were analyzed in MBH. Whereas no changes in mRNA expression levels for ER stress markers were appreciated in WT mice-fed HF diet for 7 days, significant increases were shown in MBH of CPT1C KO mice (Figure 4E–G), in keeping with their impaired thermogenic responses, leptin signaling, and more prone obese phenotype.

3.4. Expression of CPT1C in the VMH is enough to restore short-term diet-induced response in CPT1C KO mice

Due to the importance of CPT1C in the MBH region (which includes the VMH) in the regulation of energy homeostasis [21,24], and considering the crucial role of the VMH in the control of BAT thermogenesis [10], we evaluated if the expression of CPT1C in this hypothalamic area was able to restore the phenotype observed after short-term HF feeding in KO mice. Lentiviral vectors expressing CPT1C-GFP or empty vector (EV)-GFP were microinjected in the VMH of WT and CPT1C KO mice and, after 7 days, mice were fed SD or HF diet for 7 days (see experimental protocol illustrated in Figure 5A). Injection site was confirmed by direct fluorescence of GFP in brain sections or by CPT1C expression analysis by western blot in the MBH (Figure 5B). The expression of CPT1C in the VMH was enough to reverse the body weight gain (Figure 5C), the hyperleptinemia (Figure 5D) and the expression of gene thermogenic markers in BAT (Figure 5E) of KO mice fed a HF diet for 7 days. Stereotaxic injection of adeno-associated viruses expressing the EV-GFP or CPT1C in the VMH of CPT1C KO mice (see Supplementary Methods) also revealed a significant restoration of iBAT temperature and body weight gain in response to 7 days HF diet feeding (Fig. S.3).

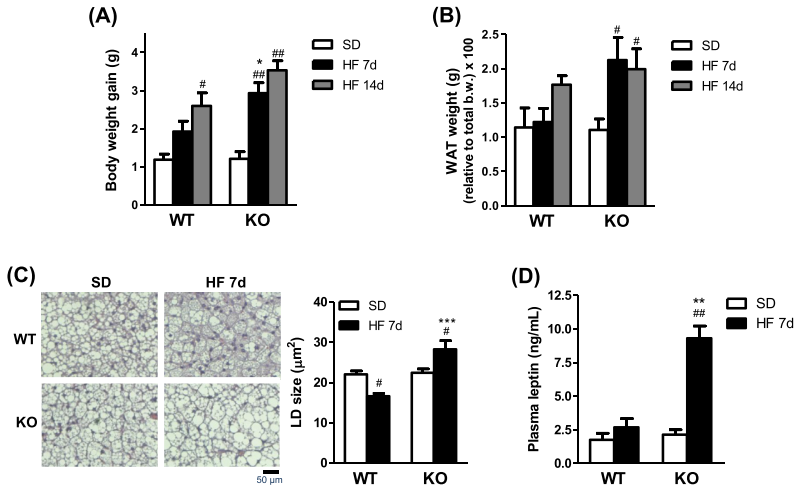

Figure 2: CPT1C KO mice show an earlier obesogenic phenotype compared to WT. (A and B) Body weight gain (A) and visceral WAT weight (B) of WT and KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 and 14 days. (C) Representative histological H&E staining and quantification of the unilocular lipid droplets (LD) size of interscapular BAT of WT and KO mice fed a SD or a HF diet for 7 days. (D) Plasma leptin levels of WT and KO mice fat a SD or HF diet for 7 days. Data are expressed as mean ± SEM (n = 5–7). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* WT with the same diet; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* SD within the same genotype.

These data confirm a key role of CPT1C in this hypothalamic area during diet-induced thermogenesis.

We also investigated the role of malonyl-CoA in the impaired hypothalamic function of CPT1C null mice. For this purpose, lentiviral vectors expressing a mutant CPT1C insensitive to malonyl-CoA (CPT1CM589S, see Methods), were used and compared to vectors expressing EV and CPT1C. Expression of the mutated isoform of CPT1CM589S in VMH of KO mice was not able to fully restore body weight gain (Figure 5C), leptinemia (Figure 5D), and expression of gene thermogenic markers in BAT (Figure 5E) in response to HF diet. These data indicate that malonyl-CoA sensing by CPT1C is relevant to regulate short-term diet-induced responses.

3.5. Selective inactivation of AMPKα in the VMH was not able to induce BAT thermogenesis and body weight loss in CPT1C KO mice

To assess whether CPT1C is a downstream factor in the AMPKα-mediated regulation of energy balance, we selectively inactivated AMPK in VMH of WT and KO mice by stereotaxic delivery of a dominant-negative AMPKα1+α2 isoforms (AMPK-*DN*) [11–13,34]. This inactivation was confirmed by reduced hypothalamic protein levels of pACC (Fig. S.4) [34]. Previous data demonstrated that AMPK-*DN* delivery into the VMH increased malonyl-CoA concentrations in the ventral hypothalamus, inducing weight loss and increased expression of BAT thermogenic markers, without altering food intake [11,12]. As illustrated in Figure 6, selective inactivation of AMPK in the VMH of WT mice involved a substantial reduction of body weight gain (Figure 6A) with a significant increase in interscapular temperature adjacent to the BAT depot (Figure 6B), elevated UCP1 protein expression levels (Figure 6C) and increased gene expression of thermogenic markers in BAT (Figure 6D). Notably, CPT1C KO mice showed a significant attenuation in all these parameters compared to WT mice (Figure 6A–D).

Recent data from our group have shown that the inhibition of AMPK in the VMH promotes decreased hepatic AMPK signaling through the vagus nerve and subsequently increased lipogenesis [12]. Our data showed that while in WT recapitulated that response, it was totally blunted in CPT1C KO mice (Figure 6E–F). Altogether, AMPK-*DN*-mediated effects within the VMH in body weight change, BAT and liver were impaired in mice lacking CPT1C. This is of importance because, it has been recently demonstrated that increased lipogenesis after VMH inhibition of AMPK is demanding for BAT thermogenesis. Therefore, CPT1C KO mice, which show impaired BAT function, also display altered associated liver responses.

4. DISCUSSION

Development of and progression to obesity are mediated by short-term neurological changes in response to nutritional status that progressively impair hypothalamic neuronal functions and therefore body weight regulation. In the last few years, several investigations have been directed towards the identification of proteins involved in the temporal dysregulation of neuronal functions to control aspects of energy balance beyond food intake, during the development of diet-induced obesity [2,7–9].

The present research demonstrates that the neuron-specific CPT1 isoform, CPT1C, plays a critical role in hypothalamic regulation of BAT thermogenesis, particularly in response to metabolic challenges activating BAT, such as short-term diet and central leptin. Considering the importance of the canonical pathway dependent on AMPK in the VMH to regulate BAT thermogenesis during the development of diet-induced obesity [10,12,13], our study also reveals that CPT1C might be a crucial factor in this canonical pathway.

Although CPT1C is still the most unknown CPT1, and its neuronal function is uncertain, our group and others have demonstrated its critical role in energy homeostasis [18]; also, it has been suggested to be a key indicator of the energetic status of neurons by sensing malonyl-CoA, a canonical signal of energy surplus [16,35]. The present study reveals that the obesogenic phenotype and acute alterations in metabolic flexibility already described in CPT1C KO mice [20,22–24] are related to impaired hypothalamic regulation of BAT

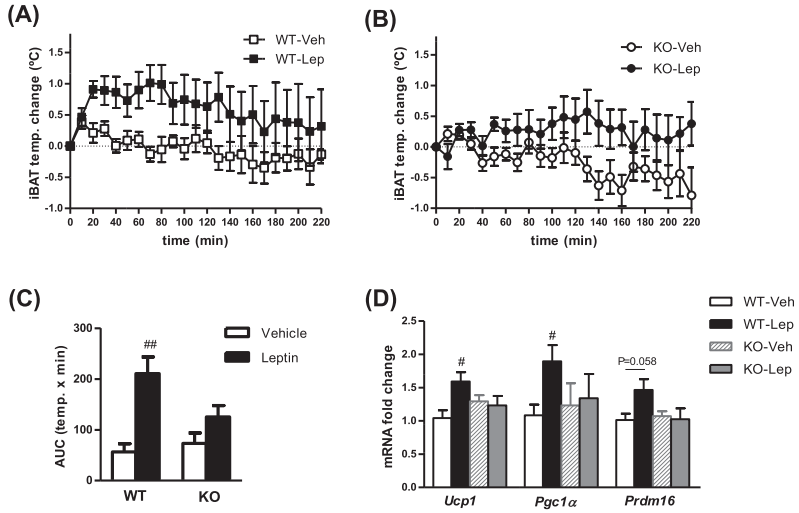

Figure 3: Impaired leptin-induced thermogenesis in CPT1C KO mice. (A–C) Quantification of interscapular temperature changes adjacent to the BAT depot (iBAT) after ICV leptin treatment in WT (A) and CPT1C KO mice (B) compared with ICV vehicle. (C) Area under the curve (AUC) of iBAT temperature during 220 min. (D) Gene expression analysis of thermogenic markers in BAT of WT and KO mice after ICV leptin. Data are expressed as mean ± SEM (n = 5–8). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* Vehicle within the same genotype.

thermogenesis, as exposed in response to short-term diet or central leptin administration. These metabolic challenges imply an increase in hypothalamic levels of malonyl-CoA [16,35,36] that need to be sensed by CPT1C. We show that, under short-term HF diet feeding (7 days), a robust activation peak of BAT thermogenesis was appreciated in WT mice, which helps mice to maintain normal body weight, adiposity and leptinemia, thus counteracting obesity development. Previous studies analyzing initial hypothalamic events during development of diet-induced obesity in mice [7–9,37] have also demonstrated that C57BL/6J mice show hypothalamic compensatory changes at early time points in response to HF diet (from 2 days to 7 days), but they may not be able to maintain them (from 14 days onwards). This could contribute to their obese phenotype after a prolonged period of HF diet. Our study describes a pronounced activation of BAT thermogenesis after 7 days of feeding a HF diet, which could be directly related to the short-term hypothalamic compensatory changes that have been previously described to counteract obesity. In contrast, HF feeding over 7 days resulted in a diminished activation of BAT thermogenesis, higher body weight gain, hyperleptinemia, and adiposity in CPT1C KO mice. This indicates that the lack of neuronal CPT1C determines an early obesogenic phenotype in response to fat-rich diets. In relation to this result, acute activation of BAT thermogenesis in response to central leptin administration was also attenuated in mice lacking CPT1C. These data could correlate with the fact that CPT1C KO mice are resistant to the satiety effect of central leptin (Fig. S.5 and [23]).

Development of obesity has been linked to increased plasma levels of leptin that positively correlate to high adiposity and body weight gain and an altered hypothalamic leptin signaling [38]. Evaluation of molecular mediators of leptin signaling during initial exposure to HF diets in MBH revealed that WT mice showed transient reduced levels of pSTAT3 with increased levels of SOCS3 levels after 7 days of HF diet. These results are in line with previous findings analyzing hypothalamic responses after short-term administration of fat-rich diets [8,9]. Transitory hypothalamic changes observed in WT animals could be more related to a compensatory response to the positive energy surplus that contributes to maintaining stable body weight during initial stages of fat-rich diets administration, as suggested by others [7–9]. In contrast to WT mice, CPT1C KO mice fed a HF diet for 7 days had increased hypothalamic pSTAT3 levels and unchanged expression of SOCS3. The opposite response in the hypothalamus of KO mice during initial stages of diet-induced obesity could indicate a lack of compensatory changes at early time points of HF diet feeding and therefore an earlier obesogenic phenotype.

In addition to these findings, MBH of mice deficient in CPT1C fed a HF diet showed significant increases in ER stress markers. The hypothesis that hypothalamic ER stress is causally linked with leptin resistance and obesity has gained substantial support in the recent years [39]. Although the exact mechanisms by which HF diet feeding can directly perturb hypothalamic neuronal function remain unclear, a number of investigations associate hypothalamic lipotoxicity derived from exposure to HF diet with ER stress as a possible explanation for the onset of obesity [31–33,40,41]. CPT1C is suggested to act as a sensor of hypothalamic malonyl-CoA levels fluctuations and also as a main regulator of the metabolism of complex lipids such as ceramides in neurons [18,20,42]. In addition, hypothalamic ER stress induced by lipotoxicity has been shown to impair the BAT thermogenic process [31–33]. A plausible hypothesis would be that the lack of CPT1C is determining an inaccurate lipid sensing, leading to hypothalamic lipotoxicity and subsequently ER stress, an idea that will require data to be confirmed.

It is known that the AMPK pathway is dysregulated in hypothalamus in obese states resulting from chronic HF feeding and that lack of dynamic responsiveness of this pathway is crucial in the pathophysiology of leptin resistance during diet-induced obesity [37]. In our study, administration of a HF diet during 7 days did not induce significant changes in pAMPK and pACC in MBH of WT or KO mice. This result agrees with previously reported data, showing that short-term administration of a HF diet (1–3 weeks) to rats did not modify hypothalamic AMPK phosphorylation [43,44]. Longer periods of HF feeding (from 3 weeks onwards) induced increased levels of the active

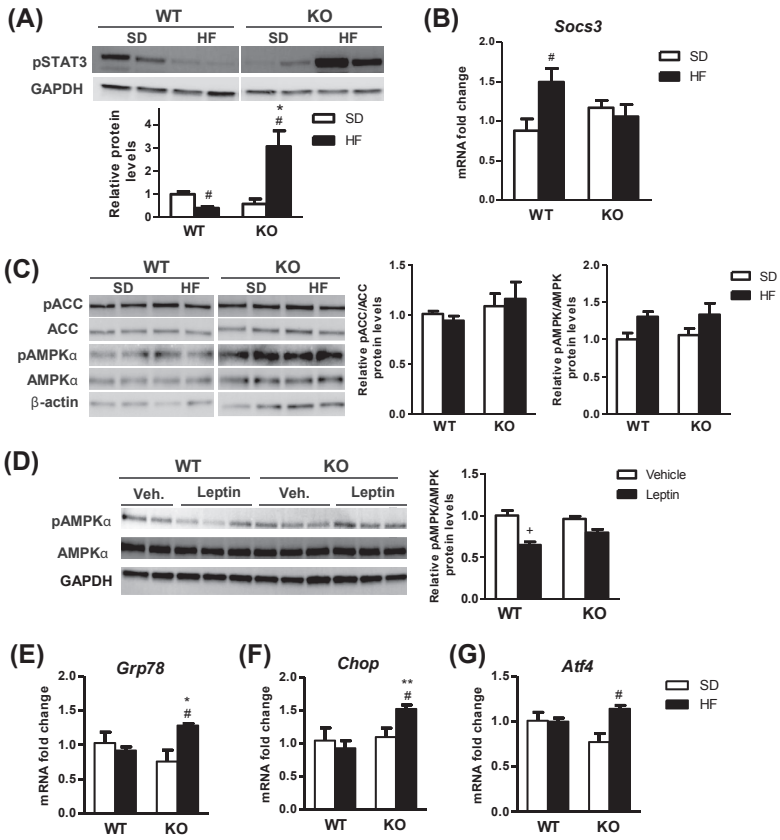

Figure 4: CPT1C KO mice show an altered expression of markers of leptin signaling and ER stress in the mediobasal hypothalamus after short-term administration of a HF diet. (A–C) Protein levels of pSTAT3 (A), mRNA levels of SOCS3 (B) and protein expression of pAMPKα, pACC, AMPK, and ACC (C) in the mediobasal hypothalamus of WT and CPT1C KO mice fed a standard diet (SD) or a high fat (HF) diet for 7 days. (D) Protein levels of pAMPKα and AMPK in the mediobasal hypothalamus of WT and CPT1C KO mice after ICV administration of leptin or vehicle. (E) mRNA levels of ER stress markers in the mediobasal hypothalamus of WT and CPT1C KO mice fed a SD or a HF diet for 7 days. Data are expressed as mean ± SEM (n = 5–7). $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* WT with the same diet; $^{\#}P < 0.05$ *versus* SD within the same genotype; $^{+}P < 0.01$ *versus* vehicle within the same genotype.

phosphorylated form of AMPK in the hypothalamus of rats [43] and mice [37], mediating the interplay between hypothalamic and peripheral response to diet. When analyzing central administration of leptin, we found a significant attenuation of pAMPK expression levels in MBH of WT mice after leptin injection, whereas this attenuation was not evidenced in CPT1C KO mice. Considering the findings that leptin has a role in SNS-mediated activation of BAT thermogenesis [45], and that inhibition of hypothalamic AMPK activity by leptin implies sympathetic activation to BAT and WAT [6], we suggest that the lack of changes in pAMPK in the MBH of CPT1C KO mice could be related to the impaired leptin-induced thermogenesis in these animals.

To further demonstrate if CPT1C, particularly in the VMH, is a factor involved in the AMPK-SNS-BAT axis, specific strategies were achieved in this study. Firstly, considering the importance of the VMH in the control of BAT thermogenesis [6,10], we showed that the lentiviral expression of CPT1C in the VMH was enough to restore the phenotype observed after short-term HF feeding in KO mice. In addition, the phenotype was not fully restored when expressing the mutated isoform of CPT1C insensitive to malonyl-CoA in the VMH. This result suggests that sensing malonyl-CoA by CPT1C is relevant to regulate short-term diet-induced responses in this hypothalamic area. Secondly, our virogenetic approaches showed that BAT thermogenesis and body weight of CPT1C KO mice did not respond to selective inactivation of AMPK in the VMH, indicating that CPT1C is a crucial factor in the AMPKα(VMH)-mediated regulation of BAT thermogenesis. Our data are in line with recent investigations proposing CPT1C as a downstream factor of AMPK in different hypothalamic nuclei to regulate feeding. A study from our group demonstrated the existence of a downstream pathway to SIRT1/p53/pAMPK axis in response to ghrelin, involving CPT1C, triggering acute changes in ceramide levels to regulate food intake by the modulation of NPY/AgRP expression in the ARC [22]. Interestingly, a recent investigation from Minokoshi's group [25] found that activation of an AMPK-CPT1C pathway in a subset of CRH-positive neurons in the PVH mediates the fasting-induced increase in high-carbohydrate diet selection. Our current investigation shows for the first time a role of CPT1C in the AMPK-brown fat axis to regulate thermogenic program in the VMH. These data suggest CPT1C as a downstream factor of hypothalamic AMPK to maintain energy

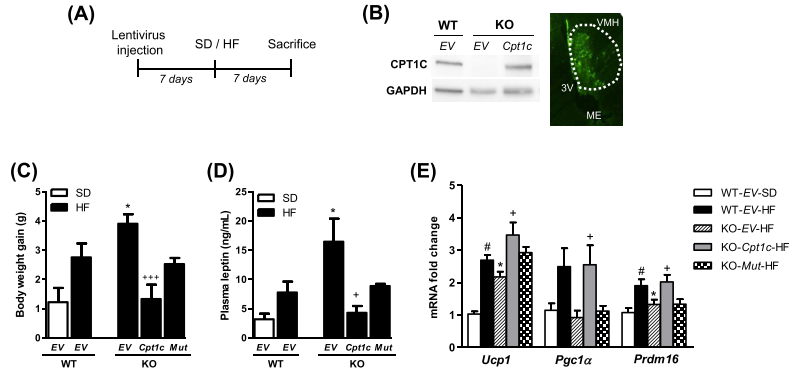

Figure 5: Expression of CPT1C in the VMH restores short-term diet-induced response in CPT1C KO mice. (A) GFP (empty vector, *EV*) or CPT1C-GFP (*Cpt1c*)-expressing lentiviruses were microinjected in the VMH of WT and CPT1C KO mice and after 1 week, mice were fed a standard diet (SD) or a high fat (HF) diet for 7 days. (B) Injection site was confirmed by direct fluorescence of GFP in brain sections or by CPT1C expression analysis by western blot in the ventral hypothalamus. (C–E) Body weight gain (C), plasma leptin (D) and gene expression analysis of thermogenic markers in BAT of WT-*EV*, KO-*EV*, and KO expressing CPT1C (KO-*Cpt1c*) or CPT1CM589S (KO-*Mut*) fed SD or HF diet for 7 days. Data are expressed as mean ± SEM (n = 6–8). $^{*}P < 0.05$ *versus* WT-*EV*-HF; $^{\#}P < 0.05$ *versus* WT-*EV*-SD; $^{+}P < 0.05$, $^{+++}P < 0.001$ *versus* KO-*EV*-HF.

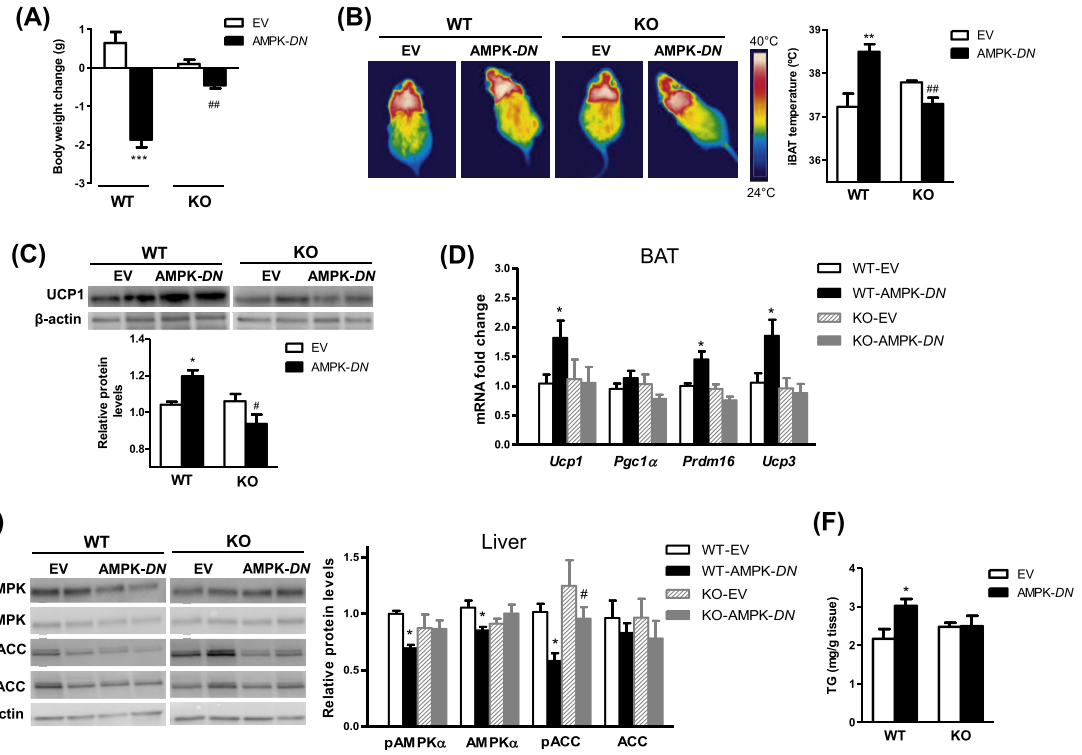

Figure 6: CPT1C KO mice show impaired AMPK-mediated effects within the VMH on body weight change, BAT thermogenesis and liver. (A) Body weight change of WT and CPT1C KO mice treated with adenoviruses encoding GFP (Empty vector, EV) or AMPK-*DN* in the VMH. (B–D) Representative infrared thermal images and quantification of interscapular temperature adjacent to the BAT depot (B), protein levels of UCP1 in BAT (C) and gene expression analysis of thermogenic markers in BAT of WT and KO mice treated with EV or AMPK-*DN* in the VMH (D). (E and F) Protein levels of the AMPK pathway (E) and TG levels in the liver of mice treated with EV or AMPK-*DN* in the VMH (G). Data are expressed as mean ± SEM (n = 6–7). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* WT-EV; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* WT-AMPK-*DN*.

homeostasis. Despite these results, using a whole-body CPT1C KO mouse is a limitation in our study, and therefore the importance of other hypothalamic nuclei (e.g. PVH or ARC) in these thermogenic responses cannot be excluded. Further work will be necessary to determine the specific neuronal VMH population mediating these effects. An interesting candidate could be SF1 neurons, as we have recently demonstrated that the specific ablation of AMPKα1 at these levels promotes a lean feeding-independent, but thermogenic-dependent phenotype that protects against HF-induced obesity [12,13].

Overall, the present investigation demonstrates that CPT1C in the VMH is necessary for the activation of BAT thermogenesis in response to central leptin and short-term HF diet administration. Also, we demonstrate that the role of CPT1C in adaptive thermogenesis is throughout the canonical pathway dependent on AMPK in the VMH. This study underscores the importance of CPT1C to provide metabolic adaptation during short-term consumption of fat-rich diets and during obesity development.

5. CONCLUSIONS

A better understanding of the neuronal pathways mediating short-term hypothalamic changes in response to nutritional status would provide valuable information about obesity development and progression. Therefore, identification of potential targets involved in these hypothalamic pathways to control aspects of energy balance beyond food intake, such as the BAT thermogenic activity, has gained relevance in the last few years.

The neuron-specific CPT1C, the most enigmatic CPT1 isoform, seems to play a key role in central regulation of energy homeostasis, mostly in terms of fuel selection and food preference during fasting or in response to ghrelin by AMPK-dependent mechanisms. The present investigation reveals that mice lacking CPT1C show an impaired activation of BAT thermogenesis in response to short-term HF feeding and central leptin administration. In this phenotype, expression of CPT1C, by sensing malonyl-CoA, in the VMH is enough to restore diet-induced thermogenesis and counteract body weight gain. Considering the importance of the canonical pathway dependent on AMPK in the VMH to regulate BAT thermogenesis during the development of diet-induced obesity, our study also demonstrates for the first time that CPT1C is a crucial factor in this canonical pathway. The link between hypothalamic CPT1C and adaptive thermogenesis by the AMPK-brown fat axis could explain the obesogenic phenotype characteristic of CPT1C KO mice and also emphasize the role of CPT1C in the VMH to provide metabolic adaptation during short-term consumption of fat-rich diets. Altogether, this study underscores the importance of CPT1C in the development and progression of obesity and could add insight into the understanding of the mechanisms underlying diet-induced obesity.

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CRediT authorship contribution statement

Rosalía Rodríguez-Rodríguez: Data curation, Writing - original draft. **Cristina Miralpeix:** Data curation. **Anna Fosch:** Data curation. **Macarena Pozo:** Methodology. **María Calderón-Domínguez:** Methodology. **Xavier Perpinyà:** Data curation. **Miquel Vellvehí:** Data curation. **Miguel López:** Writing - original draft. **Laura Herrero:** Writing - original draft. **Dolors Serra:** Writing - original draft. **Núria Casals:** Writing - original draft.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2018.10.010.

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✤ *Author's Choice*

Hypothalamic endocannabinoids inversely correlate with the development of diet-induced obesity in male and female mice[S]

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Abstract The endocannabinoid (eCB) system regulates energy homeostasis and is linked to obesity development. However, the exact dynamic and regulation of eCBs in the hypothalamus during obesity progression remain incompletely described and understood. Our study examined the time course of responses in two hypothalamic eCBs, 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamine (AEA), in male and female mice during diet-induced obesity and explored the association of eCB levels with changes in brown adipose tissue (BAT) thermogenesis and body weight. We fed mice a high-fat diet (HFD), which induced a transient increase (substantial at 7 days) in hypothalamic eCBs, followed by a progressive decrease to basal levels with a long-term HFD. This transient rise at early stages of obesity is considered a physiologic compensatory response to BAT thermogenesis, which is activated by diet surplus. The eCB dynamic was sexually dimorphic: hypothalamic eCBs levels were higher in female mice, who became obese at later time points than males. The hypothalamic eCBs time course positively correlated with thermogenesis activation, but negatively matched body weight, leptinemia, and circulating eCB levels. Increased expression of eCB-synthetizing enzymes accompanied the transient hypothalamic eCB elevation. Icv injection of eCB did not promote BAT thermogenesis; however, administration of thermogenic molecules, such as central leptin or a peripheral β3-adrenoreceptor agonist, induced a significant increase in hypothalamic eCBs, suggesting a directional link from BAT thermogenesis to hypothalamic eCBs. This study contributes to the understanding of hypothalamic regulation of obesity.—Miralpeix, C., A. Fosch, J. Casas, M. Baena, L. Herrero, D. Serra, R. Rodríguez-Rodríguez, and N. Casals. **Hypothalamic endocannabinoids inversely correlate with the development of diet-induced obesity in male and female mice. *J. Lipid Res.* 2019.** 60: **1260–1269.**

Supplementary key words hypothalamus • sexual dimorphism • brown adipose tissue

The endocannabinoid (eCB) system is a highly conserved lipid-derived signaling system that plays a critical role in the control of energy homeostasis and body weight (1). The most well-known eCBs are 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamine (anandamide; AEA). eCBs are synthetized on demand in the brain and peripheral tissues, where they can act in an autocrine or paracrine manner or be secreted to the bloodstream (2). In peripheral tissues, such as liver, fat, pancreas, and muscle, eCBs exert a wide range of metabolic effects, including the modulation of food digestion, energy expenditure, lipid storage,

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Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; BAT, brown adipose tissue; DIO, diet-induced obesity; eCB, endocannabinoid; HFD, high-fat diet; OEA-d2, *N*-oleylethanolamine-d2; SD, standard diet.

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and glucose homeostasis (3, 4). The overall action of eCBs in the periphery favors energy intake and storage, promoting obesity development (1, 2). In humans, evidence demonstrates that circulating 2-AG and/or AEA levels are increased in people with obesity, and these levels specifically correlate with visceral fat mass, either in female or male patients (5, 6).

The eCB signaling is particularly critical in the brain, where it modulates neurotransmitter release and provides neuroprotection (7, 8). The eCB system is widely expressed in brain areas associated with the regulation of energy homeostasis, like the hypothalamus, the brainstem, and the cortico-limbic system (7). In the hypothalamus, 2-AG levels are increased in different genetic models of obesity: Zucker rats, *db/db* mice, or *ob/ob* mice (9). In line with these evidences, specific deletion of the main eCB receptor (CB1) in the hypothalamus resulted in increased energy expenditure and brown adipose tissue (BAT) thermogenesis, leading to a reduction in body weight, while food intake remained unchanged (10). These studies, together with others on genetic animal models (11–13), suggest that the activation of the eCB system in the hypothalamus leads to reduced energy expenditure and promotes obesity (1).

Despite these findings in genetic models, the exact dynamic of eCB levels and their modulation in the hypothalamus during diet-induced obesity (DIO), the model that best resembles human obesity, has been poorly explored, and the scarce results in response to a high-fat diet (HFD) are contradictory. For instance, hypothalamic 2-AG levels were increased in rats after long-term exposure to an HFD (24 weeks) (14), whereas they were not changed in another study performed with mice fed an HFD for 19 weeks (15). In both studies, AEA levels remained unchanged. Moreover, the time-course fluctuations and regulation of the eCB profile in the hypothalamus during obesity development remains incompletely described and understood.

In addition to this unsolved issue, recent evidence showed some differences in brain eCB levels between male and female mice after long-term administration of an HFD (16), in line with the sexually dimorphic brain response in obesity (17–19). However, eCB dynamics in the hypothalamus between male and female animals during DIO development have not been explored.

In the present study, we have analyzed hypothalamic 2-AG and AEA levels in male and female mice at different stages of DIO development. Our results demonstrate that 2-AG and AEA levels transitorily increase in both genders, with maximum levels at 7 days of HFD administration, followed by a gradual decline to levels similar to those observed in control groups. These changes positively correlate with BAT thermogenesis and inversely correlate with bodyweight gain. Acute activation of BAT thermogenesis under different stimuli also increased eCB levels in the hypothalamus, indicating early rises in hypothalamic eCBs as a compensatory response to the increased thermogenesis. This is the first study revealing the exact dynamic of hypothalamic 2-AG and AEA during DIO development and its potential link to BAT activation in male and female mice.

METHODS

Animals, diets, and sample collection

Male and female C57BL/6J mice (8 weeks old) were used for the experiments. All animals were housed on a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room and were allowed free access to water and standard laboratory chow. Animals were placed on an HFD (60% kcal from fat; catalog no. D12492, Research Diets, New Brunswick, NJ) or standard diet (SD) (10% kcal from fat; catalog no. D12450B, Research Diets) for 7, 14, 28, 60, or 90 days. Diets were administered in two different sets of animals: *1*) mice fed an SD or HFD for 7, 14, or 28 days; and *2*) mice fed an SD or HFD for 60 or 90 days. At the end of the studies, animals were fasted for 1 h and euthanized by cervical dislocation, and tissues were collected for further molecular and biochemical analysis. For each animal, the hypothalamus and interscapular BAT were quickly removed, weighed, and stored at −80°C. Plasma was obtained after blood centrifugation. Tissue processing and analysis from both sets of animals were simultaneously performed. All animal procedures were performed in agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Local Ethical Committee (Procedure ref. 9659, Generalitat de Catalunya).

Extraction and analysis of eCBs

Hypothalamic and plasma eCBs from both sets of animals were simultaneously extracted and analyzed as previously described by Gong et al. (20). Hypothalamus (6–8 mg wet tissue) was dounce-homogenized in 200 µl of ice-cooled deionized water containing a final concentration of 0.362 µM *N*-oleylethanolamine-d2 (OEA-d2) (Cayman Chemicals, Ann Arbor, MI) as internal standard for 2-AG and AEA calibration, 100 µM PMSF, and 0.01% butylated hydroxytoluene (BHT) (Sigma-Aldrich, Madrid, Spain), followed by a brief sonication. After that, half of the homogenized sample was kept at −20°C for protein quantification, and the other half was mixed with 400 µl of ethyl acetate/*n*-hexane (9:1, v/v) and vortexed for 5 min. After centrifugation (14,000 *g*, 4°C, 5 min), the upper layer was collected and evaporated using a nitrogen evaporator.

Plasma (25 µl) was mixed with 0.362 µM OEA-d2, 100 µM PMSF, and 0.01% BHT per sample. Lipid extraction was made with 250 µl of ethyl acetate/*n*-hexane (9:1, v/v), following the same protocol as for hypothalamus extraction.

eCB levels were analyzed by LC/MS/MS, following the protocol described by Gong et al. (20). Briefly, 2-AG, AEA, and OEA-d2 (Cayman Chemicals) were used for the calibration curve in an Acquity ultra-high-performance liquid chromatography (UPLC) (Waters, Singapore) system connected to a Xevo-TQS triple-quadropole Detector (Waters, Ireland) and controlled with Waters/Micromass MassLynx software. Chromatographic separation was performed on an Acquity UPLC BEH C_{18} column (1.7 µm particle size, 100 mm × 2.1 mm; Waters) with an isocratic mobile phase of formic acid 0.1% in water-acetonitrile (30:70, v/v). The flow rate was 0.3 ml/min. Detection was performed with an electrospray interface operating in the positive ion mode. The capillary voltage was set to 3.1 kV, the source temperature was 150°C, and the desolvation temperature was 500°C, acquiring the following selected reaction monitoring transitions: OAE-d2: 328.2–62.2 Da, cone voltage 50 V, and collision energy 10 eV; AEA: 348.2–62.2 Da, cone voltage 50 V, and collision energy 10 eV; and 2-AG: 379.2–287.1 Da, cone voltage 50 V, and collision energy 10 eV. eCB levels from each experimental group were normalized to its corresponding control group (SD).

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Analysis of plasma leptin levels

Plasma levels of leptin were determined by a mouse ELISA Kit (Crystal Chem, Zaandam, The Netherlands), following the manufacturer's instructions.

RNA preparation and quantitative RT-PCR

Total RNA was extracted from tissues using Trizol Reagent (Fisher Scientific, Madrid, Spain). Retrotranscription and quantitative RT-PCR was performed as previously described (21). SYBR Green or Taqman Gene Expression assay primers were used (IDT DNA Technologies, Leuven, Belgium) (supplemental Table S1). Relative mRNA levels were measured using the CFX96 Real-time System, C1000 Thermal Cycler (Bio-Rad, Madrid, Spain).

BAT temperature measurements

Skin temperature surrounding BAT was visualized using a high-resolution infrared camera (FLIR Systems) and analyzed with a specific software package (FLIR-Tools-Software, FLIR, Kent, UK), as described (22, 23). Thermal images were acquired the day of euthanization.

Icv administration of leptin and 2-AG + AEA combination

Two different experiments were performed: *1*) icv leptin administration followed by evaluation of BAT thermogenesis and hypothalamic eCB levels; and *2*) icv administration of a mixture of eCBs that consists of combinations of 2-AG + AEA in different dosages to evaluate BAT thermogenesis. For both experiments, cannulae were stereotaxically implanted into the lateral cerebral ventricle under ketamine/xylazine ip anesthesia, as previously described (23). Lean mice fed a chow diet were individually caged and allowed to recover for 5 days before the experiment.

For leptin injection experiment. On the experimental day, lean male mice received an icv administration of 2 µl of either vehicle (aqueous buffer containing 0.1% BSA) or leptin (0.1 µg/µl) (PeproTech, London, UK) 3 h after lights on (23). At 4 h after the injection, mice were euthanized by cervical dislocation, and hypothalamus and BAT were collected for further analysis.

For eCBs injection experiment. On the experimental day, lean male mice received an icv administration of 2 µl of either vehicle (saline buffer containing 5% DMSO) or the eCB combination 2-AG + AEA (Cayman Chemicals) in two different dosages: dose 1 (0.5 µg of 2-AG + 0.005 µg of AEA) and dose 2 (2 µg of 2-AG + 0.02 µg of AEA). The doses of 2-AG and AEA were selected based on previous publications using icv or intrahypothalamic administration of these eCBs separately (24, 25) and also considering the different range of concentration found in the hypothalamic region under standard conditions (×50–100 higher concentrations of 2-AG compared with AEA).

β3-adrenergic agonist-induced thermogenesis activation

Hypothalamic eCB levels were determined after adrenergic stimulation of BAT thermogenesis with the selective β3-adrenergic agonist CL 316,243 (Tocris Bioscience, Bristol, UK) in lean mice, as previously described (26, 27). At 4 h after ip injection of either CL 316,243 (10 mg/kg) or vehicle (aqueous buffer) (26), mice were euthanized by cervical dislocation, and hypothalamus and BAT were collected for further analysis.

Statistical analysis

All results are expressed as mean ± SEM (n = 8–12). Analysis was conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Statistical analysis was determined by one-way ANOVA when different diet groups within the same gender were compared and two-way ANOVA when groups between different genders were compared. In both cases, ANOVA was followed by a post hoc two-tailed Bonferroni test. For the analysis of the relation between variables, parameters were mathematically log-transformed to improve symmetry, and correlation was analyzed by Pearson's test and lineal regression. $P < 0.05$ was considered significant.

RESULTS

Progression of DIO was delayed in female compared with male mice

Age-matched male and female mice were fed an SD or an HFD for 90 days. During this period, progression of body weight gain, plasmatic levels of leptin, food, and caloric intake were evaluated (**Fig. 1**, supplemental Figs. S1, S2). Males fed an HFD for a period of administration equal to or longer than 28 days gained significantly more weight than controls (Fig. 1A, supplemental Fig. S1). In female mice, a significant increase in body weight gain was not appreciated until 60 days of administration of an HFD compared with an SD (Fig. 1A, supplemental Fig. S1). In line with these results, plasmatic levels of leptin were significantly increased in male mice after 28 days of HFD feeding compared with control diet, whereas female mice did not show hyperleptinemia until 60 days of HFD administration, and leptin levels were considerably lower at this point than those observed in male mice (Fig. 1B). These data show that male mice became obese and hyperleptinemic at earlier time points of HFD feeding than females, and the final body weights remained higher in comparison to female mice (Fig. 1A, B, supplemental Fig. S1).

As expected, total caloric intake in male and female mice was increased when the animals were fed an HFD, whereas food intake was decreased in HFD animals of both sexes compared with SD (supplemental Fig. S2). In addition, both caloric and food intake was significantly lower in female mice fed an SD or HFD in comparison to diet-matched male animals (supplemental Fig. S2).

Short-term administration of an HFD induced a transient increase of both thermogenesis activation and hypothalamic eCB levels in male and female mice

The induction of thermogenesis in the interscapular BAT of male and female mice was analyzed at different time points during 90 days of HFD feeding, compared with SD (Fig. 1C–F). A substantial activation peak was reached in gene expression of thermogenic markers (Fig. 1C–E) and interscapular temperature (Fig. 1F) in the BAT of male and female mice after 7–14 days of an HFD when compared with an SD. Longer administration periods of HFD (from 28 days onwards) were not able to induce such a considerable activation of BAT thermogenesis (Fig. 1C–F). The activation of gene expression of thermogenic markers was higher in the BAT of female compared with male mice, particularly after 7, 14, and 28 days of an HFD (Fig. 1C–E). In addition, an increased basal expression of specific thermogenic genes (Fig. 1C, D) and basal interscapular BAT temperature (Fig. 1F) was appreciated in female mice in comparison to male mice, as described previously in the literature (28, 29).

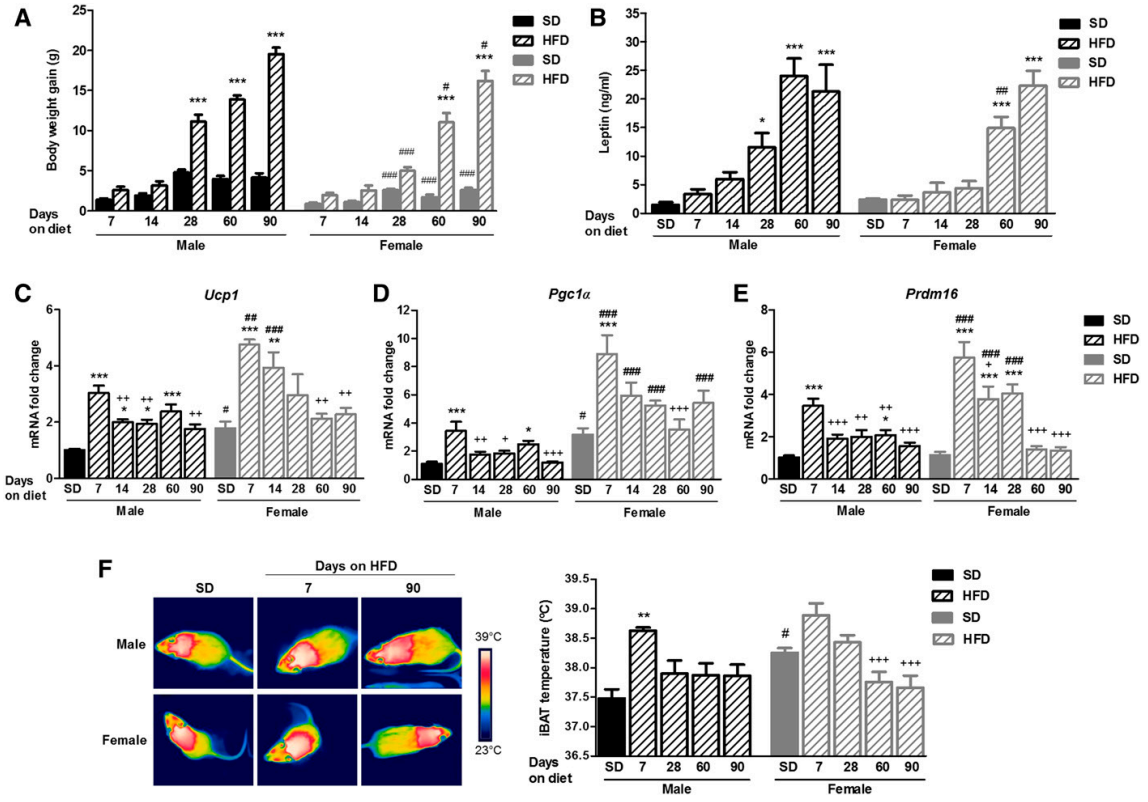

Fig. 1. DIO development of male and female mice fed an SD or HFD for 90 days. A: Body weight gain. B: Plasmatic levels of leptin. C–E: Relative mRNA expression of the thermogenic markers UCP1 (C), PGC1α (D), and PRDM16 (E) in BAT of male and female mice fed an SD or HFD for 90 days (n = 8–10). F: Representative infrared thermal images and quantification of interscapular temperature adjacent to the BAT depot of male and female mice fed an SD or HFD for 7, 28, 60, and 90 days (n = 5). Statistical significance was determined by ANOVA and Bonferroni posttest. Error bars represent SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus its corresponding SD; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus male under the same diet conditions; $^{+}P < 0.05$; $^{++}P < 0.01$; $^{+++}P < 0.001$ versus same gender fed an HFD for 7 days.

Analysis of hypothalamic eCB levels also revealed a pronounced transitory increase after short-term administration of an HFD (**Fig. 2**). Hypothalamic 2-AG levels were significantly increased after 7 days of HFD feeding in both male and female mice, and these values were progressively attenuated, reaching basal levels in a time-dependent manner (Fig. 2A). AEA levels in the hypothalamus of male and female mice remained elevated during 7–28 days of HFD feeding, whereas they were significantly reduced after longer exposure to an HFD (Fig. 2B). Interestingly, transitory hypothalamic increases in 2-AG and AEA were substantially higher in female than in male mice, and decreases were more pronounced in female than in male mice (Fig. 2). Hypothalamic concentrations of 2-AG and AEA (ng of eCB/mg of tissue) derived from the two different sets of animals during dietary administration are shown in supplemental Table S2.

In summary, short-term exposure to an HFD induced a transitory activation of BAT thermogenesis and a transient increase in hypothalamic 2-AG and AEA in mice, and these responses were sexually dimorphic (Fig. 1, 2).

Hypothalamic eCB levels correlated with body weight gain, leptinemia, and brown fat thermogenesis

First, we analyzed the relationship between hypothalamic eCB levels and body weight gain or plasma leptin levels in the animals fed the experimental diets. 2-AG and AEA levels in the hypothalamus showed a negative correlation with both body weight gain (**Fig. 3A–**D) and leptinemia (supplemental Fig. S3) in male and female mice. Then, evaluation of the relationship between eCBs in the hypothalamus and the mRNA expression levels of BAT thermogenesis activation revealed that hypothalamic 2-AG and AEA levels positively correlated with mRNA levels of Ucp1, Pgc1α, and Prdm16 in the BAT of female mice (Fig. 3F, H, supplemental Fig. S4). In male mice, this correlation was only significantly appreciated when analyzing 2-AG levels (Fig. 3E, G, supplemental Fig. S4).

These results indicate a negative association between eCB levels in the hypothalamus and obesity progression in male and female mice, but a positive association of hypothalamic eCBs with thermogenic activation in response to an HFD, particularly evidenced in female mice.

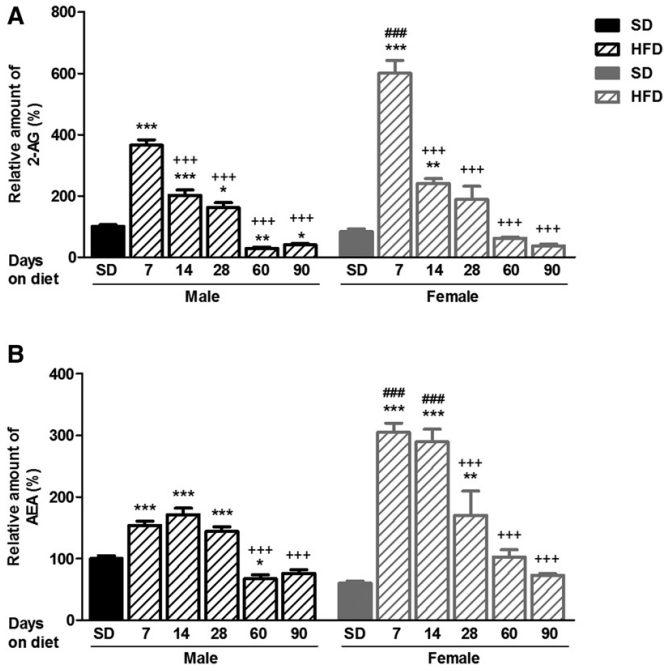

Fig. 2. Time profile of hypothalamic eCB levels during the development of DIO in male and female mice. A: Relative amount of 2-AG to male SD. B: Relative amount of AEA to male SD. Statistical significance was determined by ANOVA and Bonferroni posttest. Error bars represent SEM (n = 8–10). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus its corresponding SD; $^{\#\#\#}P < 0.001$ versus male under the same diet conditions; $^{+++}P < 0.001$ versus same gender fed an HFD for 7 days.

Plasmatic eCBs increased after longer administration periods of an HFD and showed a negative correlation to hypothalamic eCB levels

In contrast to the hypothalamus, plasmatic levels of 2-AG and AEA remained unchanged after short-term administration of an HFD compared with SD in both male and female mice (**Fig. 4A**, B). However, exposure to an HFD for 60 or 90 days revealed an increase in plasmatic 2-AG in both male and female mice (Fig. 4A) and a substantial increase in plasmatic AEA, particularly observed in female mice (Fig. 4B). In addition, 2-AG and AEA levels in plasma evidenced a positive correlation with body weight gain in

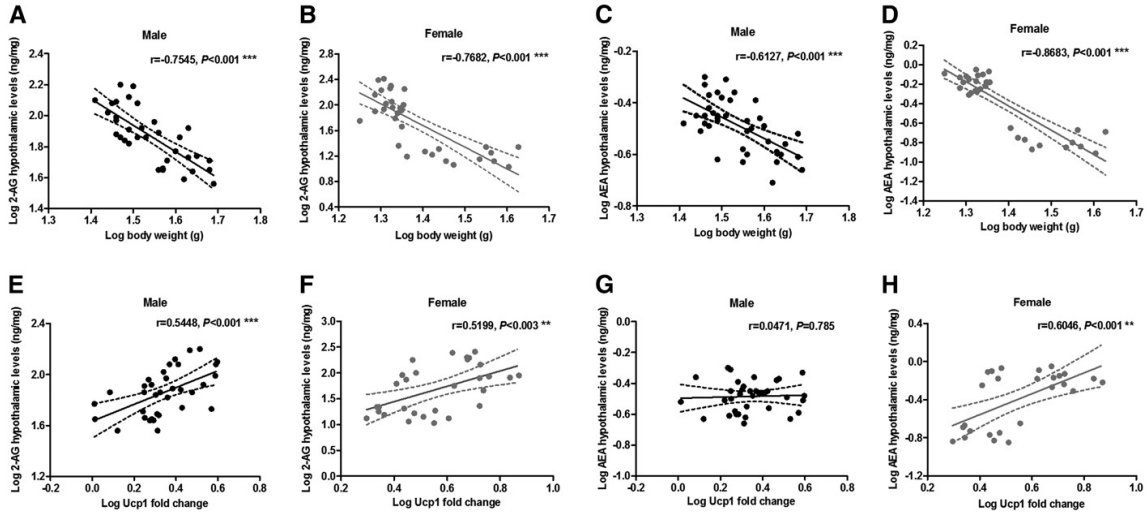

Fig. 3. Correlation between hypothalamic eCBs and body weight or UCP1 mRNA expression in BAT. Hypothalamic 2-AG levels negatively correlate with body weight at time of euthanization in both male (A) and female (B) mice. Hypothalamic AEA levels negatively correlate with body weight at time of euthanization in both male (C) and female (D) mice. Hypothalamic 2-AG levels positively correlate with UCP1 mRNA expression in BAT of male (E) and female (F) mice. Hypothalamic AEA levels do not correlate with UCP1 mRNA expression in BAT of male mice (G), but they positively correlate in BAT of female mice (H). Statistical significance and correlation was determined by Pearson correlation coefficients (*xy* values = 30–40). $^{**}P < 0.01$; $^{***}P < 0.001$.

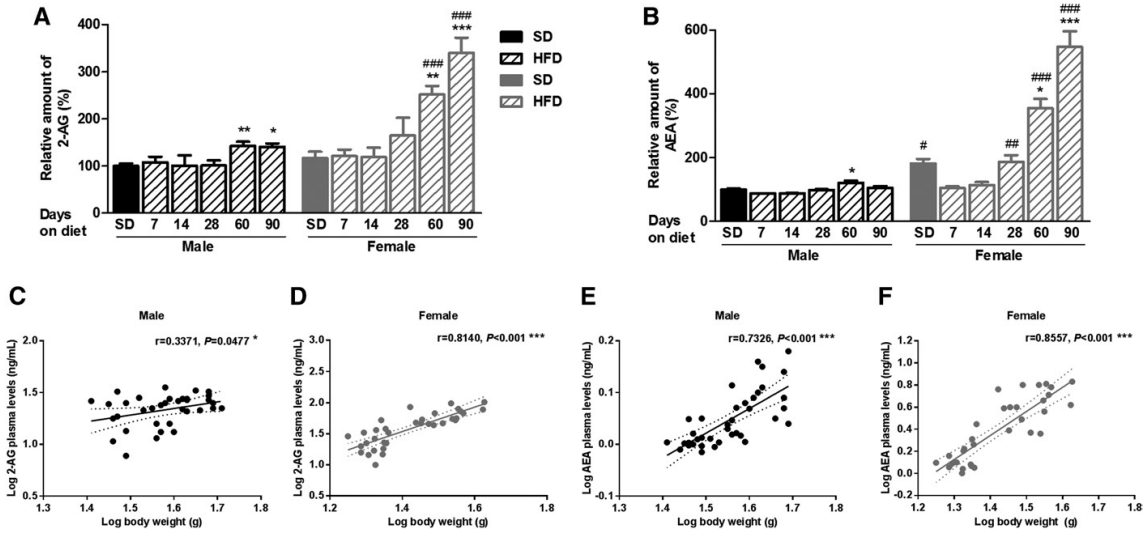

Fig. 4. Time profile of plasmatic eCBs levels during the development of DIO and correlation to body weight in male and female mice. A: Relative amount of 2-AG compared with values found in male SD. B: Relative amount of AEA compared with values found in male SD. Plasmatic 2-AG levels positively correlate with body weight at time of euthanization in both male (C) and female (D) mice. Plasmatic AEA levels positively correlate with body weight at time of euthanization in both male (E) and female (F) mice. Statistical significance was determined by ANOVA and Bonferroni posttest, and correlation was determined by Pearson correlation coefficients (*xy* values = 33–39). Error bars represent SEM (n = 8–10). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus its corresponding SD; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus male mice under the same diet conditions.

both male and female mice (Fig. 4C–F). Analysis of the relationship between plasmatic and hypothalamic eCB levels revealed that plasmatic eCBs negatively correlated with those levels in the hypothalamus of male and female mice fed an HFD (supplemental Fig. S5). Plasmatic concentrations of 2-AG and AEA (ng of eCB/ml of plasma) derived from the two different sets of animals during dietary administration are shown in supplemental Table S3.

Short-term HFD feeding increased gene expression of eCB synthesis enzymes in the hypothalamus

We analyzed gene expression of the enzymes responsible for the synthesis (*Daglα*, *Daglβ*, and *Nape*) and degradation (*Mgll*, *Abhd6*, and *Faah*) of 2-AG and AEA in the hypothalamus of mice fed an SD or HFD for 7 days. This analysis revealed a significant increase in the expression of the hypothalamic enzymes synthetizing 2-AG and AEA after short-term HFD administration, whereas this increase was not evidenced in the expression of degradation enzymes (**Fig. 5**). Overall, this result shows that the transient increase in hypothalamic 2-AG and AEA over 7 days of HFD feeding concurs with an enhanced expression of synthesis enzymes in the hypothalamus.

Acute leptin and a β3-adrenergic agonist both induced a thermogenic response with an increase in hypothalamic eCB levels

We decided to measure the levels of hypothalamic eCBs in another condition well known to activate BAT thermogenesis, such as central leptin administration and ip administration of a β3-adrenergic agonist. In agreement with our previous results (23), 4 h of leptin icv increased gene expression of thermogenic markers in BAT (**Fig. 6A**). Under these experimental conditions, hypothalamus of the same animals evidenced a significant increase in both 2-AG and AEA concentrations (Fig. 6B).

To compare the effects of central leptin versus peripheral adrenergic activation of BAT, mice received ip injection of the potent and selective β3-adrenergic agonist CL 316,243, an agonist that has demonstrated minimal access to the brain after peripheral injection (30). In line with leptin experiment, CL 316,243 induced adrenergic activation of BAT thermogenesis marker genes (Fig. 6C), with a substantial increase in both 2-AG and AEA in the hypothalamus (Fig. 6D), revealing an association between hypothalamic eCBs and thermogenesis activation.

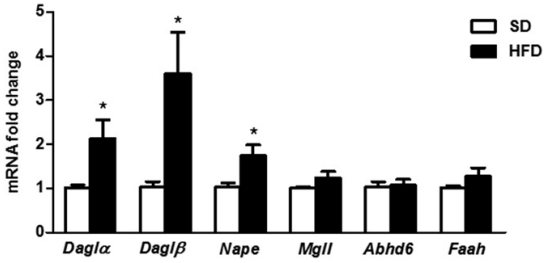

Fig. 5. Relative mRNA expression of 2-AG and AEA synthesis and degradation enzymes in hypothalamus of male mice fed an SD or HFD for 7 days. Statistical significance was determined by ANOVA and Bonferroni posttest. Error bars represent SEM (n = 5–8). $*P < 0.05$ versus SD.

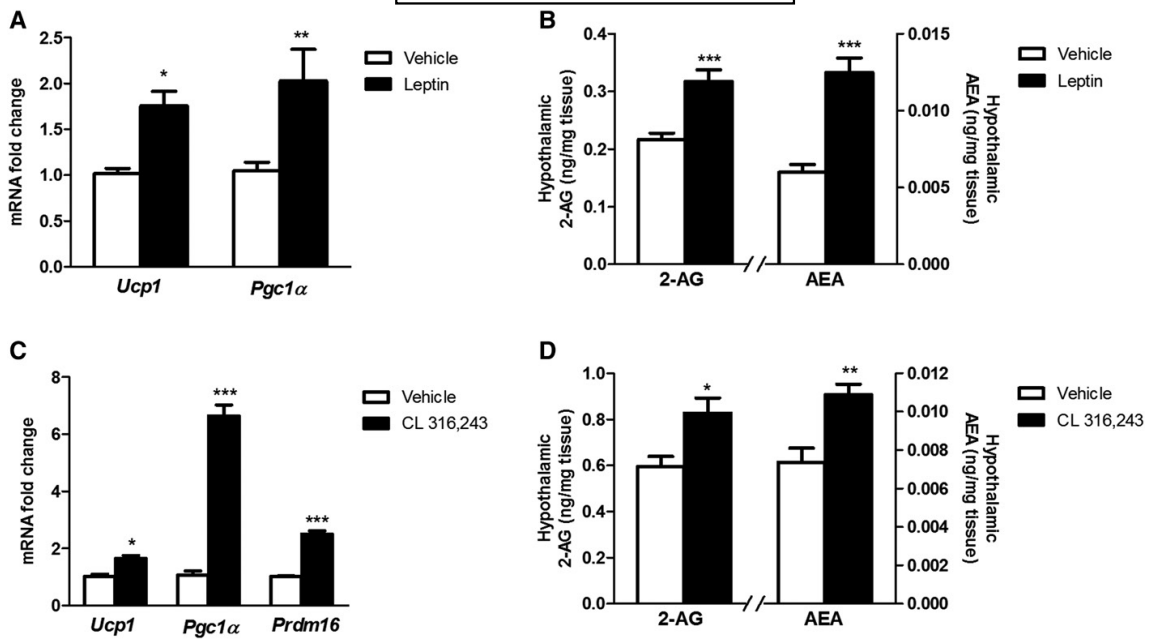

Fig. 6. Hypothalamic eCB levels and BAT thermogenesis in response to acute icv leptin (A, B) or to ip injection of the β3-adrenoreceptor agonist CL 316,243 (C, D) in lean mice fed a chow diet. Relative mRNA expression of the thermogenic markers in BAT samples after 4 h of icv leptin (A) or ip CL 316,243 administration (C). Concentration of 2-AG and AEA in the hypothalamus of mice after 4 h of icv leptin (B) or ip CL 316,243 administration (D). Statistical significance was determined by ANOVA and Bonferroni posttest. Error bars represent SEM (n = 8–12). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus vehicle.

Acute central administration of 2-AG + AEA did not induce BAT thermogenesis

Icv administration of the mixture of the eCBs, 2-AG + AEA, in two different doses for 4 h, was not able to induce a significant alteration of either interscapular BAT temperature (**Fig. 7A**) or gene expression levels of thermogenic markers in BAT (Fig. 7B). Food intake was not significantly altered in response to icv injection of the doses of these eCBs (data not shown), in agreement with a previous publication (25).

DISCUSSION

Despite the well-established function of the eCB system on energy homeostasis, our knowledge on its exact dynamic and regulation under dietary conditions leading to obesity and associated complications is still limited. Our current study presents for the first time the temporal profile of hypothalamic eCB changes during the development of DIO and its association with BAT thermogenesis activation and leptin response in male and female mice. This is an intriguing finding, considering the very few and contradictory evidence that exists in the literature on hypothalamic eCBs in response to an HFD, its relation to BAT activation, and the potential contribution of this association to sexual dimorphism in obesity.

One of the most remarkable results in our study was the transitory and substantial increase in hypothalamic eCBs after short-term administration of an HFD in both male and female mice, while these levels were progressively attenuated under long-term exposure to this diet. The transient increase was particularly pronounced on 2-AG levels after 7 days of HFD feeding (four to six times higher than the basal levels). At this time point, the early rise on eCB levels was sustained by a significant increase in the hypothalamic expression of the enzymes responsible for the synthesis of 2-AG and AEA, without significant alterations in degrading enzyme expression.

It has been reported that eCB levels become deregulated in the hypothalamus during obesity (7, 9). Di Marzo et al. (9) were the first detecting elevated levels of 2-AG in genetically obese Zucker rats and *ob/ob* mice and of both 2-AG and AEA in the hypothalamus of *db/db* mice, compared with lean controls. Because those genetic models are either leptin-deficient or express mutated forms of the leptin receptor, the increased eCB levels in the hypothalamus were suggested as an additional component of leptin-sensitive regulatory mechanisms (9). Studies on DIO animals, the model that best resembles human obesity, are scarce and controverted in terms of hypothalamic eCB changes. Moreover, some of these investigations are limited to their analysis after long-term HFD administration (19 weeks onward) and thereby when obesity has been already established (14, 15). Our current data show that hypothalamic 2-AG and AEA levels are not increased when DIO is established (at 60 or 90 days of an HFD), but they are at earlier stages (7–28 days) of high-fat feeding, in contrast

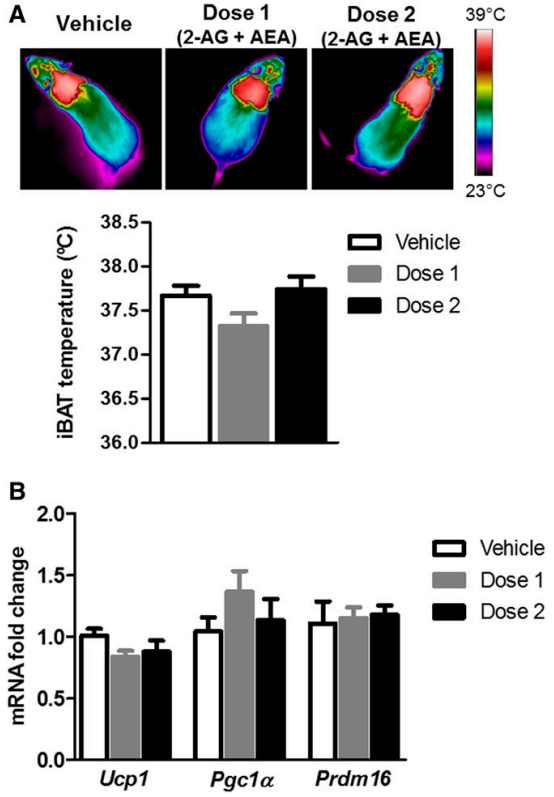

Fig. 7. BAT thermogenesis in response to acute icv injection of the eCBs 2-AG and AEA to lean mice, in two different dosages: dose 1 (0.5 μg 2-AG + 0.005 μg AEA) and dose 2 (2 μg 2-AG + 0.02 μg AEA). Representative infrared thermal images and the corresponding quantification of interscapular temperature adjacent to the BAT depot (iBAT) (A) and relative mRNA expression of the thermogenic marker UCP1 (B) in BAT samples of mice after 4 h of icv injection of 2-AG + AEA in two different dosages. Statistical significance was determined by ANOVA and Bonferroni posttest. Error bars represent SEM (n = 8–10).

to that observed in plasma. These data indicate that the eCB dynamic in the hypothalamus is different in genetic animal models of obesity compared with diet-induced obese models. Furthermore, this is the first time that a negative association between hypothalamic eCBs and body weight during DIO development is demonstrated.

Our group has recently reported that the robust activation peak of BAT thermogenesis observed under 7 days of HFD feeding matches with a transient preservation of normal body weight, adiposity, and leptinemia in the initial phases of DIO (23). The fact that the transitory increase in hypothalamic eCBs after short-term HFD administration found in the present investigation correlated with the activation peak of BAT thermogenesis led us to propose this early rise in hypothalamic eCBs as a physiological compensatory response (found in the hypothalamus, but not in plasma) to BAT thermogenesis activation triggered by diet surplus. These data also suggest the existence of a cross-talk between BAT and hypothalamic eCBs in the initial stages of obesity.

The link between the increase in hypothalamic 2-AG and AEA levels and BAT thermogenesis activation was further evaluated by measuring hypothalamic eCBs following acute thermogenic activation by different stimuli. Acute administration of either central leptin or ip injection of CL 316,243, a selective and peripherally restricted β3-adrenergic agonist, induced BAT thermogenesis activation with a substantial increase in hypothalamic eCB levels. This is the first study revealing that acute stimulation of BAT enhances eCB levels in the hypothalamus. In relation to these findings, it was recently demonstrated that in vivo stimulation of β3-adrenoreceptors in brown adipocytes by CL 316,243 (acutely or chronically injected) elevated the levels of the eCBs, 2-AG and AEA, in BAT and were suggested to act as local autocrine negative-feedback regulators (27). Our assumption that the hypothalamic eCB elevation is a compensatory response, and not a cause, to BAT thermogenesis activation was also supported by the absence of stimulation of thermogenesis under icv administration of different doses of the combination of 2-AG + AEA. The lack of BAT activation by central eCB injection, at least with the selected dosages, suggests the unidirectional link from BAT to hypothalamic eCBs. However, further research is needed to unravel the molecular mechanisms by which BAT is possibly signaling the hypothalamic eCB system, particularly in early stages of DIO development.

Therefore, the present investigation shows evidence that, in early stages of obesity, when BAT thermogenesis is more active, there is a higher increase in hypothalamic eCBs, as a physiological compensatory response to BAT activation. However, when DIO is already established, the decline of thermogenesis activation is accompanied by a decrease in hypothalamic eCB levels. Further investigation will be needed to study the specific role of hypothalamic eCBs in response to BAT activation.

Interestingly, we can find in the literature other examples of short-term changes in the hypothalamus in response to nutritional surplus suggested to be compensatory mechanisms in obesity (31–33). These changes have also been described as processes preceding insulin/leptin sensitivity disruption and inflammation in peripheral tissues and therefore promoting positive energy balance (34, 35). Therefore, we might also understand the transitory increase in hypothalamic eCBs as an early indicator to precede leptin resistance and peripheral obesity.

Despite the discrepancy on central eCBs during DIO, the dysregulation of circulating eCB levels in metabolic diseases have been widely investigated (1–3). To date, these data are more conclusive, and the results on plasmatic eCBs in rodents are similar to those observed in humans (1, 36–38). Circulating eCBs positively correlate with markers of obesity and metabolic disorders, such as BMI, waist circumference, visceral fat mass, and insulin resistance (5, 6, 37, 39, 40). Our data on circulating eCBs agree with those previously reported—that is, plasmatic 2-AG and AEA levels were increased, particularly in female mice, after long-term exposure to an HFD, but not at early stages of DIO. Interestingly,

these results negatively correlated with those concentrations in the hypothalamus. In line with these evidences, Kuipers et al. (41) recently demonstrated that there is a link between eCB metabolism in adipose tissues and plasmatic eCBs during DIO development. They showed that long-term HFD feeding increases circulating eCBs, accompanied by increased synthesis enzymes in adipose tissue (particularly BAT) of DIO mice (41). The authors also suggest that adipose tissues are likely important organs that release 2-AG and AEA levels in HFD-induced obesity (41).

An important finding in the current investigation was the difference in eCB levels observed depending on the gender. Relevant differences were appreciated between male and female mice, particularly when analyzing the transient increase of hypothalamic eCBs and the peak of activation of thermogenesis after a short-term HFD, which were substantially higher in females. In addition, obesity progression was delayed and less severe in females, suggesting a sexual dimorphism in hypothalamic eCB systems that could determine obesity progression. The relationship of central eCB systems and sexual dimorphism in obesity has been poorly explored (16). Recent findings on hypothalamic dimorphism in fatty acid concentration, chain length, and saturation, in response to HFD feeding were associated with protection to obesity and cardiovascular diseases in female compared with male mice (17, 19). Our data are the first comparing the dynamic of hypothalamic eCBs between male and female mice during DIO development. These results are therefore contributing to elucidate the relevance of central lipid metabolism in the sexual dimorphism in obesity.

Overall, this is the first study revealing the exact dynamic of hypothalamic eCBs during the development of obesity in DIO models, and these temporal changes correlated positively to BAT thermogenesis and negatively to circulating eCB, leptin, and body weight. Our data evidence a transitory elevation in hypothalamic eCBs after short-term HFD feeding accompanied by increased expression of 2-AG and AEA synthesis enzymes, understood as a physiological compensatory response to BAT thermogenesis activation triggered by diet surplus. The link between hypothalamic eCBs and BAT thermogenesis activation was also supported by a substantial upregulation of eCB in the hypothalamus following acute thermogenic activation by central leptin or peripheral β3-adrenergic stimulation. Our findings could add significant insight into the understanding of the hypothalamic mechanisms regulating obesity progression and its relationship to BAT function.

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