Hypothalamic Ceramide Levels regulated by CPT1C mediate the Orexigenic effect of Ghrelin

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DEPARTAMENTO DE CIENCIAS BÁSICAS, ÁREA DE BIOLOGÍA MOLECULAR Y CELULAR, FACULTAD DE MEDICINA

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Memoria de la tesis doctoral presentada por Sara Ramírez Flores, para optar al grado de Doctora por la Universitat Internacional de Catalunya.

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ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
ACS	Acyl-CoA synthetase
AgRP	Agouti related peptide
AICAR	5-Aminoimidazole-4-Carboxamide Riboside
Akt	Protein kinase B
AMPK	AMP-activated kinase
ARC	Arcuate nucleus
ATF4	Activating transcription factor 4
ATF6β	Activation transcription factor 6β
BiP	ER chaperone-binding immunoglobulin protein
BSA	Bovin serum albumin
bsh	Drosophila brain-specific homeobox gene
BSX	Brain-specific homeobox
C1PP	Ceramidase-1-phosphatase phosphatase
C6:0	Ceramide C6:0
CACT	Carnitine acylcarnitine translocase
CAMKK 1/2	Ca^{2+}/calmodulin-dependent protein kinase 1 or 2
CB1	Cannabinoid receptor 1
CBP	Creb Binding Protein
CD	Catalytic domain
Cdase	Ceramidase
cDNA	Complementari DNA
CerK	Ceramide kinase
CerS	Ceramide synthase
CERT	Ceramide transfer protein
CHOP-C	C/EBP homologous protein C
CNS	Central nervous system
CPT1 (a,b,c)	Carnitine palmitoyltransferase 1 (a,b,c)
CPT1C KO	CPT1C Knock down
CPT2	Carnitine palmitoyltransferase 2
CrAT	Carnitine acetyltransferase
CREB	cAMP response-element
CrOT	Carnitine octanoyltransferase
CRS	Cerebrosidase
DAG	Diacylglycerol
DEPC	Diethyl Pyrocarbonate
DES	Dihydroceramide desaturase
DMEM	Modified Eagle's medium
DMH	Dorsomedial nucleus
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced green fluorescence protein
ER	Endoplasmic reticulum
ESC	Embryonic stem cells
FAO	Fatty acid oxidation
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FoxO1	Forkhead box O1
GABA	Gamma-aminobutyric-acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gc	Glucocorticoids
GCS	Glucosylceramide synthase
GHS-R1 (a,b)	Growth hormone secretagogue receptor 1 (a,b)
GLTP	Glycolipid transfer protein gene
GLUT 4	Glucose transporter 4
GnRH	Gonadotropin-releasing hormone
GOAT	O-acyl transferae
GPBP	Goodpasture antigen binding protein
GR	Glucocorticoid receptor
GRE	Gc response elements
GSH	Glutathione
GSSG	Glutathione disulfide
HDAC	Histone Deacetylase
HFD	High fat diet
HIV-1	Inmunodeficiency virus 1
HPLC	High-performance liquid chromatography
HRP	Horse Radish Peroxidase
hTERT	Human telomerase reverse transcriptase
ICV	Intracerebroventricular
IgG	Immunoglobulin G
IKKβ	IkB kinase β
IMM	Inner mitochondrial membrane
IP	Intraperitoneal
LAG1	Longevity assurance homolog 1
LC-CoA	Long chain acyl-CoA

LHA	Lateral hypothalamic nucleus
MAM	Mitochondria-associated membranes
MBH	Mediobasal hypothalamus
MBN	Mediobasal nuclei
MCD	Malonyl-CoA carboxilase
MLV-RT	Moloney murine leukemia virus reverse transcriptase
MP	Micropunch
MRM	Multiple reaction monitoring
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Regulatori domain
NaCl	Sodium chloride
NFkb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP-40	Nonylphenol Ethoxylate
NPY	Neuropeptide Y
O/N	overnight
OMM	outer mitochondrial membrane
ORF	Open reading frame
pACC	Phosphorilated acetyl-CoA carboxylase
pAMPK	Phosphorilated AMP-activated kinase
PAT	Palmitoyl-acyl transferase
PC	phosphatidylcholine
PCR	Polimerase chain reaction
pEIF2α	Phosphorilated eukaryotic initiator factor 2α
pIKKαβ	Phosphorilated IkB kinase αβ
POMC	Pro-opiomelanocortin
pPERK	Phosphorilated RNA-dependent protein kinase-like ER kinase
PVDF	Polyvinylidene difluoride
PVN	Paraventricular nucleus
qPCR	quantitative PCR
RNA	Ribonucleic Acid
RNAse out	Ribonuclease inhibitor
ROS	Reactive oxigen species
RT	Reverse transcription
S1P	Sphingosie-1-phosphatase
S1PP	S1P phosphatase
SC	Standard chow
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Measurement
SIRT1	Sirtuin 1
SM	Sphingomyelin
Smase	Sphingomyelinase
SMS	Sphingomyelin synthase
SNAT2	Amino acid transporter 2
SP(1,3)	Specificity protein (1,3)
Sphk (1,2)	Sphingosine Kinase
SPT	Serine palmitoyltransferase
TLR4	Toll-like receptor 4
TM (1,2)	Transmembrane domain (1,2)
TNF	Tumor necrosis factor
TRAIL	TNF-related aopotosis-inducing ligand
TSA	Trichostatin A
UCP2	Uncoupling protein 2
VMH	Ventromedial nucleus
WT	Wild type
α-MSH	α-melanocyte stimulating hormone
mg	Milligram
ml	Milliliter
mM	Millimolar
µg	Microgram
µl	Microliter
µM	Micromolar
ng	Nanogram
nM	Nanomolar

CHAPTER 1

INTRODUCTION

1.FOOD INTAKE AND ENERGY HOMEOSTASIS

1.1.HYPOTHALAMIC REGULATION OF FOOD INTAKE AND ENERGY HOMEOSTASIS

The regulation of food intake and energy balance involves a number of processes that include homeostatic mechanisms as well as motivational and cognitive factors. The complexity of these mechanisms is demonstrated by the amount of brain regions involved in regulating these processes. The hypothalamus has been identified as a critical component of the homeostatic regulation of food intake. Key nuclei such as the arcuate nucleus (ARC), lateral hypothalamic area (LHA), ventromedial nucleus (VMH), dorsomedial nucleus (DMH) and the paraventricular nucleus (PVN) are all involved in the regulation of feeding (Fig. 1). The ARC in particular is recognized as a critical center for control of energy balance. Neurons within the ARC respond to peripheral factors such as ghrelin, insulin and leptin since ARC axon terminals have direct contact with the bloodstream (Peruzzo et al., 2000). Furthermore, the ARC contains two sets of neuronal populations. The first set of neurons produces neuropeptide Y (NPY), a peptide that potently increases food intake (Tatemoto et al., 1982). Interestingly, NPY neurons also produce a second orexigenic peptide known as agouti related peptide (AgRP) (Asakawa et al., 2002). The second set of neurons in the ARC, melocortin neurons, produce the α-melanocyte stimulating hormone (α-MSH) which is an anorectic peptide formed from propiomelanocortin protein (POMC). In addition, α-MSH binds to MC3/4 receptors in the lateral hypothalamus resulting in the reduction of food intake, and promotes satiety (Cone et al., 2001). NPY/AgRP and α-MSH neurons project to the PVN, as well as other hypothalamic nuclei such as, DMH, LHA and VMH to regulate energy expenditure and food intake keeping in balance energy homeostasis (Elmquist, 2001).

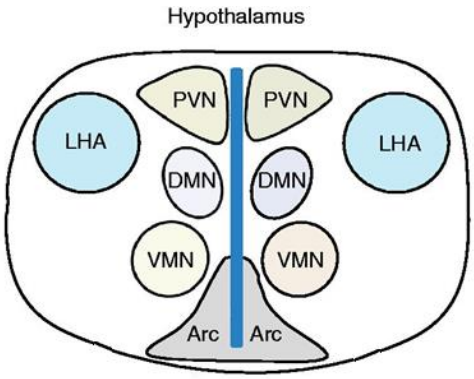


Figure 1. The different nuclei of hypothalamus. Lateral hypothalamic area (LHA), paraventricular nucleus (PVN), dorsomedial nucleus (DMN), ventromedial nucleus (VMN) and arcuate nucleus (ARC).

Energy homeostasis is controlled by a complex system in which factors such as food intake, hormonal regulation and sensory input all help to keep the system in balance. Energy provided from food consumption allows for the maintenance of biological function. When nutrient levels are low, organisms seek out food for energy, and enter

a metabolic state of catabolism where stored nutrients are oxidized to provide sufficient energy to maintain tissue function. Energy from fat and carbohydrates provide the largest amount of energy, which can be consumed to be utilized or stored for later use. At the onset of a meal, metabolism is biased towards the oxidation of carbohydrates and excess energy is stored producing an anabolic state. Thus, energy homeostasis is achieved not only by changes (increases or decreases of food intake), but also by alterations in metabolism and energy utilization.

Hormones play an important role in conveying metabolic signals from the body to the brain. These signals provide feedback to key brain regions that regulate food intake, body weight, body temperature, blood glucose levels, and fat storage. Changes in hormones are detected by cells in the hindbrain and ventral hypothalamus, both regions of the brain where the blood brain barrier is more porous (Van der Lely et al., 2004).

As shown in figure 2, there are two key hormones that play an important role in the regulation of food intake: leptin and ghrelin.

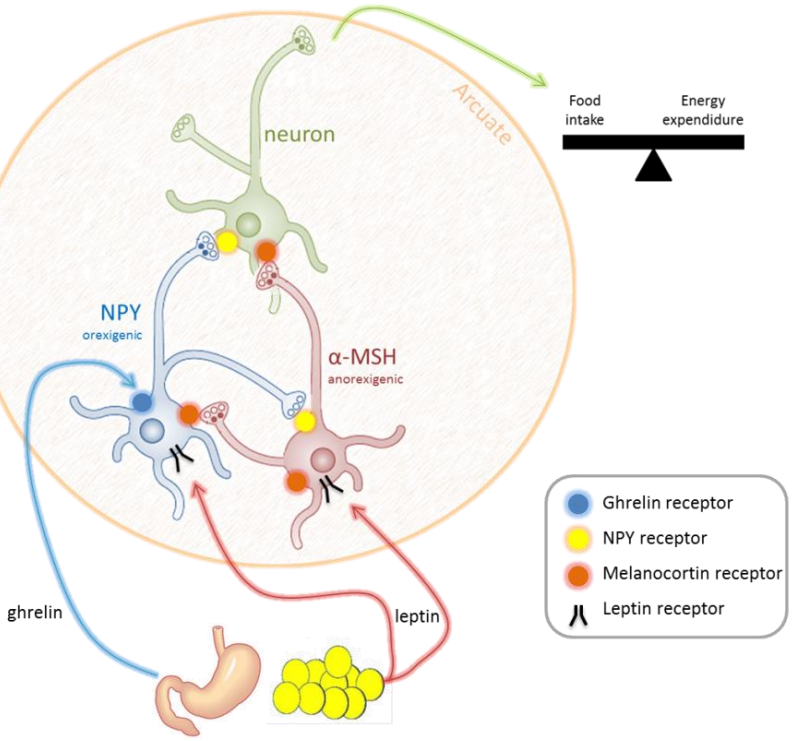


Figure 2. Hormones that control eating. In the ARC two sets of neurosecretory cells receive hormonal input and relay neuronal signals to peripheral cells. Leptin is released from adipose tissue and act on anorexigenic neurons to trigger release of α-MSH. Leptin also acts on orexigenic neurons to inhibit the release of NPY, reducing hunger. Ghrelin is released from gut and stimulates appetite by activating NPY neurons.

Leptin is a protein produced and secreted by adipocytes. Its secretion targets the brain to decrease appetite and increase energy expenditure (Zhang et al., 1994).

Leptin travels through the bloodstream, past the blood brain barrier (BBB) and binds to leptin receptors in a number of hypothalamic nuclei including the ARC, providing information about the status of the fat stores of the body (Golden, Maccagnan, & Pardridge, 1997; Schwartz et al., 1996). Leptin targets and stimulates α-MSH neurons, while inhibiting NPY/AGRP neurons resulting in the decrease of food intake as well as an increase in the expenditure of energy. The other important hormone is ghrelin, in some way, it has the opposite effects of leptin. Ghrelin stimulates appetite as well as energy expenditure, in a way that leads to the accumulation and preservation of energy stores (Tschöp et al., 2000).

1.2.THE HORMONE GHRELIN

Ghrelin exerts wide physiological actions throughout the body, including the secretion of growth hormone, the modulation of gastric acid and secretion of motility, the stimulation of appetite and food intake, the modulation of the endocrine and exocrine pancreatic secretions, glucose homeostasis, cardiovascular functions, anti-inflammatory functions, reproductive functions, bone formation… (Fig. 3) In this thesis we will focus on the role of ghrelin in food intake.

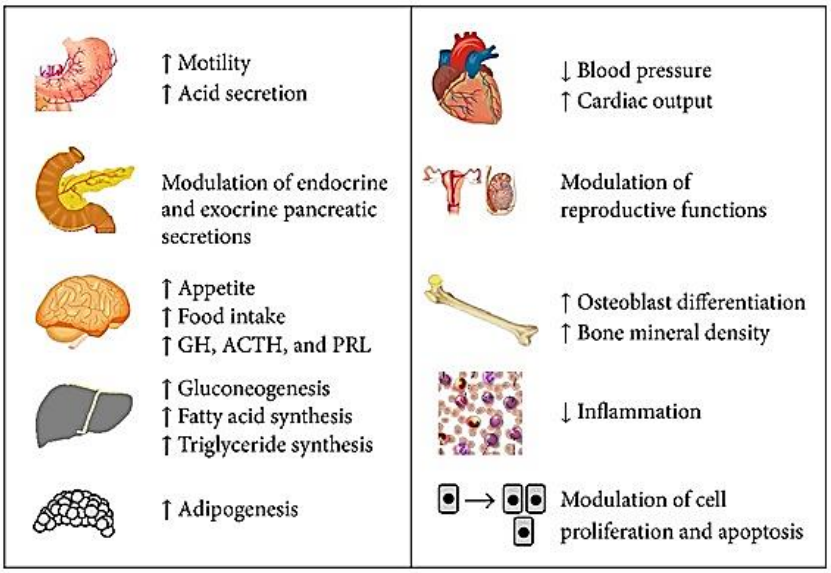


Figure 3. Main physiological functions of ghrelin (From Delporte, 2013).

Ghrelin is a gastric peptide of 28 amino acid (AA) that was first purified from rat stomach in 1999 (Kojima et al., 1999). It is mainly produced by a subset of stomach cells, the endocrine X/A-like cells of the fundus mucosa, and also by the hypothalamus, the pituitary, and other tissues (Date et al., 2000; Rindi et al., 2002). It is a natural ligand for the growth hormone secretagogue (GHS) receptor (GHS-R) (Howard et al., 1996).

Ghrelin is subjected to acylation in the endoplasmic reticulum (ER) of the hydroxyl group of the Ser3 (Kojima et al., 1999), essentially by an octanoyl group (C8:0) and more rarely by a decanoyl (C10:0) or decanoyl (C10:1) group (Hosoda et al., 2003). The enzyme responsible for ghrelin acylation is ghrelin O-acyl transferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008). Ghrelin and GOAT colocalize in gastric X/A like cells (Gutierrez et al., 2008). Ingestion of either medium-chain fatty acids or medium-chain triglycerides increases ghrelin acylation (Nishi et al., 2012). Acyl-CoA are the presumed donors of acyl group, even though it remains unclear how acyl-CoA could get into the ER lumen (Yang et al., 2008).

Circulating ghrelin consists of more than 90% of desacyl ghrelin and less than 10% acyl ghrelin (Patterson et al., 2005).The high desacyl/acyl ghrelin ratio in the circulation can be explained by the shorter half-life of ghrelin compared to desacyl ghrelin (Akamizu et al., 2005). However, the acyl group is essential for its binding to GHS-R and the concomitant activation of the inositol triphosphates/calcium pathway (Kojima et al., 1999; Kojima et al., 2001).

1.2.1.GHRELIN RECEPTORS

The ghrelin receptor, GHS-R, belongs to G-protein coupled receptors (GPCR) superfamily, characterized by seven transmembrane helix domains (Howard et al., 1996). Human GHS-R is located on chromosome 3 and has been identified two splice variants of this receptor: GHS-R1A and GHS-R1B. GHS-R1A is a 366 AA protein containing seven transmembrane helix domains, while GHS-R1B is a 289 AA protein containing five transmembrane helix domains (McKee et al., 1997).

Coupling of GHS-R1A to G-protein involves the 3 rd intracellular loop. GHS-R1B is lacked of the 3 rd intracellular loop and that prohibits it from coupling G-proteins. GHS-R1A activation leads to the subsequent activation of phospholipase C, inositol triphosphates, and intracellular calcium pathways (Smith et al., 1999). At physiological concentrations, only acyl ghrelin binds to GHS-R1A (Gauna et al., 2007; Staes et al., 2010). It has been suggested that the cell membrane acts as "catalyst" for ghrelin binding to its receptor. Indeed, acyl ghrelin and desacyl ghrelin are electrostatically attracted to membranes by their basic residues, but acyl ghrelin penetrates deeper due to its acyl group (Staes et al., 2010). The acyl group of ghrelin is assumed to favor ghrelin partitioning into the lipids to increase the local concentration of ghrelin in the vicinity of the receptor, to bring ghrelin to the membrane where its binding pocket is present, and to optimize the conformation of ghrelin for improving its docking to GHS-R1A (Staes et al., 2010). Noteworthy is that GHS-R1A possesses constitutive level activity (Holst et al., 2003) that appears to be conferred by the presence of 3 aromatic AA located in the 6 th and 7 th transmembrane helix domains.

The proposed model supposes that these AA ensure proper docking of the extracellular end of the 7 th transmembrane helix domain into the 6 th transmembrane helix domain, thereby mimicking agonist activation and stabilizing the receptor in its active conformation (Holst et al., 2003). The high basal signaling of GHS-R1A has also been demonstrated in vivo in the hypothalamus (Petersen et al., 2009).

It has been proposed that ghrelin exerts mostly of its feeding effects through the GHS-R1A receptor. The relevance of the receptor GHS-R1A is further supported by anatomical data showing that GHS-R1A is highly expressed in hypothalamic nuclei that regulate feeding and body weight homeostasis, such as ARC, VMN and PVN (Bennett et al., 1997; Zigman et al., 2005).

1.2.2.FOOD INTAKE AND ENERGY BALANCE

Ghrelin is secreted in a pulsated manner as its levels increase before the onset of meal, during fasting, and decrease after feeding (Cummings and Schwartz, 2003; Tschöp et al., 2001). This pulsatile secretion of ghrelin suggests that ghrelin may act as a signal for meal initiation. However, it appears that peaks of ghrelin concentrations are related to meal patterns and may rise in anticipation of eating rather than elicit feeding (Frecka and Mattes, 2008). Central and peripheral administration of ghrelin to rats induces food intake stimulation and energy expenditure reduction accounting for body weight increase (Kamegai et al., 2001; Nakazato et al., 2001; Shintani et al., 2001; Tschöp et al., 2000; Wren et al., 2001). Ghrelin stimulates appetite by central and peripheral pathways and via the vagus nerve.

Ghrelin has also been related with energy homeostasis as is involved in long-term body weight regulation. Its levels decrease with weight gain (overfeeding, pregnancy, or high fat diet) (Williams et al., 2006; Palik et al., 2007; Hosojima et al., 2006) and increase with weight loss (food restriction, long-term chronic exercise but not acute exercise, cachectic states in anorexia nervosa, lung, breast and colon cancer) (Purnell et al., 2007; Kraemer and Castracane, 2007; Soriano-Guillén et al., 2004; Shimizu et al., 2003; Wolf et al., 2006).

This hormone is able to active orexigenic neurons in the ARC and VMH nuclei where the GHS-R1A receptor is expressed (Guan et al., 1997), producing an increase of mRNA levels of the neuropeptides AgRP and NPY (Kamegai et al., 2001; Nakazato et al., 2001; Lage et al., 2010). It has also been reported that ghrelin inhibits the firing of POMC neurons by increasing the release of γ-aminobutyric acid (GABA) (Andrews et al., 2008). To confirm the effects of ghrelin in the neuropeptides, studies with AgRP KO or NPY KO mice were done. AgRP KO and NPY KO mice responded normally to

ghrelin but a double KO, an AgRP/NPY KO failed to respond to ghrelin indicating the existence of redundancy among these two neuropeptides as mediators of ghrelin's orexigenic action (Chen et al., 2004).

The molecular mechanisms by which ghrelin exerts its orexigenic effects are not totally elucidated, however it is known that hypothalamic AMP-activated protein kinase (AMPK) plays a pivotal role in the effects of ghrelin on appetite and food intake (Kola et al., 2008; Anderson et al., 2004).

- **AMP-activated protein kinase**

AMPK is a serine/threonine protein kinase that senses the energy status of the cells and regulates fuel availability by stimulating ATP producing pathways (Hardie, 2008). Following ATP depletion, AMP rises and induces the activation of AMPK through its phosphorylation in Thr^{172} (Woods and Dickerson, 2005).

Ghrelin has been shown to stimulate AMPK by phosphorylation via calmodulin kinase-kinase 2 (CaMKK2) activated in response to rise in intracellular calcium concentration induced by GHS-R1A signaling (Cowley et al., 2003; Kola and Korbonits, 2009).

It has been proposed other mechanisms by which ghrelin activates AMPK. For instance, It has been shown that ghrelin activates AMPK by the hypothalamic Sirtuin 1 (SIRT1)/p53 (Velázquez et al., 2011). SIRT1 is a deacetylase activated in response to calorie restriction that acts through the tumor suppressor p53. SIRT1 and p53 are required for ghrelin-induced AMPK activation and consequent orexigenic action.

Furthermore, it has been proposed that the cannabinoids pathway in the hypothalamus is required for the effect of ghrelin on AMPK (Kola et al., 2008). It is possible that the ghrelin receptor and the cannabinoids receptor (CB1) work coordinately generating heterodimers between them.

Whatever the mechanism, activated AMPK (pAMPK) is a counter-regulatory response to avoid ATP depletion, leading to a switching off ATP-consuming processes (such as fatty acid synthesis), while switching on catabolic processes that produce ATP (such as fatty acid β-oxidation) (Lage et al., 2008; Martínez de Morentin et al., 2010).

- **Fatty acid pathway**

Activated AMPK (pAMPK) by ghrelin in the hypothalamus induces the phosphorylation of acetyl-CoA carboxylase (ACC). The phosphorylation of ACC decreases its activity (to catalyze the carboxylation of acetyl-CoA to malonyl-CoA)(Dowell et al., 2005; López et al., 2007; Lage et al., 2008; Martínez de Morentin et al., 2010). Thus, decreases malonyl-CoA levels and the flux of substrates in the fatty acid biosynthesis pathway. As malonyl-CoA is an allosteric inhibitor of CPT1 (McGarry and Brown, 1997) its reduction produces an increase of CPT1 activity (to enter acyls-CoA into the mitochondria) and increases fatty acid oxidation (Fig. 4).

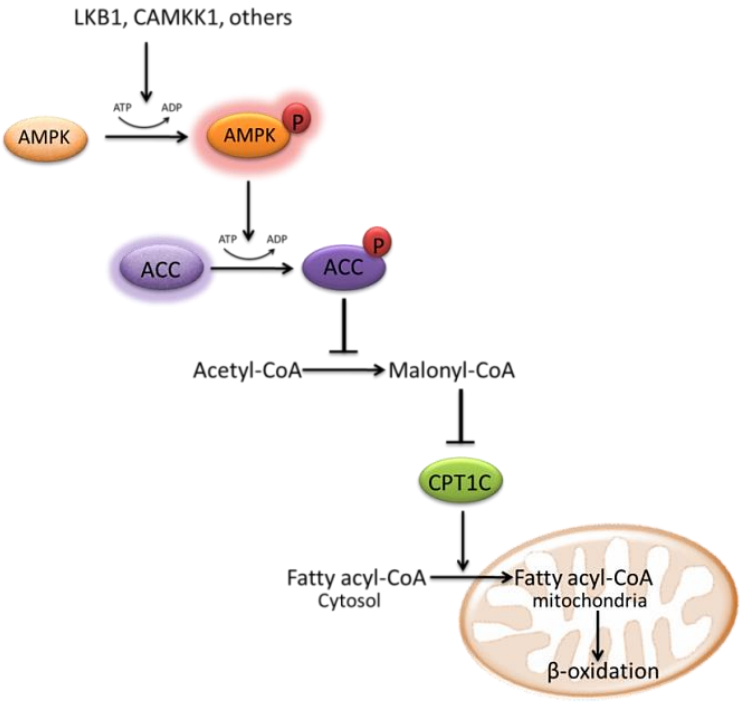


Figure 4. The fuel sensor AMPK. Activation of AMPK leads to the inhibition of ACC by phosphorylation. AMPK increases fatty acid oxidation via the regulation of malonyl CoA levels, which is both a critical precursor for biosynthesis of fatty acids and a potent inhibitor of CPT-1, the shuttle that controls the transfer of LCA-CoA into the mitochondria.

During fatty acid β-oxidation, reactive oxygen species (ROS) are generated and in order to scavenge them, increases uncoupling protein-2 (UCP2) which promotes ROS scavenging (Andersson et al., 2004). Concordant with this, ghrelin increases ROS production in UCP2-KO mice but not in WT mice (where ROS production are stimulated but then buffered by UCP2). It has been reported that this mechanism is necessary for the effect of ghrelin in AgRP and NPY. In UCP2 KO mice the upregulation that produces ghrelin of AgRP, NPY is blunted (Andrews et al., 2008).

It is thought that hypothalamic lipid metabolism and UCP2 influence neuropeptide gene expression through their transcription factors (BSX, FOXO1, and pCREB) (Andersson *et al.* 2004; Kola et al., 2008; Andrews *et al.* 2008; López et al., 2008; Lage *et al.* 2010) but the molecular events connecting lipid metabolism-ROS-UCP2 axis with the transcription factors involved in the gene expression of NPY and AgRP remain totally unclear.

- **Transcription factors in ghrelin pathway**

It has been reported that the transcription factor brain-specific homeobox (BSX) is highly expressed in orexigenic neurons in the ARC and that it regulates the expression of AgRP and NPY in rodents (Sakkou et al., 2007; Nogueiras et al., 2008; Lage et al., 2010). Both genes share BSX as a common transcriptional factor but BSX needs to interact with another two transcriptional factors: cAMP response-element binding protein (CREB) and forkhead box O1 (FoxO1). BSX interacts with phospho-CREB and FoxO1 to modulate the expression of AgRP and NPY respectively (Sakkou et al., 2007) (Fig. 5).

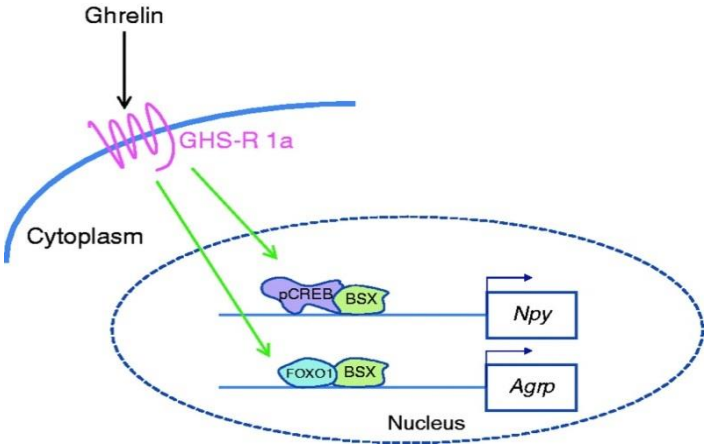


Figure 5. The ghrelin orexigenic effect. Ghrelin, acting on GHS-R1A, stimulates the expression of hypothalamic homeobox domain transcription factor (BSX), forkhead box O1 (FoxO1), and the phosphorylated cAMP response-element-binding protein (pCREB). Subsequently, agouti-related peptide (AgRP) and neuropeptide Y (NPY) gene expressions are increased in the ARC of the hypothalamus (From Varela et al., 2010).

The transcription factor CREB is active when it is phosphorylated, but the transcription factor FoxO1 is more complicated. The protein FoxO1 is located in the cytoplasm but when it is the phosphorylated, it translocates to the nucleus where it can act as a transcription factor (Kim et al., 2006). We can say that FoxO1 is the inactive form and phospho-FoxO1 the active form. However, ghrelin produces an increase of both FoxO1 and phospho-FoxO1 (Lage et al., 2010), hence the physiological relevance of increased phospho-FoxO1 is unclear.

Little is known about the physiological mechanism by which BSX, pCREB and FoXO1 are activated. Recently it has been elucidated that mTOR plays an important role in the activation of pCREB and FoXO1 in the ghrelin pathway. mTOR is a serine/threonine protein kinase that regulates cell fate in addition to sensing energy. It activates when the ratio AMP/ATP decreases in states of high energy (Woods et al., 2008). Surprisingly, in the ARC of the hypothalamus, mTOR is activated by AMPK (Villanueva et al., 2009) and activation mediates the orexigenic action of ghrelin (Martins et al., 2012; Villanueva et al., 2009; Stevanovic et al., 2003). Indeed, ghrelin-

mediated mTOR activation induces the increase of pCREB and FoXO1 leading to an increase of NPY and AgRP and finally generating hunger (Martins et al., 2012).

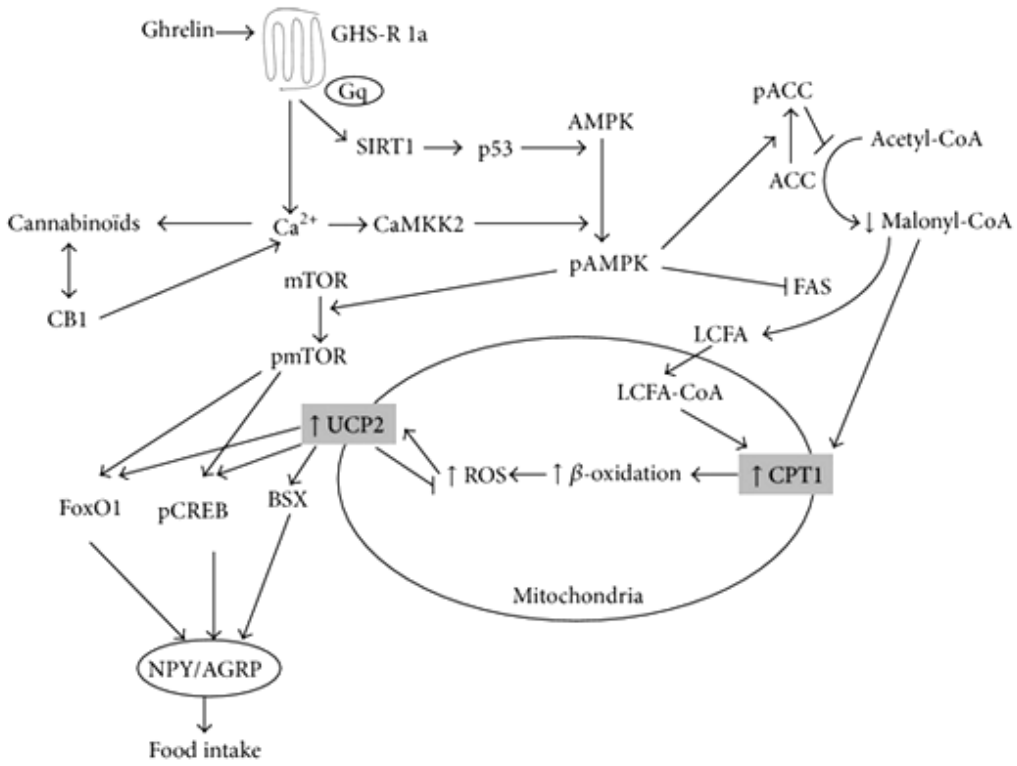


Figure 6. Molecular mechanisms leading to ghrelin-induced food intake in the hypothalamus. Intracellular signaling pathways and mitochondrial metabolism resulting in NPY/AGRP secretion consecutive to ghrelin receptor activation are schematized. ACC: acetyl coenzyme A carboxylase; AGRP: agouti-related protein; AMPK: 5′ adenosine monophosphate-activated protein kinase; BSX: brain-specific homeobox transcription factor; CaMKK2: calmodulin kinase-kinase 2; CB1: cannabinoid receptor type 1; CPT1: carnitine-palmitoyltransferase-1; CREB: cyclic adenosine 3′,5′ monophosphate response element-binding protein; FAS: fatty acid synthase; FoxO1: forkhead box protein O1; GHS-R1a: growth hormone secretagogue receptor type 1a; Gq: Gq protein; LCFA: long chain fatty acid; LCFA-CoA: long chain fatty acyl coenzyme A; mTOR: mammalian target of rapamycin; NPY: neuropeptides Y; p: phosphorylated state; ROS: reactive oxygen species; SIRT1: sirtuin 1; and UCP2: uncoupling protein-2 (From Delporte, 2013).

2.CARNITINE PALMITOYLTRANSFERASE 1

2.1.CARNITINE ACYLTRANSFERASES

To the cell, mitochondrial β-oxidation is the main pathway for degrading fatty acid and obtaining energy. The main step in this pathway is the transport of long chain fatty acyl groups into the mitochondria matrix. The transport of fatty acids is performed by a group of enzymes with acyltransferase activity (carnitine acyltransferase family) and a transporter in the inner mitochondrial membrane (McGarry et al., 1989). Different enzymes belong to the carnitine acyltransferase family, each of them having specificity for a determinate length of the fatty acyl group used as a substrate. Carnitine acetyltransferase (CrAT or CAT) uses acetyl-CoA as a substrate (Bieber, 1988), Carnitine octanoyltansferase (CrOT or COT) facilitates transport of fatty acids (C8-C10) from peroxisomes to mitochondria and Carnitine palmitoyltransferases (CPTs) 1 and 2 facilitate transport of long chain fatty acids (C16-C20) to the mitochondrial matrix where they will be β-oxidized (McGarry et al., 1989).

2.2.CARNITINE PALMITOYLTRANSFERASE

The carnitine palmitoyltransferase system (CPT) permits the entry of long chain fatty acyl-CoA (LC-CoA) into the mitochondria for β-oxidation by consecutive transesterificactions involving different activities (Kerner and Hoppel, 2000). The first component of this system is carnitine palmitoyltransferase 1 (CPT1), and integral transmembrane protein of the mitochondrial outer membrane that catalyzes the transfer of acyl moieties from CoA to carnitine. The acylcarnitine product can then traverse the inner membrane by means of a carnitine-acylcarnitine translocase (CACT). CPT2, on the matrix side of the inner membrane, reverses CPT1 reaction, retransferring the acyl group to CoA-SH (Fig. 7). The acyl-CoA then undergoes β-oxidation and ultimately yields acetyl-CoA (Zammit, 2008).

The reaction catalyzed by CPT1 is the key regulatory site controlling the flux through β-oxidation, by virtue of its inhibition by malonyl-CoA, an intermediate in fatty acid biosynthesis. This reaction is not only central to the control of fatty acid oxidation, but it also determines the availability of long chain acyl-CoA for other processes, notably the synthesis of complex lipids.

The CPT1 enzyme exists in at least three isoforms in mammals:

- **CPT1A** has also been referred to as the liver isoform or L-isoform (L-CPT1). It is the most ubiquitously expressed isoform and it is found not only in liver

mitochondria, but also in mitochondria of the pancreas, kidney, lung, spleen, intestine, brain and ovary (Esser et al., 1993; Britton et al., 1995; McGarry and Brown, 1997).

- **CPT1B** is also known as the muscle isoform (M-CPT). It was first identified in skeletal and cardiac muscle mitochondria, but also occurs in adipose tissue and the testis (Yamazaki et al., 1995; Esser et al., 1996).
- **CPT1C** has been more recently reported and is a brain-specific protein that shows high sequence homology with the other isoforms but its function remains unknown (Price et al., 2002). CPT1C unlike the other isoforms is located in the endoplasmic reticulum (ER) of cells (Sierra et al., 2008).

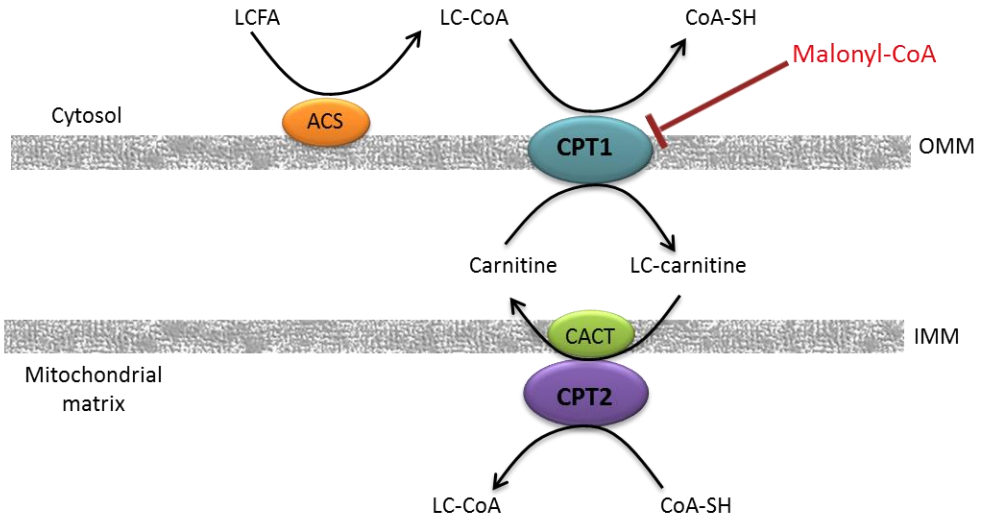


Figure 7. LCFA translocation into the mitochondria by the carnitine palmitoyltransferase system. LCFA (long chain fatty acids) are activated to LC-CoA by the action of the acyl-CoA synthetase (ACS). Transport of LC-CoA from the cytosol to the mitochondria matrix involves the conversion of LC-CoA to acylcarnitines by CPT1, translocation across the mitochondrial inner membrane by the carnitine acylcarnitine translocase (CACT) and reconversion to LC-CoA by CPT2. The physiological inhibition of CPT1 enzymes by malonyl-CoA is also represented (in red). OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane.

2.3.CPT1A AND CPT1B

CPT1A and CPT1B have been extensively studied since they were cloned for the first time. The identity in amino acid residues is high (62 %), but both isozymes present significantly different kinetic and regulatory properties: CPT1A displays higher affinity for its substrate carnitine and lower affinity for the physiological inhibitor malonyl-CoA than the muscle isoform (Esser et al., 1996; McGarry and Brown, 1997; Zammit, 2008). This differential sensitivity to the reversible inhibitor of the enzyme is probably involved in the finer regulatory of fatty acid oxidation in heart and skeletal muscle, in

comparison to liver. It has been published that the interaction between N- and C-terminal domains are critical for malonyl-CoA binding and sensitivity (López-Viñas et al., 2007).

CPT1A and B are both localized in the outer mitochondria membrane with two transmembrane domains (TM1 and TM2) and the N- and C- termini facing the cytosolic side (Fraser et al., 1997).

CPT1 is the most physiologically important regulatory step in mitochondrial fatty acid oxidation (McGarry and Foster, 1980). This process allows the cell to signal the relative availability of lipid and carbohydrate fuels in liver, heart, skeletal muscle, and pancreatic β-cells (Zammit, 1999).

2.4.CPT1C

2.4.1.A NEW ISOFORM OF CPT1

Performing *in silico* database searches based on human CPT1A cDNA nucleotide or protein sequence, a new gene was identified. This new gene was designated CPT1C due to its high similarity to the other isoforms of CPT1 (Price et al., 2002). The new isozyme contains an extended tail (of approximately 30 AA) at the C-terminal end in contrast to the other isoforms.

The study of the phylogram from the three isozymes indicates that three CPT1 genes diverged approximately at the same time, and presumably, two independent duplication events occurred, close together in evolutionary time (Price et al., 2002).

When looking for CPT1C EST sequences from other species, no orthologous sequences could be found in species other than mammals, while the other isoforms are expressed in organisms like birds, fishes, reptiles, amphibians or insects. This suggests that CPT1C has a specific function in more evolved brains (Price et al., 2002; Sierra et al., 2008).

2.4.2.LOCALIZATION OF CPT1C

Expression studies indicate that CPT1C is widely expressed throughout the central nervous system (CNS) (Fig. 8) including the hypothalamus, hippocampus, cortex, and amygdale. CPT1C is enriched in neural feeding centers of the hypothalamus perhaps to regulate energy homeostasis (Price et al., 2002).

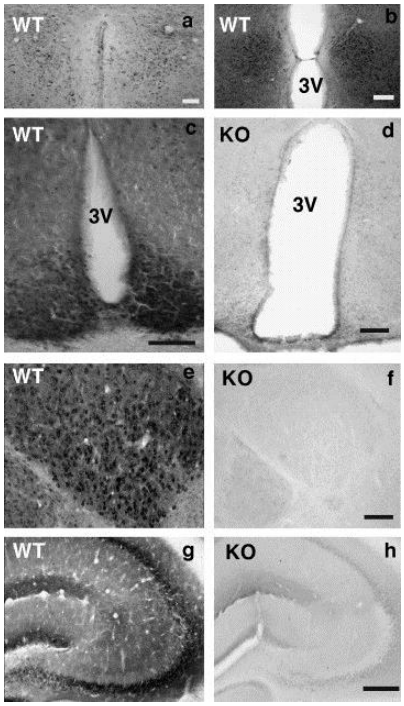


Figure 8. Immunohistochemical localization of CPT1C in mouse brain :including the hypothalamus (a-d), amygdale (e), and hippocampus (g). Coronal sections (50 µm) from mouse brain (a) PVN, (b) VMH, and Arc (c,d) were stained with anti-CPT1C antibody. CPT1C is also densely expressed in the amygdale (e) and hippocampus (g). The expression is specific as CPT1C immunoreactivity is absent in CPT1C knockout mice (d, f and g). WT refers to wild-type mice and KO to CPT1C knockout mice (d,f and h). Bar: 100 µm (From Dai et al., 2007).

To identify the cell types expressing CPT1C in the brain, co-localization studies with a neuronal marker (Neuronal Nuclei, NeuN), or an astrocyte marker (Glial Fibrillary Acidic Protein, GFAP) were performed by our group. The figure 9 shows co-labeling of CPT1C with NeuN confirming that CPT1C is expressed mainly in neurons. No co-localization was detected between CPT1C and GFAP indicating that CPT1C is not present in brain astrocytes (Sierra et al., 2008).

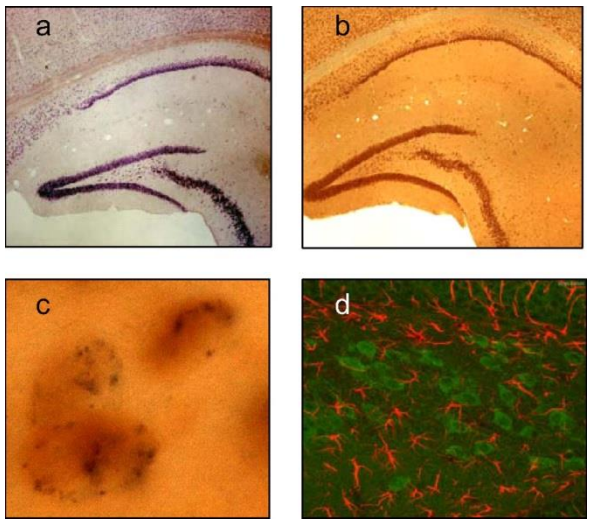


Figure 9. Co-localization studies of CPT1C mRNA with NeuN and GFPA proteins in brain sections. Brain sections were processed suing in situ hybridization with CPT1C antisense Riboprobe (a) or immunocytochemistry with NeuN primary antibodies and biotinylated secondary antibodies (b) or both methods (c). (d) Mouse adult brain sections were processed by double immunocytochemistry with CPT1C antibodies (green stain) and GFAP (red stain). (From Sierra et al., 2008).

CPT1C has a high sequence similarity to CPT1A and CPT1B. Therefore, it has been proposed that CPT1C would have the same intracellular localization, which is the outer mitochondrial membrane (Price et al., 2002) but in 2007, our group demonstrated that CPT1C is located in endoplasmic reticulum of neurons. In order to demonstrate the localization of CPT1C there were designed different strategies. The first approach was to transfect fibroblast with pCPT1A-EGFP or pCPT1C-EGFP. They show different fluorescence pattern, cells transfected with pCPT1A-EGFP express a punctuate manner while cells transfected with pCPT1C-EGFP show a reticular manner, indicating a different subcellular localization between the isozymes (Sierra et al., 2008). The second approach to verify the subcellular localization of CPT1C in the endoplasmic reticulum was co-localization studies with Mito Tracker, a potential-sensitive dye that accumulates in mitochondria or with antibodies against calnexin, an ER integral protein. As shown in the figure 10, CPT1C is localized in the ER membrane and not in mitochondria. It is also described that the N-terminal region of CPT1C contains a putative microsomal targeting responsible for ER localization (Sierra et al., 2008).

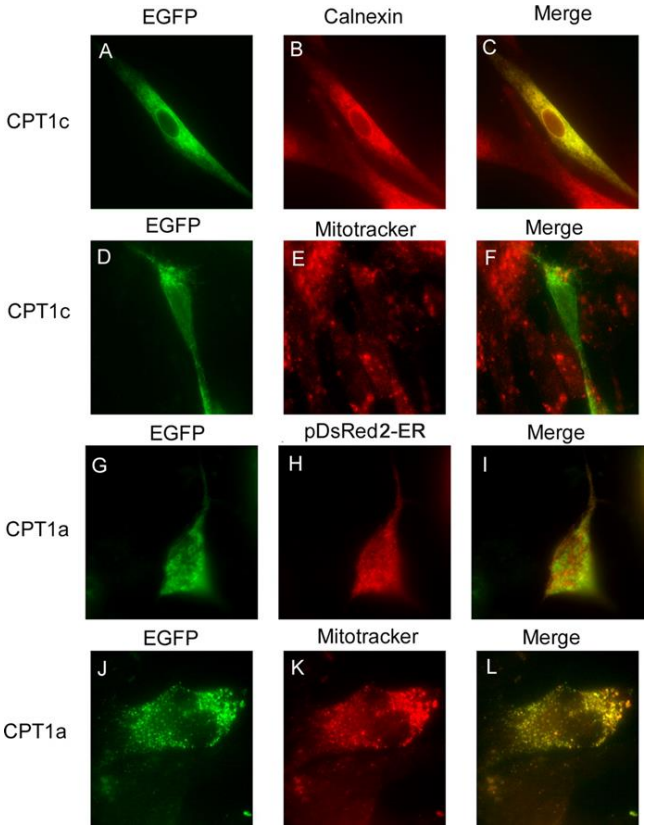


Figure 10. Co-localization studies of CPT1C in mitochondria and ER. Fibroblasts were transfected with pCPT1C-EGFP (*A–F*) or pCPT1A-EGFP (*G–L*) and incubated with anti-calnexin as primary antibody (*B*) or stained by Mito-Tracker (*E* and *K*) or co-transfected with pDsRed2-ER (*H*). Images were taken by confocal microscopy with a filter to see *green* emission, *red* emission, or the *merged image* (*C*, *F*, *I*, and *L*) (From Sierra et al., 2008).

2.4.3.CPT1C ENZYME ACTIVITY

Analysis of the amino acid sequence of these proteins reveals that all residues important for carnitine acyltransferase activity are conserved in CPT1C, as well as the malonyl-CoA binding site.

Despite this, when performing CPT1 radiometric activity assays, no catalytic activity was found even when assaying a large range of acyl-CoA, which are good substrates for CPT1A and CPT1B (Price et al., 2002; Wolfgang et al., 2006).

Trying to elucidate why CPT1C has a very low activity, it has been recently published a comparison of the structural regulatory and catalytic domains between the CPT1A and CPT1C. In CPT1A, the regulatory domain (N), adopts an inhibitory or a non-inhibitory state, Nα or Nβ, respectively, which differ in their association with the catalytic domain (CD) (Fig.11). When the state is Nβ, the N- terminal interacts with the CD domain and the enzyme is active, has carnitine palmitoyltransferase activity. When the levels of malonyl-CoA rise or there is a specific fluidity or curvature of the outer mitochondrial membrane (OMM) the Nβ state transforms to Nα state and the affinity of N for the CD terminal disappear inactivating CPT1A. In relation with CPT1C, the inhibitory Nα state was found to be structurally homologues whereas the non-inhibitory Nβ state was severely destabilized (Fig. 12), suggesting a change in overall regulation that may contribute to the low catalytic activity of CPT1C (Samanta et al., 2014).

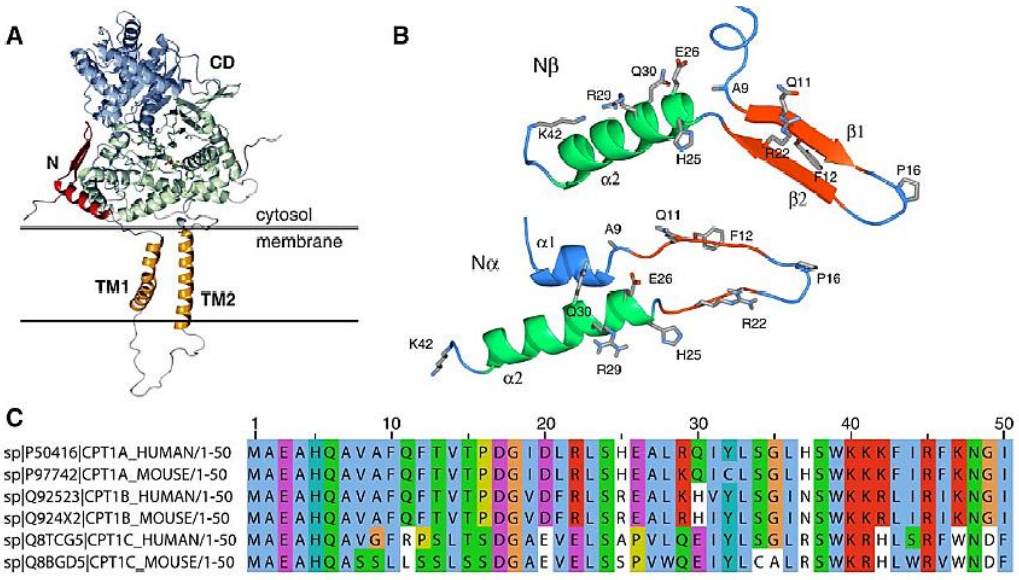


Figure 11. Overview of carnitine palmitoyltransferase 1 enzymes. A: Structural model of the human CPT1A enzyme. The structure of the CPT1A regulatory domain, termed N, in the non-inhibitory Nβ state (PDB ID 2LE3) is shown in complex with modeled transmembrane and catalytic domains, termed TM1/TM2 and CD, respectively. B: A model of the Nα state and the structure of the Nβ state of human CPT1A are depicted in cartoon representation. Amino acids that are substituted in CPT1C are shown in stick representation. C: Sequence alignment of N for the three

mammalian CPT1 isoforms. Conserved amino acids are colored by the Jalview multiple alignment editor using the ClustalX color scheme (From Samanta et al., 2013).

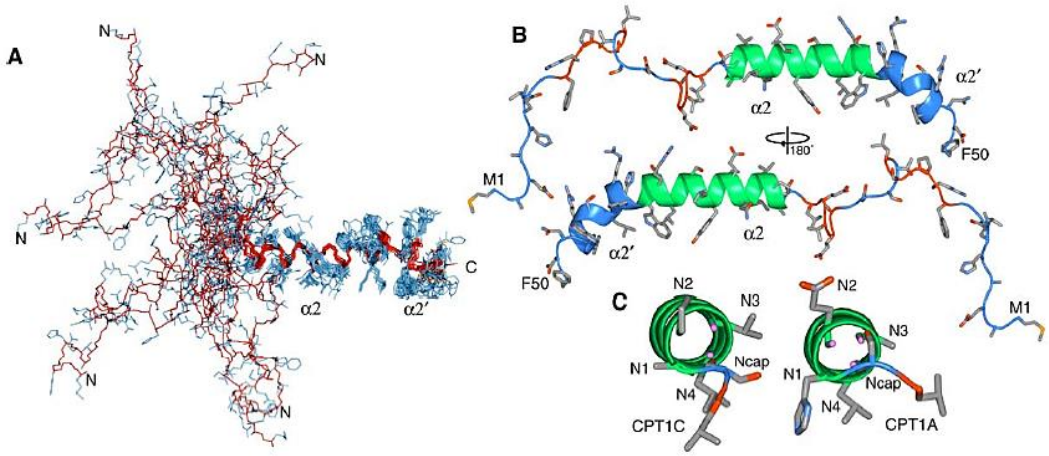


Figure 12. Structure of the Nβ state of human CPT1C. A: Ensemble of 20 calculated simulated annealing structures, showing the backbone in red and side chains in cyan. The structures were superimposed in the heavy atom backbone coordinates of the well-structured Leu23-Trp47 residues. The folded nature of helices α2-α2′ contrasts the dynamically unstructured conformations of residues Met1-Glu22. B: Cartoon representations of the lowest energy ensemble structure. The polar and apolar faces of helices α2-α2′ are oriented toward the top and bottom of the page, respectively. The shown orientations are related by a rotation of 180º about the y-axis. C: Illustration of differences in N-terminal helix α2 capping between CPT1C and CPT1A. Ncap denotes the capping residue Ser24 and N1 to N4 represent the first four residues of the helix. The H^N atoms of N2-N4 are shown in violet (From Samanta et al., 2013).

The ability of CPT1C to bind malonyl-CoA has been demonstrated, and it has been suggested that CPT1C regulates malonyl-CoA availability in the brain (Price et al., 2002; Wolfgang et al., 2006). More recently, it has been suggested based on the structural properties of the N terminal that CPT1C must be more sensitive compared to CPT1A to malonyl-CoA, perhaps in relation to a sensory instead of catabolic function in neurons (Samanta et al., 2013).

2.4.4.ROLE OF CPT1C IN ENERGY HOMEOSTASIS

In order to elucidate the role of the CPT1C isozyme, the first CPT1C knockout (KO) mice model was developed by Lane and Colleagues in 2006.

CPT1C KO mice showed no apparent developmental abnormalities or alterations in any organ size, and did not show differences in body temperature when compared with their wild type littermates. When fed a standard chow (SC), CPT1C KO mice showed a reduction in whole body weight, approximately 15%, and a decrease in food intake by approximately 25% (Fig. 13). When fed a high fat diet (HFD), CPT1C KO mice were more susceptible to obesity (increased weight gain despite reduced food intake)

(Fig. 14), became mildly insulin-resistant and showed lower peripheral energy expenditure (Wolfgang and Lane; 2006). Additionally, overexpression of CPT1C in the hypothalamus was sufficient to protect CPT1C KO mice from body weight gain when fed a high fat diet (Fig. 15) (Dai et al., 2007).

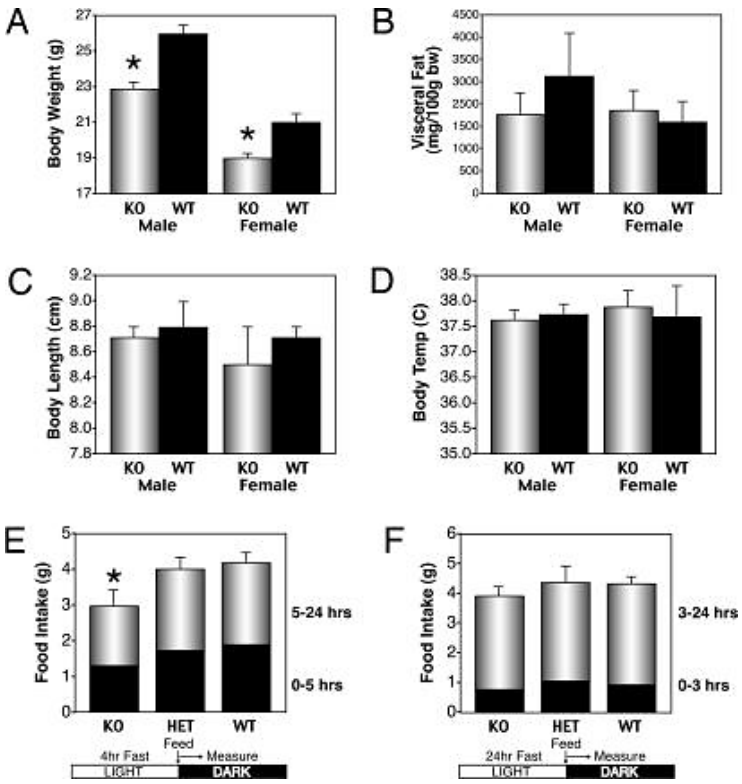


Figure 13. CPT1C KO mice are hypophagic and have a lower body weight than WT mice. (*A*) Body weight was significantly lower in CPT1C KO males (*, $P < 0.0001$; n=10 per group) and females (*, $P < 0.005$; n=10 per group).(*B*) CPT1C KO male mice have a trend toward increased visceral adipose tissue (n=6 per group). The CPT1C KO does not affect body length (*C*) or temperature (*D*) (n = 6 per group). (*E*) CPT1C KO mice exhibit lower food intake than WT or heterozygous littermates (*, $P < 0.05$; n = 6 per group) after a short (4-h) fast just before the dark cycle. (*F*) Effect of CPT1C KO on food intake after a 24-h fast. (From Wolfgang et al., 2006).

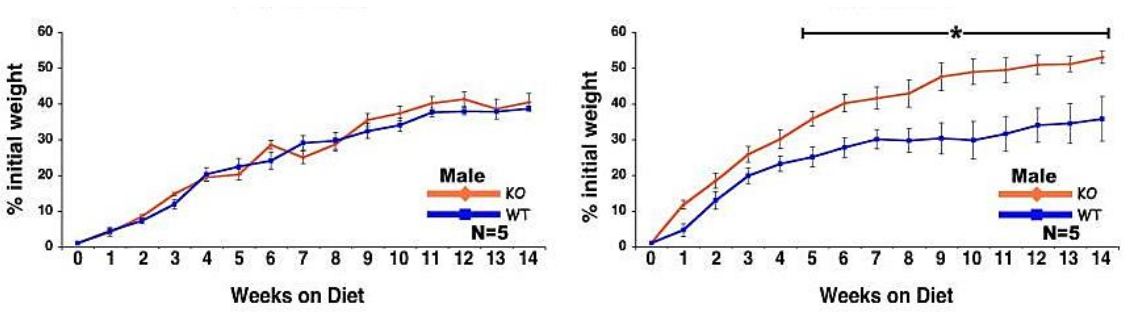


Figure 14. CPT1C KO mice are more susceptible to the effect of a high-fat diet. WT and CPT1C KO mice (n = 5 per group) were fed a control diet (10% of total kcal from fat; 1 kcal = 4.18 kJ) (*A*) or a high-fat diet (45% of total kcal from fat) (*B*) for 14 weeks. Animals were weighed weekly at 1400 hours. Initial body weights (average grams) were as follows: male WT, 25.9 g; male KO, (*, $P < 0.05$) (From Wolfgang et al., 2006)

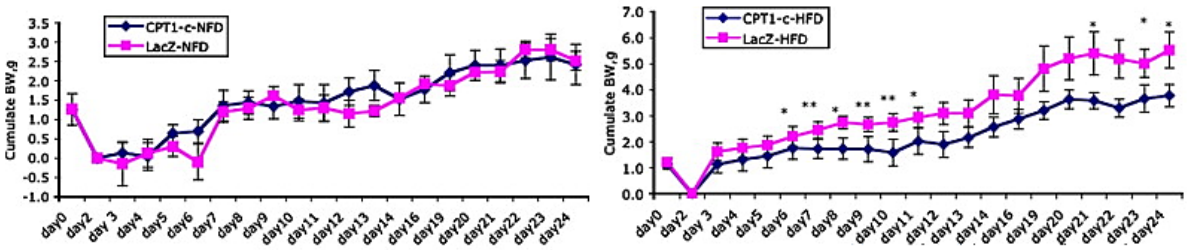


Figure 15. Effect of bilateral stereotactic injection of adenoviral CPT1C or LacZ (control) vectors into the ventral hypothalami on body weight and food intake of mice (three mice/treatment) fed a normal (NFD) or a high-fat (HFD) diet. Cumulative body weight gain was measured daily for two weeks beginning at day 1 after stereotactic injection. Asterisks refer to the level of statistical significance ($*p < 0.05$ or $**p < 0.001$) (From Dai et al., 2007).

Wu and colleagues three years after that, developed another CPT1C KO model that displayed a similar basic phenotype to that of the previous model. In addition, they found that the liver and muscle expression of genes promoting fatty acid oxidation (such as CPT1A) was markedly decreased in CPT1C KO mice on a high fat diet compared to WT mice. In line with these results, CPT1 activity and palmitate oxidation was also lower in CPT1C KO mice on HFD (Gao et al., 2009).

More recently, some studies have been done with an animal model that allowed the conditional expression of CPT1C in a specific tissue. Exogenous expression of CPT1C specifically in brain, displayed severe growth retardation in the postnatal period and a reduction of the brain weight (microcephaly). When these mice fed a high diet, they were protected from weight gain (Reamy and Wolfgang, 2011).

Taken together these preliminary results of CPT1C suggest that CPT1C is protective against the effect of fat feeding on body weight and that CPT1C is necessary for the regulation of energy homeostasis.

2.4.5.OTHER ROLES OF CPT1C

CPT1C has been related with food intake and energy homeostasis as it is located in the hypothalamus, but CPT1C is also located in other brain regions and recently different roles of CPT1C have been elucidated.

- **Dendritic spine maturation and cognition**

Studies from our group in primary hippocampal cultured neurons of CPT1C KO mice reveled that CPT1C deficiency altered dendritic spine morphology by increasing immature filopodia and reducing mature mushroom and stubby spines. Total protrusion density and spine head area in mature spines were unaffected. Treatment

of cultured neurons with ectopic overexpression of CPT1C reverted the KO phenotype indicating a role in spine maturation. They also performed the hippocampus-dependent Morris Water maze test on mice and results showed that CPT1C deficiency strongly impairs spatial learning (Carrasco et al., 2012).

- **Motor function**

More recently, a battery of neurological tests on CPT1C KO mice showed some motor disturbances. A detailed study of motor function done by our group revealed impaired coordination and gait, severe muscle weakness, and reduced daily locomotor activity. Analysis of motor function in these mice at ages of 6-24 weeks showed that motor disorders were already present in young animals and that impaired increased progressively with age. Analysis of CPT1C expression in different motor brain areas during development revealed that CPT1C levels were low from birth to postnatal day 10 and then rapidly increased peaking at postnatal day 21, which suggests that CPT1C plays a relevant role in motor function during and after weaning (Carrasco, et al., 2013).

- **Cancer**

A role in cancer has been attributed to CPT1C by Zaugg and colleagues (Zaugg et al., 2011). A role in promoting cancer cell survival and tumor growth. They analyzed an extensive panel of breast cancers xenografs determining CPT1C mRNA expression, mTOR activation status and rapamycin sensitivity (rapamycin is an inhibitor of mTOR). CPT1C mRNA expression was most strongly correlated with mTOR activation. Rapamycin-sensitive tumors consistently displayed lower CPT1C mRNA expression, whereas rapamycin-resistant tumors were more likely to show high levels of CPT1C mRNA.

On one hand, they overexpressed CPT1C in vitro in breast cancer cells and found increased ATP synthesis and fatty acid oxidation (FAO). On the other hand, they used a siRNA against CPT1C that produced an increase of rapamycin sensitivity to the cells. Cells without CPT1C were also more sensitive to other stresses like low glucose or hypoxia conditions. All these data together apparently indicates that CPT1C might be supplementing the high energy needs of cancer cells via FAO, and that cancers lacking CPT1C could not take advantage of this supplementation of energy. CPT1C seems to be a stress-responsive protein.

Further evidence for a role of CPT1C in stress response has been provided for some analysis of the sequence of the CPT1C mRNA. These studies revealed that CPT1C contains an upstream open reading frame (uORF) in the 5'-UTR that is repressing the main open reading frame (mORF) and that this repression is relieved in response to specific stress stimuli, namely glucose deprivation and palmitate-BSA treatment.

Furthermore the AMPK inhibition can relieve this uORF-dependent repression. (Lohse et al.,2011).

- **Ceramide**

Recently, CPT1C has been implicated in ceramide metabolism. It has been described that the lack of CPT1C produces a decrease in the ceramide levels of some brain areas like: cerebellum, stratum and motor cortex (Carrasco et al., 2013).

The involvement of CPT1C has been also proved in some experiments where they overexpress CPT1C in primary hippocampal neurons cultured neurons and that effect increases ceramide levels, whereas in CPT1C-deficient neurons, ceramide levels are diminished. Furthermore, CPT1C deficiency alters dendritic spine morphology by increasing immature filopodia and reducing mature mushroom and stubby spines. Treatment of cultured neurons with exogenous ceramide revert the KO phenotype indicating that CPT1C regulation of spine maturation is mediated by ceramide (Carrasco et al., 2012).

Additionally some other studies done in rats also demonstrate that the overexpression of CPT1C, in this case in the ARC, increase ceramide levels. When mice are treated with myriocine, an inhibitor of the *de novo* synthesis of ceramide, the increase in ceramides produced by CPT1C is prevented (Gao et al., 2011). The same group also demonstrated that there is an up-regulation of ARC ceramide levels in fasting and that increase is dependent of CPT1C as the change in ceramides is negated in the mice with deletion of CPT1C (Gao et al., 2011).

There is sufficient evidence confirming the existence of a relationship between CPT1C and ceramides.

3.CERAMIDE

Ceramides are a family of lipid molecules that consist of sphingoid long-chain linked to an acyl chain via an amide bound. Ceramides differ from each other by length, hydroxylation, and saturation of both the sphingoid base and fatty acid moieties. Sphingoid bases are of three general chemical types: sphingosine, which has a trans-double bond at the c4-c5 position; dihidrosphingosine or sphinganine, the inactive precursors of ceramide which presents a saturated sphingoid backbone; and phytosphingosine, the yeast counterpart of the mammalian ceramide which has a hydroxyl group at the C4 position (Pruett et al., 2008).

The acyl chains of ceramide vary widely in composition. The acyl chain lengths range from 14 to 26 carbon atoms, although the most common fatty acids are palmitic (C16:0) and stearic (C18:0) non-hydroxy fatty acids. The fatty acids are commonly saturated or mono-unsaturated. α-Hydroxylated fatty acids at C2 position and ω-hydroxy fatty acids at the C terminal position are often present as well (Kolesnick, 2002).

Ceramides are the core structure of a class of complex lipid called sphingolipids, ubiquitous components of eukaryotic cell membranes (Zachowski, 1993). Sphingolipids have long been regarded as inactive and stable structural components of the membrane, however nowadays they are recognized to be biologically active.

Sphingolipids are very heterogeneous and are classified depending on their structural combinations in long-chain (sphingoid) bases, amide-linked fatty acid and hundreds of headgroups variants (Karlsson, 1970; Sullards et al.,2007). The headgroup is attached at the primary alcohol group of the ceramide molecule. Depending on the type of polar group, two major classes are defined: Phosphosphingolipids, the major one is sphingomyelin (SM) and Glycosphingolipids (GSL). The GSL are very heterogeneous (cerebrosides, gangliosides, globosides…).

3.1.CERAMIDE GENERATION

The two major via by which ceramide can be produced in cells are the *de novo* synthesis or the hydrolysis of complex sphingolipids (Sandhoff and Kolter, 2003). The activation of different catabolic enzymes yields ceramide within a few minutes whereas the *de novo* synthesis produces ceramide in several hours (Hannun et al., 1996). Different extra- and intra- cellular stimuli dictate the pathway used for ceramide generation resulting in distinct subcellular localization of ceramide and different biochemical and cellular responses.

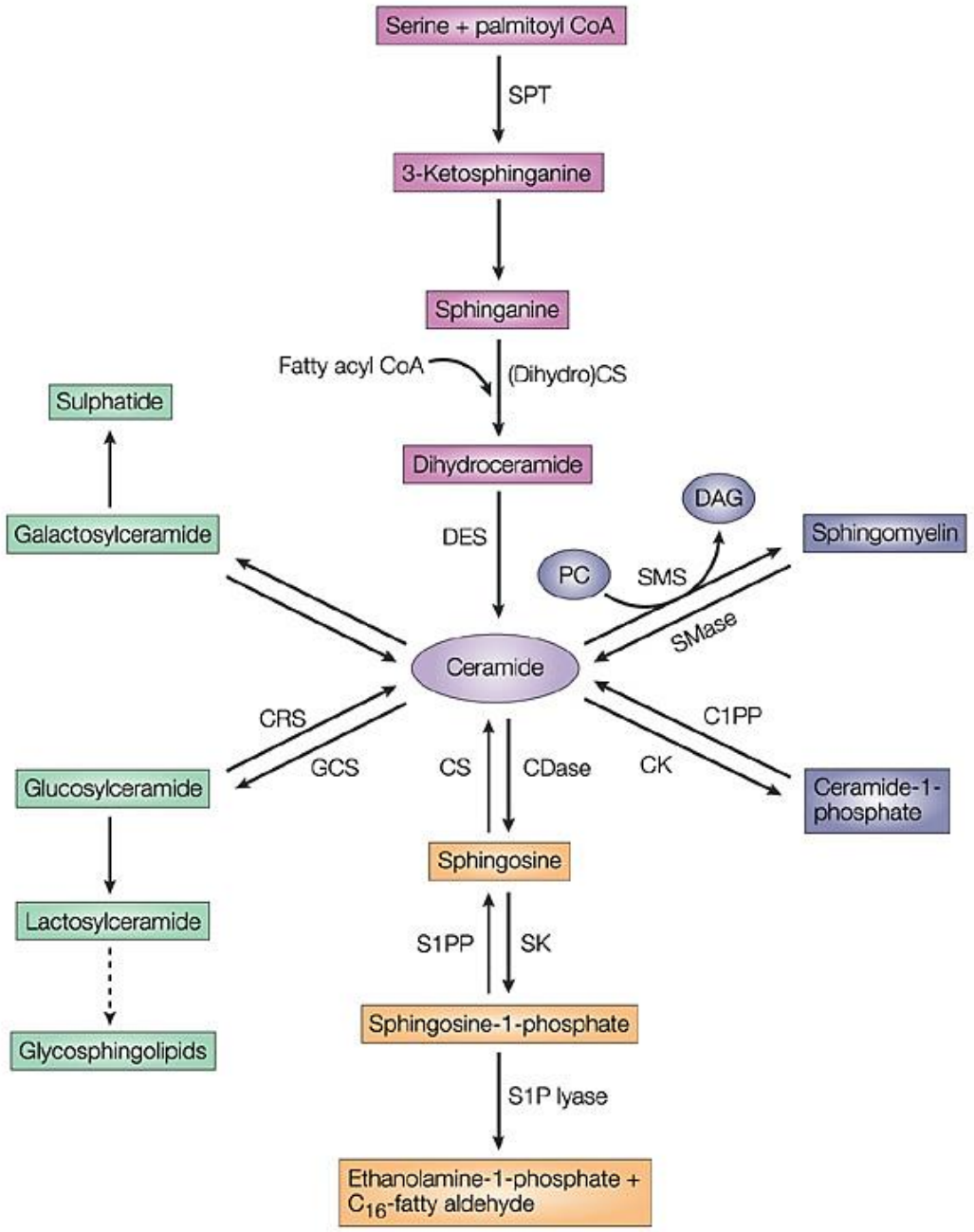


Figure 16: Ceramide can be formed *de novo* (pink) or from hydrolysis of sphingomyelin (blue) or cerebrosides (green). Conversely, ceramide can be phosphorylated by ceramide kinase to yield ceramide-1-phosphate, or can serve as a substrate for the synthesis of sphingomyelin or glycolipids. Ceramide can be metabolized (orange) by ceramidases (CDases)to yield sphingosine, which in turn is phosphorylated by sphingosine kinases (SKs) to generate sphingosine-1-phosphate (S1P). S1P can be cleared by the action of specific phosphatases that regenerate sphingosine or by the action of a lyase that cleaves S1P into ethanolamine-1-phosphate and a C_{16}-fatty-aldehyde. C1PP, ceramide-1-phosphate phosphatase; CRS, cerebrosidase; CK, ceramide kinase; CS, ceramide synthase; DAG, diacylglycerol; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; PC, phosphatidylcholine; S1PP, S1P phosphatase; SMS, sphingomyelin synthase; SMase, spingomyelinase; SPT, serine palmitoyl transferase (From Ogretmen and Hannun, 2004).

3.1.1.THE *DE NOVO* SYNTHESIS

The *de novo* synthesis of ceramide is produced on the cytoplasmic face of the smooth endoplasmic reticulum (ER) (Michel and Echten-Deckert, 1997). The synthesis begins with the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, through the action of serine palmitoyl transferase (SPT). Then 3-ketosphinganine is reduced to the sphingoid base sphinganine, which is subsequently N-acetylated by dihydro-ceramide synthase (CerS) to form dihydroceramide. The enzyme dihidro-ceramide desaturase introduces a bond to the position C_4 to form mammalian type ceramides (Michel et al., 1997).

It has been identified, in vertebrates and plants, six different types of CerSs (Pewzner-Jung et al., 2006). Each CerS regulates the *de novo* synthesis of endogenous ceramides with a high degree of fatty acid specificity. In line with the presence of multiple CerSs, ceramides occur with a broad fatty acids length distribution inside the cell. Although some CerSs are ubiquitously expressed, other isoforms present a very specific distribution among tissues, according to the need of each tissue for specific ceramide species (Lahiri and Futerman 2005). CerS1 specifically generates C18 ceramide and is highly expressed in the brain and skeletal muscles but is almost undetectable in other tissues (Ben-David et al., 2010). CerS2 mainly generates C20-26 ceramides and has been found to have the highest expression of all CerSs in oligodendrocytes and Shwann cells especially during myelination. The selectivity of different CerS isoforms to synthesize different ceramide species is important since ceramide with specific acyl chain lengths might mediate different responses within cells (Pewzner-Jung et al., 2006).

The high hydrophobicity and low polarity of ceramide moiety limit free ceramide to circulate inside the cell or more generally in solution. This may explain the occurrence of several izoenzymes of ceramide biosynthesis at different subcellular sites and supports the view that the site of ceramide formation might determine its function. But the cell needs to transport these new ceramides formed by *de novo* from the luminal face of the ER to the Golgi compartment to generate complex sphingolipids via the classical vesicular route (Hanada et al., 2003). The step-wise addition of sugar groups to ceramides is catalyzed by membrane bound glycosyltransferases and it is restricted to the ER-Golgi complex (Sandhoff and Echten, 1993). The synthesis of most GSLs begins with glycosylation of ceramide to form GlcCer, at the cytosolic surface of the Golgi (Jeckel et al., 1992).

Ceramides destined for the formation of SM reach the Golgi carried by the ceramide transfer protein (CERT) in a non-vesicular manner (Hanada et al., 2003). CERT mediates the transfer of ceramides containing C14-C20 acyl chain SM in many tissues and cell lines whereas GSLs are formed by longer ceramides. CERT, works as mediator of sphingolipids homeostasis. It has an alternatively spliced isoform characterized by

the presence of an additional 26 amino acids domain, responsible for its localization at the plasma membrane and consequent secretion to the extracellular milieu, named $CERT_L$ or Goodpasture antigen binding protein (GPBP) (Mencarelli et al., 2010). Both isoforms can be detected in adult brain (Mencarelli et al., 2009).

Once delivered to the Golgi apparatus, ceramide spontaneously translocate from the cytosolic to the luminal leaflet for SM synthesis. Formation of SM from ceramide is catalyzed by sphingomyelin synthase (SMS) (Ullman and Radin, 1974) that transfers the phosphocholine headgroup from phosphatidylcholine onto ceramide yielding SM as a final product and diacylglycerol (DAG) as a side product (Huitema et al., 2004). Whether the DAG generated by SMS regulates cellular processes remains unclear. SMS exists in two isoforms:

- **SMS_1,** faces the lumen of the *cis*/medial Golgi (Jeckel et al., 1990) and it is responsible for the *de novo* synthesis of SM (Futerman et al., 1990).
- **SMS_2,** which resides in the plasma membrane (Huitema et al., 2004), could instead play a more specific role in signal transduction events.

In neural cells the *de novo* SM is mostly synthesized at the plasma membrane and the production at the *cis* medial Golgi is less prominent (Linke et al., 2001; van Echten et al., 1990). This indicates that the subcellular localization of SM formation is cell type specific and that SMS activities may be involved in different biological processes.

3.1.2.CATABOLIC PATHWAYS FOR CERAMIDE PRODUCTION

Significant contribution to intracellular ceramide levels occur also through hydrolysis of complex sphingolipids by activation of different hydrolases (Perry and Hannun, 1998).

Ceramides derived from SM catabolism require the activation of sphingomyelinases (SMase) (Reynolds et al., 2004), specific forms of phospholipase C, which hydrolyze the phosphodiester bond of SM yielding water soluble phosphorylcholine and ceramide (Okazaki et al., 1994). Several SMases have been characterized and classified by their pH optimum, subcellular distribution and regulation:

- **aSMase** is the acid sphingomyelinase, which exhibits an optimal enzymatic activity at pH 4.5–5 (Schissel et al., 1998). This lipase is localized in lysosomes and is required for the turnover of cellular membranes (Bamholz et al., 1966).

- **nSMase** is the neutral sphingomyelinase, these are membrane bound enzymes with an optimal activity at a neutral pH. Several isoforms have been characterized.
    - **nSMase 1** is localized in the membranes of the ER, and it is ubiquitously expressed and highly enriched in kidney (Tomiuk et al., 2000).
    - **nSMase 2** is localized in the plasma membrane and is specifically highly expressed in brain (Gatt et al., 1976; Marchesini et al., 2003).
    - **nSMase 3** is ubiquitously present in all cell types and distributed mainly in the ER and Golgi membrane (Krut et al., 2006).nSMases are further classified as Mg2+/Mn2+ dependent or independent.
- An alkaline SMase exists only in intestinal cells and it is activated by bile salts (Cheng et al., 1999).

The function of these multiple isoforms is still elusive; however their membrane localization has led to speculation that they may contribute to the modification of local microdomains in the membrane organization during vesicle formation, transport, and fusion (Ybe et al., 2000).

3.1.3.SALVAGE PATHWAY

Ceramides can be generated by an alternative acyl-CoA-dependent route, which is called the "salvage pathway". This pathway relies upon the reverse activity of the enzyme ceramidase (CDase), since catabolic fragments are recycled for biosynthetic purposes (Smith and Merrill, 1995; Gillard et al., 1998). CDase catalyzes the hydrolysis of ceramide to generate free sphingosine and fatty acid. Together with ceramide production, CDase regulates also sphingosine levels. In fact, it is important to note that whereas sphinganine is generated by *de novo* sphingolipid biosynthesis, free sphingosine seems to be derived only via turnover of complex sphingolipids, more specifically by hydrolysis of ceramide (Michel et al., 1997). The catabolism of ceramide takes place in lysosomes from where sphingosine can be released (Riboni et al., 1998) in contrast to ceramide, which does not appear to leave the lysosome (Chatelut et al., 1998). Free sphingosine is probably trapped at the ER-associated membranes where it undergoes re-acylation (condensation with a fatty-acylCoA) to again generate ceramide. This "reverse" activity is carried out by the same CDase (Chatelut et al., 1998).

Different CDases have been identified associated with different cellular compartments according to the pH at which they achieve optimal activity:

- **Acid CDases** (aCDase) are lysosomal (Gatt, 1963).
- **Neutral/alkaline CDases** (nCDase and alCDase) have been purified from mitochondria (El Bawab et al., 2000) and nuclear membranes (Shiraishi et al., 2003).
- **Pure alkaline CDase** has been localized to the Golgi apparatus and ER (Mao et al., 2001). This variability in CDases subcellular localizations and distribution in tissues suggests that these enzymes may have diverse functions in the biology of the cell.

n/a CDases have been shown to catalyze the reverse reaction to generate ceramide from sphingosine and fatty acids (Mao et al., 2001; El Bawab et al., 2001) whereas the acid isoform resides in lysosome. Mitochondria are also capable of generating ceramide via the action of reverse CDase (Bionda et al., 2004; El Bawab et al., 2000).

3.2.DIFFERENT ROLES OF CERAMIDES

Ceramides are one of the component lipids that make up sphingomyelin, the major lipids in the lipid bilayer. Contrary to previous assumptions that ceramides and other sphingolipids found in cell membrane were purely structural elements, ceramide can participate in a wide variety of cellular signaling.

3.2.1.LIPID RAFT

Biological membranes are typically in a fluid or liquid-disordered state at physiological temperatures. Combinatorial interactions between specific lipids drive the formation of dense liquid-ordered domains called lipid rafts (Kolesnick et al., 2000; Brown and London, 1998; Simons and Vaz, 2004). Lipid rafts are generally enriched in lipids, especially SM and cholesterol which pack tightly within the lipid bilayer (London, 2002).

Ceramide presumably acts at the level of lipid rafts in transducing external signal. Rafts are the primary site of action of the enzyme SMase that releases ceramide from SM (Bollinger et al., 2005). The tight interaction between SM and cholesterol serves as the basis for raft formation. Ceramides, on the other hand, mix poorly with cholesterol

and have a tendency to self-associate and segregate into highly ordered microdomains (Kolesnick et al., 2000). The nature of ceramide has a strong impact on membrane structure. In fact, long-chain saturated ceramide molecules are intermolecular stabilized by hydrogen bonding and van der Waal forces (Nurminen et al., 2002) and form a liquid ordered domains that induce lateral phase separation of fluid phospholipid bilayers into regions of liquid-crystalline (fluid) phases. Moreover, the small size of ceramide polar headgroup results in a low hydration and allows ceramide molecules to pack tightly avoiding any interference with surrounding lipids (Megha et al., 2007). In fact it has been shown that as little as 5 mol% ceramide is sufficient to induce ceramide partitioning in the lipid bilayer and to drive the fusion of small inactive rafts into one (or more) larger active ceramide-enriched membrane platforms (Nurminen et al., 2002).

The ceramide-enriched membrane platforms serve as clustering components to achieve a critical density of receptors involved in signaling. This high density of receptors seems to be required for effective transmission of the signal into cells. The neuronal plasma membrane is particularly enriched in lipid rafts (Ledesma et al., 1999). Lipid and protein raft composition differs according to neuronal developmental stage. Mature neuron lipid raft content is higher than that of immature neurons and astrocytes. Synaptic proteins such as synaptophysin or synaptotagmin localize in lipid rafts (Gil et al., 2005) and lipid rafts are critical for maintaining the stability of synapses and dendritic spines (Hering et al., 2003). Neurotransmitter signaling seems to occur through a clustering of receptors and receptor-activated signaling molecules within lipid rafts. Aberrant organization of SM and cholesterol in rafts has been linked to loss of synapses and changes in nerve conduction (Hering et al., 2003). Depletion of sphingolipids or cholesterol leads to gradual loss of inhibitory and excitatory synapses and dendritic spines (Hering et al., 2003). Rafts also play an important role in neuronal cell adhesion, localization of neuronal ion channels, and axon guidance…

3.2.2.STRESS AND APOPTOSIS

The basal ceramide concentrations in the cells are low and may change during cellular differentiation or progression through the cell cycle. Various inducers of cellular stress however lead to an accumulation of ceramide that promotes apoptotic and also mediates the onset of a specific response.

Apoptosis is an essential process for normal embryonic development and to maintain cellular homeostasis within mature tissues. A proper balance between regulation of normal cell growth and cell death is the basis of life.

It has been reported that an increase of ceramide levels leads to cell death (Obeid et al., 1993; Novgorodov et al., 2005). In contrast, depletion of ceramide can reduce the progression of apoptosis (Bose et al., 1995; Dbaibo et al., 2001). Ceramide levels inside the cell determine its dual role: protection and cell sustenance at low concentrations but death and threat when over produced. This outlines the importance for cells to maintain a strict ceramide balance by a tight regulation of sphingolipid based signaling networks.

Ceramide can induce apoptosis via different routes and different intracellular organelles are the target of its action. SM hydrolysis by neutral and/or acid SMases is known to be a very important pathway for production of pro-apoptotic ceramides (Tepper et al., 2000). However, the *de novo* synthesis pathway has also been reported to be relevant in the generation of a signaling pool of ceramide leading to cellular apoptosis (Son et al., 2007; Blazquez et al., 2000) These two pathways can induce apoptosis independently or jointly.

Ceramide production occurs in response to diverse apoptotic stimuli and with different mechanisms. Many inducers of cell death activate one or more ceramide generation pathways. SM hydrolysis generates a rapid and transient increase of ceramide and results in formation of ceramide-enriched membrane platforms. In contrast, the ceramide *de novo* pathway requires multiple enzymatic steps and it is responsible for a slow but robust accumulation of ceramide over a period of several hours. SMase activation occurs in response to stimulation of cell surface receptors of the tumor necrosis factor (TNF) upon the binding with specific ligands such as TNF alpha, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligands.

SM hydrolysis in response to TNF signals involves both nSMase. Although aSMase and nSMase seem to induce death receptor dependent and independent mediated apoptosis through apparently separate mechanisms, both enzymes are activated by the same stimuli (UV light, hypoxia, radiation, TNF-related apoptosis-inducing ligands and the DNA-damaging drug doxorubicin. Instead, exposition to cannabinoids or retinoic acid triggers a large increase in ceramide levels formed specifically through the *de novo* pathway (Mencarelli and Martinez-Martinez, 2013).

In contrast with the apoptotic role of ceramides, sphingosine-1-phosphate (S1P), a sphingolipid derived from ceramide, has been implicated in cell growth and survival (Fig. 17). S1P promotes cell growth and inhibits apoptosis, while its precursors, ceramide and sphingosine, inhibit cell growth and induce apoptosis (Futerman and Riezman, 2005). Thus, the dynamic balance between S1P and ceramide and the consequent regulation of opposing signaling pathways, is an important factor that determines cell fate and to prevent diseases including cancer and inflammation (Hannun and Obeid, 2008). S1P is produced intracellularly by two sphingosine kinases

(Sphk1 and Sphk2) and is the ligand for a family of five G protein-coupled receptors, named S1P1-5. It produces many of its effects by acting as a ligand for one or more of its five receptors. However, although intracellular targets for both ceramide and sphingosine have been identified, indicating they are both bona fide second messengers, intracellular targets for S1P have remained elusive.

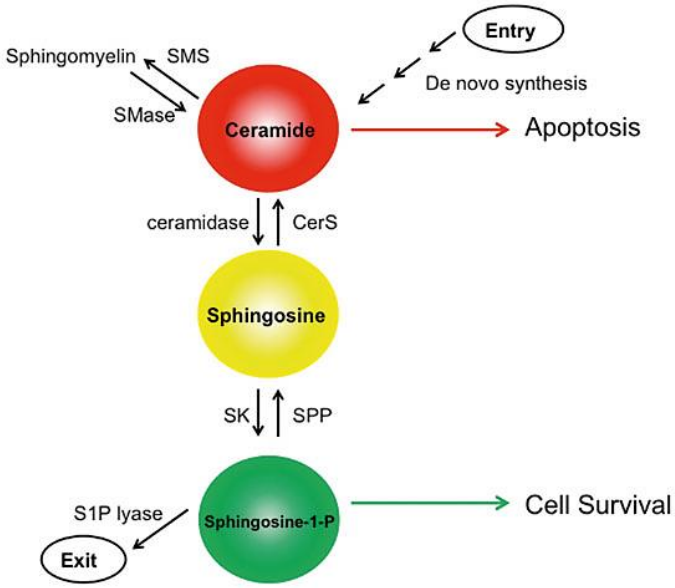


Figure 17. Sphingolipid metabolism and homeostasis. Ceramide is synthesized *de novo* and can also be generated through breakdown of complex sphingolipids. Ceramide can also be further metabolized to sphingosine, which is then phosphorylated to generate S1P. Degradation of S1P by S1P lyase marks the exit from sphingolipid pathway. Ceramide has been linked to anti-proliferative responses including apoptosis while S1P is important for survival. Homeostasis is maintained through interconversion of ceramide and S1P though sphingosine. Abbreviations used: SMase — sphingomyelinase, SMS — sphingomyelin synthase, CerS — ceramide synthase, SK — sphingosine kinase, SPP — sphingosine-1-phosphate phosphatase, S1P lyase — sphingosine-1-phosphate lyase (From Tirodkar and Voelkel-Johnson, 2012).

3.2.3.METABOLIC DISORDERS

A lot of stress stimuli: inflammatory cytokines, glucocorticoids, chemotherapeutics... induce sphingolipid synthesis, leading to the accumulation of ceramides and ceramide metabolites. Some studies suggest that a primary consequence of ceramide accumulation is an alteration in metabolism (Guenther and Edinger,2009). The metabolic impairments caused by ceramide likely contribute to the tissue dysfunction underlying the numerous diseases associated with obesity. Recent studies reveal that tissue ceramide levels are regulated by hormonal signals that modulate rates of synthesis and degradation. Inflammatory agonists, which are upregulated in obesity and contribute to insulin resistance and other complications of obesity, are important drivers of ceramide production (Holland et al., 2011).

- **Nutrient handling**

Recent attention has been placed on the impact of ceramide on cellular metabolism as a mechanism driving its action on cell survival. In particular, ceramide was recently shown to kill cells by starving them of nutrients, at least partially by downregulating nutrient transport proteins. Guenther and colleagues treated cells with methyl pyruvate, a membrane-permeant molecule that can be taken into cells independently of nutrient transporters and oxidized to produce ATP, and this alternative fuel source protected cells from ceramide-induced cell death (Guenther et al., 2008). The literature reveals that ceramide indiscriminately affects cellular uptake of all three key dietary nutrients in a variety of cell types and organisms.

The effect of ceramide as an antagonist of insulin-stimulated glucose uptake has been demonstrated in a variety of cell culture and rodent models of lipid oversupply and obesity. Short-chain ceramide analogs impair insulin-stimulated glucose uptake by blocking the insulin-stimulated translocation of the GLUT4 glucose transporter to the plasma membrane (Summers et al., 1998). Interestingly, the effect is specific for saturated fatty acids, as unsaturated fatty acids antagonize insulin action via a ceramide-independent mechanism.

Despite requiring intracellular fatty acids for its formation, ceramide nonetheless appears to exert a negative effect on lipid uptake. CD36, also referred to as fatty acid translocase, has been implicated in actively transporting fatty acids across the plasma membrane (Schwenk et al., 2010). Some other experiments found that treatment of cells with short-chain ceramide inhibits CD36 cell-surface expression in a dose- and time-dependent manner (Luan et al., 2006).

When talking about proteins, some experiments observed that both exogenous and endogenous ceramides attenuate basal and insulin-stimulated amino acid transporter in muscle cells by reducing plasma membrane levels of sodium-coupled neutral amino acid transporter 2 (SNAT2) while leaving total cellular abundance untouched (Guenther et al., 2008).

- **Inhibition of Akt.**

A major mechanism through which ceramide alters cellular metabolism is the inhibition of Akt (Summers et al., 1998). When talking about the mediating nutrient handling function of Akt, it activates anabolic pathways while inhibiting catabolism, leading to a marked upregulation in nutrient storage. Akt also activates prosurvival and inhibits autophagy enzymes, leading to a net increase in cellular growth and stability.

Ceramide has been shown to oppose nearly all of these Akt actions (Stoica et al., 2003). Both exogenous and endogenous ceramides inhibit Akt phosphorylation and activation without blocking any upstream signaling events (Zhou et al., 1998).

- **ROS production**

Ceramide is known to interact with mitochondria in inducing apoptotic pathways via increased membrane permeability (Siskind, 2005). It has been reported that the ceramide-induced perturbation of mitochondrial membrane structure elicits a disruption of electron transport. Not surprisingly, the disruption of electron transport by ceramide results in elevated reactive oxygen species, which can have a wide range of deleterious consequences, including the induction of insulin resistance (Houstis et al., 2006).

3.2.4.AGING

Sphingolipids (lipids where ceramide is the core) hold a major role in regulating development and lifespan (Mattson et al., 2002). A close connection between ceramide levels and aging comes from studies carried on *Saccharomyces cerevisiae* where a gene involved in ceramide synthesis was identified as a regulator of yeast longevity. This gene is called longevity assurance homolog 1 (LAG1) and its lost correlates with a marked increase in yeast lifespan (D'Mello et al., 1994). The human homolog LAG1Hs (CerS1) is highly expressed in the brain, testis and skeletal muscles and specifically generates C18-ceramide (Pewzner-Jung et al., 2006). This conclusion seems to be supported by cell culture studies where overexpression of CerS1 with increased C18-ceramide generation resulted in apoptosis (Koybasi et al., 2004). Interestingly, C18-ceramide generated by CerS1 was found to downregulate the expression of the enzyme telomerase (Wooten-Blanks et al., 2007). Telomerase functions by elongating the end of existing chromosomes and thus preventing cellular senescence. Since cellular aging is dependent on cell division, these enzymes play a critical role in long-term viability of highly proliferative organ systems (Lee et al., 1998). Therefore, besides the telomeric roles, telomerase was found to protect the post-mitotic neuronal cells from stress-induced apoptosis and may serve a neuron survival-promoting function in the developing brain and be important for regulating normal brain functions. Thus, the regulation that C18-ceramide seems to exert on telomerase expression may contribute to increase neuronal vulnerability of the adult brain in various age-related neurodegenerative disorders.

Several studies support the role of ceramide in inducing senescence and in activating biochemical pathways involved with aging. Accumulation of ceramide occurs normally

during development and aging in single cells (Venable et al., 1995) and young cells treated with exogenous ceramide exhibit a senescent-like phenotype (Venable and Yin, 2009)

In addition, a significant change in ceramide metabolic enzyme activities seems to occur in specific organs or even in specific cell types with aging (Venable et al., 1995; Venable et al., 2006). The activities of the sphingolipid catabolic enzymes (SMase and CDase) seem to change more robustly than that of the anabolic enzymes (SMS and CerS).

ASMase and nSMase activity significantly increase in rat brain during aging demonstrating that there is an increase SM turnover in aging. CDases activities are increased specifically in brain tissue from aging rats and among the isoforms of CDases, alCDase shows the highest activity. Increase in the CDase activity in brain indicates an increase in the production of sphingosine and its contribution toward aging in these tissues. In contrast, CerS shows a lower activity, suggesting a minor contribution of ceramide *de novo* synthesis to ceramide accumulation (Sacket et al., 2009).

3.2.5.SP TRANSCRIPTION FACTORS

The Sp transcription factors are part from a bigger family called Sp and Krüppel-like factors (Sp/KLF). That family contains at least twenty identified members which include Sp1-4 and numerous krüppel-like factors. Members of the family bind with varying affinities to sequences designated as "Sp sites" like CG-boxes as they are lacked of TATA box. Family members have different transcriptional properties and can modulate activity of each other by a variety of mechanisms.

Sp1 and Sp3 are the most interesting factors for us since they have been related with ceramides.

Sp1 and Sp3 are very similar, but with different transcriptional activity. They could function as transcriptional activators or transcriptional repressors depending on the environment and the promoter structure (Yu et al., 2003). Sp3 is able to produce additive and synergic effect with Sp1 but also is able to repress the activity of Sp1 in the case of promoters that contain multiple adjacent binding sites (Nicolas, 2003). Sp3 is also able to activate itself (Tapias et al., 2005).

In connection with Sp3 and ceramide, recently, it has been published by several groups of research a link between ceramides and the acetylated state of Sp3. Experiments with cells have shown that treatment with exogenous ceramide produces

an increase of the binding affinity of Sp3 to the promoter and that effect is mediated by a decrease in the acetylated levels of Sp3. In some cases, like the glycolipid transfer protein gene (GLTP), the binding of Sp3 to the promoter triggers an increase of expression (Zou et al., 2011) and in other cases, like the human telomerase reverse transcriptase (hTERT), that effect triggers a decrease of expression (Wooten-Blancks et al., 2007).

As shown in figure 18, in the case of hTERT, C18-ceramide is able to mediate a negative regulation of hTERT promoter by promoting the association of histone deacetylase 1 (HDAC1) with Sp3 and the concomitant recruitment to the promoter (Wooten- Blancks et al., 2007).

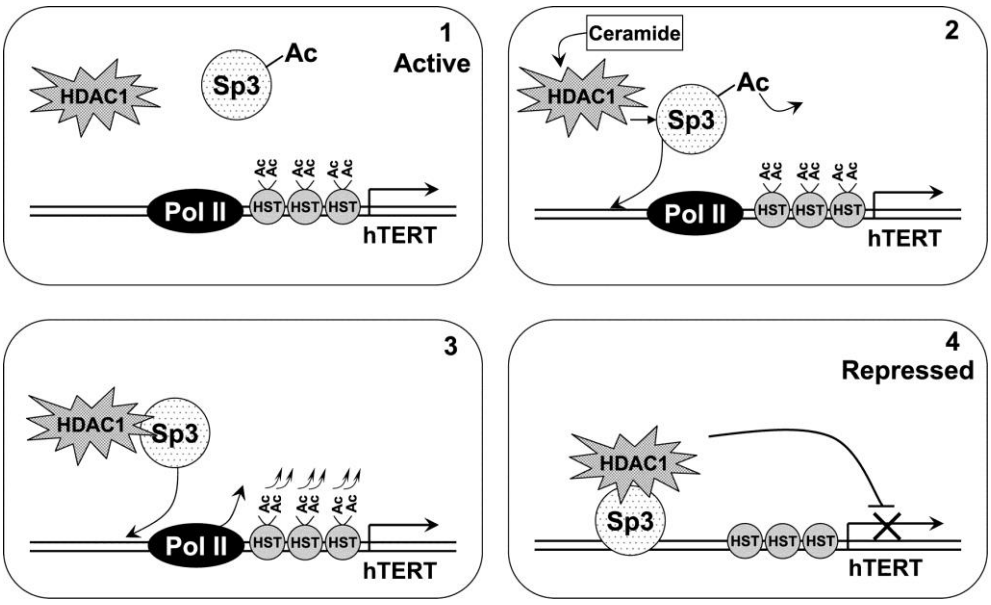


Figure 18. Schematic model of the mechanism involved in repression of the hTERT promoter by deacetylation of Sp3 via ceramide signaling. The data presented here revealed a novel mechanism for ceramide-mediated repression of the hTERT promoter, which is active in most resting cancer cells (panel 1), by deacetylation of Sp3. Specifically, these results suggest that exogenous C6-ceramide or *de novo* generation of C18-ceramide by CerS1 results in rapid association between Sp3 and HDAC1, which leads to the deacetylation of Sp3 (panel 2). Deacetylation of Sp3, therefore, plays a major role in recruiting HDAC1 to the hTERT promoter by its increased association with the promoter (panel 3). Recruitment of HDAC1 results in local histone H_3 deacetylation, which facilitates the closed chromatin complex formation and prevents the association of RNA polymerase II with the promoter, leading to its repression (panel 4) (From Wooten- Blancks et al., 2007).

4.THE BRAIN-SPECIFIC HOMEOBOX

4.1.THE TRANSCRIPTION FACTOR BSX

Homeobox genes are an evolutionarily conserved class of transcription factors that play important roles in regionalization, pattering, and cell differentiation during embryogenesis and organ development (Krumlauf et al., 1993; Panganiban and Rubenstein, 2002). They contain a homeobox, which is a segment of DNA that encodes a DNA-binding homeodomain of about 60 amino acids. The homeodomain includes a helix-turn-helix motif consisting of three α-helices and short loops that link them. All domeodomain transcription factors bind to DNA TAAT/ATTA motifs.

Homeobox genes were first identified in Drosophila by large scale forward genetic screens (Scott and Weiner, 1984). Brain-specific homeobox (BSX) is the mammalian homologue of the Drosophila brain-specific homeobox gene, bsh (Jones and McGinnis, 1993) and was first described and named at 2004 by Cremona and colleagues (Cremona et al., 2004).

Up to now, there are only a few studies with the mammalian homeobox, all of them done with the mouse homologue. At any rate, the sequence between human and mouse is highly conserved (Cremona et al., 2004).

It has been recently published that the mouse BSX promoter has several putative binding sites for transcription factors (Fig. 19), including NRSE for REST and the CG box for the transcription factor Sp1 (Park et al., 2007).

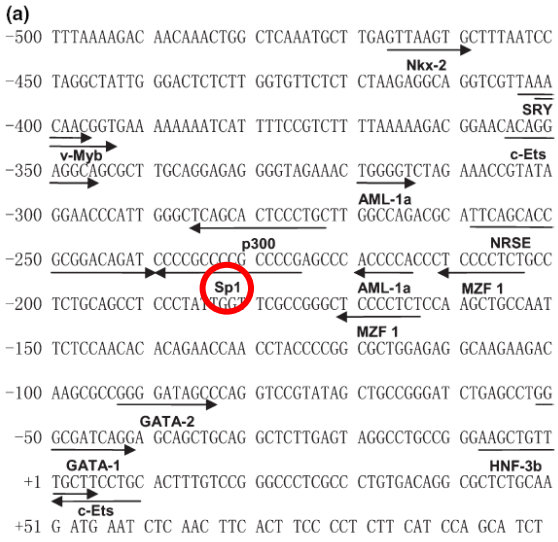


Figure 19. The mouse BSX promoter. Putative cis regulatory elements in the mouse BSX promoter. This sequence is numbered from the computer-aided transcription initiation site (+1). Putative binding sites for several trans-acting factors are additionally indicated (From Park et al., 2007).

4.2.LOCALIZATION OF BSX

Mammalian BSX is expressed in the ARC, pineal gland, septum and mammillary body in the embryo and after birth (Fig. 20) (Cremona et al., 2004). In adult, expression of BSX is seen in the ARC and the DMH (Sakkou et al., 2007; Nogueiras et al., 2008).

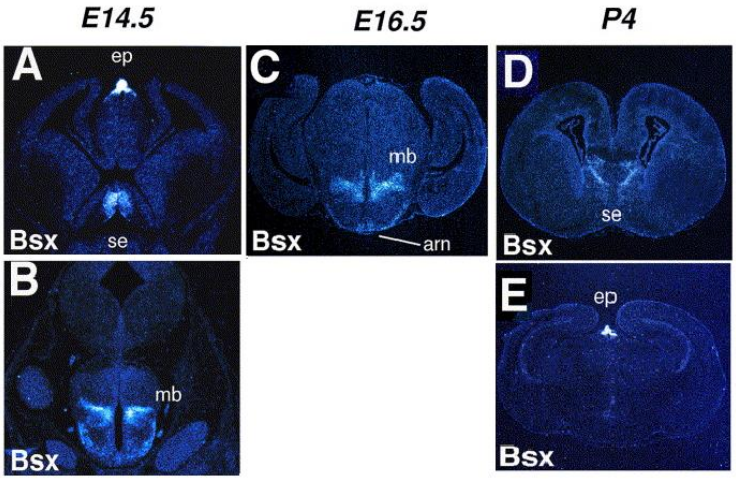


Figure 20. Pre- and post-natal *BSX* expression in the hypothalamus and septum. (A) Coronal section through the telencephalon showing *Bsx* expression in the medial regions of the septum. This expression is maintained, although weaker, at post-natal stages (E). (B) Coronal sections at the level of the ventral hypothalamus comparing Bsx. *Bsx* is detected in the ventral mammillary body and the ventral area of the hypothalamus. (C) Coronal sections of E16.5 brains showing *Bsx* expression domains overlapping in the arcuate nucleus and ventral mammillary body. (D) P4 brain coronal sections with *Bsx* staining in the septal area (D) and in the epiphysis (H). arn, arcuate nucleus; ep, epiphysis; mb, mammillary body; se, septum (From Cremona et al., 2004).

Chu and colleagues identified a splice variant of mouse Bsx (Bsx1a), designated Bsx1b, which retains the N-terminal region but lacks the homeodomain and C terminus of Bsx1a. Transfection experiments showed that Bsx1a and Bsx1b localize in the nucleus and cytoplasm, respectively (Chu and Ohtoshi, 2007).

4.3.PHYSIOLOGICAL FUNCTION OF BSX

It has been demonstrated that NPY and AgRP neuropeptides expression as well as hypothalamic control of locomotor behavior depend on BSX function as mice lacking BSX exhibited lower expression of NPY and AgRP (Fig. 21) and reduced locomotor activity.

These mice also exhibited increase in body temperature, increase in fat mass, attenuated physiological responses to fasting, lack of food-seeking behavior, and reduced rebound hyperphagia (Sakkou et al., 2007).

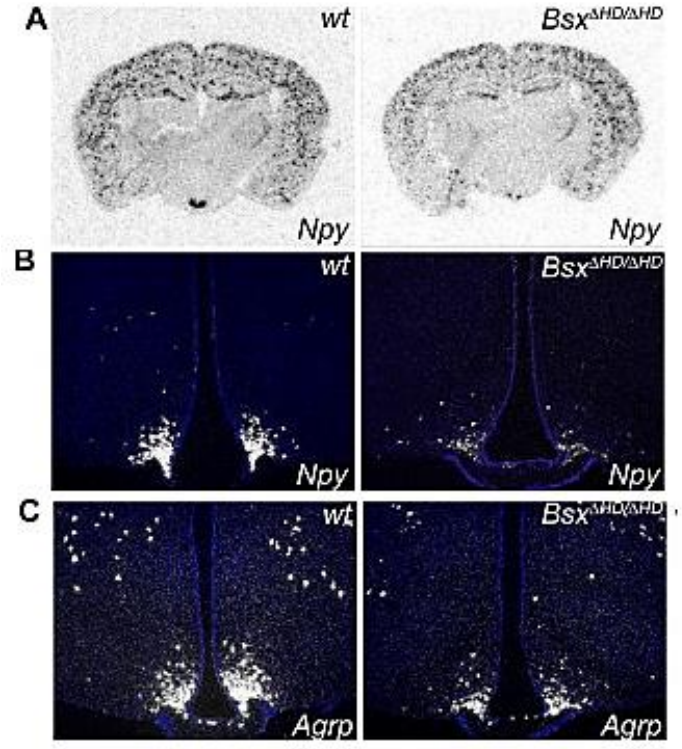


Figure 21. Downregulation of Npy and Agrp Expression in Bsx Mutant Mice. (A) 35S in situ hybridization demonstrates downregulation of Npy expression specifically in the arcuate of Bsx mutant brains. (B) NPY levels are consistent with (A), also reduced. Detection and comparison of (C) Agrp (From Sakkou et al., 2007).

Using pull-down experiments it has been found that BSX binds, in the NPY and AgRP promoter region with CREB and FoxO1 respectively, to increase their transcriptional levels (Sakkou et al., 2007).

In keeping with the topic of feeding, it has been shown that fasting and feeding can alter the expression of BSX in the ARC (Nogueiras et al., 2008). The level of BSX expression becomes high during fasting while refeeding returns expression to the normal low level. In addition, ghrelin and leptin alter the expression of BSX also in the ARC. ICV injection of leptin decreases fasting-induced BSX expression whereas the injection of ghrelin increases it. Moreover, ghrelin receptor (GHS-R) blockade decreases the expression of BSX (Fig. 22) (Nogueiras et al., 2008). It has been shown that a high fat diet increases BSX mRNA expression in the ARC (Nogueiras et al., 2008).

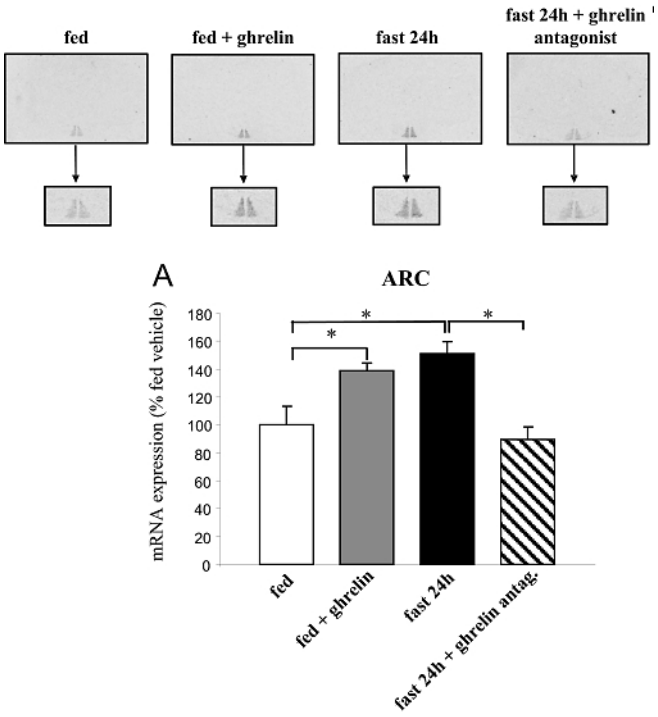


Figure 22. Representative pictures of BSX mRNA expression in the ARC of ad libitum-fed and fasted rats after ghrelin injection. A) Effect of ghrelin and ghrelin antagonist on BSX expression in the ARC. n = 6–7 animals per group. *, P < 0.05 (From Nogueiras et al., 2008).

In line with the connection between BSX and neuropeptides, very recently has been published that in states of fasting, BSX together with the glucocorticoid receptor (GR), produces a synergic effect activating the AgRP transcription in the ARC (Lee et al., 2013).

Glucocorticoid (Gc) is a well-known peripheral orexigenic signal. Fasting increases plasma levels of Gc and activates expression of NPY and AgRP (Fehm et al., 2004). Gc interacts with GR that is in the cytoplasm and then translocates to the nucleus where interacts with Gc response elements (GRE) to regulate the expression of target genes. The results of this group demonstrate that in AgRP neurons, the peripheral Gc signal triggers a synergistic activation of AgRP expression by GR and BSX via a GRE in AgRP (Lee et al., 2013).

All these findings suggest that BSX plays a physiological role in energy balance control systems. Its expression is regulated by changes in energy balance, by nutrient signals such as ghrelin or leptin, by exposure to dietary lipids and by changes in glucocorticoid levels.

Some other roles have been proposed for BSX apart from being important in feeding and locomotor behavior.

In 2007 McArthur and Ohtoshi found that BSX was essential for proper postnatal growth and nursing. They generated a BSX mutant mouse and found that there was a growth retardation caused by the lack of BSX and a premature involution of mammary glands (McArthur and Ohtoshi, 2007).

4.4.RELATIONSHIP BETWEEN BSX AND CPT1

It has been demonstrated the importance of BSX for ghrelin and leptin actions.

Lage and colleagues corroborate that central administration of ghrelin increases BSX mRNA expression in the hypothalamus and also increases the levels of the orexigenic neuropeptides NPY and AgRP, the related transcription factors pCREB, FoxO1 and the components of the fatty acid synthesis *de novo* (pAMPK, pACC). They also demonstrated that the administration of ghrelin increases the activity of CPT1 in the hypothalamus and conclude that the orexigenic effect of ghrelin is mediated by the stimulation of hypothalamic CPT1 (Lage et al., 2010)

If fatty acid β-oxidation is blocked with etomoxir (an inhibitor of CPT1), ghrelin induced increase of BSX in prevented. So BSX is downstream of fatty acid oxidation induced by ghrelin connecting it with AgRP and NPY (Lage et al., 2010).

More recently it has been reported a relationship between BSX, the specific brain isoform of CPT1 (CPT1C) and leptin. The adenoviral overexpression of CPT1C in hypothalamic arcuate nucleus of rats increases food intake and concomitantly up-regulates NPY and BSX. In addition, CPT1C overexpression also blocks leptin-induced down-regulation of NPY and BSX (Gao et al., 2011).

Moreover, ceramide metabolism appears to play a role in leptin's central control of feeding. Leptin treatment decreases ARC ceramide levels, with the decrease being important in leptin-induced anorectic actions and downregulations of NPY and BSX. These data indicates that leptin impacts ceramide metabolism in part through CPT1C, and ceramide acts downstream of CPT1C mediating the effects on feeding and expression of NPY and BSX (Gao et al., 2011).

CHAPTER 2

OBJECTIVES

GENERAL OBJECTIVE:

To study the physiological function of CPT1C in the control of food intake.

SPECIFIC OBJECTIVES:

1. To determine the involvement of CPT1C in the hypothalamic ghrelin signaling pathway.

2. To determine the involvement of ceramide in the hypothalamic ghrelin signaling pathway.

3. To study the role of ceramide in the increase of brain-specific homeobox transcription factor trough the activation of deacetylases in the hypothalamic cell line GT1-7.

4. To identify the effect of lacking CPT1C in lifespan.

CHAPTER 3

EXPERIMENTAL PROCEDURES

1. ANIMALS

1.1 ANIMAL PREPARATIONS

Animals used in the following experiments were adult (25-30 g) wild-type (WT) and their littermate Knock-out mice for the gene *Cpt1c* (CPT1C KO). These CPT1C KO mice were generated in collaboration with the Centre de Biotecnologia Animal I Teràpia Gènica (CBATEG) at the Universitat Autónoma de Barcelona (UAB). The generation of KO mice was through a genetic mutation introduced by homologous recombination. The exons from 12 to 15 were deleted, and then the open reading frame for the protein CPT1C changed thereby producing a truncated protein. The construct was electroporated into 129SvEv embryonic stem cells (ESC) and the two positive ESC clones obtained during the analysis were expanded. Then they were microinjected into C57BL/6 mouse blastocysts, which were transferred into foster mothers. The mice born from these procedures were referred to as chimeras, as they were composed of cells derived from the C57BL/6 embryo and the 129/SV ES cells. The chimeric mice capable of germline transmission were selected to obtain heterozygous mice. We intercrossed them to generate homozygous knockout mice. For our experiments we used the homozygous mice from the 6^{th} backcross.

Mice were housed in a room with a 12:12 hour light-dark schedule with lights on at 8:00 a.m., in controlled environmental conditions of temperature (22ºC + 2°C) and humidity (60%) with food and water ad libitum. The numbers of animals used for each test were from 4 to 10 mice per group depending on the experiment.
All animal procedures met the guidelines of European Community Directive 86/609/EEC and were approved by the Local Ethics Committee.
All mice were individually housed for 1 week prior to conducting a study with access to water bottles and normal chow. Bedding consisted of bet-o-cob bedding.

1.2. CANNULATION SURGERY

Direct access to the CNS via a cannula implanted in the cranium is useful in experimental situations where the test compound has effects on the CNS. Significant doses can be administered directly to the brain using this technique, which can eliminate the uncertainty of systemic kinetic variables. The guide cannula was placed into the brain at predetermined coordinates through a hole drilled in the skull. The cannulas used were of two types. The first one, purchased in Bilaney (C317GS-4/spc Mouse Guide 2.2 mm plus C316DCS-4/spc Dummy Specify length 2.2 mm guide w/o projection) and the other ones were handmade.

The handmade cannula must be of these dimensions and shape:

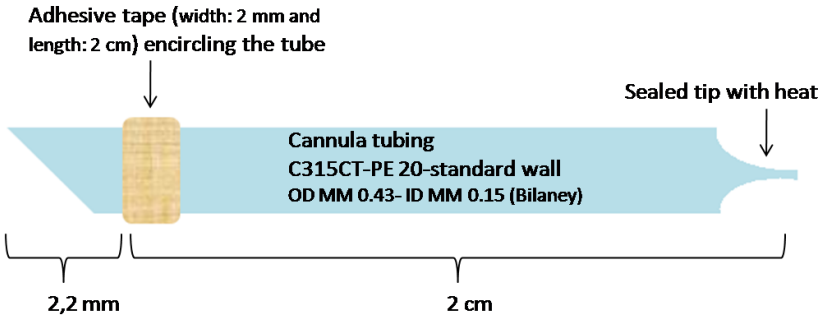


Figure 23. Schematic handmade cannula.

1. 3. SURGICAL PROCEDURE

The surgical procedure to insert the cannula was:

Step 1. Anesthetize the mouse using either an inhalant injection anesthetic Xylazine plus Ketamine (ketamine 75 mg/kg body weight plus xylazine 10 mg/kg body weight). Fit the mouse into a stereotaxic apparatus.
Step 2. Shave and wash the scalp. Starting slightly behind the eyes, make a midline sagittal incision and expose the skull. With the rounded end of a spatula, lightly scrape the exposed skull area and pat it dry. Scraping should remove the periosteal connective tissue which adheres to the skull, permitting good adhesion of the dental cement which is later used to secure the cannula.
Step 3. Identify the bone suture junctions bregma and lambda. With these as reference points, determine and mark the location for cannula placement using the stereotaxic coordinates, in this case, the lateral ventricle (0.58 mm posterior to bregma; 1 mm lateral to the midsagittal suture and to a depth of 2.2 mm; and with bregma and lambda at the same vertical dimension). Drill a hole through the skull at the marked, stereotaxically correct, location. This hole will receive the cannula.
Step 4. Insert the cannula through the skull.
Step 5. Completely dry the skull surface and cover the cannula and the entire implantation site with cement (Superglue®).
Step 6. Close the scalp wound before the cement completely dries (about 1 min).
Step 7. Remove the animal from the stereotaxic apparatus and place it back into its cage. The animal requires no restraint or handling during the delivery period.
Step 8. Animals have to be allowed to recover for 1 week before experiments and they have to receive postoperative medicine base on analgesics dissolved in the water.

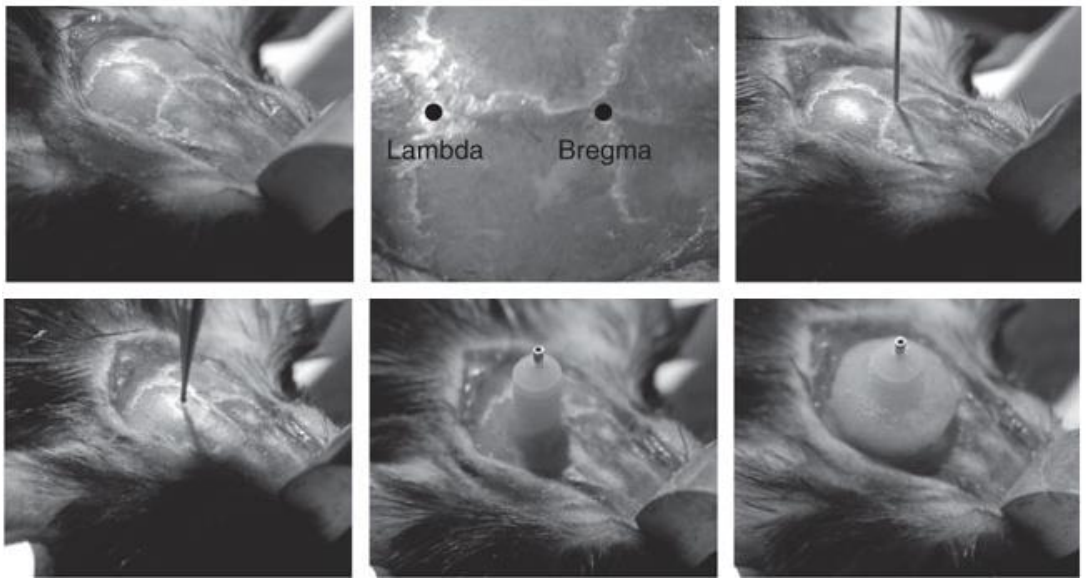


Figure 24. Serial photos exemplifying the cannula placement.

1. 4. VERIFYING CANNULA PLACEMENT

Two techniques were used to verify the cannula placement. One of them post mortem (Blue staining) and the other one with live animals (Angiotensin II) enabling later use of them.

- **Blue staining**

To verify the accuracy of the surgical technique and the placement of the cannula, it was used the Evans Blue (0.5 µl) staining:

Step 1. Sacrifice the mouse.
Step 2. Fix the brain with a suitable fixative (e.g., 4% formaldehyde).
Step 3. Remove the jaw and roof of the mouth of the mouse and expose the floor of the brain.
Step 4. Inject a dye (Evans Blue) through the cannula.
Step 5. Examine the dye stains to confirm its placement by sectioning the brain.

- **Angiotensin II**

To verify the placement of the cannula, one day before performing the experiments, it was used the ICV injection of angiotensin II (100 ng, 2 µl) through the cannula. Angiotensin II produces a thirsty effect, and when the cannula is correctly placed, the mice, within some minutes, start drinking. Only the mice that responded to angiotensin II were used in the experiments.

1. 5. INTRACEREBROVENTRICULAR AND INTRAPERITONEAL TREATMENTS

Injections were made through a 30-gauge microsyringe (Hamilton®). In the case of the handmade cannulas, at the moment of the administration, the tip of the tube had to be cut, and then the microsyringe inserted into the tub. The microsyringe must have some air inside to produce pressure when injecting the substance.

- **Ghrelin**

For the ghrelin (Bachem, Bubendorf, Switzerland) experiments, mice received an intracerebroventricular (ICV) administration of 5 µg (dissolved in 2 µL of physiological serum) or an IP administration of 10 µg (dissolved in 20 µL of physiological serum) of ghrelin.
The research group of Miguel López had previously demonstrated that ICV ghrelin exerts a dose-dependent effect on food intake and hypothalamic fatty acid metabolism, with the dose of 5 µg being the one that results in a greater response (López et al., 2008). The dose of 10 µg ghrelin IP has been previously used in the literature to induce food intake and produces serum ghrelin levels in the range observed in fasted mice (Sun et al., 2004).

- **Myriocin**

For the myriocin (Sigma, St. Louis, MO) experiments, mice received an ICV administration of 4 µg myriocin (dissolved in 1:3 DMSO:saline).

- **Ceramide C6:0**

For the Ceramide C6:0 (N-hexanoyl-D-sphingosine; Sigma) experiments, mice received an ICV administration of 2.5 µg Ceramide C6:0 (dissolved in 1:3 DMSO:saline).

Ghrelin and Ceramide C6:0 were administered at the beginning of the light cycle when mice were satiated. When indicated, myriocin was administrated 1 h before ghrelin administration. Mice were killed by cervical dislocation and tissue was collected and saved at 80ºC.

1. 6. FOOD INTAKE

Food dispensed into animal cages was initially weighed, then substances were injected and at the end, form 30 minutes to 3 hours depending on the experiment, food was subsequently reweighed.

1.7. SAMPLE RECOLLECTION

The hypothalamus was used for Western blotting and ceramide measurements.
To obtain the hypothalamus, the brain was placed ventrally. The hypothalamus was dissected out along the limits of the *black square*.

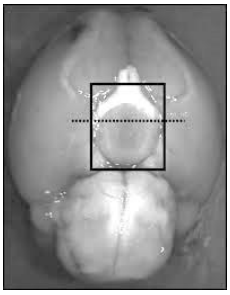


Figure 25. Ventral view of a mouse brain. The section framed in the black box is the hypothalamus.

The mediobasal hypothalamus (MBH) was used for real-time PCR analysis and ceramide measurements.

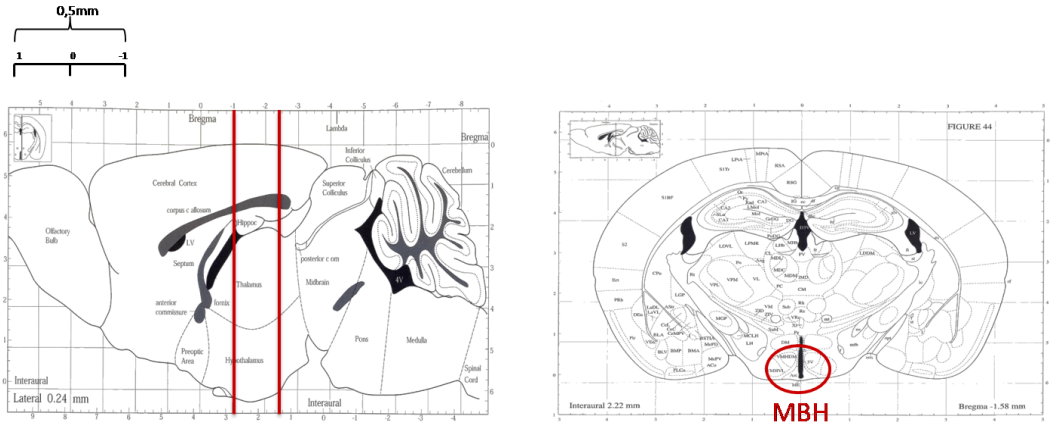


Figure 26. Sagittal schematic representation of the brain section containing the MBH and coronal representation of the section micropunched to obtain the MBH. (Drawns have been taken from Paxinus Atlas).

To dissect the MBH:

Step 1. The brain has to be carefully dissected and removed from the *calvarium*, then it has to be placed in a previously cooled matrix for sectioning (Roboz Surgical Instrument, Gaithersburg, MD).
Step 2. The brain is sectioned from bregma 21 mm to bregma 22.5 mm using two blades. The section has to be determined using a mouse brain stereotaxic atlas.
Step 3. Then, a 1-mm-diameter tissue collector* is used to obtain the MBH from each section.

Step 4. The MBH tissue recollected is then placed in an eppendorf at -80ºC for further utilization in real time PCR, western blot or HPLC experiments.

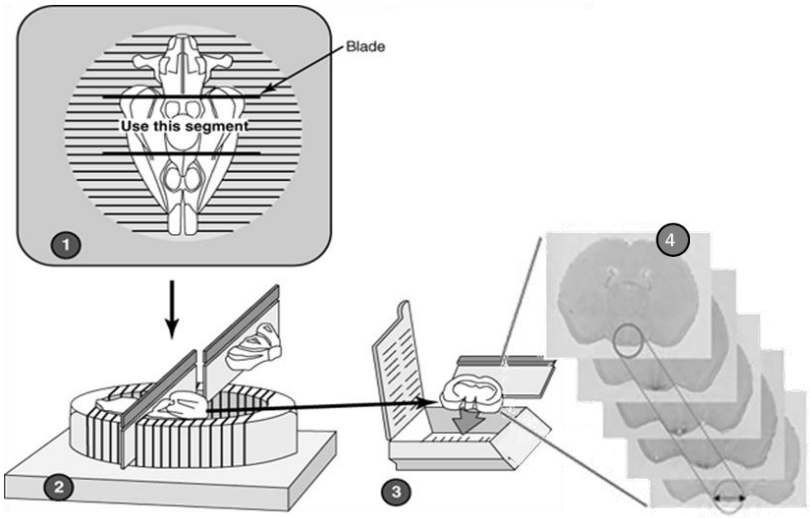


Figure 27. Schematic representation of the steps needed to obtain MBH.

(*) Some experiments analyzing the neuropeptide NPY by real time PCR were performed to decide which tissue collector was the most appropriated one. The 1-mm-tissue collector got the highest levels of NPY demonstrating the accurate obtaining of the MBH.

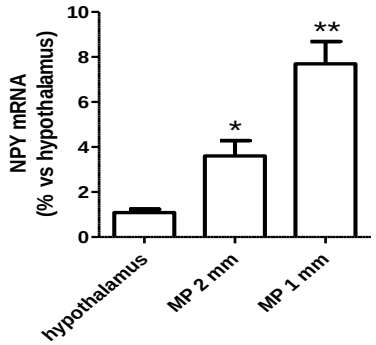


Figure 28. NPY mRNA levels of the hole hypothalamus and the MBH collected with a micro-punch of 2-mm of diameter (MP 2mm) or with a micro-punch of 1-mm of diameter (MP 1mm).

1.8. ANALYSIS OF LIFESPAN

Mice were allowed to live out their life, caged in groups of ten and the lifespan for individual mice was determined by recording the age of spontaneous death. Mean, minimum and maximum survivals were calculated for each group. Animals that appeared to be near death (listless, unable to walk, and cold to the touch) or had tumors were euthanized, and the date of euthanasia was considered the date of death.

2. CELL LINES

2.1 NEURO-2A

Clone Neuro-2a was established by R.J. Klebe and F.H. Ruddle from a spontaneous tumor of neuroblastoma of a strain albino mouse. This tumor line, designated C1300, was obtained from the Jackson Laboratory, Bar Harbor, Maine.

2.2 N-41 CELL LINE

The cell line N41 is an embryonic mouse hypothalamus cell line that has been immortalized from mouse embryonic day 15, 17, 18 hypothalamic primary cultures by retroviral transfer of SV40 T-Ag. These cell lines have been found to express an ever expanding array of neuropeptides, enzymatic markers and biologically active receptors. This cell line was purchased in CELLutions Biosystems Inc.

2.3. GT 1-7 CELL LINE

GT1-7 is a hypothalamic neuronal mouse cell lines producing gonadotropin-releasing hormone (GnRH), with a lot of receptors like the one for dopamine, GABA, glutamate, ghrelin…
The cells GT1-7 are clones derived from the cell line GT1. GT1 were produced by Pamela and Colleagues in 1990 by genetically targeting tumorigenesis to specific hypothalamic neurons in transgenic mice using the promoter region of the gonadoptropin-releasing hormone (GnRH) gene to express the SV40 T-antigen oncogene, they produced neuronal tumors and developed clonal, differentiated, neurosecretory cell lines.

This cell line was provided by Pamella Mellon from University of California.

2.3.1 MAINTENANCE OF GT1-7 CELLS

Cells were grown at 37ºC with 5% CO_2 in complete medium composed of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, #41965-039) containing 4.500 mg/L D-glucose and supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2mM glutamine, and penicillin/streptomycin (100 U/ml/100 µg/ml). The maintenance culture was passaged twice a week and the medium was changed every 2-3 days.

To trypsinize the cells, trypsine preheated to 37ºC was used. Cells were first washed in PBS, also preheated to 37ºC and then the trypsine was added during 3-5 min and the cells were detached by simply up and down pippeting.

2.3.2 PROTOCOL FOR SEEDING GT 1-7 CELLS

The protocol followed for the experiments was:

Step 1. For optimal adherence of the cells, it was necessary to do a pretreatment of the 6 well culture plates with poly-L-lysine (500 µl). Wells were treated with poly-L-lysine and then were placed in the incubator at 37ºC at least 2 hours up to 12 (O/N). Then the plates were cleaned 3 times with sterile water and were dried during a minimum of 30 min at 37ºC.
Step 2. Cells were grown until confluence.
Step 3. Cells were trypsinized and counted. Then, $2 \cdot 10^5$ cell/ml were seeded into the 2-ml wells.
Step 4. Cells were FBS removed during 48 hours until the performance of the experiments. Without FBS GT1-7 cells stopped growing and started to differentiate to neurons.

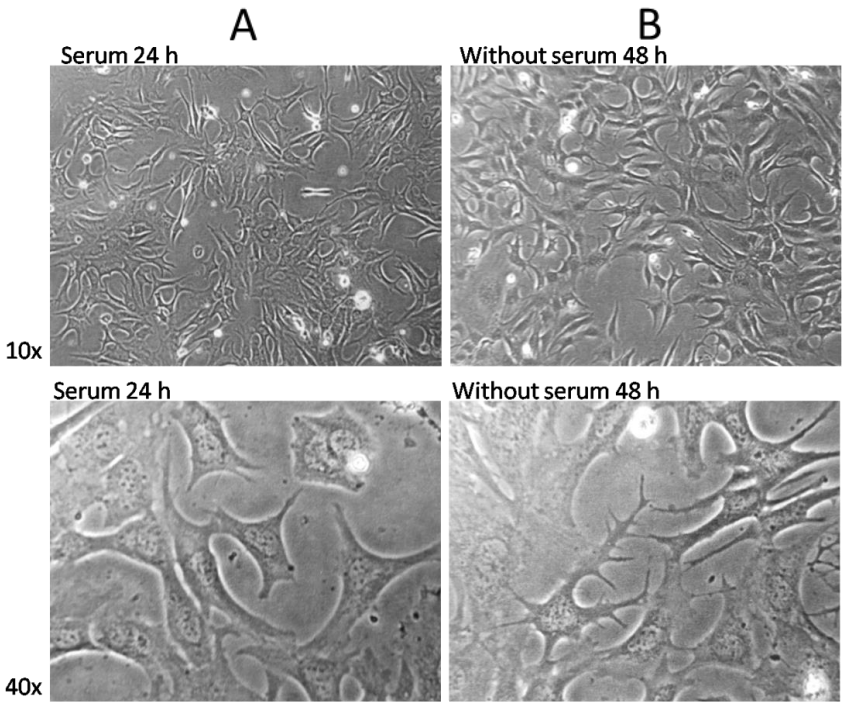


Figure 29. Morphology of the cells with (A) and without serum (B). **A:** 24 h before the placement of the cells at 10 and 40 magnification. **B:** 48 h after the remove of serum of the media at 10 and 40 magnification. The differentiation process and the new ramifications in cells without serum are highly visible in figure B at 40 magnifications.

2.3.3 CELL TREATMENTS

- **Ghrelin**

For the ghrelin (Bachem, Bubendorf, Switzerland) experiments, cells were treated with 6 nM of ghrelin (dissolved in physiological serum).

- **Myriocin**

For the myriocin (Sigma, St. Louis, MO) experiments, cells were treated with 10 µM myriocin (dissolved in DMSO).

- **Ceramide C6:0**

For the Ceramide C6:0 (N-hexanoyl-D-sphingosine; Sigma) experiments, cells were treated with 1µM Ceramide C6:0 (dissolved in DMSO).

- **Trichostatin A**

For the trichostatin (TSA) (Sigma, St. Louis, MO) experiments, cells were treated with 100 ng/ml of TSA (dissolved in ethanol).

- **Mithramycin**

For the mithramycin (Sigma, St. Louis, MO) experiments, cells were treated with 500 nM of mithramycin (dissolved in PBS 1x).

2.3.4 LENTIVIRAL TRANSDUCTION

Lentiviral vectors derived from the human immunodeficiency virus (HIV-1). The advantageous feature of lentivirus vectors is the ability to mediate potent transduction and stable expression into dividing and non-dividing cells both in vitro and in vivo. Essential lentiviral (HIV-1) genes must be expressed in cells to allow the generation of lentiviral particles. Some days after transfection of the cells, the cell supernatant contains recombinant lentiviral vectors, which can be used to transduce the target cells. Once in the target cells, the viral RNA is reverse-transcribed, imported into the nucleus and stably integrated into the host genome. Some days after the integration of the viral RNA, the expression of the recombinant protein can be detected.
The protocol followed for the experiments in GT1-7 cells was:

Day 0. Seed cells at appropriate density, 4.10^4 cell/ml.
Day 1. Remove half of the growing media and add 20 MOI* of lentivirus (pCPT1C-EGFP and pWpi-EGFP) to cells. At the end of the day remove media and replace with fresh one and without serum.
Day 4. Replace media with fresh one.
Day 6. Remaining cells population should emit fluorescence.

(*) **MOI** is the multiplicity of infection. The units of MOI are TU/ml where TU stands for transducing units. MOI=1, means that the ratio of the number of infectious virus

particles is 1:1 (1 virus: 1 cell). Ideally, one cell will be infected by one virus particle. However, in reality, multiple virus particles may infect the same cells leaving some others uninfected.

We used 2 types of transfer vectors in our experiments: pWpi and pCPT1C. The two transfer vectors shared the same backbone,pWpi, it is shown in figure 8. This bicistronic vector allows the expression of the fluorescent protein EGFP alone (pWpi) or a simultaneous expression of a transgene, in this case CPT1C cloned in PmeI, and the fluorescence protein EGFP (pCPT1C). EGFP was used to facilitate the tracking of transduced cells.

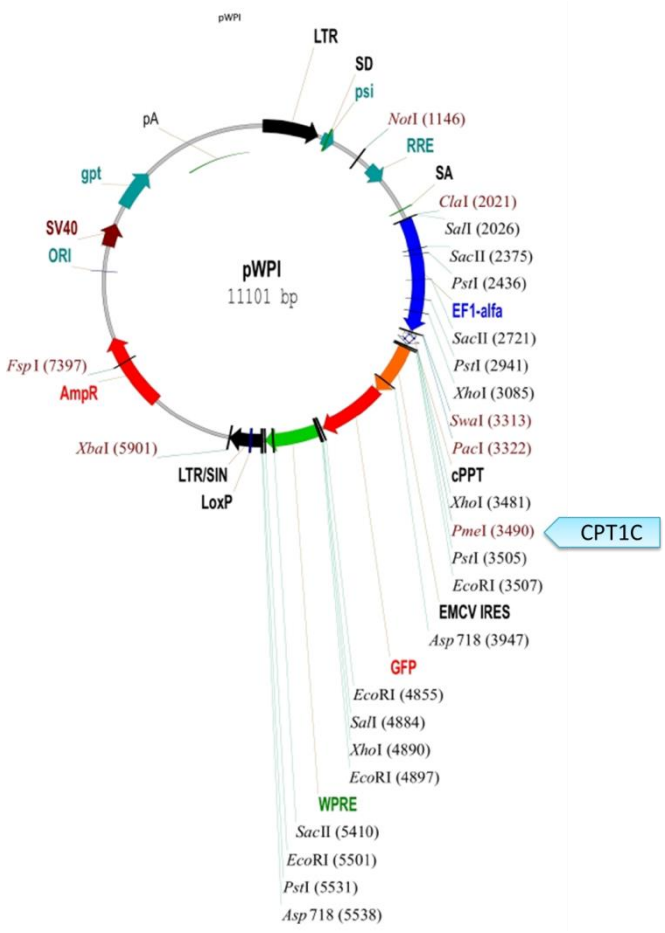


Figure 30. View map of pWpi plasmid.

2.3.5 SAMPLE RECOLLECTION

At the end of the treatments, the medium was removed and cells were scrapped (with a scrapper from Sarsted) in ice with tryzol® (for the real time PCR experiments), with a protein lysis buffer supplemented with phosphatase and protease inhibitors (for western blot assays) or with a HPLC buffer (for HPLC analysis).

3. DNA AND RNA

3.1 RNA ISOLATION AND QUANTIFICATION

Total RNA isolation from tissue or cells was performed using Trizol Reagent (Life Technologies, California) following manufacturer's instructions. Tissue or cells were homogenized 10 times on ice, using an ultrasonic homogenizer with 1ml of Trizol Reagent per 50-100 mg of tissue.

Then RNA was quantified in a multi-mode microplate reader (Biotech Synergy^{HT}) measuring the absorbance at 260 and 280 in a multi-volume plate.

3. 2 cDNA SYNTHESIS

Reverse Transcription (RT reaction) is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA, a reverse transcriptase enzyme (Moloney Murine Leukemia Virus Reverse Transcriptase: M-MLV RT (Invitrogen,California), primers, dNTPs and an RNase inhibitor. The PCR reaction used for cDNA synthesis was:

Component	Volume/well (20µl)	Final concentration
Buffer 5X	4 µl	1X
DTT 0.1 M	2 µl	10 mM
dNTPs 10 mM each	1 µl	500 µM
Random primers 50 ng/µl	5 µl	250 ng
RNAase out 40 U/µl	0.5 µl	20 U
MLV-RT 200 U/µl	1 µl	200 U
RNA	--	200 ng – 1 µg
$H_2$0 DEPC	--	Up 20 µl

The reaction was put in the Thermocycler machine at 37ºC during 1 hour.
For the analysis of all genes we use 1 µg of RNA, except in the case of BSX, that we use 200 ng of RNA to obtain the cDNA.

3.3 REAL TIME PCR

A quantitative polymerase chain reaction (qPCR), also called real-time polymerase chain reaction, is based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more

specific sequences in a DNA sample, quantitative PCR enables both detection and quantification at the same time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for the detection of products in quantitative PCR are:

1. **DNA binding dyes as reporters**: Non-specific fluorescent dyes that intercalate with any double-stranded DNA.
2. **Fluorescent reporter probes**: Sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence to quantify mRNA and non-coding RNA.

Both methods were used.
The protocol used for the reaction using the first method,SYBR green, was:

Component	**Volume/well (20 µl)**	**Final concentration**
Sso Advanced SYBR green supermix 2X	10 µl	1X
Primer mix*	8 µl	500 nM each
cDNA	2 µl	--
Total	20 µl	--

(*) The primer mix was prepared with forward primer at 100 µM, reverse primer at 100 µM and RNase-free water. (For 100 µl of primer mix: 2.5 µl forward primer + 2.5 µl reverse primer + 95 µl RNase-free water).

The thermal cycling protocol used was:

1. 95 ºC for 0:30
2. 95 ºC for 0:05
3. 60 ºC for 0:20

Plate read

4. GOTO2, 39 more times
5. 95 ºC for 0:10
6. Melt curve 65 ºC to 95 ºC: increment 0.5 ºC for 0:05

Plate read

The protocol used for the reaction using the second method, Taqman assay, was:

Component	Volume/well (20 µl)	Final concentration
SsoFast ProbesTaqman supermix 2X	10 µl	1X
Assay on demand 20X *	1 µl + 1 µl	1X
cDNA	8 µl (2 µl cDNA + 6 µl H_2O DEPC)	--
Total	20 µl	--

(*) We put in the same well: 1 µl of the assay on demand of a gene labeled with one fluorochrome (FAM) and another 1 µl of the assay on demand of the housekeeping gene labeled with a different fluorochrome (VIC).

The thermal cycling protocol used was:

1. 95 ºC for 0:30

Plate read

2. 95 ºC for 0:05
3. 60 ºC for 0:30

Plate read

4. GOTO2, 39 more times

The primers used for the SYBR green technique were:

- **CPT1C** 5′- TATGCAGTCGCCCTTCCT-3′ and 5′-ACATCAATCAGGTGTGTCTGC-3′
- **BSX** 5′-CGCTTTCGGTTCGTCTTG-3′ and 5′ -CACTGCCCTCAGCCTCTC-3′
- **GAPDH** 5′-TCCACTTTGCCACTGCA-3′ and 5′-GAGACGGCCGCATCTTCTT-3′

Taqman probes used for the experiment were the following:

- **AgRP** Mm00475829_g1 (FAM)- Applied biosystem
- **NPY** Mm00445771_m1 (FAM) - Applied biosystem
- **UCP2** 63705740 (FAM)- Integrated technologies
- **ATF4** Mm00515324_m1 (FAM) - Integrated technologies
- **Nfkb** Mm00445273_m1 (FAM) - Integrated technologies
- **GAPDH** 4352339E (VIC) - Applied biosystem

FAM and VIC are fluorocromes.

Relative abundance of mRNA was calculated after normalization to GADPH mRNA in both real time PCR techniques.

4. PROTEIN QUANTIFICATION AND DETECTION

4.1 CELL AND TISSUE LYSIS

Tissue or cells were lysed in chilled lysis buffer* (containing freshly added phosphatase inhibitor cocktail and protease inhibitor cocktail (Roche)). Tissue or Cells were placed on an ice tray. In the case of cells, they were washed once with cold PBS and collected from the culture dish in the appropriate volume of lysis buffer (e. g. 500 µl/ 10 cm Ø dish) using cell scraper. Tissue was homogenized in chilled lysis buffer (150 µl) using a glass homogenizer (B.Braun, Melsulneg AG) or an ultrasonic homogenizer. Lysates were collected in tubes on ice and fixed on a spinning wheel at 4°C for minimum 10 minutes to let the lysis proceed. Thereafter, the lysates were cleared from cell debris by centrifugation at 4°C, 5 minutes, and the protein content was determined.

(*) Lysis buffer composition:

Components	Final concentration
Tris 1M pH=7.4	0.02 M
EDTA 0,5 M pH=8	0.005 M
NP-40 10%	1%
NaCl 1M	0.15 M
H_2O miliQ	Sufficient for the final volume
Phosphatase inhibitor cocktail	Depending on the product
Protease inhibitors	Depending on the product

4.2 WESTERN BLOT ASSAY

Protein levels were measured according to the manufacturer's instructions (BioRad protein assay, #500-001 MT), using BSA (Sigma, #P0834) as a protein standard in the range of 2-25 µg/ml. BSA standard stock was prepared in water at 0.1 mg/ml and the absorbance of the blank, standard curve or the samples were measured at 595 nm in a multi-mode microplate reader (Biotech Synergy^{HT}).

Then protein lysates were subjected to SDS-PAGE, electrotransferred on a PVDF membrane, and probed with the following antibodies:

Primary antibodies:

- **Anti phospho-ACC:** rabbit pACC polyclonal antibody detects endogenous levels of ACC only when phosphorylated at Ser79. Diluted 1:1000 in BSA 3%. (Cell Signaling, 3661)
- **Anti phospho-AMPKα**: rabbit pAMPKα monoclonal antibody against AMPKα only when phosphorylated at Thr172. The antibody detects both α1 and α2 isoforms of the catalytic subunit. Diluted 1:1000 in BSA 3%. (Cell Signaling, 2535)
- **Anti CPT1C**: rabbit CPT1C polyclonal antibody against the last 14 amino acids of the *Mus musculus* sequence of CPT1C (Sigma Genosys). Diluted 1:500 in blocking solution.
- **Anti phospho-CREB**: rabbit pCREB polyclonal antibody against CREB when it is phosphorylated at serine 129. Diluted 1:1000 in BSA 3%. (Santa Cruz, sc-101662)
- **Anti FoxO1**: rabbit FoxO1 monoclonal antibody against FoxO1 protein. Diluted 1:1000 in blocking solution. (Cell Signaling, 2880)
- **Anti Sp3:** rabbit Sp3 polyclonal antibody against a peptide mapping at the C-terminal of Sp3. Diluted 1:500 in blocking solution. (Santa Cruz, sc-664)
- **Anti Tubulin:** mouse tubulin monoclonal antibody. Diluted 1:1000 in blocking solution. (Sigma, #T5201)

Secondary antibodies:

- **Donkey anti-rabbit HRP IgG:** A dilution of 1:5000 in blocking solution was used. (Amersham Biosciences, ref. NA 9340).
- **Sheep anti-mouse HRP IgG**: was used for detecting tubulin primary antibodies. A dilution of 1:10.000 in blocking solution was used. (Jackson immunoresearch, #515-035-003).

5.HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

5.1 HPLC ANALYSIS

High-performance liquid (HPLC) is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

We used this technique to analyze and quantify ceramides. Were extracted and analyzed them using an API 3000 (PE Sciex) liquid chromatography-electrosrapy ionization tandem mass spectrometer in positive ionization mode. A Kinetex 2,6 µm (75x3 mm C18) column was used.
Concentrations were determined by multiple reaction monitoring (MRM) with N-hepatodecanoyl-d-erythro-sphingosine (c17-ceramide) as internal standard (50 ng/ml). The method was linear over a range of 2-600 ng/ml.

5.2 CERAMIDE EXTRACTION PROTOCOL

The protocol use for ceramide extraction was:

Step 1. Add between 100-150 µl of PBS for each 1 mg of weight and sonicate at 100%, 0'6.

Step 2. Take 20 µl of samples for Bradford protein assay.

Step 3. Add 250 µl of methanol and 500 µl of chloroform (1:2) per tube, and 50 ng/ml C17:0 ceramide as internal standard.

Step 4. Incubate 1h at 48°C in the bath

Step 5. Put the tubes at room temperature and add 50 µl of KOH 1M in water and sonicate another time.

Step 6. Incubate at 37°C, 1h.

Step 7. Sonicate again.

Step 8. Separate the 2 phases by centrifugation at 2000 X g during 5 minutes.

Step 9. Take the lower phase.

Step 10. Nitrogen evaporation.

Step 11. Dissolve the ceramides with 300 µl of HPLC buffer*.
Step 12. Filter the samples with a teflon filter of 0.22 µm.
Step 13. Store samples at -80ºC.

(*) HPLC buffer composition:

Components	Final concentration
Methanol	74%
Formic acid	1%
Ammonium formate	5mM
H20-miliQ	25%

6. STATISTICAL ANALYSIS

Data are expressed as means ± SEM in relation to the control group in each case. Statistical significance was determined by Student *t* test when two groups were compared and by ANOVA with post hoc two-tailed Bonferroni test when more than two groups were compared. Values of $P<0.05$ were considered significant. All the analyses were performed using the statistical package Statgraphics Plus.

CHAPTER 4

RESULTS

PART I

INVOLVEMENT OF CPT1C AND CERAMIDES IN THE GHRELIN PATHWAY IN THE HYPOTHALAMUS

Recently, ghrelin has been shown to exert orexigenic action through the regulation of the hypothalamic AMPK pathway (López et al., 2008), leading to a decline in malonyl-CoA levels and the disinhibition of CPT1A (Andrews et al., 2008; López et al., 2008), which increases mitochondrial fatty acid oxidation and ultimately enhances the expression of the orexigenic neuropeptides AgRP and NPY (Sakkou et al., 2007). However, it is unclear whether CPT1C, which is located in the endoplasmic reticulum of neurons, may play a role in this action.

In addition, CPT1C has been shown to play a role in the anorexigenic actions of leptin through the ceramide levels in the ARC. The over-expression of CPT1C in ARC up-regulates NPY and BSX and blocks the effects of leptin on NPY and BSX. Moreover, leptin treatment decreases NPY, BSX, and ceramide levels in the ARC and this effect is mediated by CPT1C (Gao et al., 2011).

With these findings in minds, the present study aimed to determine whether CPT1C plays a role in the orexigenic actions of ghrelin, and if so, whether such an effect is mediated through ceramide levels.

1. GHRELIN ADMINISTRATION DOES NOT INCREASE FOOD INTAKE IN CPT1C KO MICE

To establish whether CPT1C forms part of the hypothalamic ghrelin signaling pathway, we analyzed the orexigenic effect of ghrelin by comparing WT and CPT1C KO mice.

Ten µg of ghrelin IP was injected at the beginning of the light cycle, when mice were satiated, and food intake was analyzed for 2 hours. We also tested food-seeking behavior, which consisted of determining the elapsed time before mice begin to eat after the administration of 10 µg of ghrelin IP. We found that ghrelin injection in WT mice increased food intake 2-fold (Fig. 31A) and highly reduced the time before eating commenced (Fig. 31B). Both effects were completely blocked in CPT1C KO mice (Fig. 31A,B). In order to elucidate the effect of ghrelin in the central nervous system and to eliminate peripheral effects, we repeated the experiment using an ICV injection of 5

µg of ghrelin and again found that CPT1C KO mice failed to respond to ghrelin treatment (Fig. 31C).

We conclude that CPT1C plays a role in the orexigenic effect of ghrelin.

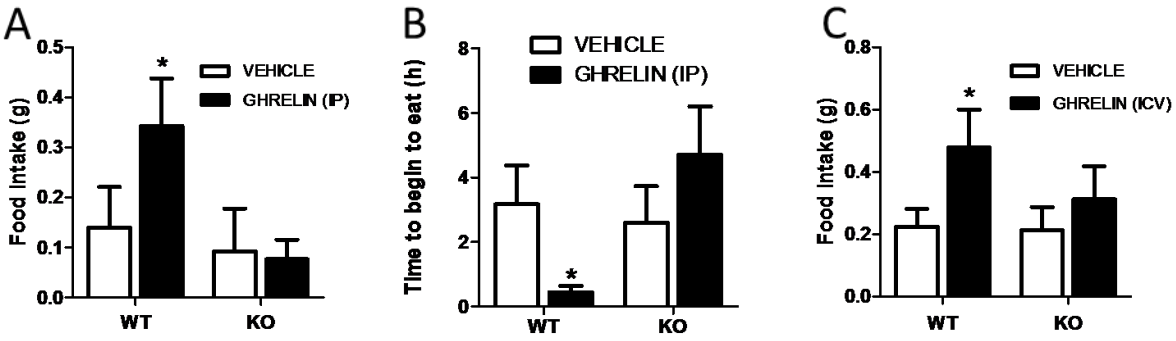
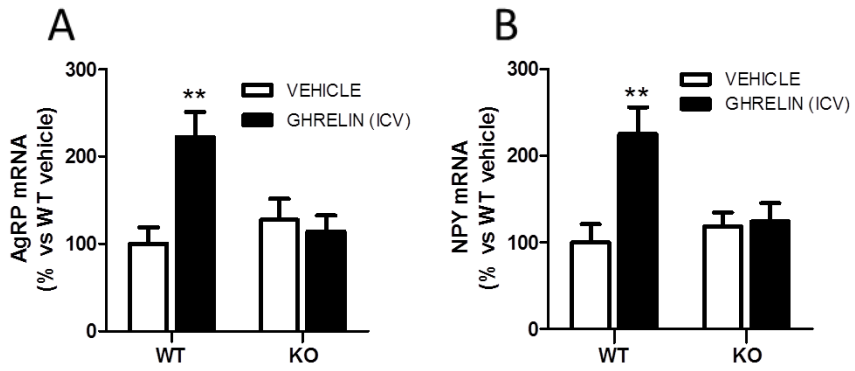


Figure 31. Ghrelin does not induce orexigenic effects in CPT1C KO mice. **A:** The 2-h food intake in WT and CPT1C KO mice treated with a vehicle (white bars) or with 10 µg ghrelin IP (black bars). **B:** Time before eat commenced following IP injection of either the vehicle (white bars) or 10 µg of ghrelin (black bars). **C:** The 2-h food intake in WT and CPT1C KO mice treated with ICV vehicle (white bars) or 5 µg ICV ghrelin (black bars).*$P^{<}$0.05 vs. WT mice treated with vehicle. n= 8-10 per group.

2. GHRELIN ADMINISTRATION DOES NOT INCREASE THE EXPRESSION OF OREXIGENIC NEUROPEPTIDES IN CPT1C KO MICE

In the next experiment, we treated WT and CPT1C KO mice with 5 µg of ICV ghrelin at the beginning of the light cycle. After 2 hours, mice were sacrificed and the expression levels of the orexigenic neuropeptides AgRP and NPY were analyzed using *in situ* hybridization (these experiments were performed by the Miguel López research group at the University of Santiago de Compostela). The ghrelin-induced increase in AgRP and NPY levels present in WT mice was blunted in CPT1C KO mice (Fig. 32A-C), which correlated with the lack of ghrelin-induced orexigenic effect in those mice.

These results indicate that CPT1C is involved in the expression of AgRP and NPY.



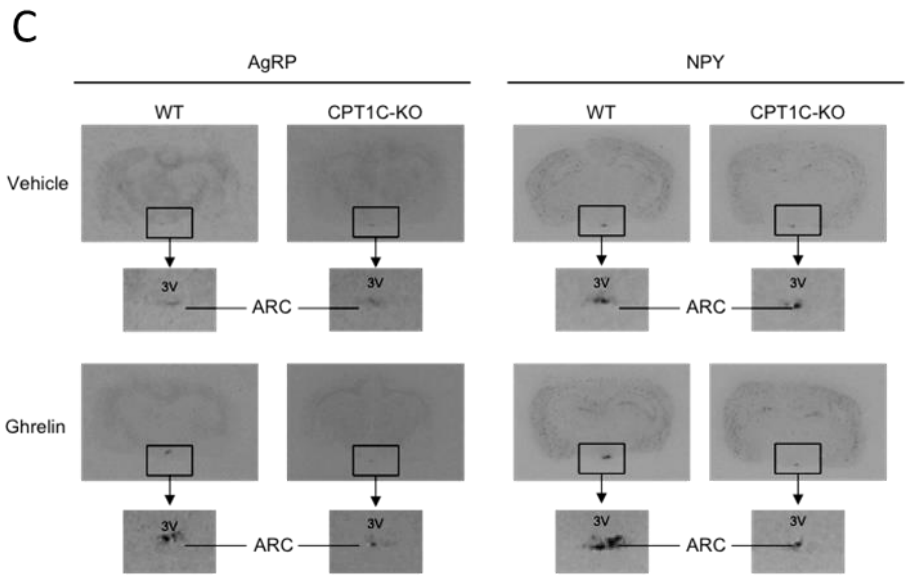


Figure 32. Ghrelin does not induce increased levels of AgRP or NPY in CPT1C KO mice. **A-C:** ARC mRNA levels of AgRP and NPY of WT and CPT1C KO mice treated with vehicle (white bars) or 5 µg ghrelin (black bars) using an ICV technique. Samples were obtained 2 h after treatment. ***P* < 0.01 vs. WT mice treated with vehicle. n=6 per group.

3. GHRELIN DOES NOT MODIFY THE mRNA LEVELS OF CPT1C

Having demonstrated that CPT1C is involved in the increased expression of the orexigenic neuropeptides AgRP and NPY in response to ghrelin, our next objective was to determine if this effect is mediated by an increase of CPT1C expression. WT and CPT1C KO mice received an ICV injection of 5 µg of ghrelin at the beginning of the light cycle, and 2 hours later mice were sacrificed. CPT1C levels in MBH were analyzed by real time PCR. We did not observe changes between mice treated with the vehicle and those treated with ghrelin (Fig. 33A).

These data suggest that the effects of CPT1C on AgRP and NPY are not due to an increase in the mRNA levels of CPT1C.

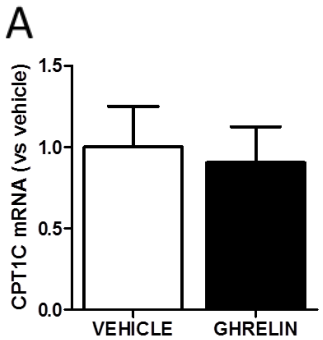


Figure 33. mRNA levels of CPT1C are not modified by ghrelin administration. **A:** MBH mRNA levels of CPT1C in WT mice treated with ICV vehicle (white bars) or 5 µg of ghrelin (black bars). n=4 per group.

4. THE CANONICAL GHRELIN SIGNALING PATHWAY IS IMPAIRED IN CPT1C KO MICE

Ghrelin produces the phosphorylation of AMPK and pAMPK becomes active and phosphorylates and inactivates ACC, causing a decrease in malonyl-CoA levels, the physiological inhibitor of CPT1 enzymes. This action produces disinhibition of the CPT1A enzyme, an increase in fatty acid oxidation, and an accumulation of ROS, which are mainly buffered by UCP2. Previous studies demonstrated that all these metabolic changes occur in the VMN (López et al., 2008; Gao et al., 2013), and although not fully understood, they ultimately activate transcriptional events by eliciting increased levels or activation of key transcription factors, such as pCREB, FoxO1, and BSX. These transcription factors are responsible, in part, for the increase of orexigenic neuropeptides AgRP and NPY in the ARC. CPT1C is more expressed in the brain than CPT1A. Although it has very low CPT1 activity it is known to bind malonyl-CoA as CPT1A does. Thus, we proceeded to analyze the ghrelin signaling pathway in the hypothalamus of CPT1C KO mice to determine if CPT1C plays a role in this pathway.

We injected the mice with central ghrelin (5 µg) and after 2 hours of treatment we obtained the hypothalamus and analyzed the components of the canonical ghrelin pathway. Our data showed a marked stimulatory effect on the levels of pAMPK (Fig 34A,C), pACC (Fig. 34B,C), UCP2 (Fig. 34H), and the transcription factors FoxO1 (Fig. 34D, F), pCREB (Fig. 34E, F), and BSX (Fig. 34G) in WT mice, in line with previous literature (Lage et al., 2010). However, these effects were impaired in CPT1C KO mice. Basal levels of pAMPK and pACC, putative upstream factors of CPT1C, were significantly increased in CPT1C KO mice when compared with WT mice, suggesting that the AMPK pathway is constitutively activated in the hypothalamus of CPT1C KO mice. Notably, in keeping with the altered levels of pAMPK and pACC, the expression of UCP2, a downstream factor of this pathway related to mitochondrial fatty acid oxidation, and the levels of transcription factors FoxO1 and pCREB also were increased in the hypothalami of CPT1C KO mice when compared with vehicle-injected WT mice. The basal levels of the transcription factor BSX in CPT1C KO mice were not significantly higher in comparison with the basal levels of WT mice, although they showed a tendency to increase.

These data suggest that the canonical signaling pathway of ghrelin is activated in CPT1C KO mice in basal conditions.

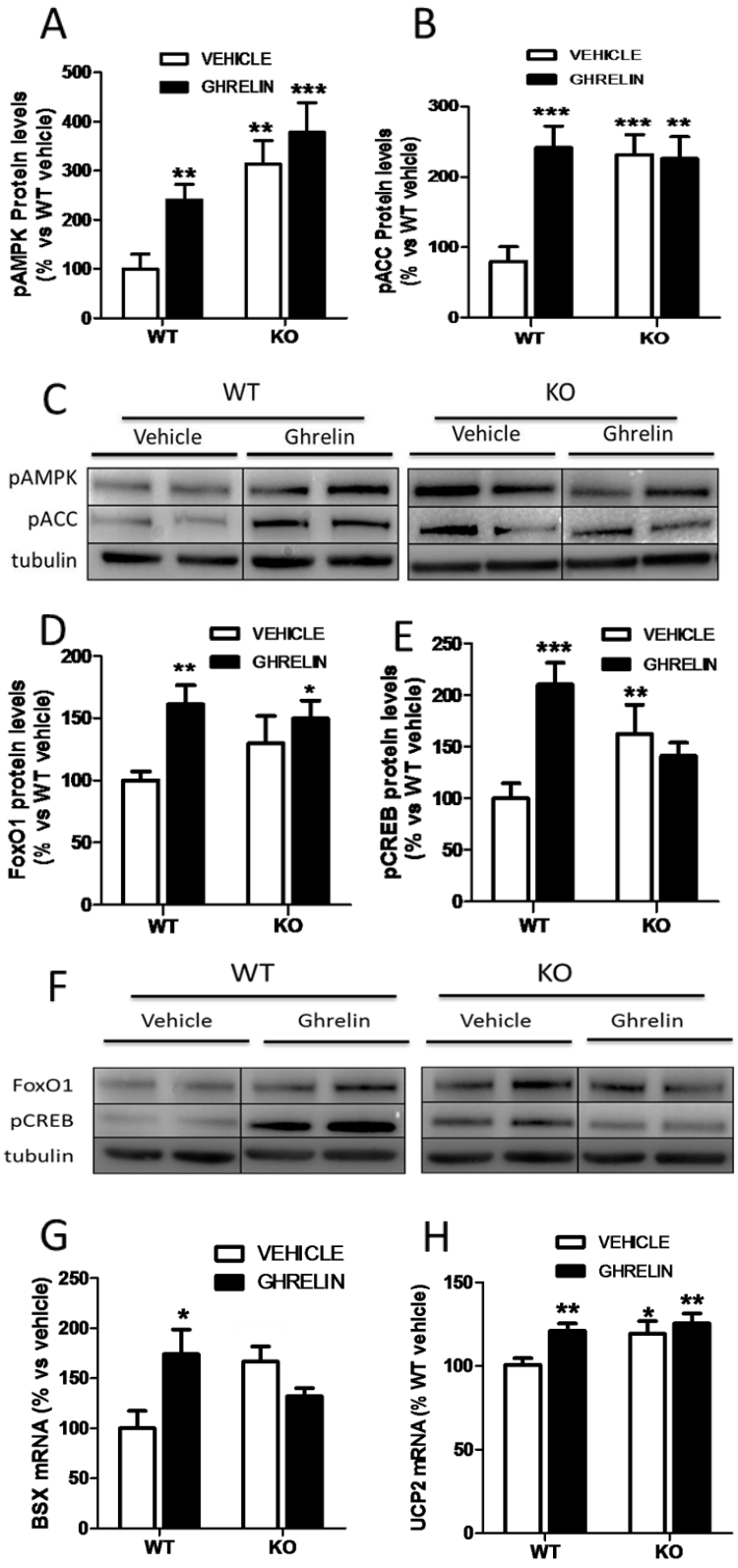


Figure 34. The ghrelin signaling pathway in WT and CPT1C KO mice. **A-C:** Hypothalamic protein levels of pAMPK and pACC. **D-F:** hypothalamic protein levels of FoxO1 and pCREB. **G and H:** MBH mRNA levels of BSX and UCP2 measured by real time in WT and CPT1C KO mice 2 h after ICV injection of vehicle (white bars) or 5 μg ICV injection of ghrelin (black bars). **P* < 0.05, ***P* < 0.01 vs., ****P*< 0.001. WT mice treated with vehicle. n=10-15 per group.

5. HYPOTHALAMIC AND MBH CERAMIDE LEVELS IN CPT1C KO ARE DECREASED IN COMPARISON WITH WT MICE

As previously stated, we aimed to determine whether CPT1C plays a role in ghrelin-induced orexigenic actions mediated through ceramide levels in MBH.

Current evidence from our group has demonstrated that CPT1C KO mice have reduced levels of ceramides in the cerebellum, striatum, and motor cortex (Carrasco et al., 2013). We set out to determine whether the reduction in ceramide levels also occurs in the hypothalamus. Our next step was to determine the levels of the different ceramide species between WT and CPT1C KO mice in the whole hypothalamus. Our data shows that CPT1C KO mice have reduced levels of ceramide in basal conditions in the hypothalamus for almost all ceramides (Fig. 35A). Because the hypothalamus is very heterogeneous and composed of different nuclei with different functions, we proceeded to analyze what occurs specifically in the MBH, our area of interest because it is related with the effects of ghrelin. We found that levels of ceramide, at least in C18:0 and C20:0, were lower in CPT1C KO mice in comparison with WT mice (Fig. 35B). We concluded that the lack of CPT1C causes a decrease in the ceramide levels in the whole hypothalamus and also in the MBH.

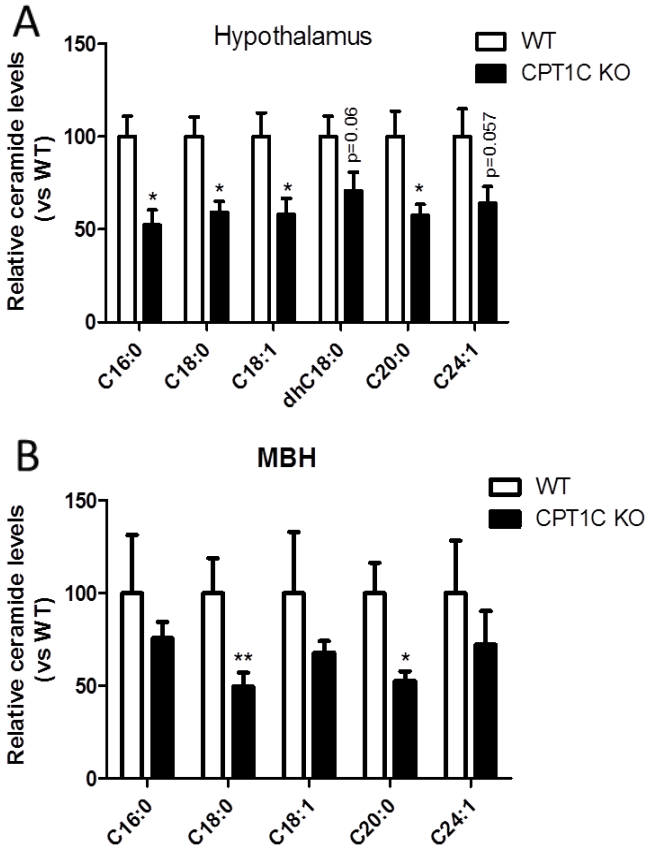


Figure 35. Levels of ceramide between WT and CPT1C KO mice. **A:** Ceramide levels in the whole hypothalamus. **B**: Ceramide levels in the MBH between WT (white bars) and CPT1C KO (black bars). *P < 0.05, **P < 0.01 vs. WT mice. n=8-10 per group.

6. CENTRAL ADMINISTRATION OF GHRELIN INCREASES CERAMIDE LEVELS IN THE MBH OF WT MICE, NOT IN CPT1C KO MICE.

C18:0 ceramide is the most abundant ceramide in the brain and in neurons (Ben-David et al., 2010). C18:0 was also the most abundant ceramide in the MBH (Fig. 36A). To determine whether ghrelin has any impact on MBH ceramide concentrations, we measured the levels of C18:0 in WT mice at different time points after ghrelin administration (5 µg, ICV). Our data showed that central ghrelin promoted a marked stimulatory and transitory action in C18:0 ceramide levels in the MBH, evident from with the maximal effect detected at 30 and 60 minutes after ghrelin injection (Fig. 36B). We also measured other ceramide species, however, none increased significantly (Fig. 36C,D). We then performed a dose-response curve and found that MBH ceramide levels at 30 min after ghrelin administration increased progressively with the dose of ghrelin. This increase became statistically significant at a dose of 5 µg (Fig. 36E). Next, we analyzed the same action in CPT1C KO mice. Contrary to WT mice, 5 µg ghrelin failed to induce any effect in C18:0 ceramide levels of CPT1C KO mice 30 min after its administration (Fig. 36F), indicating that CPT1C is needed for the stimulatory effects of ghrelin on ceramide content in the MBH.

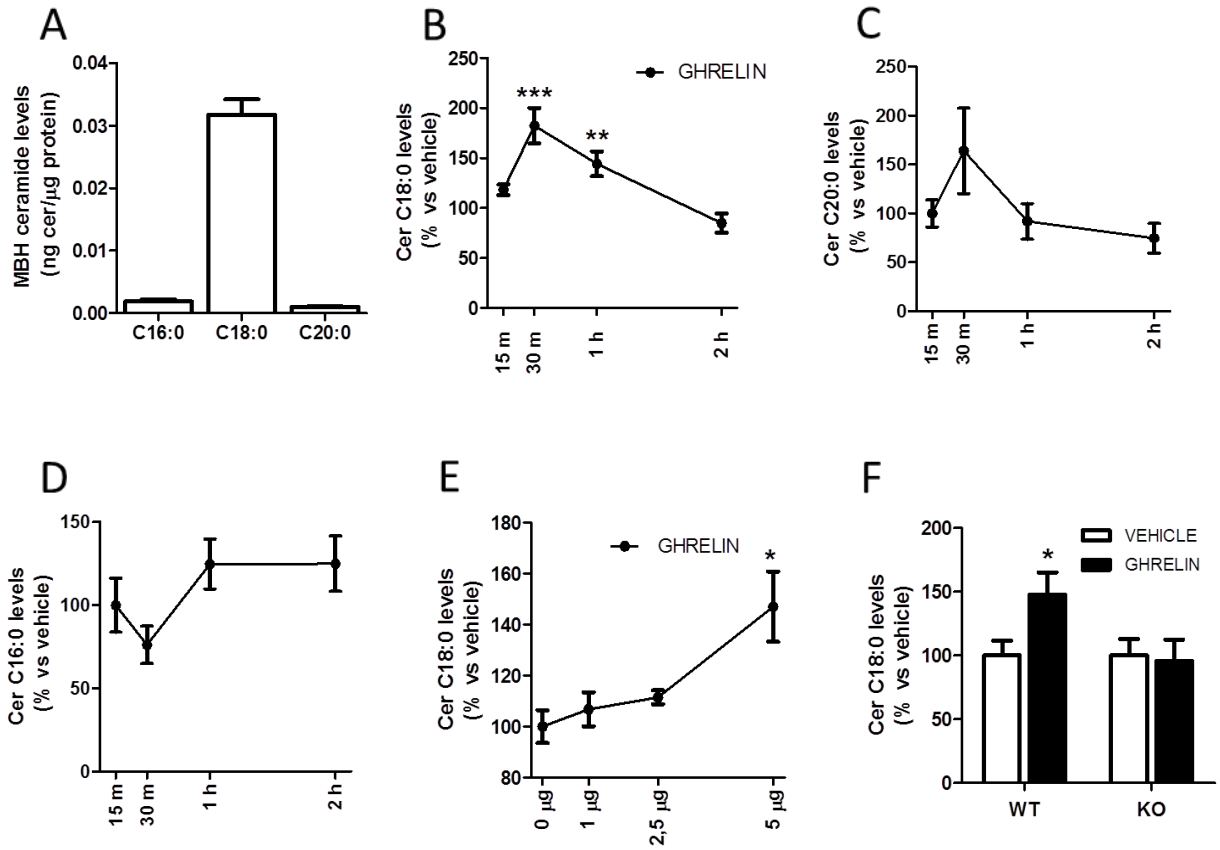


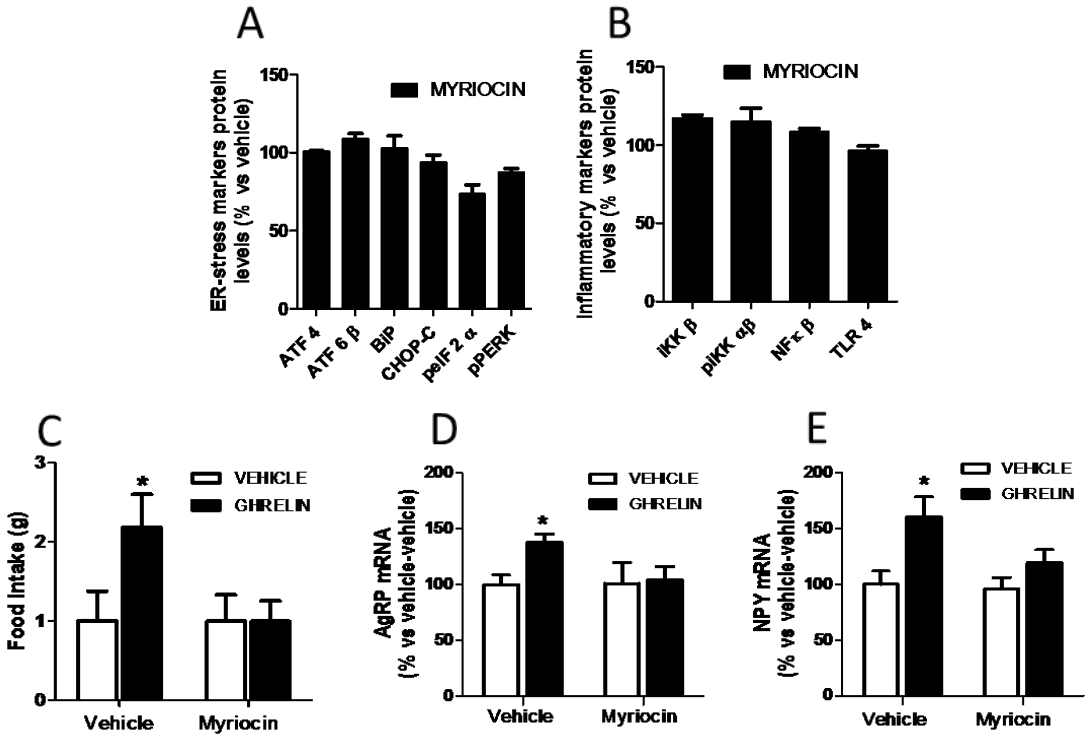
Figure 36. MBH ceramide levels in response to ghrelin. **A:** MBH ceramide levels of WT mice. **B:** Time course of MBH C18:0, C20:0, and C16:0 levels in WT mice after ghrelin administration (ICV, 5 µg). The percentage of respective increase in vehicle-treated mice is represented. **C**: MBH C18:0 levels in WT mice 30 min after ICV administration of different doses of ghrelin. **D:** MBH C18:0

levels of WT and CPT1C KO mice 30 min after administration of ICV vehicle (white bars) or 5 µg of ICV ghrelin (black bars). *P < 0.05, **P < 0.01,***P < 0.001 vs. WT vehicle-administered mice. n=7-10 per group.

7. THE INHIBITION OF HYPOTHALAMIC CERAMIDE SYNTHESIS BLOCKS THE OREXIGENIC EFFECT OF GHRELIN

To investigate the existence of any mechanistic link between the orexigenic effect of ghrelin and the activation of ceramide synthesis, we studied the effects of 4 µg of myriocin, a specific inhibitor of the *de novo* synthesis of ceramide, on ghrelin action. This dose had already been used by our group in earlier studies (Gao et al., 2011). The selected dose of myriocin did not induce an anorectic effect *per se* at any evaluated time, nor did it induce illness or malaise, ER stress (Fig. 37A), or hypothalamic inflammation (Fig. 37B) (Figures 7 A,B were produced by the Miguel López group). Although this dose of myriocin was subeffective when injected alone, our data show that an ICV injection of myriocin 1 hour before ghrelin administration decreases the orexigenic effect of ghrelin (Fig. 37C) and its stimulatory effect on neuropeptides AgRP and NPY (Fig. 37D-F) and transcription factors FoxO1, pCREB, and BSX (Fig. 37G-J).

These results indicate that ceramide synthesis is a required component of the ghrelin hypothalamic signaling pathway.



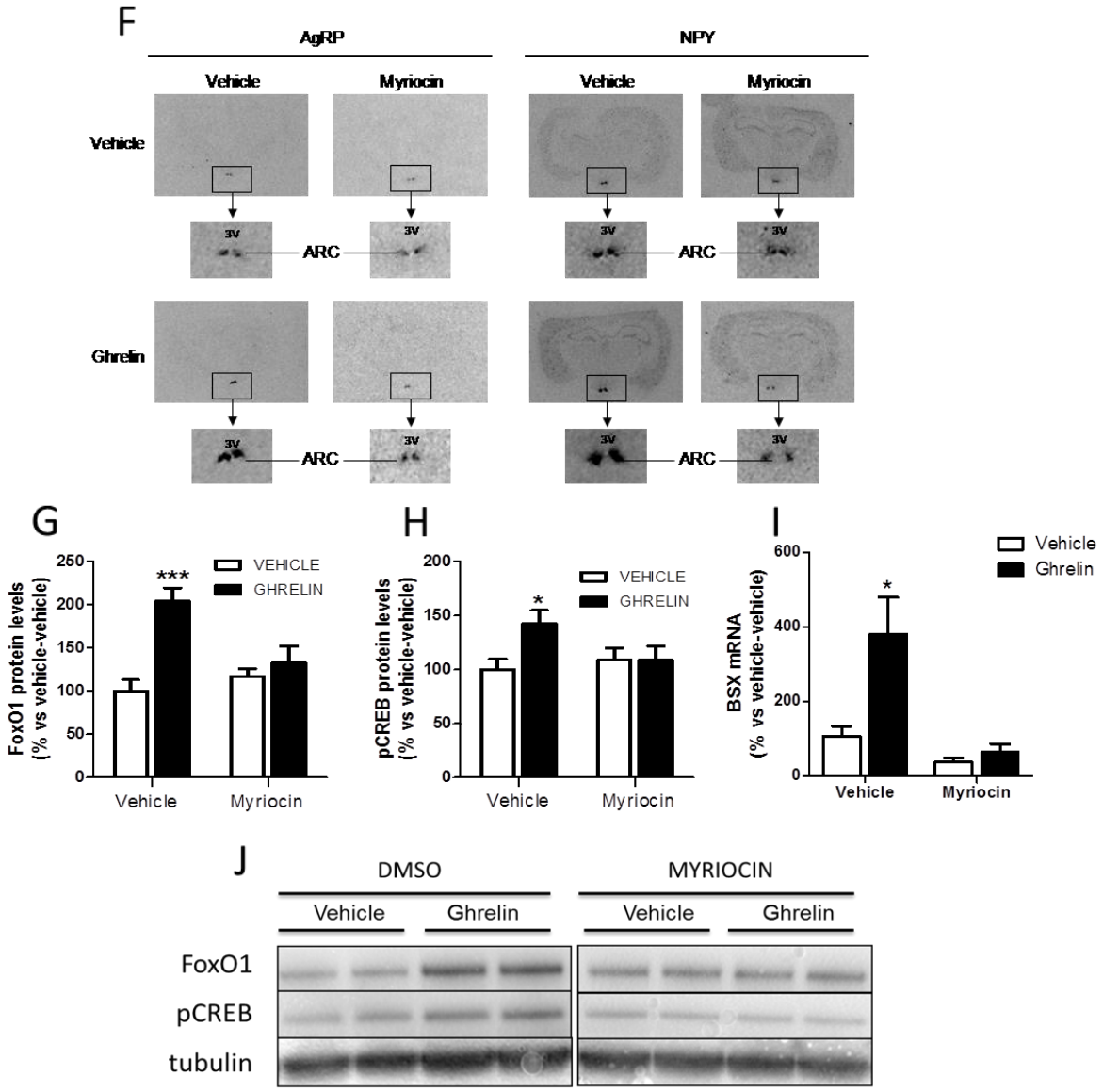


Figure 37. Myriocin injection blocks the orexigenic effect of ghrelin. WT mice were pretreated with ICV vehicle or 4 µg ICV myriocin 1 h before ICV administration of vehicle (white bars) or 5 µg ICV administration of ghrelin (black bars). **A, B:** ICV injection of myriocin does not cause ER stress or inflammation in the hypothalamus. Hypothalamic ER stress and inflammatory markers were measured by Western blot. ATF4, activating transcription factor 4; ATF6β, activation transcription factor 6β; BiP, ER chaperone-binding immunoglobulin protein, also known as glucose-regulated protein 78 KDa, GRP78; CHOP-C, C/EBP homologous protein C; pEIF2α, phosphorylated eukaryotic initiator factor 2α; pPERK, phosphorylated RNA-dependent protein kinase-like ER kinase; IKKβ, IκB kinase β; pIKK αβ, phosphorylated IκB kinase α β; NFκB, nuclear factor κB; TLR4, Toll-like receptor 4. **C**:Food intake after 2 h of ghrelin injection. **D-F**: ARC mRNA levels of AgRP and NPY measured by in situ hybridization. **G, H and J:** Hypothalamic protein levels of FoxO1 and pCREB measured by Western blot. **I**: MBH mRNA levels of BSX measured by real time PCR.*P < 0.05, ***P < 0.001 vs. vehicle-vehicle-treated mice. For the experiments of ER-stress and inflammatory markers n=4, for the rest of experiments n=8-10 per grou

8. CERAMIDE ADMINISTRATION INCREASES FOOD INTAKE IN CPT1C KO MICE

After demonstrating that ceramides are necessary mediators for the orexigenic effect of ghrelin, and that CPT1C KO mice do not increase ceramide levels in MBH after ghrelin treatment, we wanted to study whether ICV ceramide injection could reverse the phenotype of CPT1C KO mice and induce food intake. We used Ceramide-C6:0, a cell-penetrating ceramide that is converted to long chain ceramides inside the cell (Ogretmen et al., 2002; Sultan et al., 2006). We used the submaximal dose of 2.5 µg, which blocks the satiating effects of leptin but lacks an orexigenic effect *per se* in WT animals (Gao et al., 2011). We injected Ceramide-C6:0 by ICV and after 2 hours the mice were sacrificed. The selected dose of Ceramide-C6:0 induced neither hypothalamic inflammation (Nfkb), nor ER stress (ATF_4) (Fig. 38A). Ceramide, which was injected at the beginning of the light cycle when animals were satiated, increased food intake (Fig 38B) and the expression of AgRP and NPY in CPT1C KO mice but, as expected, it had no effect on WT mice (Fig. 38C, D). These results indicate that ceramide can be used to rescue feeding patterns when the canonical ghrelin signaling pathway (pAMPK/pACC/CPT1A/UCP2) is previously activated, as occurs in CPT1C KO mice. By contrast, in fed WT mice in which the ghrelin canonical pathway is not activated ceramide alone is unable to induce orexigenic neuropeptide expression and food intake.

These results support the idea of 2 parallel signaling pathways in ghrelin, with the involvement of CPT1C and ceramide in 1 of the branches. We propose that ghrelin must activate 2 parallel pathways, the mitochondrial pathway (with the activation of CPT1A and fatty acid oxidation) and the ER pathway (with the activation of CPT1C and ceramide synthesis), for its orexigenic effect to be effective.

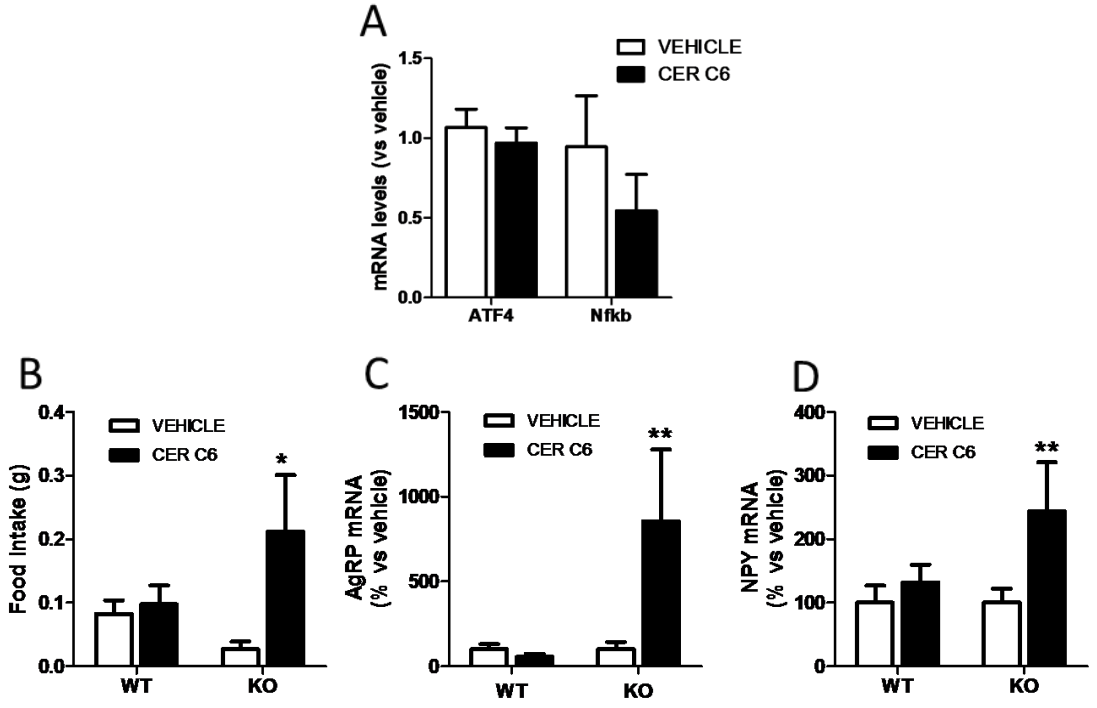


Figure 38. Ceramide induces food intake in CPT1C KO mice. WT and CPT1C KO mice were treated with ICV vehicle (white bars) or 2.5 µg of ICV Ceramide-C6:0 (black bars) at the beginning of the light cycle. **A:** ICV injection of Ceramide-C6:0 does not cause ER stress or inflammation in MBH. MBH ER stress and inflammatory markers were measured by real-time PCR. $P < 0.05$, $^{**}P < 0.01$ vs. vehicle-vehicle-treated mice. **B:** Food intake was measured 3 h after the injection. **C, D**: AgRP and NPY mRNA levels in MBH were measured by real-time PCR in samples obtained 3 h after treatment. For the experiments of ER stress and inflammation markers, n=4, for all other experiments, n=8-10.

9. CERAMIDE ADMINISTRATION INCREASES THE BSX MRNA LEVELS IN WT MICE

As previously explained, ceramide increases food intake and the expression of orexigenic neuropeptides in CPT1C KO mice, which have the ghrelin canonical pathway activated. However, for WT mice, in which the canonical pathway is not activated, ceramides alone are unable to induce any effect in food intake. Nevertheless, it is obvious that ceramide plays a role in the ghrelin signaling pathway because the inhibition of ceramide synthesis blocks the increase of AgRP and NPY. With this in mind, we proceeded to determine whether ceramide injection had any effect on the levels of FoxO1, pCREB, or BSX, the transcription factors responsible for increases in orexigenic neuropeptides in WT mice. We injected 2.5 µg of Ceramide-C6:0, as in the previous experiment, at the beginning of the light cycle when animals were satiated. After 2 hours of treatment the mice were sacrificed. The injection of ceramide had no effect on FoxO1 or pCREB protein levels (Fig. 39B,C), but it increased the BSX mRNA levels by 50% (Fig. 39A).

These results indicate that ceramides are involved in the expression of BSX, the transcription factor shared by neuropeptides AgRP and NPY.

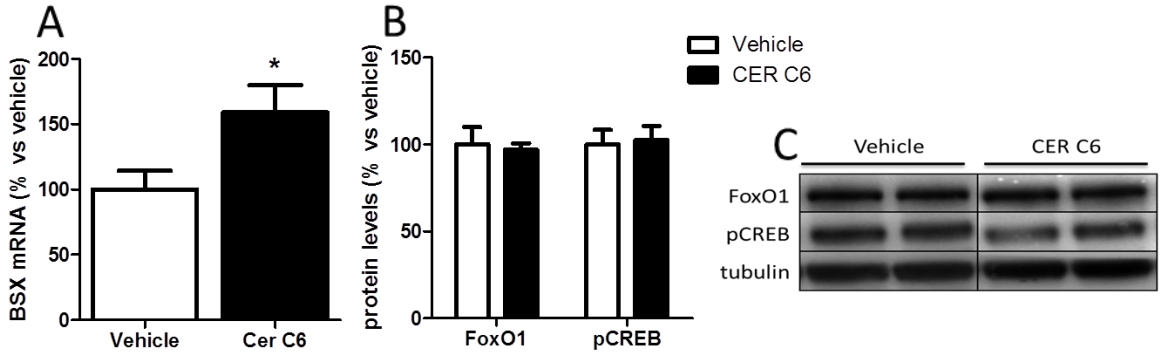


Figure 39. mRNA levels of BSX are increased in WT mice after Ceramide-C6:0 administration. WT mice were treated with ICV vehicle (white bars) or 2.5 µg of ICV Ceramide-C6:0 (black bars) at the beginning of the light cycle **A:** BSX mRNA levels in MBH were measured by real-time PCR in samples obtained 2 h after the treatment. **B, C**: Hypothalamic protein levels of FoxO1 and pCREB measured by Western blot.* $P < 0.05$ vs. vehicle-vehicle-treated mice. n=4-6.

PART II

THE INVOLVEMENT OF CERAMIDES IN THE INCREASE OF BSX EXPRESSION THROUGH THE ACTIVATION OF DEACETYLASES

BSX is a transcription factor with a highly restricted expression pattern. It is mostly expressed in feeding centers of the hypothalamus (Cremona et al., 2003; Nogueiras et al., 2008). We performed a real-time PCR experiment to corroborate these data and analyzed the mRNA levels of BSX in different parts of the brain. Our results show that BSX is expressed approximately 10 times more in MBH nuclei than in other areas of the brain (Fig. 40).

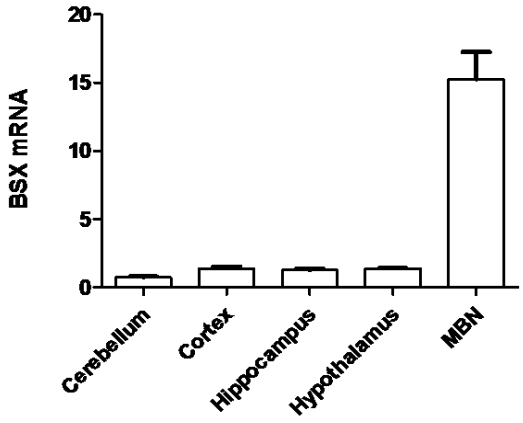


Figure 40. BSX mRNA levels at different parts of the brain: the cerebellum, cortex, hippocampus, whole hypothalamus, and mediobasal nuclei (MBN) of the hypothalamus (MBH). n=3.

It has been reported that BSX and pCREB physically interact in the NPY promoter region to increase transcription, and that BSX and FoxO1 cooperate to induce AgRP expression (Sakkou et al., 2007). Furthermore, it is known that the BSX promoter contains a putative binding site for the transcription factors Sp1/3 (Park et al., 2007). Sp1/3 factors can be regulated by phosphorilation, sumoylation, and acetylation, and Sp1 may also be regulated by glycosidation. Sp1/3 can be acetylated by CBP and p300 (Suzuki et al., 2003) and it can be deacetylated by HDACs (Ferrante et al., 2003). Previous studies have reported that ceramides enhance the association of HDAC with Sps in order to deacetylate them, thus producing changes in the binding of Sps to the promoters of genes, an event that could produce the subsequent up-regulation or down-regulation of these genes (Zou et al., 2011; Wooten-Blanks et al., 2011). Combining these data, we hypothesize that ceramides activate HDACs, which deacetylate Sp1/3, allowing Sp1/3 to interact with its putative binding site in the BSX promoter to induce transcription.

To corroborate this hypothesis with a simpler model, we decided to move from mice models to a hypothalamic cell line. After testing different cells lines, we chose the cell line GT1-7 as our experimental model.

10. MOVING FROM MICE TO A CELL LINE. IS ANY CELL LINE SUITABLE FOR OUR EXPERIMENTS?

One requirement in selecting the cell line, given that CPT1C is only expressed in neurons, was that the line originate from neuronal cells. A second essential requirement was that cells express BSX, which would increase in response to ghrelin or ceramides.

10.1 Neuro-2a cell line

The Neuro-2a cell line was generated from a spontaneous neuroblastoma. This cell line had been used in some published articles as hypothalamic cell model (Mohammad et al., 2007; Gomes et al., 2013). Experiments performed in our lab by real time PCR showed that Neuro-2a expressed very low levels of BSX making impossible its use for our purposes.

10.2 N-41 cell line

The N-41 cell line is an embryonic mouse hypothalamic cell line. As the transcription factor BSX expresses mainly in the hypothalamus, we thought that this cell line would be a good choice. Additionally, some published articles indicate that the N-41 cell line responds to ghrelin treatment in some ways (Stevanovic et al., 2013). We performed some experiments to corroborate the suitability of these cells.

- **Ghrelin and AICAR treatment in N-41 cells**. We tested whether the cell line N-41 responded to ghrelin or AICAR (an activator of AMPK) by increasing the levels of BSX or the orexigenic neuropeptides (NPY and AgRP). We found that ghrelin (6 nM) and AICAR (0.5 mM) did not increase the levels of BSX or the neuropeptides after 2 and 24 hours after treatment, respectively. mRNA levels in AgRP were almost inappreciable in this cell line (data not shown).

- **N-41 dose-response to Ceramide-C6:0**. We found that administering 10 µM of Ceramide-C6:0 produced an increase in the BSX mRNA levels after a 2-hour treatment (Fig 41A). We also tested the levels of BSX 1 hour and 24 hours after treatment and no statistical differences were observed (data not shown). We

performed a MTT assay with cells treated with vehicle or 10 µM of Ceramide-C6:0 and the viability percentage remained optimal (Fig. 41B).

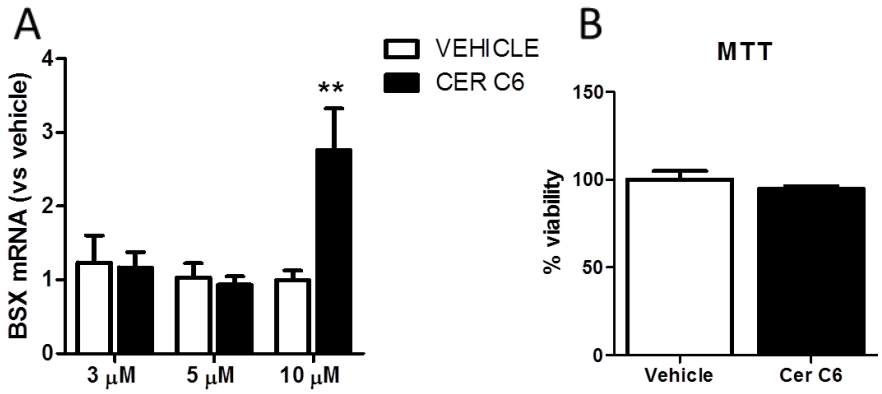


Figure 41. BSX mRNA levels of N-41 cells A: Dose-response experiment. Cells treated with vehicle (white bars) or with Ceramide-C6:0 (black bars). **B:** MTT assay, cells treated with vehicle (back bars) or with 10 µM of Ceramide-C6:0 (black bars). In both cases cells were collected 2 h after treatment. $^{**}P < 0.01$ vehicle treatment. n= 4 per group.

We rejected this cell line because it did not respond to ghrelin treatment and because the 10 µM-dose of Ceramide-C6:0, the necessary dose to induce BSX expression, was too high. Although the MTT assay demonstrated the viability of the cells after 2 hours of treatment, some articles have demonstrated that this dose could produce apoptosis (Mitoma et al., 1998).

10.3 The GT1-7 cell line

The GT1-7 cell line is a hypothalamic neuronal mouse cell line. Recently, these cells have been reported to respond to ghrelin treatment, with increasing the levels of pAMPK and pACC (Komori et al., 2012). We tested whether these cells also respond to ghrelin and ceramide by increasing BSX levels.

Two days before performing all experiments, the serum of the media was removed to promote the cell differentiation.

We tested 2 doses of ghrelin: 6 nM and 12 nM. Data showed that 6 nM of ghrelin increased BSX levels after 2 hours of treatment (Fig. 42A). We then analyzed the BSX levels after ceramide treatment. A dose-response experiment was carried out using 1 µM, 2.5 µM, and 5 µM of Ceramide-C6:0. We found that 1 µM and 2.5 µM of C6:0-ceramde produced an increase in BSX mRNA levels after 2 hours of treatment (Fig. 42B). We selected the lowest effective dose, 1 µM, because at higher doses ceramides are known to produce apoptosis.

After obtaining the correct doses of ghrelin and Ceramide-C6:0 to increase BSX levels, we tested whether 10 µM of myriocin could block ghrelin action as observed in the hypothalami of mice. The administration of myriocin 30 minutes before ghrelin appeared to decrease the effect of ghrelin on BSX (Fig. 42C), confirming that ceramide increase is necessary for ghrelin to induce BSX mRNA levels, as we previously demonstrated in mice.

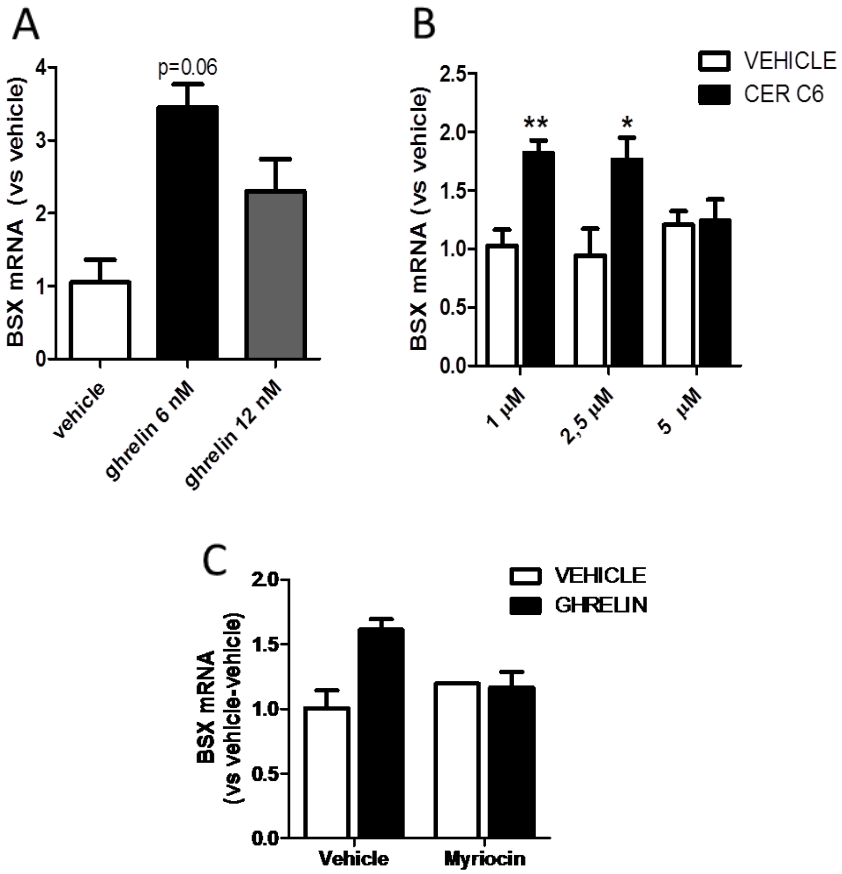


Figure 42. Ghrelin and ceramide increase BSX mRNA levels in the hypothalamic GT1-7 cell line. GT1-7 levels of BSX mRNA after vehicle treatment (white bar) and different doses of ghrelin (black and gray bars) (A), and after vehicle (white bar) and different doses of Ceramide-C6:0 (Black bar) (B). In both cases cells were collected after 2 h of treatment. **C**: GT1-7 cells were pretreated 30 min before ghrelin administration (6 nM) with vehicle or 10 µM of myriocin. After 2.5 hours cells were collected. $*P < 0.05$, $**P < 0.01$ vs. vehicle treated cells or vs. vehicle-vehicle treated cells. Experiment with ghrelin, n=2-3. Experiment with Cer C6, n=4. Experiment of myriocin, n=2.

11. THE OVER-EXPRESSION OF CPT1C INCREASES BSX IN GT1-7 CELLS

To determine whether CPT1C *per se* has any effect on the mRNA levels of BSX, CPT1C was over-expressed in GT1-7 cell lines using lentivirus vectors. Lentivirus has the ability to transduce non-dividing cells or slowly dividing cells. The serum was removed from our cells 2 days previously to stop growth and initiate differentiation; in this context, lentivirus was the best choice. We used a modified pWpi vector, which carries CPT1C and the fluorescence protein, EGFP (pCPT1C), and as a control we used a pWpi vector, which carries only the EGFP (pWpi). The EGFP was used to facilitate the tracking of transduced cells. The percentage of cells expressing EGFP after 6 days of transduction was 80% to 90% (Fig. 43A). CPT1C over-expression was confirmed by real-time PCR (Fig 43B). CPT1C mRNA levels were increased 30-fold in cells transduced with pCPT1C viruses when compared to control cells (transduced with pWpi) (Fig. 43B). We then analyzed the BSX levels to determine if an increase in CPT1C could modulate BSX mRNA levels. We found that BSX expression has a tendency to increase in cells over-expressing CPT1C (Fig. 43C).

These data suggest that CPT1C is upstream of BSX in the ghrelin signaling pathway.

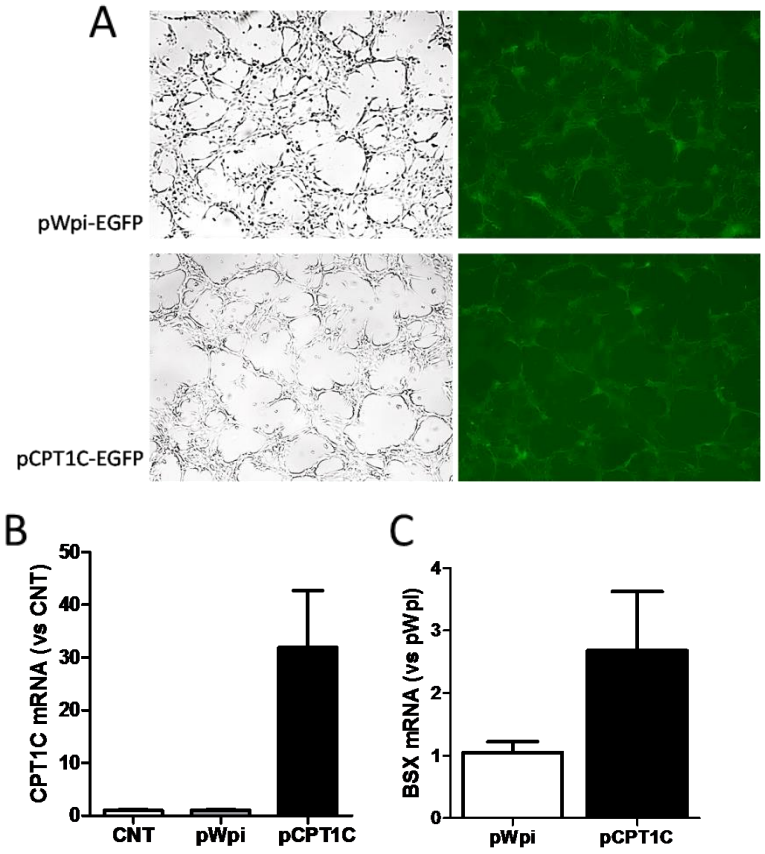


Figure 43. Over-expression of CPT1C in the GT1-7 cell line. A: Transduction efficiency. Six days after transduction approximately 80-90% of the GT1-7 cells showed green fluorescence. Images were taken with 20 X magnification in a fluorescent microscope with either white light (left) or

filter for fluorescence (right). **B:** Real-time PCR was used to confirm the mRNA levels of CPT1C after 6 days of transduction: control cells (white bars), cells transduced with pWpi (gray bars) and cells transduced with pCPT1C (black bars). **C:** BSX mRNA levels of cells transduced with 20 MOI of virus containing pWpi (white bar) or pCPT1C (black bars) determined by real-time PCR. n= 2.

12. THE INHIBITION OF HDAC BLOCKS THE INCREASE IN BSX mRNA LEVELS MEDIATED BY CERAMIDE

Acetylation of Sp1 and Sp3 is known to regulate the binding of these proteins to the promoter sequences of genes (Ammanamanchi et al., 2003; Zou et al., 2011) and ceramide treatment can alter Sp acetylation status (Wooten and Ogretmen, 2005; Wooten-Blanks et al., 2007). We therefore hypothesized that ceramides could increase BSX expression by modulating the acetylation status of Sp1/3.

First, we tested the effects of Ceramide-C6:0 on Sp3 expression levels. We wanted to analyze whether ceramides are able to increase the protein expression of Sp3 in addition to the deacetylation of it. A significant 2-fold increase was observed in cells treated with Ceramide-C6:0 (Fig. 44A,B). This result indicates that ceramides are able to increase the protein levels of Sp3.

To further study the effects of ceramide on Sp acetylation levels, we pretreated cells with trichostatin A (TSA), an inhibitor of the HDAC deacetylases, 30 minutes before Ceramide-C6:0 treatment. We wanted to determine if TSA is able to block the increase of BSX mRNA levels induced by ceramide. A dose of 100 ng/ml was used in line with earlier studies (Wooten and Ogretmen, 2005; Wooten-Blanks et al., 2007; Zou et al., 2010). Inhibition of the Sp deacetylation produced by TSA blunted the increase of BSX mRNA levels produced by ceramide (Fig. 44C).

Our results suggest that ceramides produce an increase in BSX expression due to a decrease of the acetylated levels of Sps. Decreasing the acetylated levels of Sp1 or Sp3, or both, could produce an increase in BSX. On the other hand, ceramides are able to increase the Sp3 expression. It has been reported that Sp3 is able to autoregulate because it acts as an activator and increasing levels on its own. Furthermore, it is important to note that Sp1 may also act as an activator of Sp3 (Tapias et al., 2008).

It is possible that HDAC modulation also produces changes in the acetylation state of other transcription factors or histones that may be responsible in part for the observed changes in the expression of transcription factor BSX.

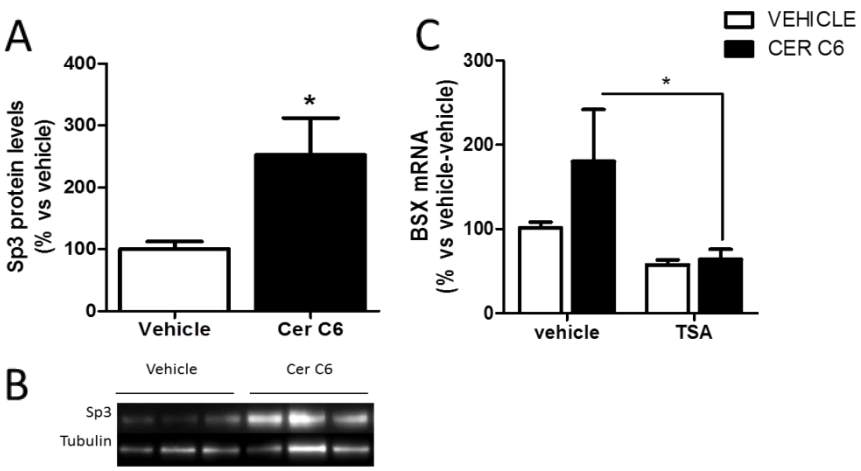


Figure 44. TSA treatment blocks the increase of BSX and Sp3 produced by ceramide. GT1-7 cells were treated with vehicle (white bars) or ceramide Ceramide-C6:0 (Black bars). **A,B:** Sp3 protein levels were measured by Western blot and samples were obtained 1 h after the treatment. **C**:BSX mRNA levels were determined by real-time PCR in cells treated with vehicle or 100 ng/ml TSA 30 min before treatment with 1 μM Ceramide-C6:0. Samples were obtained 2.5 h after treatment. **P* < 0.05 vs. vehicle or vehicle-vehicle-treated cells. n= 3.

13. SP INVOLVEMENT IN CERAMIDE-INDUCED UP-REGULATION OF BSX

As seen in previous experiments, if HDAC is inhibited the effect of ceramides on BSX expression is blunted. However, the inhibition of HDAC does not produce a specific action on Sp3 because HDAC has several different targets, such as Sp1. Therefore, our next step was to identify Sp1 or Sp3 as an activator or inhibitor. Although there are many exceptions under normal conditions, Sp1 acts as an activator and Sp3 acts as an inhibitor.

For this experiment, we uses mithramycin, a drug that binds GC-rich regions of DNA and blocks Sp binding to the promoter sequence of DNA (Blume et al., 1991). We treated the cells with 500 nM of mithramycin, the dose that had already been used in earlier studies (Zou et al., 2011), 30 minutes before ceramide-C6:0 administration.

As shown in Figure 45, due to the inability of Sp1/3 to bind CG-boxes placed in promoter sequences, the mRNA levels of BSX increase even more than with ceramide treatment (Fig. 45A). This result shows that Sp1 or Sp3 normally acts as a constitutive inhibitor and if it is not able to bind the promoter sequence, BSX is then able to increase its expression.

We analyzed the Sp3 levels after mithramycin treatment. Mithramycin blocked the effect of ceramides in BSX, a result that is consistent with previous data showing that Sp3 is able to upregulate levels by itself. However, if Sp3 is not able to bind its GC-boxes then it is not able to autoregulate (Fig. 45B,C).

What mithramycin treatment does, in fact, is block transcription factors that act through GC-boxes, including Sp3 and Sp1. Thus, it is important to be able to discern between these 2 transcription factors and understand their role in increasing BSX expression. It is very common that Sp1 and Sp3 work together to modulate the levels of different genes.

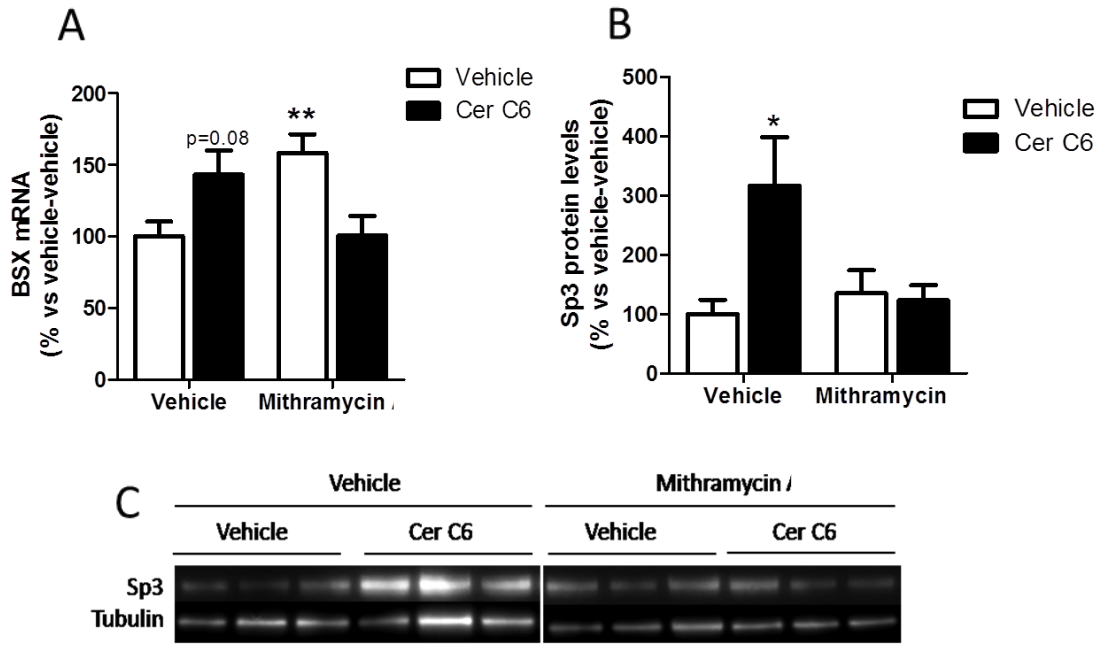


Figure 45. Mithramycin treatment increases BSX expression and decreases the effect of ceramides on Sp3. Cells were treated with vehicle or mithramycin 30 min before treatment with 1 μM Ceramide-C6:0. **A:** Cells were collected 2.5 h after treatment and BSX mRNA levels were analyzed by real-time PCR. **B, C:** cells were collected 1.5 h after treatment and Sp3 protein levels were analyzed by Western blot. *P< 0.05 vs., **P < 0.01 vs. vehicle-vehicle-treated cells. BSX experiment, n= 8. Sp3 experiment, n=3.

It is possible that deacetylated Sp1 acts as an activator and acetylated Sp3 acts as an inhibitor. It is also possible that acetylated Sp3 constitutively represses the BSX promoter. Thus, when there is an increase in ceramides, they could activate HDAC deacetylases, triggering a decrease in acetylation levels of both transcription factors, Sp1 and Sp3. At the same time, deacetylated Sp3 could lose its binding affinity to the promoter, allowing deacetylated Sp1 to bind the promoter activating the BSX transcription.

PART III

CPT1C AND LIFESPAN

Recently, a metabolomic analysis comparing WT and CPT1C mouse brains indicated that CPT1C could play a role in neuronal oxidative metabolism: a decrease in oxidized glutathione (GSSG) in CPT1C KO mice was observed, indicating a loss of antioxidant capacity (Lee and Wolfgang, 2012). A large number of studies indicate that a decreased ratio of GSH/GSSG is present during aging, producing shorter lifespan (Chen et al., 1989; Suh et al., 2005). These data combined suggest that if a lack of CPT1C produces a decreased GSH/GSSG ratio, then GSH is not able to produce an antioxidant effect to lower ROS, thus increasing ROS levels in CPT1C KO mice leading to oxidized proteins and lipids, and a DNA-produced accumulation of oxidative stress generating damage and aging (Gupta et al., 1991; Hamilton et al., 2001).

We hypothesize that CPT1C KO mice, having higher levels of GSSG, suffer more oxidative stress and thus have reduced survival rates. To study this idea, we performed a lifespan experiment and compared longevity between WT and CPT1C KO mice.

LONGEVITY

Survival data were collected on WT mice (10 females) and CPT1C KO mice (10 females) housed in groups of 10 throughout their life. Animals that appeared to be near death (listless, unable to walk, and cold to the touch) or had tumors were euthanized, and the date of euthanasia was considered to be the date of death. Longevity was significantly decreased in CPT1C KO mice (P = 0.0005). The percentage of survival is summarized in Figure 46A and mean, minimum, and maximum lifespans are summarized in Figure 46B. Mean lifespan was decreased by 53.3% in CPT1C KO mice. The survival distribution of WT and CPT1C KO mice is represented in Figure 46C.

From these data we concluded that a lack of CPT1C produced a significant reduction in lifespan.

A

Life span (in months)			
	WT	CPT1C KO	% decrease
Mean	15	8	53,3%
Minimum	10	3	
Maximum	20	14	

B

Time (in months)	WT % survival	CPT1C KO % survival
0	100 %	100 %
2	100 %	90 %
4	100 %	70 %
6	100 %	40 %
8	100 %	40 %
10	80 %	30 %
12	60 %	10 %
14	60 %	0 %
16	20 %	0 %
18	10 %	0 %
20	0 %	0 %

C

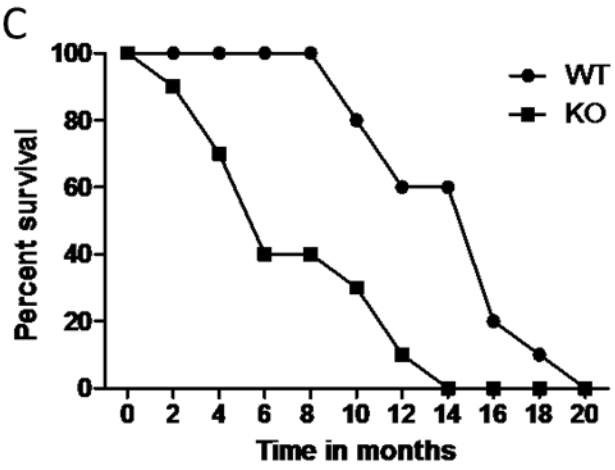


Figure 46. Decreased longevity in CPT1C KO mice. A: Statistic summary. **B,C:** Survival distribution summarized in a table (B) and expressed in percentages of survival in a graph (C). WT mice (circles) and CPT1C KO mice (squares). n= 10 per group.

CHAPTER 5

DISUSSION

The findings obtained in this study and in published literature support the concept that CPT1C has a completely different function from those of the other 2 known isoforms, CPT1A and CPT1B. This hypothesis is supported mainly by the following 2 facts:

1) CPT1C is located in the ER membrane, not the outer mitochondrial membrane, which is the known location for the other isoforms.

2) Although CPT1C contains all of the key residues known to be important for CPT1A catalytic activity and could potentially catalyze the same reaction with similar kinetics characteristics, the findings published by our group (Sierra et al., 2008) show that CPT1C is not involved in the oxidation of palmitate, at least in PC12 or HEK 293 cells.

CPT1C function may not be related to energy production from fatty acids, but it probably plays a role that is linked to ER-specific functions.
CPT1C is expressed in the ER of neurons throughout the whole brain; thus, it would seem that its function is related to some requirement found in all neurons in brain cells. In the present study, however, I aim to elucidate the role of CPT1C in the pathway of ghrelin in the hypothalamus. Indeed, it is only a small piece of the whole pie, but this is how scientific investigation works.

I will discuss the findings of the present study sequentially: first I will discuss the role of CPT1C in the ghrelin pathway in the hypothalamus, second I will explain how CPT1C appears to produce an increase in ceramides due to ghrelin activity, third I will discuss how these ceramides impact the brain-specific homeobox transcription factor BSX, which is responsible, in part, for an increase in orexigenic neuropeptides, and finally I will comment on CPT1C and its role in aging.

1. CPT1C IS PRESENT IN THE GHRELIN PATHWAY IN THE HYPOTHALAMUS

The first finding in the present study was that CPT1C is involved in the ghrelin pathway. Deletion of CPT1C blunts the effect of ghrelin in the hypothalamus, increasing orexigenic neuropeptides and food intake. This result is in line with earlier published findings, which had already pointed to CPT1 as being related to the central regulation of food intake. These previous works have been the driving force for the present work.

1) In the first instance, certain studies related CPT1A, the other isoform expressed in the brain, with food intake: a) Obici and colleagues inhibited CPT1A in the whole hypothalamus, which suppressed food intake (Obici et al., 2003); b) López and colleagues pretreated rats with an inhibitor of CPT1s (etomoxir) before ghrelin administration and saw a decrease in ARC levels of the orexigenic neuropeptides (AgRP and NPY), thus preventing food intake (López et al., 2010). However, these authors used a nonspecific inhibitor of CPT1s and analyzed the increase of CPT1 activity after ghrelin administration. Since CPT1C is not active, or is minimally active, one can conclude that the effect observed was due to CPT1A. For the first time, they reported a relationship between CPT1A and the ghrelin orexigenic pathway.

2) In the second instance, a work that suggested that CPT1C is part of the ghrelin pathway in the hypothalamus involved CPT1C with leptin satiating actions (Gao et al., 2011). The authors showed that adenoviral overexpression of CPT1C in the ARC nucleus of rats increased food intake and concomitantly upregulated orexigenic NPY. Moreover, they demonstrated that this overexpression antagonizes the anorectic actions of central leptin. CPT1C overexpression also blocks leptin-induced down-regulations of NPY, and furthermore, anorectic actions of central leptin are impaired in mice with CPT1C-deleted brains.

Taking all these data into account, I hypothesize that

1) CPT1A and CPT1C work coordinately to allow adequate ghrelin function in the hypothalamus.

2) Leptin and ghrelin may both require the presence of CPT1C in order to function properly, although in opposing ways.

In this section the first hypothesis will be discussed, while the relationship between leptin and ghrelin will be examined in subsequent sections.

- **THE 2 PARALLEL PATHWAYS OF GHRELIN**

Recently, a great deal of effort has been made to distinguish between the various roles played by ghrelin in the different nuclei of the hypothalamus. As the receptor of ghrelin is mainly in the VMN and in the ARC, these 2 localizations have been studied most.

a) VMN effects: it has been shown that ghrelin regulates feeding through the inhibition of *de novo* fatty acid synthesis and the increase of β-oxidation in the VMN in an AMPK-dependent manner (López et al., 2008).
b) ARC effects: it has been shown that ghrelin directly stimulates NPY/AgRP neurons in the ARC, increasing its synaptic plasticity and firing through an AMPK-dependent manner (Kohno et al., 2007; Andrews et al., 2009).

These different effects of ghrelin have been associated with the 2 different brain isoforms of the CPT1. Specifically, CPT1A has been associated with the VMN effects of ghrelin while CPT1C has been associated with its ARC effects. Inhibition of CPT1A in VMN produces a decrease in food intake, whereas overexpression of CPT1A in VMN produces an increase. Overexpression of mutant CPT1A (constitutively active) in the VMN of mice prevents the decrease of food intake produced by etomoxir. The same experiment performed in the ARC showed that CPT1C was unable to revert etomoxir effects, indicating that CPT1A is active only in the VMN (Gao et al., 2013). On the other hand, the same authors overexpressed CPT1C in the ARC and observed an increase in food intake in the following hours, indicating that CPT1C is active, at least in the ARC nucleus (Gao et al., 2011).

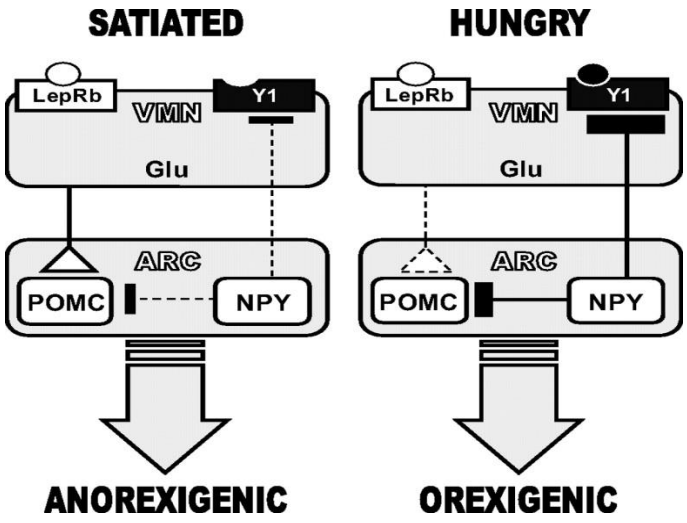


Figure 47. Schematic diagram of the network circuitry between VMN and ARC. Under satiated conditions (left), the NPY neurons in the ARC are inactive, and the tonically present leptin maintains activity in glutamatergic VMN efferent neurons that innervate ARC POMC neurons. During hunger (right), neurons in the ARC are actively releasing NPY onto the VMN. The tonic effect of leptin is overcome by robust, NPY-mediated inhibition of VMN neurons. This will decrease the VMN-mediated excitation of ARC POMC neurons and contribute to the net orexigenic effect of NPY (Chee et al., 2010).

These 2 nuclei are connected (Fig.47) but have different receptors and neuron types. Nevertheless, both have ghrelin receptors and in both cases the effect of ghrelin is

related to an activation of the AMPK energy sensor. Together, this indicates that ghrelin exerts differential effects, most probably synergic, in the VMN through CPT1A and in the ARC through CPT1C. Furthermore, they appear to need to work coordinately for ghrelin to be effective.
However, another possibility exists: both isoforms may be present in both nuclei, each being predominantly activity depending on the site.
It is important to note that the experiments in the present study have been performed with mediobasal nuclei (MBN), which includes the ARC and the VMH, and thus it is not possible to distinguish between the CPT1C effects in these 2 nuclei.

A lot of effort is still needed to elucidate these intricate and poorly understood mechanisms in ghrelin. However, the results of the present study clearly demonstrate that CPT1C is needed for ghrelin to induce food intake.

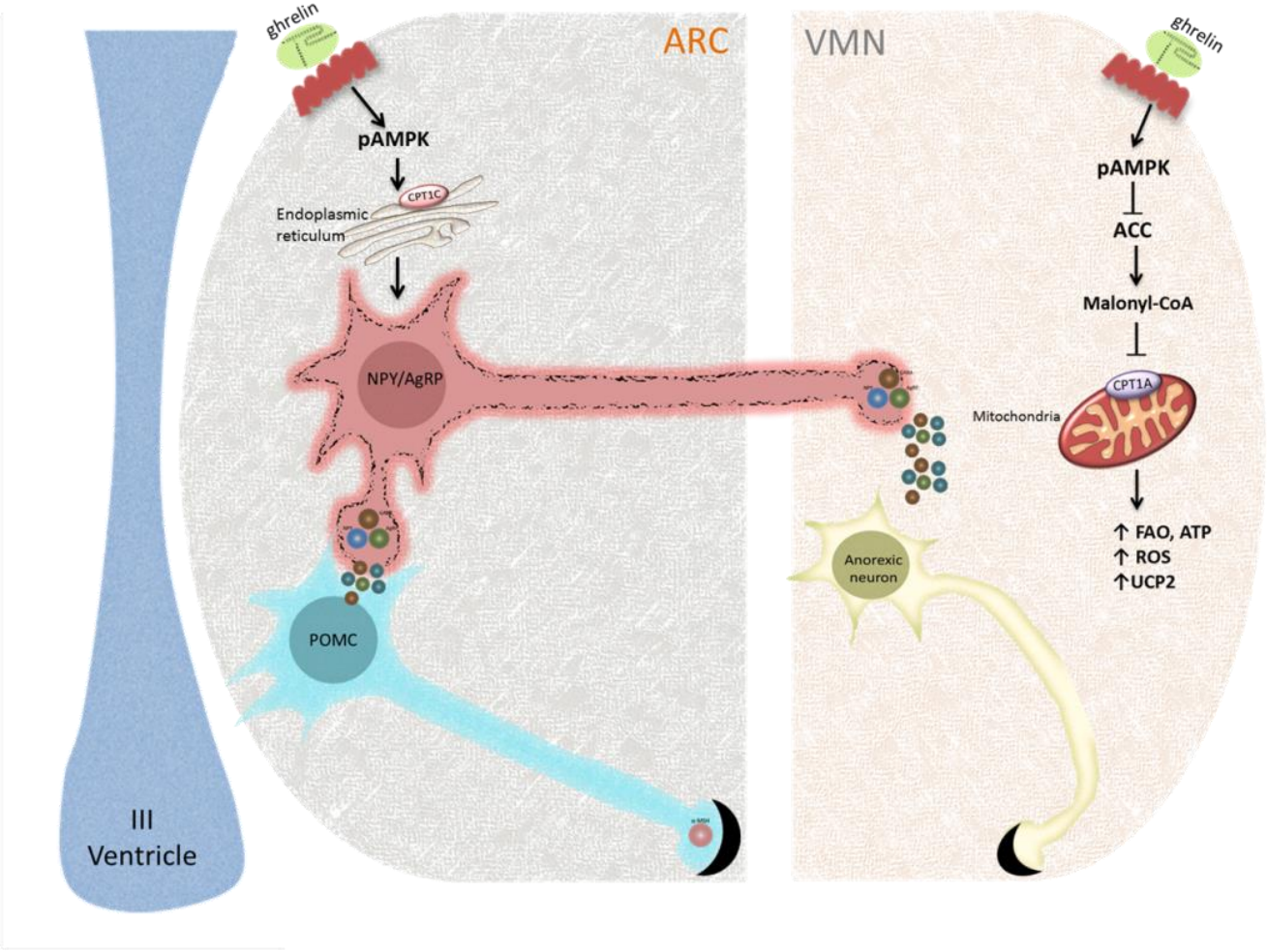


Figure 48. **Theoretical representation of the effect of ghrelin on ARC and VMN nuclei**. Ghrelin activates CPT1C in the ARC that triggers an increase in the NPY/AgRP firing neurons that connect ARC and VMN nuclei. In VMN, ghrelin activates fatty acid metabolism, increasing β-oxidation to obtain energy from fat.

- **THE ROLE OF UCP2 AND ROS IN GHRELIN SIGNALLING**

It is known that after ghrelin treatment, there is an increase in non-esterified fatty acid in the blood that provides a source of long chain fatty acids-CoA to the hypothalamus. Mitochondrial β-oxidation then activates and ROS is generated. Lower levels of ROS act as signaling molecules, but at high cellular levels ROS react with lipids, proteins, and DNA, causing cellular damage to organelles (Miao and St.Clair, 2009). For that reason, when ROS is generated there is also an increase in the mitochondrial protein UCP, and, UCP2 in the case of neurons, which are responsible for scavenging ROS and preventing an excess of oxidative stress (Korshunov et al., 1997).

The following has been demonstrated previously:

1) UCP2 is necessary for the firing of orexigenic neurons produced by the effect of ghrelin (Andrews et al., 2008).

2) Our results show that CPT1C KO mice have increased basal levels of UCP2 in the MBH when compared to WT mice.

3) A recently published metabolic study reveals that the GSH/GSSG ratio, the main scavenger of ROS, is decreased in the brains of CPT1C KO mice (Lee and Wolfgang, 2012). Although these authors did not explain the cause, their finding suggests that CPT1C KO mice may have increased basal ROS levels.

In this context, it is possible to hypothesize that CPT1C KO mice have increased levels of UCP2 as an attempt to scavenge ROS levels, and the altered levels of UCP2 could be the cause of the lack of response to ghrelin treatment. In fact, this effect could involve both AgRP/NPY and POMC neurons because a) some studies indicate that AgRP/NPY neurons need to rapidly and efficiently buffer ROS production to properly function (Hernandez-Fonseca et al., 2008) and b) it is known that increased ROS production stimulates POMC activity inducing satiety (Kuo et al., 2011). This indicates that ghrelin may not be functioning properly due of the high levels of ROS produced by the lack of CPT1C.

An alternative explanation to these observations is that the increased levels of UCP2 in CPT1C KO mice cause ROS to be excessively scavenged, lowering their levels in the MBH excessively. Since ROS acts as signaling molecule at low levels, it is possible that it may not be able to perform this function in the ghrelin pathway, and consequently the firing of neurons is prevented.

- **CPT1C AS A REGULATOR OF THE PALMITOYL-CoA POOL**

In order to explain the role of CPT1C in the pathway of ghrelin and in the increase of neuropeptides NPY and AgRP, I postulate that CPT1C could have an important role in mitochondria-associated membranes (MAMs), which are necessary for the correct expression of neuropeptides.

MAMs are ER and mitochondrial contacts that mediate the exchange of ions, lipids, and metabolites between these 2 compartments (Hayashi et al., 2009). It has been shown that some ER membrane proteins need to be palmitoylated to be targeted in MAMs (Lynes et al., 2011). Palmitoylation consists of the addition of a palmitate to a cysteine residue of the target protein through the formation of a thioester bond. The incorporation of palmitate into proteins is usually performed by a series of enzymes, generally named palmitoyl-acyl transferases (PATs), some of which are present in the brain. The incorporation of this lipid to proteins facilitates membrane targeting, resulting in changes in their subcellular localization or changes in their function.

A recent study by Claret and colleagues demonstrated that MAMs are necessary for correct neuropeptide production and processing. Their data showed that the lack of these contacts in POMC neurons generates ER stress, leptin resistance, defective POMC processing, reduced energy expenditure, hyperphagia, and obesity (Schneeberger et al., 2013).

Preliminary unpublished results from our group reveal that CPT1C could be located in MAMs as well as in the ER (Jacas and Casals, unpublished results). Tacking all these data together, it is possible to hypothesize that CPT1C is active in MAMs regulating the pool of the substrate (pamitoyl-CoA) available for palmitoylation reactions (fig. 49). If no CPT1C is present, certain necessary proteins cannot target MAMs, preventing the normal functions of these membranes, which could produce ER stress and the subsequent metabolic phenotype seen in CPT1C KO mice (no increased NPY/AgRP in response to ghrelin, reduced energy expenditure, hypophagia, and susceptibility to HFD).

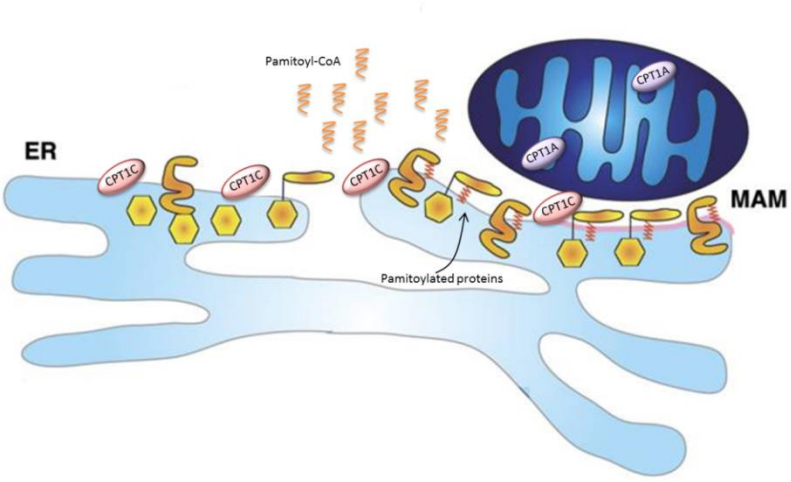


Figure 49. Model for palmitoylation dependent MAM enrichment of ER proteins. CPT1C in MAMs acting as a regulator of the palmitoyl-CoA poll. Palmitoylated proteins (indicated with the orange modifications) target to the MAM (pink domine) (Modified from Lynes et al., 2012).

2. CPT1C PRODUCES AN ACUTE INCREASE IN THE CERAMIDES NECESSARY FOR GHRELIN ACTIVITY

One of the most relevant contributions in the present work is the second finding. I have elucidated that CPT1C produces an acute increase in the ceramides necessary for the effect of ghrelin in the hypothalamus. This result is consistent with the previous work of Gao and colleagues, which showed that leptin impacts ceramide metabolism and downregulates it, in part through CPT1C action (Gao et al., 2011). Therefore, it is possible, on one hand, that CPT1C and decreased ceramides are involved in the leptin anorexigenic pathway, and that on the other hand CPT1C is involved in the pathway of ghrelin, increasing ceramides levels and triggering an orexigenic effect.

A number of potential mechanisms have been proposed to explain how CPT1C regulates ceramide levels. One is that CPT1C could modulate *de novo* synthesis of ceramide, as CPT1C is located in the ER, where the first enzyme of *de novo* synthesis, SPT, resides. It was hypothesized that CPT1C physically interacted with SPT to modulate its activity or that CPT1C acted as a shutter of palmitoyl-CoA to SPT. However, our group does not agree with this hypothesis since what we have observed is an acute and fast increase of ceramides, suggesting that ceramides are generated by acute catabolic pathways (SMases) rather than slow anabolic ones (*de novo* synthesis). Moreover, previous experiments by our group in primary hippocampal neurons of WT and CPT1C KO mice showed that the *de novo* synthesis of ceramides was not increased in CPT1C overexpressing neurons and were not reduced in CPT1C

KO neurons, indicating that CPT1C acts through some other metabolic pathway (Carrasco et al., 2012).

A handful of studies suggest that the activity of SMases and the *de novo* pathway may be regulated in a coordinated fashion by the same stimulus, resulting in the transient generation of distinct waves of ceramide increases that could serve to diversify the biological effects of ceramide. The transient increase of ceramides, within minutes, has been shown to be associated with SMases (Haimovitz-Friedman et al., 1994), while the accumulation of ceramides several hours after the stimulus is considered to be related more with *de novo* synthesis (Vit and Rosselli, 2003).

I postulate that the effect of CPT1C in the acute generation of ceramides occurs through 1 type of NSMase, perhaps NSMase 3, the neutral sphingomyelinase that is located in the ER and which produces fast peaks of ceramides. There is very little information about this neutral sphingomyelinase. To date, it is known that it is a ubiquitously expressed sphingomyelinase (mostly in heart and muscle tissue but also in the brain), has an ER localization, and has been associated with cellular stress responses (Krut et al., 2006). NSMase 2, which has been studied most, has been identified as a redox-sensitivity protein because it may be activated when the ratio of GSH/GSSG is low. Some other studies indicate that redox sensitivity could be a common property of neutral SMases (Martin et al., 2007).
These findings tie in to what has been previously discussed in the present work about the role of ROS. It is possible that CPT1C works coordinately with NSMase to sense oxidative stress.

3. CERAMIDE IMPACT ON THE LEVELS OF BSX EXPRESSION

The third discovery of this thesis is that CPT1C-mediated increases in ceramide results in an increase of the expression levels of BSX. This result is coherent with the previous work of Gao and colleagues, which showed that an overexpression of CPT1C in the ARC produces an increase in the mRNA levels of BSX. Furthermore, they demonstrated that myriocin, an inhibitor of the *de novo* synthesis of ceramides, decreases BSX levels. Additionally, treatment with leptin, the counterpart of ghrelin, produces a decrease in BSX levels (Gao et al., 2011). This is 1 more demonstration of the *yin-yang* effect of leptin and ghrelin. While on one hand leptin decreases BSX expression by diminishing ceramide levels, it has been demonstrated in the present study that ghrelin upregulates BSX expression by increasing ceramide levels.

BSX is a transcription factor that works coordinately with phosphorylated CREB and with FoxO1 to increase NPY and AgRP, respectively. In fact, ghrelin can induce NPY

and AgRP expression due to the increase of BSX, phosphorylated CREB, and FoxO1 (Nogueiras et al., 2008). However, the exact mechanisms by which these transcription factors are activated are not completely elucidated. The following is a proposed hypothesis to explain the ceramide-mediated increase of BSX in the ghrelin pathway based on both previous published data and our results.

Initially, ceramides were regarded as purely structural components, but this group of molecules is now recognized as a key signaling and regulatory element involved in differentiation, proliferation, apoptosis, and neuronal plasticity and able to regulate the function of various kinases, phosphatases, and deacetylases, among others (Hannun et al., 2008).
Recent findings have demonstrated that ceramides produce changes in acetylated levels of the transcription factor Sp1/3, favoring or preventing its binding to the promoter sequences of different genes and in turn activating or repressing them. Recently, the Ogretmen research group described the involvement of deacetylated Sp3 in inhibiting the human telomerase reverse transcriptase (hTERT) promoter with ceramide. Specifically, they showed that C18-ceramide produces an activation of HDAC1 that deacetyles Sp3, leading to a repression of the promoter (Wooten-Blanks et al., 2007). Nevertheless, chromatin immunoprecipitation assays of other groups have demonstrated that an increase of Sp3 binding to the promoter occurs when it is acetylated (Steiner et al., 2004). These results are contradictory; the regulatory mechanisms of Sps appear quite arduous and puzzling, and depend on the gene involved.
In the same line, recent evidence from another group implicated ceramide in Sp activity, in this case, human glycolipid transfer protein (hGLTP)-induced expression (Zou et al., 2010). The treatment of cells with ceramide C6:0 produces a significant increase in hGLTP expression, altering the binding affinity of Sp1 and Sp3 to the promoter by changing its acetylation grade. Ceramide produces a decreased binding of Sp1 and an enhanced binding of Sp3 to the promoter, which results in an increase of transcription levels. These results show hGLTP expression regulation via Sp1/3 through a complex mechanism that responds to elevated ceramide levels.

In another study, the BSX promoter was found to contain several putative binding sites of transcription factors, including the GC box for the transcriptional factors Sp1 and Sp3 (Park et al., 2007). However, no evidence has been found to link the increase of BSX with these transcription factors. In this context, the present study aimed to determine whether ceramide increases BSX expression by modulating the acetylation grade of SP1/3.

The results presented indicate that ceramide produced in the ER activates cytosolic HDACs, which migrate to the nucleus to deacetylase the transcription factors Sp1/3.

Changes in the acetylation of Sp1/3 could modulate their affinity for the promoter sequence, producing an increase or decrease in the mRNA levels of BSX.

In the present work, it is hypothesized that deacetylated Sp1 acts as an activator and acetylated Sp3 acts as an inhibitor. It is possible that acetylated Sp3 constitutively represses the BSX promoter, in which case an increase of ceramides would activate HDAC deacetylases and possibly trigger a decrease of acetylation levels in both Sp1 and Sp3. At the same time, deacetylated Sp3 would lose its binding affinity to the promoter, allowing deacetylated Sp1 to bind the promoter activating the transcription of BSX (Fig. 50). Consequently, when cells were treated with the HDAC inhibitor TSA and the acetylation levels of Sp1 and Sp3 increased, BSX mRNA levels did not increase. In this case, Sp3 was acetylated and therefore coupled to the promoter inhibiting it. Additionally, when cells were treated with mithramycin, an inhibitor of Sps binding to GC sequences, the constitutive binding of the repressor Sp3 was prevented and an increase of BSX mRNA levels was observed.

It must be taken into account that another more soluble sphingolipid derivated from ceramide, the sphingosine-1-phosphate (S1p), has also been shown to regulate HDAC activity. The insolubility of ceramide suggests that once generated, ceramide is likely to exert its effects at the place of its synthesis until metabolized to other sphingolipids. Consequently, the increase in ceramide concentration during ghrelin response is compartmentalized and might affect targets only in the ER. The molecule producing the increase of BSX could be S1P, which can move between different compartments and enter into the nucleus. Some findings have demonstrated that S1P inhibits the activity of HDAC1 and HDAC2 in the histones (Riccio, 2010), resulting in an increase of histone acetylation and transcriptional activation. This sphingolipid is the first to be described as having an epigenetic function. Thus, one cannot rule out that the increase in ceramide induced by ghrelin is ultimately transformed into S1P, which would inhibit nuclear HDACs and produce an increase in BSX expression.

Great effort is needed to elucidate whether ceramide or S1P triggers BSX expression. In the big picture, it is possible to say that CPT1C is necessary for the correct expression of BSX. In fact, CPT1C KO animals and BSX-deficient mice share some similarities. Both have hypophagia, reduced locomotor activity, growth retardation, and higher susceptibility to high fat diets. Furthermore, both are unable to increase the orexigenic neuropeptides in response to ghrelin (in CPT1C KO mice) or in response to fasting (in BSX-deficient mice) (Sakkou et al., ; Nogueiras et al.; 2008). These data combined help to corroborate the present hypothesis and demonstrate that CPT1C and BSX are closely related.

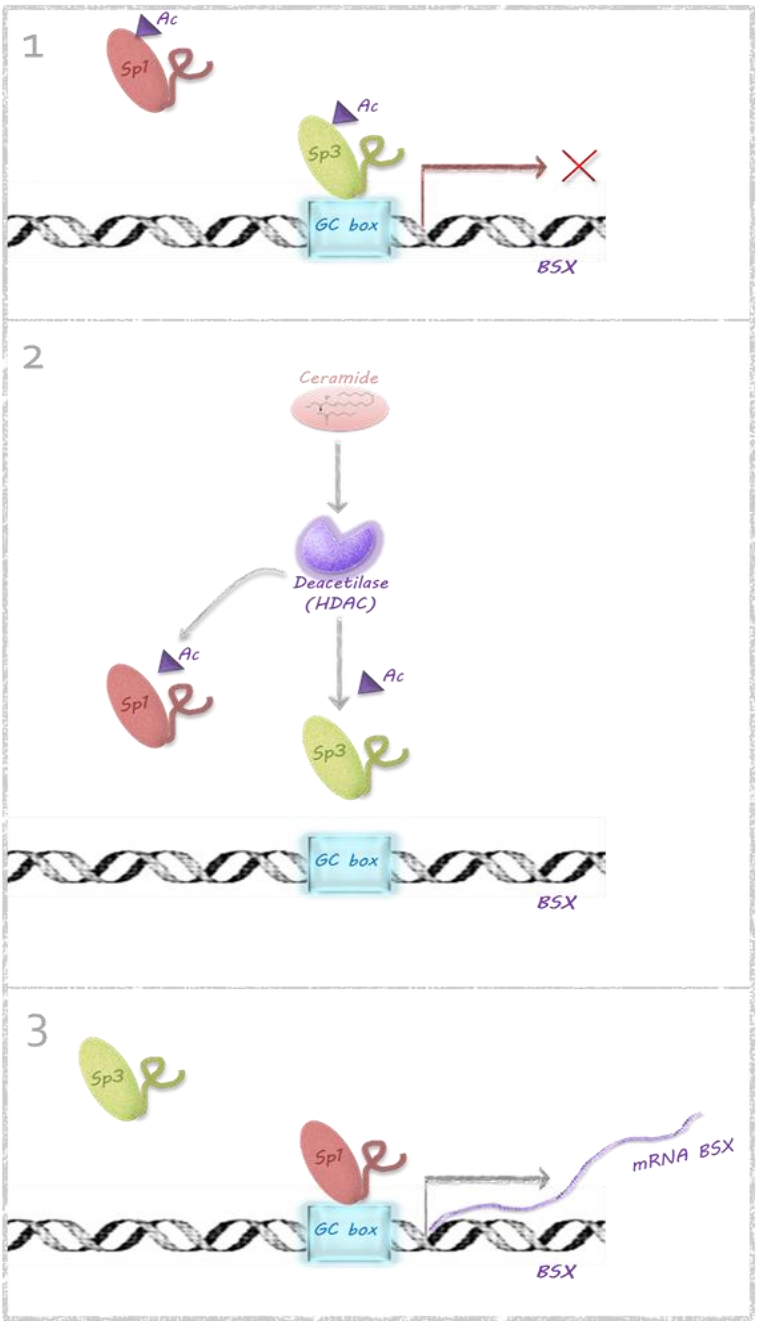


Figure 50. Hypothetical mechanism in BSX increase produced by ceramide. 1) Acetylated Sp3 is a constitutive repressor of BSX. 2) Different stimuli such as ghrelin could activate the increase in ceramides, thus producing the activation of HDACs. HDACs deacetylate Sp1 and Sp3. 3) On one hand, deacetylated Sp1 is able to bind the sequence promoter of BSX, increasing its expression. On the other hand, deacetylated Sp3 loses its ability to bind the BSX promoter.

4. CPT1C AND LIFESPAN

Based on our findings, CPT1C KO mice have a shorter lifespan than WT mice.
The extended hypothesis to explain aging is the mitochondrial theory, also known as the free-radical theory (Harman, 1956). Some studies report that young rats have small mitochondria whereas older ones have the same number of mitochondria but they are larger (Sastre et al., 1998). These larger mitochondria are not bio-energetically efficient as they have lost their functionality in terms of ATP production, increasing the levels of Ca2+ entering into the mitochondria and thus lowering ROS (Nicholls, 2004).

A metabolomic analysis comparing CPT1C KO and WT mouse brains indicates that CPT1C does not play a significant role in fatty acid oxidation, unlike other CPT1 isoforms. However, it could play an alternative role in neuronal oxidative metabolism; as previously explained, the authors found changes involving a 2-fold increase in oxidized glutathione (GSSG) in CPT1C KO mice, indicating a loss of antioxidant capacity (Lee and Wolfgang, 2012). Numerous studies have shown that GSH/GSSG is decreased in aged cells (Chen et al., 1989; Lantomasi et al., 1993; Oubidar et al., 1996; Suh et al., 2005). Bearing these data in mind, it is possible that CPT1C KO mice have increased ROS levels that oxidize proteins, lipids, and DNA, producing an accumulation of oxidative stress and generating damage and aging (Gupta et al., 1991; Hamilton et al., 2001).

Aging, in actual fact, consists of a reduced capacity to cope with the damaging effects of strong, excessive stressors.
A recent study on the role of CPT1C in cancer cells in response to metabolic stress, which produces oxidative stress and ROS, showed that CPT1C protects cells against hypoxia and hypoglycemia. Specifically, the authors postulated that CPT1C acts as an alternative supplier of energy increasing ATP from FAO, for example, in cases of limited glucose. The study suggested that fatty acids freed by CPT1C-driven events might be used as a fuel source to drive metabolic transformation and tumor growth (Zaugg et al., 2011). CPT1C could play the same role in hypothalamic neurons by promoting survival in situations of energy restriction (Fontana et al., 2010). During energy restriction, there is a relative metabolic shift from glucose to the use of other substrates, such as fatty acids in many types of tissue. This phenomenon has also been described in MBH (Taib et al., 2013).

On one hand, energy restriction produces an increase in resistance in neurons and an increase in lifespan. On the other hand, metabolic stress activates CPT1C in some way, triggering resistance to metabolic stress and thus producing an increased

survival rate. Thus, it is possible to imagine that CPT1C in neurons can be activated in a stress-induced manner, for example in caloric restriction, to produce resistance and neuron survival. Neurons lacking CPT1C are unable to fight against stress in a proper way, which could produce a decrease in longevity.

Taking into account that ceramides are considered proapoptotic lipids (Huang et al., 2011), some data do not corroborate with this overview.
Our CPT1C KO mice have decreased basal levels of ceramides in the different parts of the brain but they showed reduced lifespan. Furthermore, other authors have reported that in kidney, liver, and brain tissues, sphingolipid catabolic enzyme activities as well as the level of SMase and CDase are greater than those of anabolic ones, SMS, and CS in aged rats when compared with young rats. Specifically, aSMase and nSMase activity in the brain significantly increases during aging, demonstrating that aging is accompanied by an increase in SM turnover and an increase in ceramides (Sacket et al., 2009).
It would be useful to compare the ratio of S1P over ceramide in hypothalamus of WT and CPT1C KO mice because ceramide is antiproliferative while S1P favors survival. An additional interesting experiment would be to inject ICV S1P into CPT1C KO mice and determine whether longevity is increased.

In summary, the present study hypothesizes that CPT1C acts as a sensor of metabolically stressful situations, whereby CPT1C produces an acute increase in the synthesis of ceramides, producing changes in neurons that allow them to cope with the stress stimulus.
In the ghrelin pathway in the hypothalamus, CPT1C is responsible for the increase in transcription factor BSX mediated by ceramides, which triggers NPY and AgRP expression and the increase of food intake. A failure to combat stress in the neurons of CPT1C KO mice appears to be the cause of their decreased lifespan.

In this complex landscape, several questions remain unanswered and a number of controversies have yet to be clarified. For this reason, it is essential that research in this field continue in the future.

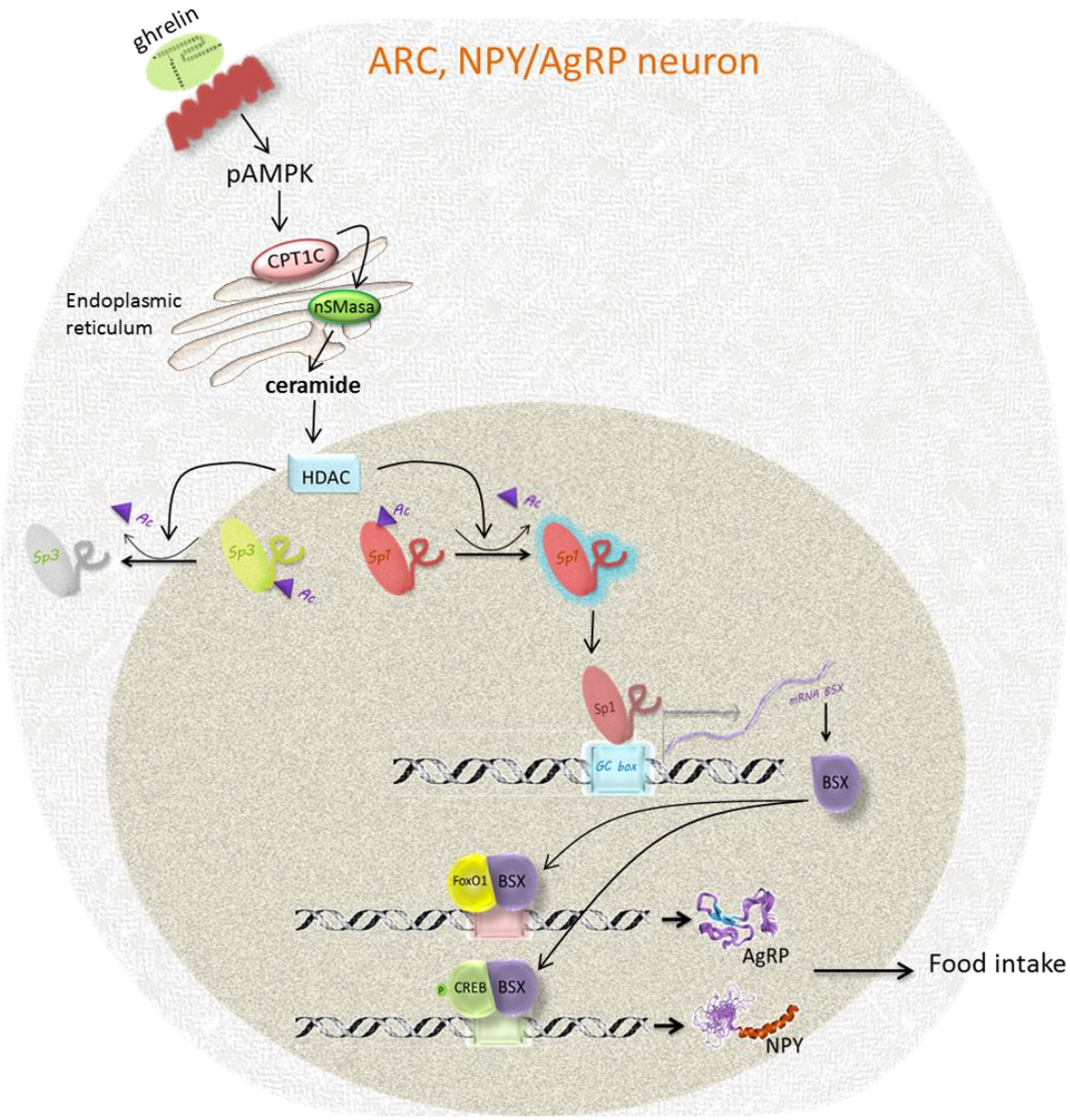


Figure 51. The involvement of CPT1C and ceramides in the ghrelin pathway in the hypothalamus. In MBH ghrelin activates the increase of ceramides through CPT1C, leading to an activation of HDACs that deacetylates the transcription factor Sp1, which binds it to the BSX promoter. BSX dimerizes with pCREB and FoxO1 to increase the orexigenic neuropeptides (NPY and AgRP) and food intake.

CHAPTER 6

CONCLUSIONS

1. CPT1C is involved in the hypothalamic pathway of ghrelin, ultimately affecting the expression of the neuropeptides NPY and AgRP and food intake.

2. Intracerebroventricular injection of ghrelin produces an acute and transient increase of ceramide levels in the MBH., necessary to induce an increase of NPY,AgRP and food intake.

3. CPT1C is required for the stimulatory effect of ghrelin on the ceramide content in the hypothalamus.

4. Intracerebroventricular ceramide injection in WT mice is not able to induce the expression of NPY and AgRP or food intake, indicating that the increase in ceramide in the MBH is necessary but not sufficient to induce an orexigenic effect.

5. CPT1C deficiency produces an increase in the basal activity of AMPK and UCP2 levels, suggesting that the route AMPK/CPT1A/UCP2 is permanently active.

6. Over-expression of CPT1C in cells suggests that CPT1C is upstream BSX in the ghrelin signaling pathway.

7. The data from mice and cells experiments indicate that ceramides are involved in the expression of the transcription factor BSX, probably by regulating Sp1/3 acetylation level.

8. Results suggest that ceramides produce an increase in BSX expression due to a decrease of the acetylated levels of Sps.

9. Data from the experiment of mithramycin indicate that Sp1 or Sp3 is normally acting as a constitutive inhibitor and if it is not able to bind the promoter sequence, then BSX is able to increase its expression.

10. The conclusion from the longevity experiment is that the lack of CPT1C produces a significant reduction in lifespan.

RESUMEN EN CASTELLANO

Datos recientes sugieren que la ghrelina ejerce su acción a través de la regulación de la vía del AMP proteína quinasa (AMPK), lo que lleva a una disminución en los niveles de malonil-CoA y posterior desinhibición de la carnitina palmitoiltransferasa 1A (CPT1A), lo que aumenta la oxidación mitocondrial de ácidos grasos y en última instancia aumenta la expresión de los neuropéptidos orexigénicos AgRP y NPY. Sin embargo, no está claro si la CPT1C, isoforma específica del cerebro, que se encuentra en el retículo endoplásmico de las neuronas, puede desempeñar un papel en esta acción. En esta tesis, demostramos que la acción orexigéncia de la ghrelina está totalmente mitigada en ratones CPT1C knockout (KO), a pesar de tener la vía de señalización canónica de la ghrelina activa. También demostramos que la ghrelina provoca una marcada regulación al alza de la ceramida hipotalámica C18:0 mediada por CPT1C. Además, la inhibición central de la síntesis de ceramida con miriocina bloquea la acción orexigénica de la ghrelina y normaliza los niveles de AgRP y NPY, así como los factores de transcripción responsables del aumento de los neuropéptidos (BSX, pCREB y FoxO1). Hicimos tratamiento central con ceramida en ratones CPT1C KO y comprobamos que el fenotipo se revertia completamente. Una vez determinado que la ghrelina provoca un aumento de ceramidas mediado por la CPT1C, quisimos saber donde actuaban las ceramidas y comprobamos que era aumentando los nivels de BSX, el factor de transcripción que comparten tanto AgRP como NPY. En conjunto, estos datos indican que, además de los mecanismos anteriormente reportados, la ghrelina también induce la ingesta de alimentos a través de la regulación de CPT1C y el metabolismo de las ceramidas en el hipotálamo, un hallazgo de potencial importancia para la comprensión y el tratamiento de enfermedades metabólicas.
Finalmente también hemos generado un vínculo entre CPT1C y longevidad. Un experimento nos demuestra claramente que los animales CPT1C KO tienen menos supervivencia que los animales salvajes. Dejamos así, otra línia abierta con respecto a la CPT1C para investigar con detalle en un futuro.

1. INTRODUCCIÓN:

La ghrelina es una hormona producida por el estómago que induce la ingesta de alimentos a través del receptor secretagogo de la hormona del crecimiento 1a (GHSR1a) en el hipotálamo. La ghrelina y las proteínas que están implicadas en la vía de señalización corriente abajo son objetivos claros para el tratamiento de la obesidad y trastornos de la ingesta de alimentos. Recientemente, se ha invertido mucho esfuerzo en estudiar el mecanismo molecular por el cual la ghrelina aumenta la expresión de los neuropéptidos proteína r-aguti (AgRP) y neuropéptido Y (NPY) en el núcleo arcuado (ARC) del hipotálamo. Se ha descrito que la unión a su receptor induce la liberación de calcio intracelular, que activa la calcio calmodulina quinasa quinasa 2 (CaMKK2) hipotalámica y la fosforilación del sensor de energía proteína quinasa

activada por AMP (AMPK). También se ha descrito que la ghrelina activa específicamente la vía hipotálamica Sirtuin1/p53 que es esencial para la fosforilación de AMPK. Uno de los principales efectos de la activación de AMPK en el hipotálamo es la modulación del metabolismo de los ácidos grasos. Cuando se activa, AMPK (pAMPK) fosforila e inactiva a la acetil-CoA carboxilasa (pACC), causando una disminución en los niveles de malonil-CoA y generando así la desinhibición de la carnitina palmitoiltransferasa 1 (CPT1). El resultado general de este efecto es el aumento de la oxidación de ácidos grasos y la acumulación de especies reactivas de oxígeno (ROS), que se tamponan principalmente por proteína desacoplante 2 (UCP2). En última instancia, todos estos cambios metabólicos, activan eventos transcripcional provocando un aumento de los niveles o la activación de factores de transcripción: como la isoforma fosforilada de la proteína de respuesta a cAMP (pCREB), la proteína Fox O1 (FoxO1) y su isoforma fosforilada (pFoxO1), y el factor de transcripción homeobox específico de cerebro (BSX). Estos son responsables, en parte, del aumento de los neuropéptidos orexigénico AgRP y NPY. Sin embargo, el mecanismo molecular exacto por el cual los cambios en el metabolismo de los ácidos grasos modulan AgRP y la expresión de NPY no se conoce totamente.

La CPT1C es una isoforma de la CPT1 específica del cerebro que, contrariamente a la isoforma mitocondrial (CPT1A), se localiza en el retículo endoplásmico (RE) de las neuronas. CPT1C tiene una actividad CPT1 muy baja, pero se ha demostrado que el inhibidor fisiológico de enzimas CPT1, el malonil-CoA es capaz de inhibirlo. En consecuencia, CPT1C ha sido propuesta en anteriores artículos como un sensor de los niveles de malonil-CoA en las neuronas del hipotálamo. A nivel fisiológico, se sabe que CPT1C en el hipotálamo está implicada en el control de la homeostasis de la energía porque ratones CPT1C KO muestran una reducción de la ingesta de alimentos así como un metabolismo periférico alterado. Sin embargo, el mecanismo molecular por el cual CPT1C en el hipotálamo regula la ingesta de alimentos sigue sin saberse.

La hipotesis de que la CPT1C podría estar implicada en la vía de señalización de la ghrelina a nivel hipotalámica modulando los nivels de ceramidas deriva de que recientemente hemos demostrado que la sobreexpresión de CPT1C en ARC bloquea los efectos anorexígenos de leptina, la hormona contraria a la ghrelina, a través de un mecanismo que implica un aumento de los niveles de ceramida. Aquí, demostramos que la CPT1C media un aumento a corto plazo en los niveles de ceramida en el hipotálamo en respuesta a la ghrelina y, en particular , que este efecto es crítico para los efectos de la ghrelina sobre la expresión de AgRP y NPY, así como en la alimentación.

También demostramos que estos efectos en los neuropéptidos son debidos a que las ceramidas aumentan el factor de transcripción que es común tanto en AgRP como en NPY, el BSX.

2. OBJETIVOS

OBJETIVO GENERAL:

Estudiar la función fisiológica de CPT1C en el control de la ingesta de alimentos.

OBJETIVOS ESPECÍFICOS:

1. Determinar la implicación de CPT1C en la vía de señalización de la ghrelina a nivel hipotalámico.

2. Determinar la implicación de la ceramida en la vía de señalización de la ghrelina hipotalámica.

3. Estudiar el papel de la ceramida en el aumento del factor de transcripción homeobox específico del cerebro (BSX) a través de la activación de las deacetilasas en la línea celular GT17 hipotalámica.

4. Identificar que efecto genera la falta de CPT1C en la longevidad.

3. RESULTADOS

3.1. La administración de ghrelina no aumentó ni la ingesta de alimentos ni la expresión de los neuropéptidos orexigénicos en ratones CPT1C KO.

Para establecer si CPT1C era parte de la vía de señalización de la ghrelina en el hipotálamo, se analizó el efecto de la ghrelina en ratones CPT1C KO. Inyectamos ghrelina IP y analizamos la ingesta de alimentos y el comportamiento al ir en busca de ellos. Encontramos que la inyección de ghrelina en los ratones WT aumento la ingesta 2 veces y redujo en gran medida el tiempo para empezar a comer. Ambos efectos fueron completamente bloqueados en los ratones CPT1C KO (Fig. 1A y B). Luego, se repitió el experimento de inyección ICV de ghrelina y otra vez encontramos que los ratones KO para CPT1C no respondieron al tratamiento (Fig. 1C). A continuación, analizamos la expresión de los neuropéptidos orexigénicos AgRP y NPY mediante hibridación in situ. El aumento inducido por la ghrelina en los niveles de AgRP y NPY presentes en los ratones WT se bloqueó completamente en ratones CPT1c KO (Fig. 1D y E), y esto se correlacionó con la falta de efecto orexigénico de la ghrelina en estos ratones. Estos resultados indican que la CPT1C está implicada en el efecto orexigénico ghrelina.

3.2-La vía de señalización canónica de la ghrelina se encuentra alterada en los ratones CPT1C KO.

A continuación, analizamos la vía de señalización canónica de la ghrelina en el hipotálamo de los ratones CPT1C KO. Nuestros datos muestran que el tratamiento central de la ghrelina indujo un efecto estimulante en los niveles de pAMPK, pACC, UCP2, y los factores de transcripción FoxO1, pCREB y BSX en ratones WT. Esos efectos se vieron bloqueados en ratones CPT1C KO (Fig. 2A- G). Los niveles basales de pAMPK y pACC, se vieron incrementados significativamente en los ratones CPT1C KO en comparación con ratones WT, lo que sugiere que la vía del AMPK está constitutivamente activa en el hipotálamo de ratones CPT1c KO (Fig. 2A - C). Es importante destacar que, de acuerdo con los niveles alterados de pAMPK y pACC, la expresión de UCP2 y los niveles de los factores de transcripción FoxO1 y pCREB también se incrementaron en el hipotálamo de ratones CPT1c KO (Fig. 2D - G). Por lo tanto, estos datos sugieren que la vía de señalización canónica de la ghrelina está basalmente activa en ratones CPT1C KO. La falta CPT1C provoca el bloqueo de la inducción por parte de la ghrelina de los neuropéptidos orexigénicos y de la ingesta de alimentos.

3.3 La administración central de ghrelina aumenta los niveles de ceramida en el hipotálamo de ratones WT pero de ratones CPT1C KO.

Nuestro grupo ha identificado a la CPT1C como un regulador clave de los niveles de ceramida en las neuronas (Carrasco et al., 2012). Para investigar si la ghrelina tenía algún impacto sobre la concentración de ceramidas hipotalámicas, se midieron los niveles de ceramida C18:0, la ceramida más abundante en el cerebro y neuronas (Ben-David and Futerman, 2010), en ratones WT a diferentes tiempos después de la administración de ghrelina. Nuestros datos muestran que la ghrelina promovió una acción estimulante y transitoria en los niveles de la ceramida C18:0 en los nucleos del MBH, con un efecto máximo detectado a los 30 y 60 min después de la inyección de ghrelina (Fig. 3A). A continuación, se realizó una curva de dosis-respuesta y se encontró que los niveles de ceramida en MBH en 30 min después de la administración de ghrelina aumentaron progresivamente con la dosis de ghrelina, con el aumento estadísticamente significativo a la dosis de 5 mg (Fig. 3B). A continuación, analizamos lo que ocurria en los ratones CPT1C KO. Al contrario a lo que sucedia en los ratones WT, 5 mg de ghrelina no indujo ningún efecto en los niveles de la ceramida C18:0 a los 30 minutos después de su administración (Fig. 3C), lo que nos indica que la CPT1C es necesaria para el efecto estimulador de la ghrelina en el contenido de ceramidas en el MBH .

3.4 La inhibición de la síntesis de ceramidas hipotalámicas bloquea el efecto orexigénico de la ghrelina.

Para poder investigar la existencia de una relación mecanicista entre el efecto de la orexigénico de la ghrelina y la activación de la síntesis de la ceramida, se investigó los efectos de 4 µg de miriocina, un inhibidor específico de la síntesis *de novo* de la ceramidas (Hanada et al., 2000), sobre la acción de ghrelina. La dosis seleccionada de miriocina no indujo ningún efecto anorexígeno de por sí (Fig. 4A) ni enfermedad o malestar, ni inflamación del hipotálamo, ni estrés del RE (Fig. 4B y C). Aunque esta dosis de miriocina era subeficaz inyectada sola, nuestros datos muestran que la inyección ICV de miriocina 1 h antes de la administración de ghrelina disminuye el efecto orexigénico de la ghrelina (Fig. 4A), así como su efecto estimulante sobre los factores de transcripción (FoxO1, pCREB, BSX) y sobre los neuropéptidos (AgRP y NPY) (Fig. 4D - I). Estos resultados indican que la síntesis de ceramidas también es un componente necesario de la vía de señalización hipotalámica de la ghrelina .

3.5 La administración de ceramidas aumenta la ingesta de alimentos en ratones CPT1c KO.

Teniendo en cuenta que se ha involucarado a la CPT1C en la síntesis de ceramidas, nuestro próximo objetivo fue inyectar ceramidas y analizar si se revertia el fenotipo de los ratones CPT1C KO. Utilizamos la ceramida C6:0, una ceramida con capacidad de penetración celular que se convierte en ceramidas de cadena larga en el interior de la célula (Mitoma et al., 1998). Se utilizó la dosis submáxima de 2,5 µg, que se había utilizado previamente para bloquear los efectos de saciedad de la leptina, pero que carece de efecto orexigénico *per se* en los animales WT (Gao et al., 2011). La ceramida, se inyectó en el inicio del ciclo de luz cuando los animales están saciados, produjo un aumento de la ingesta de alimentos y la expresión de AgRP y NPY en ratones CPT1C KO pero, como era de esperar, no tuvo ningún efecto en ratones WT (Fig. 5). Estos resultados indican que la ceramida es capaz de recuperar los patrones de alimentación cuando la vía de señalización canónica ghrelina (pAMPK/pACC/CPT1A/UCP2) está activa previamente, como ocurre en los ratones CPT1C KO (fig. 2). Por el contrario, en los ratones WT, que no tienen la vía canónica ghrelina activa basalmente, la ceramida sola no fue capaz de inducir la expresión de neuropéptidos orexigénicos ni la ingesta de alimentos. Estos resultados apuntant a que la ghrelina actua por dos vías de señalización paralelas, con la participación de CPT1C y ceramida en una de las ramas (Fig.6). Proponemos que la ghrelina debe activar dos vías paralelas, la vía mitocondrial (con la activación de CPT1A y la oxidación de ácidos grasos) y la vía del RE (con la activación de CPT1C y la síntesis de ceramidas), para que su efecto orxigénico sea eficaz.

3.6 La administración de ceramidas aumenta los nivels del factor de transcripción BSX en ratones WT.

En los ratones WT, en los que no está basalmente activada la vía canónica de la ghrelina, las ceramidas por sí solas no fueron capaces de inducir ningún efecto en la ingesta de alimentos. Sin embargo es obvio que la ceramida tiene un papel en la vía de señalización de la ghrelina ya que la inhibición de la síntesis de ceramida bloqueó el aumento de AgRP y NPY. Así que lo próximo fue analizar si la inyección de ceramida producia algún efecto en los niveles de FoxO1, pCREB o BSX, los factores de transcripción responsables del aumento de los neuropéptidos orexigénicos. Se utilizó 2,5 µg de Ceramida C6:0 y se inyectó en el inicio del ciclo de luz. Después de 2 horas se sacrificaron los ratones. La inyección de ceramida no tuvo efecto en los niveles protéicos de FoxO1 o pCREB, pero aumentó en un 50 % los niveles de mRNA de BSX. Estos resultados indican que las ceramidas están involucradas en la expresión del factor de transcripción BSX.

3.7 La sobreexpresión de la CPT1C aumenta la expresión de BSX en células GT1-7.

Para determinar si CPT1C *per se* tenia algún efecto sobre los niveles de mRNA de BSX, CPT1C se sobreexpresó en la línea celular GT1-7 utilizando vectores (lentivirus). Eliminamos el suero de las células 2 días antes, por lo que las células dejaron de crecer y se diferenciaron. Se utilizó como control el vector pWPI y otro vector derivado de pWPI pero con la secuencia de la CPT1C introducida, pCPT1C. El porcentaje de células que expresan EGFP después de 6 días de transducción era 80-90 % (Fig. 13A). La sobreexpresión de la CPT1C fue confirmada por PCR en tiempo real (B). Los niveles de mRNA de la CPT1C se incrementaron unas 30 veces (Fig. 13B). A continuación se analizaron los niveles de BSX para determinar si un aumento de CPT1C era capaz de modular los niveles de BSX. Encontramos que la expresión BSX tenía una tendencia a aumentar en las células que sobreexpresaban la CPT1C. Estos datos sugieren que CPT1C está por encima de BSX en la vía de señalización de la ghrelina y que la presencia o no de esta puede influenciar los nivels de este factor de transcripción.

3.8 La inhibición de HDACs bloquea el aumento de los niveles de mRNA de BSX mediado por ceramidas.

La acetilación de Sp1/3 puede regular su unión a secuencias promotoras de genes y también se sabe que las ceramidas pueden alterar el estado de acetilación los Sps. Esto nos lleva a la siguiente hipótesis: las ceramidas pueden aumentar la expresión BSX modulando el estado de acetilación de Sp1/3. Primero probamos los efectos de la ceramida C6:0 en los niveles de expresión Sp3. Queríamos analizar si las ceramidas eran capaces de aumentar la expresión de la proteína de Sp3 además de desacetilarla. Se observó un aumento significativo de 2 veces en las células tratadas con ceramida C6:0 (Fig. 14A, B). Ese resultado indica que las ceramidas fueron capaces de aumentar los niveles de proteína de Sp3. En el siguiente experimento tratamos las células con

tricostatina A (TSA), un inhibidor de las HDACs, 30 minutos antes de añadir la C6:0. Se utilizó una dosis de 100 ng/ml. La inhibición de la desacetilación de Sps producida por la TSA bloqueó el incremento de los niveles de mRNA de BSX producidos por las ceramidas (Fig. 14C). Los resultados sugieren que las ceramidas producen un aumento en la expresión BSX debido a una disminución de los niveles de acetilación de los Sps.

3.9 Papel de los Sps en la regulación de BSX por parte de las ceramidas.

Habíamos visto en experimentos anteriores que si inhibimos HDAC el efecto de las ceramidas en la expresión de BSX se veía impedido. Las HDACs actúan deacetilando a Sp1 pero también a Sp3. El siguiente paso fue analizar si Sp1 o Sp3 estaban actuando como activador o inhibidor. Normalmente, aunque existen excepciones, Sp1 actúa como activador y Sp3 como inhibidor. Utilizamos mitramicina, un fármaco que se une a cajas CG en el ADN y bloquea la unión de los factores de transcripción Sps (Blume et al., 1991). Se trató a las células con 500 nM de mitramicina, 30 minutos antes de la administración de ceramida C6:0. Como se muestra en la figura 15, debido a la incapacidad de Sp1/3 para unirse a las cajas CG colocadas en secuencias promotoras, los niveles de mRNA de BSX aumentan aún más que con el tratamiento con ceramida (Fig. 15A). Este resultado mostró que Sp1 o Sp3 normalmente actúa como un inhibidor constitutivo y si no es capaz de unirse a la secuencia del promotor, entonces BSX es capaz de aumentar su expresión. En realidad, con el tratamiento de mithramicina, estamos bloqueando factores de transcripción que actúan a través de cajas CG, como Sp1 y Sp3. Por lo tanto, tenemos que trabajar mucho más para poder discernir entre estos dos factores de transcripción y su papel en la expresión de BSX. De todos modos, es muy común que ambos factores, trabajen conjuntamente modulanto diferentes genes de una manera sinérgica o antagónica.

3.10 CPT1C y longevidad.

Los datos de supervivencia se recogieron en ratones WT (10 hembras) y ratones CPT1C KO (10 hembras) que fueron alojados en grupos de 10 a lo largo de su vida. Los animales que parecían estar cercana de la muerte o con tumores fueron sacrificados, y la fecha de la eutanasia se consideró la fecha de la muerte. La longevidad se redujo significativamente en los ratones CPT1C KO ($p = 0{,}0005$). El porcentaje de supervivencia se resume en la figura 16A y la los datos estadísticos también se resumen en la figura 16B. La vida media se redujo en un 53,3% en los ratones CPT1C KO. La distribución de la supervivencia de WT y KO CPT1C se representa en la figura 16C. A partir de estos datos se concluyó que hubo una reducción significativa de longevidad en ratones con carencia de CPT1C.

4. DISCUSIÓN

Los resultados obtenidos en este estudio conjuntamente con los datos que hay publicados indican que la CPT1C tiene una función completamente diferente de las otras dos isoformas conocidas (CPT1A y CPT1B). Esta hipótesis se basa principalmente por dos hechos:

1) La CPT1C no se encuentra en la mitocondria, como las otras dos isoformas, sino que se localiza en le membrana del retículo endoplásmico.

2) Aunque la CPT1C contiene todos los residuos clave para la actividad catalítica CPT1 y potencialmente podría catalizar la misma reacción con características cinética similar, los resultados publicados por nuestro grupo (Sierra et al. 2008) muestran que la CPT1C no está involucrada en la oxidación de palmitato, por lo menos, en células PC12 o 293.

En primer lugar discutiré el papel de la CPT1C en la vía hipotalámica de la ghrelina, seguido de cómo pienso que la CPT1C produce un aumento de las ceramidas debido a la actividad de la ghrelina, cómo estas ceramidas impactan en el factor de transcripción BSX y finalmente especularé sobre la CPT1C y su papel en la longevidad.

4.1. CPT1C EN LA RUTA HIPOTALÁMICA DE LA GHRELINA

Nuestro primer hallazgo fue descubrir que la CPT1C estaba implicada en la vía de señalización de la ghrelina. La supresión de la CPT1C bloqueó el efecto de la ghrelina en el hipotálamo y no se produjo un aumento de neuropéptidos orexigénicos ni de ingesta de alimentos. Este resultado concuerda con algunas publicaciones anteriores que ya apuntaban a que la CPT1A, la otra isoforma cerebral, estaba implicada en la regulación central de la ingesta de alimentos y ghrelina (Obici et al., 2003; López et al., 2010).

Por lo tanto, pienso que CPT1A y CPT1C podrían estar trabajando de forma coordinada para permitir una función adecuada de la ghrelina en el hipotálamo.

Dos caminos paralelos: Recientemente se ha hecho un gran esfuerzo para distinguir entre el papel de la ghrelina en los diferentes núcleos del hipotálamo. El receptor de la ghrelina se encuentra principalmente en el VMN y en el ARC. CPT1A se ha relacionado con los efectos de la ghrelina en VMN y CPT1C con los efectos en el ARC. Estos dos núcleos están conectados pero tienen diferentes receptores y tipos de neuronas, sin embargo, ambos tienen receptores de ghrelina y en ambos casos el efecto de la ghrelina se relaciona con una activación del sensor de energía de AMPK. Todo apunta

que la ghrelina ejerce efectos diferenciados, pero muy probablemente sinérgicos, en VMN por parte de la CPT1A y en el ARC por la CPT1C.

El papel de UCP2 y ROS en la ruta de señalización de la ghrelina: Teniendo en cuenta que: 1) UCP2 es necesaria para la activación de las neuronas orexigénicas en respuesta a la ghrelina (Andrews et al. 2008). 2) Nuestros resultados muestran que los ratones CPT1C KO tienen aumentados los niveles basales de UCP2 en el MBH en comparación con los ratones WT. 3) Un estudio metabólico reciente revela que los cerebros de los ratones CPT1C KO tienen la relación GSH/GSSG disminuida. No explican la causa, pero sugieren que los ratones CPT1C KO podrían tener niveles de basales ROS elevados (Lee y Wolfgang, 2012).

Podría ser que los ratones CPT1C KO tengan aumentados los niveles de UCP2 en un intento de disminuir los niveles de ROS, y en consecuencia, los niveles alterados de UCP2 podrían ser la causa de la falta de respuesta a la ghrelina. Otra explicación alternativa a esta observación seria que los niveles aumentados de UCP2 en ratones CPT1C KO estuvieran produciendo una disminución excesiva de ROS en el MBH. Dado que, a bajos niveles de ROS actúa como moléculas de señalización, es posible que no fueran capaces de producir sus efectos como moléculas de señalización en la vía de la ghrelina.

CPT1C como regulador de la disponibilidad de pamitoil-CoA: A fin de explicar el papel de CPT1C en la vía de la ghrelina y el aumento de NPY y AgRP, otra hipótesis sería que CPT1C podría tener un papel importante en las membranas asociadas a mitocondrias (MAM), que son necesarias para la expresión correcta de los neuropéptidos.

Los MAM median el intercambio de iones, lípidos y metabolitos entre estos dos compartimentos (mitocondrias y RE) (Hayashi et al., 2009). Se ha descrito para algunas proteínas de la membrana del RE que necesitan palmitoilación para ser dirigidas a los MAM (Lynes et al., 2011). Por otra parte, un trabajo reciente realizado por Claret y colaboradores demuestra que los MAM son necesarios para la producción y procesamiento correctos de los neuropéptidos (Schneeberger et al., 2013).

Resultados preliminares no publicados de nuestro grupo revelan que CPT1C podría estar ubicada en MAM, aparte de estar en el RE (Jacas y Casals, resultados no publicados). Por lo tanto CPT1C podría estar actuando en MAM regulando la palmitoilación.

4.2.CPT1C PRODUCE UN AUMENTO AGUDO DE LA CERAMIDAS NECESARIO PARA EL EFECTO DE LA GHRELINA

Una de las aportaciones más relevantes de esta tesis es nuestro segundo hallazgo: CPT1C produce un aumento agudo de ceramidas necesario para el efecto de la ghrelina en el hipotálamo. Este resultado concuerda con un estudo previo de Gao y colaboradores que muestran que la leptina, la hormona contrapuesta a la ghrelina, disminuye los niveles de ceramides, en parte, a través de la CPT1C (Gao et al., 2011).

Un aumento transitorio de ceramidas, en cuestión de minutos, se relaciona con la generación de ceramidas a través de las esfingomielinasas (SMasas) (Haimovitz-Friedman et al. , 1994) y la acumulación de ceramidas en varias horas se relaciona con la síntesis *de novo* (Vit y Rosselli., 2003). Ya que el efecto que observamos es en cuestión de minutos, probablemente la CPT1C está afectando o modulando la síntesis de ceramidas a través de las SMasas.

4.3.CERAMIDA EN LOS NIVELES DE EXPRESIÓN DE BSX

Otro descubrimiento ha sido que el aumento de ceramidas que se produce, genera un aumento en los niveles de BSX. Este resultado da consistencia a otro trabajo anterior de Gao y colaboradores que demostraron que una sobreexpresión de CPT1C en el ARC produce un aumento de los niveles de mRNA de BSX. Además, el tratamiento con leptina produce una disminución de los niveles de BSX (Gao et al., 2011).

Datos recientes demuestran que las ceramidas producen cambios en los niveles acetilados del factor de transcripción Sp1/3, a través de las HDACs, favoreciendo o previniendo su unión a las secuencias promotoras de diferentes genes y, a su vez, produciendo la activación o la represión de estos. Además, se ha publicado que el promotor BSX contiene varios supuestos sitios de unión de factores de transcripción, incluyendo la caja de GC para los factores de transcripción Sps (Park et al., 2007).

Nuestros resultados indican que la ceramidas producidas en el RE podrían activar HDACs citosólicas, que migrarían al núcleo para deacetilar a los factores de transcripción Sp1/3. Los cambios en la acetilación de Sp1/3 podrían modular su afinidad por la secuencia de la promotora de BSX provocando un aumento en sus niveles.

La hipótesis es que el Sp1 desacetilado actúa como un activador y Sp3 acetilado como un inhibidor permanente. Entonces, un aumento de las ceramidas activaría HDACs que podrían provocar una disminución de los niveles de acetilación de ambos factores de transcripción, Sp1 y Sp3. Por un lado, Sp3 desacetilado perdería su afinidad al promotor permitiendo Sp1 desacetilado se uniera activando la transcripción de BSX.

También se ha descrito que la esfingosina-1-fosfato (S1p) regula la actividad de las HDACs. No podemos descartar que el incremento de ceramida inducido por la ghrelina se transforme en S1p y que sea este el generador de los efectos.

4.4. CPT1C Y LONGEVIDAD

Finalmente descubrimos que los ratones CPT1C KO, en comparación con los WT, tienen una vida más corta.

Existe una hipótesis extendida para explicar el envejecimiento, la teoría mitocondrial, también conocida como la teoría de los radicales libres (Harman, 1956). Algunos estudios revelan que las ratas jóvenes tienen mitocondrias pequeñas, mientras que las envejecidas tienen el mismo número de mitocondrias, pero más grande (Sastre et al., 1998). Estas mitocondrias más grandes no son bio-energéticamente eficientes (Nicholls, 2004).

Un análisis metabolómico comparando cerebros de ratones CPT1C KO y WT indica que CPT1C podría tener un papel en el metabolismo oxidativo neuronal, ya que, como hemos explicado anteriormente, encontraron disminuido el glutatión oxidado (GSSG) en los ratones KO, lo que indicaría una pérdida de la capacidad antioxidante (Lee y Wolfgang, 2012). Una gran cantidad de estudios muestran que la disminución del ratio GSH/GSSG sucede durante el envejecimiento (Chen et al, 1989; Iantomasi et al, 1993; Oubidar et al, 1996; Suh et al, 2005). Todos estos datos nos llevan a pensar que los ratones CPT1C KO tienen aumentados los niveles de ROS produciéndose una oxidación de las proteínas, los lípidos y el ADN que generaría estrés oxidativo, daño celular y envejecimiento (Foster et al, 1996; Gupta et al, 1991; Hamilton et. al, 2001).

En realidad, el envejecimiento es la pérdida de la capacidad a hacer frente a los factores de estrés. Un estudio reciente de la función de CPT1C en cáncer muestra que la CPT1C protege a las células contra la hipoxia y la hipoglucemia (Zaugg et al., 2011). También se sabe que en situaciones de restricción calórica se produce un aumento de resistencia en las neuronas al estrés y un aumento de la supervivencia. Por lo tanto podemos imaginar que CPT1C en las neuronas se puede activar por el estrés, para producir la supervivencia y la resistencia de la neurona. Las neuronas que carecen CPT1C no serán capaces de luchar contra el estrés de una manera adecuada y esto podría producir una disminución de la longevidad.

En resumen, podría ser que CPT1C fuera un sensor de estrés metabólico, que actuara a través de un aumento agudo de la síntesis de ceramidas que produjeran un aumento de algunos genes necesarios para hacer frente al estrés.

Concretamente en la ruta hipotalámica de la ghrelina, la CPT1C sería la responsable del aumento de BSX mediado por ceramidas. En cualquier caso, la falta de CPT1C y la incapacidad resultante para lidiar con el estrés produciría una disminución de la supervivencia.

5. CONCLUSIONES

1 . CPT1C está implicada en la vía de señalización hipotalámica de la ghrelina, afectando en última instancia la expresión de los neuropéptidos (NPY y AgRP) y la ingesta de alimentos.

2 . La inyección intracerebroventricular de ghrelina produce un aumento agudo y transitorio de los niveles de ceramida en el MBH, que es necesario para inducir un aumento de NPY, AgRP y la ingesta de alimentos.

3 . Se requiere CPT1C para el efecto estimulador de la ghrelina sobre el contenido de ceramida en el hipotálamo.

4 . La inyección intracerebroventricular de ceramidas en ratones WT no es capaz de inducir la expresión de NPY, AgRP o la ingesta de alimentos, lo que indica que el aumento de la ceramida en el MBH es necesaria pero no suficiente para inducir un efecto orexigénico.

5 . La deficiencia de CPT1C produce un aumento en la actividad basal de los niveles de AMPK y UCP2, lo que sugiere que la ruta AMPK/CPT1A/UCP2 está permanentemente activa .

6 . La sobreexpresión de la CPT1C en las células sugiere que CPT1C está por encima de BSX en la vía de señalización de la ghrelina.

7 . Los datos procedentes de los experimentos con ratones y células, indican que las ceramidas están involucradas en la expresión del factor de transcripción BSX, probablemente mediante la regulación de la acetilación de Sp1/3.

8 . Los resultados sugieren que las ceramidas producen un aumento en la expresión de BSX debido a una disminución en la acetilación de los factors de transcripción Sps.

9 . Los datos del experimento de mitramicina indican que Sp1 o Sp3 puede estar actuando como un inhibidor constitutivo y si este no se puede unir a la secuencia del promotora, entonces BSX es capaz de aumentar su expresión.

10 . Del experimento es que la longevidad concluyo que la falta de CPT1c produce una reducción significativa de la supervivencia.

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PUBLICATIONS

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ORIGINAL ARTICLE

Hypothalamic Ceramide Levels Regulated by CPT1C Mediate the Orexigenic Effect of Ghrelin

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Recent data suggest that ghrelin exerts its orexigenic action through regulation of hypothalamic AMP-activated protein kinase pathway, leading to a decline in malonyl-CoA levels and desinhibition of carnitine palmitoyltransferase 1A (CPT1A), which increases mitochondrial fatty acid oxidation and ultimately enhances the expression of the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY). However, it is unclear whether the brain-specific isoform CPT1C, which is located in the endoplasmic reticulum of neurons, may play a role in this action. Here, we demonstrate that the orexigenic action of ghrelin is totally blunted in CPT1C knockout (KO) mice, despite having the canonical ghrelin signaling pathway activated. We also demonstrate that ghrelin elicits a marked upregulation of hypothalamic C18:0 ceramide levels mediated by CPT1C. Notably, central inhibition of ceramide synthesis with myriocin negated the orexigenic action of ghrelin and normalized the levels of AgRP and NPY, as well as their key transcription factors phosphorylated cAMP-response element–binding protein and forkhead box O1. Finally, central treatment with ceramide induced food intake and orexigenic neuropeptides expression in CPT1C KO mice. Overall, these data indicate that, in addition to formerly reported mechanisms, ghrelin also induces food intake through regulation of hypothalamic CPT1C and ceramide metabolism, a finding of potential importance for the understanding and treatment of obesity. ***Diabetes* 62:2329–2337, 2013**

Ghrelin is a hormone produced by the stomach that induces food intake through the growth hormone segretagogue receptor 1a in the hypothalamus (1,2). Ghrelin and the proteins that are involved in the downstream signaling pathway are clear targets for the treatment of obesity and food intake disorders. Recently, much effort has been invested in studying the molecular mechanism by which ghrelin enhances the expression of the orexigenic neuropeptides agouti-related protein (AgRP) and neuropeptide Y (NPY) in the arcuate nucleus of the hypothalamus (ARC). It has been described that ghrelin binding to its receptor induces intracellular calcium release, which activates hypothalamic calmodulin-dependent protein kinase kinase 2 and the phosphorylation of the energy sensor AMP-activated protein kinase (AMPK) (3–5). It also has been described that ghrelin specifically triggers a hypothalamic Sirtuin1/p53 pathway that is essential for AMPK phosphorylation (6,7). One of the main effects of AMPK activation in the hypothalamus is the modulation of fatty acid metabolism; when activated, phosphorylated AMPK (pAMPK) further phosphorylates and inactivates acetyl-CoA carboxylase (ACC), causing a decrease in malonyl-CoA levels and the disinhibition of carnitine palmitoyltransferase 1 (CPT1) A enzyme (4,5). The overall outcome of that effect is increased fatty acid oxidation and accumulation of reactive oxygen species, which are mainly buffered by uncoupling protein 2 (UCP2) (5). All these metabolic changes ultimately activate transcriptional events in the cell nucleus by eliciting increased levels or activation of key transcription factors, such as cAMP-response element–binding protein (CREB) and its phosphorylated isoform (pCREB), forkhead box O1 (FoxO1) and its phosphorylated isoform, and brain-specific homeobox transcription factor. These are responsible, in part, for the increase of the orexigenic neuropeptides AgRP and NPY (8). The physiological relevance of hypothalamic AMPK signaling on the orexigenic effect of ghrelin stems from the finding that genetic or pharmacological inhibition of calmodulin-dependent protein kinase kinase 2, AMPK, CPT1A, or UCP2, as well as increased concentrations of malonyl-CoA levels in the hypothalamus block ghrelin-induced feeding (3–5). Nevertheless, despite that compelling evidence, the exact molecular mechanism through which changes in fatty acid metabolism modulate AgRP and NPY expression is not completely understood.

Carnitine palmitoyltransferase 1C (CPT1C) is a brain-specific CPT1 isoform that, quite opposite to mitochondrial CPT1A, localizes in the endoplasmic reticulum (ER) of neurons (9). CPT1C has very low CPT1 activity but has been demonstrated to bind malonyl-CoA (the physiological inhibitor of CPT1 enzymes) with a K_d within the dynamic range of hypothalamic malonyl-CoA concentration in fasted and refed states (10,11). Consequently, CPT1C has been proposed to be a sensor of malonyl-CoA levels in hypothalamic neurons (12,13). At the physiological level, it is well-established that hypothalamic CPT1C is involved in the control of energy homeostasis because CPT1C knockout (KO) mice show reduced food intake and impaired peripheral metabolism (11,14). However, the hypothalamic molecular pathway through which CPT1C regulates food intake remains unclear. We recently have demonstrated that overexpression of CPT1C in ARC blocks the anorectic effects of leptin through a mechanism involving increased hypothalamic ceramide levels (15). Considering that ceramide levels in the mediobasal hypothalamus (MBH) are

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increased in response to fasting (15), a state in which circulating ghrelin levels are elevated, we hypothesized that CPT1C might be involved in the hypothalamic ghrelin signaling pathway. Here, we demonstrate that CPT1C mediates a short-term increase in hypothalamic ceramide levels in response to ghrelin and, notably, that this effect is critical for the effects of ghrelin on AgRP and NPY expression, as well as on feeding.

RESEARCH DESIGN AND METHODS

Animal preparations. All animal procedures were performed in accordance with the guidelines of European Community Directive 86/609/EEC (European Union directive 86/609, European Union decree 2001–486) and Standards for Use of Laboratory Animals A5388–01 (National Institutes of Health) and were approved by the Local Ethics Committee. We used adult (25–30 g) CPT1C KO male mice and their wild-type (WT) littermates. They were housed in a controlled (12-h light/12-h dark) environment. The animals were fed ad libitum with standard laboratory chow and water.

Cannulation surgery. Mice were anesthetized by an intraperitoneal (IP) injection of ketamine/xylazine (ketamine 75 mg/kg body weight plus xylazine 10 mg/kg body weight). Brain infusion cannulae were stereotaxicaly placed in the lateral cerebral ventricle using the following coordinates: 0.58 mm posterior to bregma; 1 mm lateral to the midsagittal suture and to a depth of 2.2 mm; and with bregma and lambda at the same vertical dimension. Former studies of our group have demonstrated that ghrelin administration by using this route does not affect fatty acid metabolism in other brain areas apart from MBH, such as amygdala, striatum, habenula, fields CA1, CA2, and CA3 of the hippocampus, hippocampus dentate gyrus, motor cortex, pyriform cortex, sensory cortex, substantia nigra, and zona incerta (thalamus) (4). Animals were individually caged and allowed to recover for 1 week before experiments.

Intracerebroventricular and IP treatments and sample recollection. For the ghrelin (Bachem, Bubendorf, Switzerland) experiments, mice received an intracerebroventricular (ICV) administration of 5 μg (dissolved in 2 μL of physiological serum) or an IP administration of 10 μg (dissolved in 20 μL of physiological serum) ghrelin. We have previously demonstrated that ICV ghrelin exerts a dose-dependent effect on food intake and hypothalamic fatty acid metabolism, with the dose of 5 μg being the one that results in a greater response (4). The dose of 10 μg ghrelin IP has been previously used in the literature to induce food intake and produces serum ghrelin levels in the range observed in fasted mice (16). For the myriocin (Sigma-Aldrich, St. Louis, MO) experiments, mice received an ICV administration of 4 μg myriocin (dissolved in 1:3 DMSO:saline). For the C6:0 ceramide (*N*-hexanoyl-D-sphingosine; Sigma-Aldrich) experiments, mice received an ICV administration of 2.5 μg C6:0 ceramide (dissolved in 1:3 DMSO:saline). Ghrelin and C6:0 ceramide were administered at the beginning of the light cycle when mice were satiated. When indicated, myriocin was administrated 1 h before ghrelin administration. Mice were killed by cervical dislocation and tissue was collected. The whole brain was used for in situ hybridization analysis, the hypothalami were used

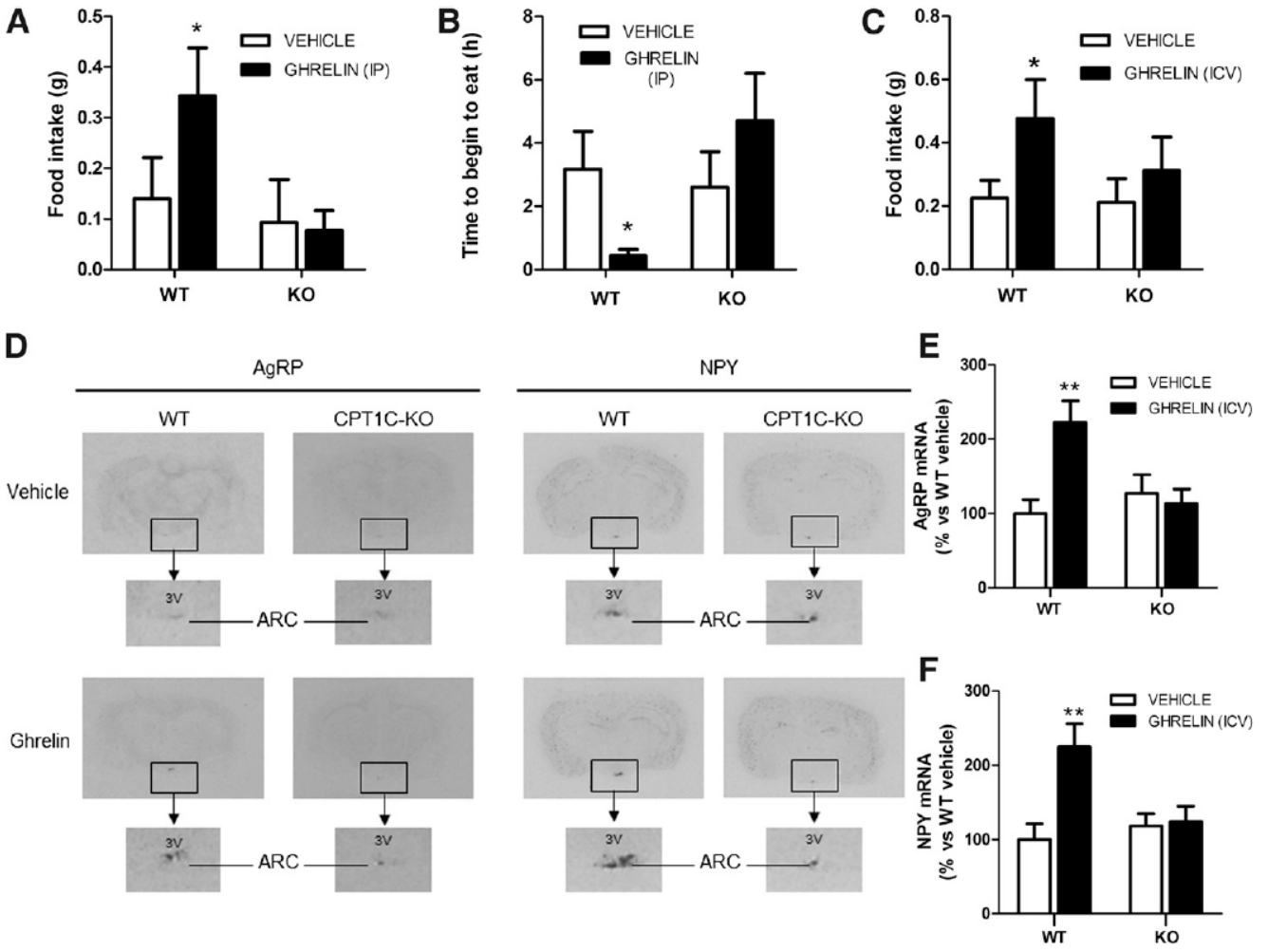

FIG. 1. Ghrelin does not induce orexigenic effects in CPT1C KO mice. *A*: The 2-h food intake in WT and CPT1C KO mice treated IP with vehicle (white bars) or with 10 μg ghrelin (black bars). *B*: Time to begin to eat after IP injection of vehicle (white bars) or 10 μg ghrelin (black bars). *C*: The 2-h food intake in WT and CPT1C KO mice treated with ICV vehicle (white bars) or with 5 μg ICV ghrelin (black bars). ARC mRNA levels of AgRP (*D* and *E*) and NPY (*D* and *F*) of WT and CPT1C KO mice treated with ICV vehicle (white bars) or 5 μg ghrelin (black bars). Samples were obtained 2 h after the treatment. *$P < 0.05$, **$P < 0.01$ vs. WT mice treated with vehicle. 3V, third ventricle.

for Western blotting, and the MBH was used for real-time PCR analysis and ceramide measurements. To dissect the MBH, brains were placed in a coronal brain matrix (Roboz Surgical Instrument, Gaithersburg, MD) and were sectioned from bregma −1 mm to −2.5 mm. Then, a 1-mm-diameter tissue collector was used to obtain the MBH from each section.

Ceramide quantification. Ceramides were extracted and analyzed via the LC-ESI-MS/MS System (API 3000 PE Sciex; Spectralab Scientific, Markham, Ontario, Canada) in positive ionization, as described previously (17). Their concentrations were measured by multiple reaction monitoring experiments using *N*-heptadecanoyl-D-erythro-sphingosine (C17 ceramide) as internal standard (50 ng·mL^{-1}). The method was linear over the range from 2 to 600 ng·mL^{-1}.

Western blotting. Hypothalamic total protein lysates (30 μg) were subjected to SDS-PAGE, electrotransferred on a polyvinylidene fluoride membrane, and probed with the following antibodies: phosphorylated ACC (pACC)α-Ser^{79} 1:1,000; pAMPKα1 1:1,000; BiP 1:1,000; FoxO1 1:1,000 (Cell Signaling, Danvers, MA); ATF-6β 1:1,000; CHOP 1:500; pCREB-Ser^{129} 1:500; peIF2α 1:2,000; pIKKα/β 1:1,000; IKKβ 1:1,000; nuclear factor-κB 1:1,000; pPERK 1:500; TLR4 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin 1:10,000; ATF4 1:1,000; and 0.2 μg/mL anti-β-tubulin III (Sigma-Aldrich). Values are expressed relative to β-actin or β-tubulin levels. The blots were developed using the ECL Western blotting system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Real-time quantitative PCR. We performed real-time PCR (TaqMan; Applied Biosystems, Carlsbad, CA) as described (18) using primers designed by Applied Biosystems (AgRP Mm00475829_g1, NPY Mm00445771_m1, and glyceraldehyde 3-phosphate dehydrogenase 4352339E) or IDT Integrated Technologies (UCP2 63705740). Values were expressed in relation to glyceraldehyde 3-phosphate dehydrogenase levels.

In situ hybridization. Coronal brain sections (16 μm) were probed with specific oligonucleotides for AgRP (5′-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3′) and NPY (5′-AGA TGA GAT GTG GGG GGA AAC TAG GAA AAG TCA GGA GAG CAA GTT TCA TT-3′) as previously published (4,18–21).

Statistical analysis. We used 8–10 animals per group in all experiments except for real-time PCR analysis, for which we used six animals. Data are expressed as mean ± SEM in relation (%) to vehicle-treated mice. Statistical significance was determined by Student *t* test when two groups were compared and by ANOVA with post hoc two-tailed Bonferroni test when more than two groups were compared. $P < 0.05$ was considered significant.

RESULTS

Ghrelin administration did not increase either food intake or the expression of orexigenic neuropeptides in CPT1C KO mice. To establish whether CPT1C was part of the hypothalamic ghrelin signaling pathway, we analyzed the orexigenic effect of ghrelin in CPT1C KO mice. We injected ghrelin IP and analyzed food intake and

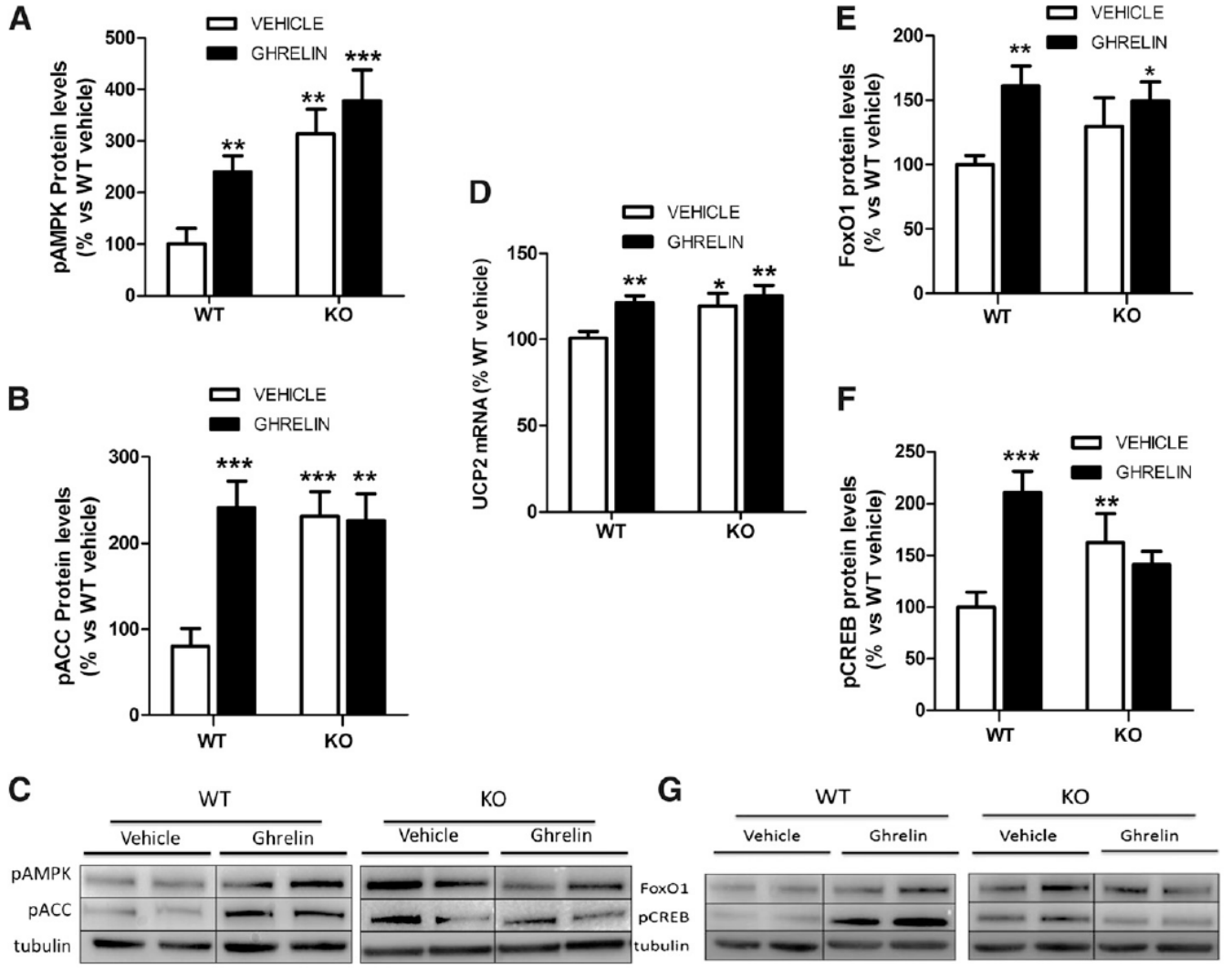

FIG. 2. The ghrelin signaling pathway in WT and CPT1C KO mice. Hypothalamic protein levels of pAMPK (*A* and *C*) and pACC (*B* and *C*), MBH mRNA levels of UCP2 measured by real-time PCR (*D*), and the hypothalamic protein levels of FoxO1 (*E* and *G*) and pCREB (*F* and *G*) in WT and CPT1C KO mice after 2 h of ICV injection of vehicle (white bars) or 5 μg ICV injection of ghrelin (black bars). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. WT mice treated with vehicle.

the food-seeking behavior. We found that ghrelin injection to WT mice increased food intake two fold and highly reduced the time to begin eating. Both effects were completely blocked in CPT1C KO mice (Fig. 1*A* and *B*). Then, we repeated the experiment ICV injection of ghrelin and again found that CPT1C KO mice failed to respond to ghrelin treatment (Fig. 1*C*). Next, we analyzed the expression of the orexigenic neuropeptides AgRP and NPY by in situ hybridization. The ghrelin-induced increase in AgRP and NPY levels present in WT mice was completely blunted in CPT1C KO mice (Fig. 1*D* and *E*), which correlates with the lack of the orexigenic effect of ghrelin in those mice. These results indicate that CPT1C is involved in the ghrelin orexigenic effect.

The canonical ghrelin signaling pathway is impaired in CPT1C KO mice. Next, we analyzed the ghrelin signaling pathway in hypothalamus from CPT1C KO mice. Our data showed that central ghrelin treatment induced a marked stimulatory effect on the levels of pAMPK, pACC, UCP2, and the transcription factors FoxO1 and pCREB in WT mice. Those effects were impaired in CPT1C KO mice (Fig. 2*A*–*G*). Basal levels of pAMPK and pACC, putative upstream factors of CPT1C, were significantly increased in CPT1C KO mice when compared with WT mice, suggesting that AMPK pathway is constitutively activated in the hypothalamus of CPT1C KO mice (Fig. 2*A*–*C*). Notably, in keeping with the altered levels of pAMPK and pACC, the expression of UCP2, a downstream factor of this pathway related to mitochondrial fatty acid oxidation, and the levels of the transcription factors FoxO1 and pCREB also were increased in the hypothalamus of CPT1C KO mice when compared with vehicle-injected WT mice (Fig. 2*D*–*G*). Therefore, these data suggest that even the canonical signaling pathway of ghrelin is activated in CPT1C KO mice. The lack of CPT1C blocks the ghrelin induction of orexigenic neuropeptides and food intake.

Central administration of ghrelin increased ceramide levels in hypothalamus of WT but not CPT1C KO mice. Current evidence from our group has identified CPT1C as a key regulator of ceramide levels in neurons (22). To investigate whether ghrelin had any impact on hypothalamic ceramide concentration, we measured the levels of C18:0 ceramide, the most abundant ceramide in brain and neurons (23), in WT mice at different times after ghrelin administration. Our data showed that central ghrelin promoted a marked stimulatory and transitory action in C18:0 ceramide levels in the MBH, evident from the maximal effect detected at 30 and 60 min after ghrelin injection (Fig. 3*A*). Then, we performed a dose-response curve and found that MBH ceramide levels at 30 min after ghrelin administration increased progressively with the dose of ghrelin, with the increase statistically significant at the dose of 5 μg (Fig. 3*B*). Next, we analyzed what happened in CPT1C KO mice. Opposite to WT mice, 5 μg ghrelin failed to induce any effect in the C18:0 ceramide levels of CPT1C KO mice 30 min after its administration (Fig. 3*C*), indicating a requirement of CPT1C for the stimulatory effect of ghrelin on ceramide content in the MBH.

Inhibition of hypothalamic ceramide synthesis blocked the orexigenic effect of ghrelin. To investigate the existence of any mechanistic link between the orexigenic effect of ghrelin and the activation of ceramide synthesis, we investigated the effects of 4 μg myriocin, a specific inhibitor of the de novo synthesis of ceramide (24), on ghrelin action. The selected dose of myriocin induced neither an anorectic effect per se at any evaluated time (Fig. 4*A* and data not shown) nor illness or malaise (data not shown), nor hypothalamic inflammation (measured as protein levels), nor ER stress (measured as protein levels), an indirect indicator of inflammation (Fig. 4*B* and *C*). Although this dose of myriocin was subeffective when injected alone, our data showed that ICV injection of myriocin 1 h before ghrelin administration decreased

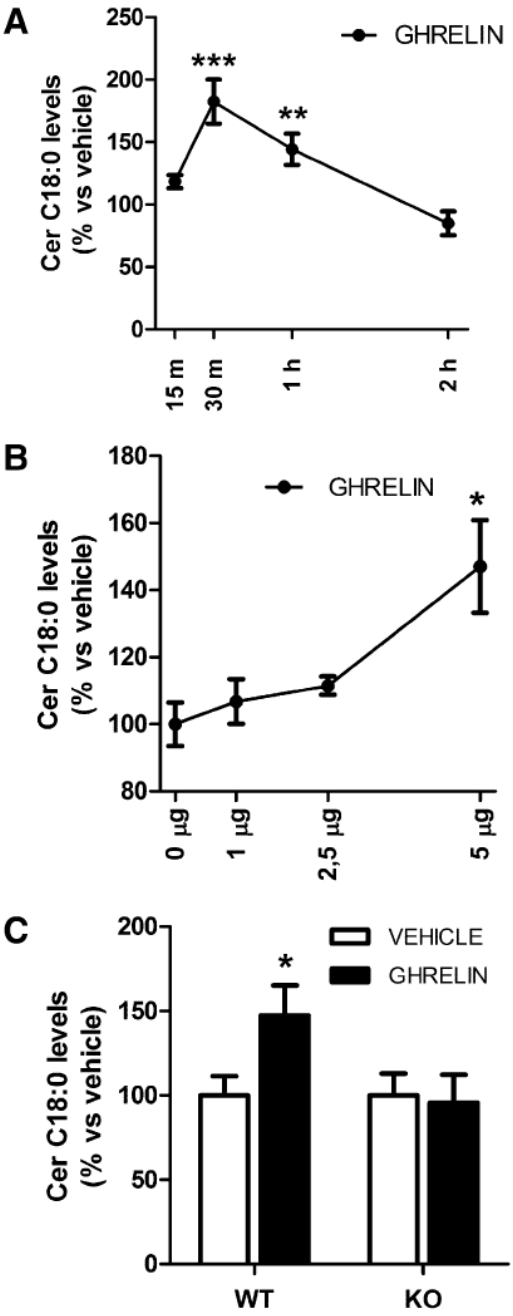

FIG. 3. MBH ceramide levels in response to ghrelin. *A*: Time course of MBH C18:0 ceramide levels in WT mice after ghrelin administration (ICV, 5 μg). Percentage of respective increase in vehicle-treated mice is represented. *B*: MBH C18:0 ceramide levels in WT after 30 min of ICV administration of different doses of ghrelin. *C*: MBH C18:0 ceramide levels of WT and CPT1C KO mice after 30 min of ICV administration of vehicle (white bars) or 5 μg ICV administration of ghrelin (black bars). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. WT vehicle. Cer, ceramide.

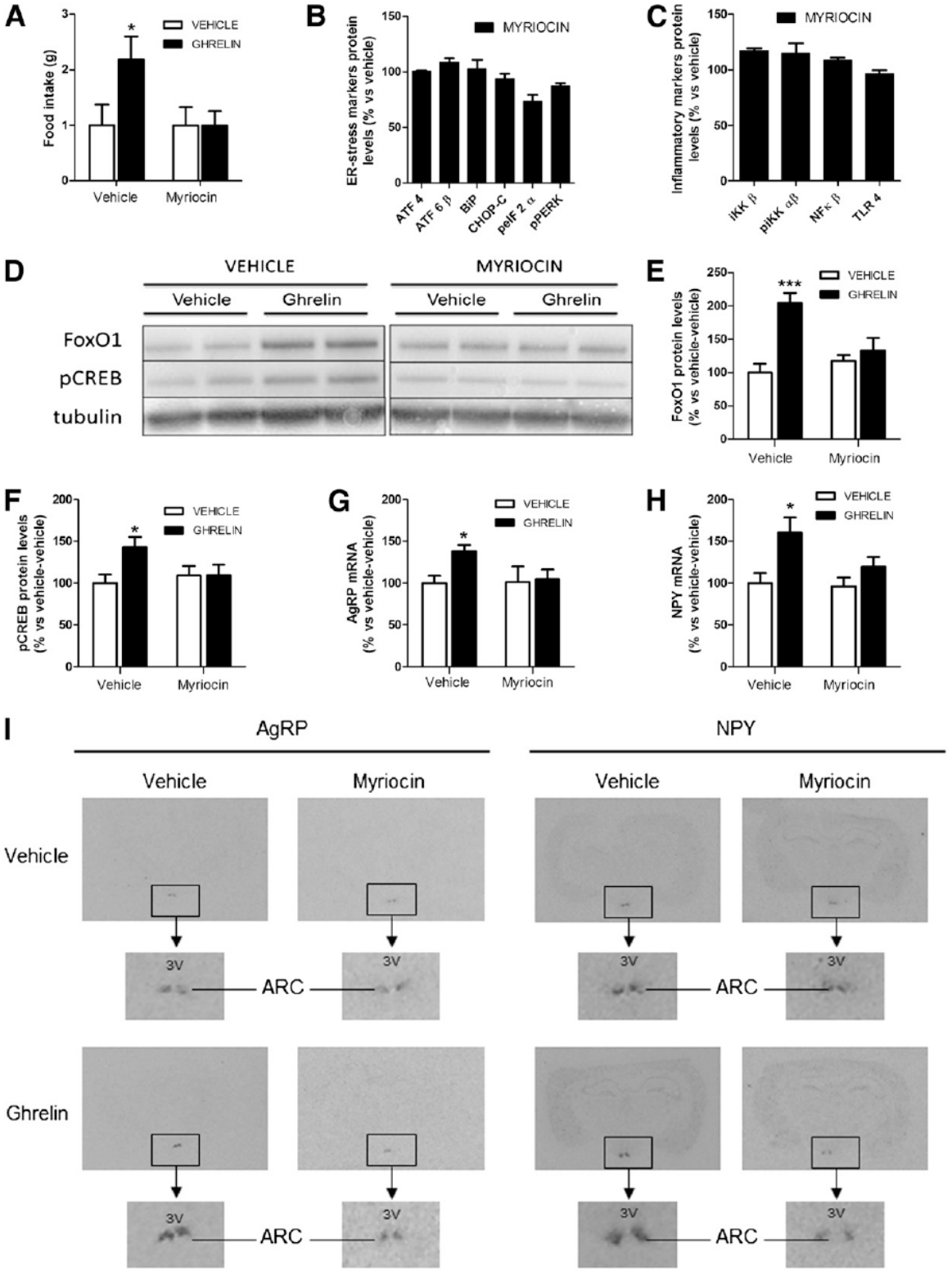

FIG. 4. Myriocin injection blocks the orexigenic effect of ghrelin. WT mice were pretreated with ICV vehicle or 4 μg ICV myriocin 1 h before ICV administration of vehicle (white bars) or 5 μg ICV administration of ghrelin (black bars). *A*: Food intake after 2 h of ghrelin injection. ICV injection of myriocin does not cause ER stress (*B*) or inflammation (*C*) in hypothalamus. Hypothalamic ER stress and inflammatory markers were measured by Western blot. ATF4, activating transcription factor 4; ATF6β, activating transcription factor 6β; BiP, ER chaperone-binding immunoglobulin protein, also known as glucose-regulated protein 78 kDa, GRP78; CHOP-C, C/EBP homologous protein C; pEIF2α, phosphorylated eukaryotic initiator factor 2α; pPERK, phosphorylated RNA–dependent protein kinase-like ER kinase; IKKβ, IκB kinase β; pIKK αβ, phosphorylated IκB kinase αβ; NFκB, nuclear factor-κB; TLR4, Toll-like receptor 4. Hypothalamic protein levels (*D–F*) of FoxO1 and pCREB measured by Western blot. ARC mRNA levels (*G–I*) of AgRP and NPY measured by in situ hybridization. *$P < 0.05$, ***$P < 0.001$ vs. vehicle-vehicle–treated mice. 3V, third ventricle.

the orexigenic effect of ghrelin (Fig. 4*A*) and its stimulatory effect on transcription factors FoxO1 and pCREB and on neuropeptides AgRP and NPY (Fig. 4*D–I*). These results indicate that ceramide synthesis also is a required component of the ghrelin hypothalamic signaling pathway.

Ceramide administration increases food intake in CPT1C KO mice. Bearing in mind that CPT1C has been involved in ceramide synthesis, we next investigated whether ceramide injection had any orexigenic effect in CPT1C KO mice; we used C6:0-ceramide, a cell-penetrating ceramide that is converted to long chain ceramides inside the cell (25). We used the submaximal dose of 2.5 μg, which has been reported to block the satiating effects of leptin but lacks of orexigenic effect per se in WT animals (15). Of note, 2.5 μg C6:0 ceramide via ICV injection produced MBH C18:0 ceramide levels in the range observed in ghrelin-treated mice (ICV ceramide: 0.53 ± 0.10 ng C18:0 ceramide/mg protein; ICV ghrelin: 0.41 ± 0.04 ng C18:0 ceramide/mg protein); in both cases, hypothalamic ceramide levels were significantly higher than those found in controls (vehicle-treated). Ceramide, which was injected at the beginning of the light cycle when animals were satiated, increased food intake and the expression of AgRP and NPY in CPT1C KO mice but, as expected, had no effect in WT mice (Fig. 5). These results indicate that ceramide is able to rescue feeding patterns when the canonical ghrelin signaling pathway (pAMPK/pACC/CPT1A/UCP2) is previously activated, as it happens in CPT1C KO mice (Fig. 2). By contrast, in fed WT mice, which have the ghrelin canonical pathway not activated, ceramide alone is unable to induce orexigenic neuropeptides expression and food intake. These results argue for two parallel signaling pathways for ghrelin, with the involvement of CPT1C and ceramide in one of the branches (Fig. 6). We propose that ghrelin must activate two parallel pathways, the mitochondrial pathway (with the activation of CPT1A and fatty acid oxidation) and the ER pathway (with the activation of CPT1C and ceramide synthesis), for its orexigenic effect to be effective.

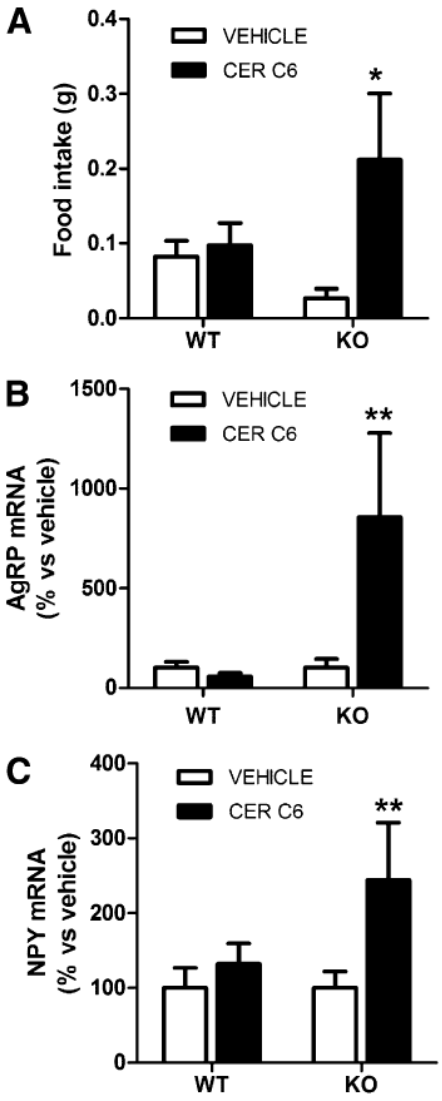

FIG. 5. Ceramide induces food intake in CPT1C KO mice. WT and CPT1C KO mice were treated with ICV vehicle (white bars) or 2.5 μg ICV ceramide C6:0 (black bars) at the beginning of the light cycle. Food intake (*A*) was measured 3 h after the injection. AgRP (*B*) and NPY (*C*) mRNA levels in MBH were measured by real-time PCR in samples obtained 3 h after the treatment. *$P < 0.05$, **$P < 0.01$ vs. vehicle-treated mice. CER, ceramide.

DISCUSSION

In this study we demonstrate that the orexigenic effect of ghrelin is coupled to an ability of ghrelin to regulate hypothalamic CPT1C and ceramide synthesis, and that this action is required for the subsequent increase in levels of AgRP and NPY mRNA expression in the ARC via modulation of the transcription factors pCREB and FoxO1. Recent data have demonstrated that the orexigenic effect of ghrelin is mediated by the selective modulation of hypothalamic SIRT1/p53/AMPK and fatty acid metabolism pathways, as well as UCP2 levels, which culminate in increased AgRP and NPY expression in the ARC (4–7). Although it is clear that the modulation of hypothalamic fatty acid metabolism is a bona fide component of ghrelin signaling, it is unclear whether complex species might be involved in that action. In fact, this is a major constraint in our current knowledge about hypothalamic lipids and energy balance, which is restricted to a small corner of lipid pathways, namely de novo fatty acid synthesis (regulated by AMPK, ACC, fatty acid synthase, and malonyl-CoA decarboxylase) and fatty acid oxidation (regulated by CPT1A).

Current evidence from our group has implicated hypothalamic ceramides and CPT1C in the actions of leptin on food intake (15). Quite opposite to CPT1A, which is located in the mitochondria, CPT1C resides in the ER of neurons (9). At the cellular level, CPT1C is involved in ceramide metabolism, which is demonstrated by the increased ceramide levels detected after CPT1C overexpression in vitro and the reduced ceramide concentration detected in neurons from CPT1C KO mice (22). Furthermore, our recent data also show that genetic (adenoviral-driven) overexpression of CPT1C in the ARC increases feeding through a mechanism involving increased ceramide levels and that this effect antagonizes the anorectic actions of leptin at central level (15). However, so far no evidence has linked the orexigenic effect of ghrelin to alterations in ceramide metabolism or hypothalamic CPT1C function. Thus, all the evidence led us to investigate the possible involvement of hypothalamic CPT1C and ceramides on the action of ghrelin.

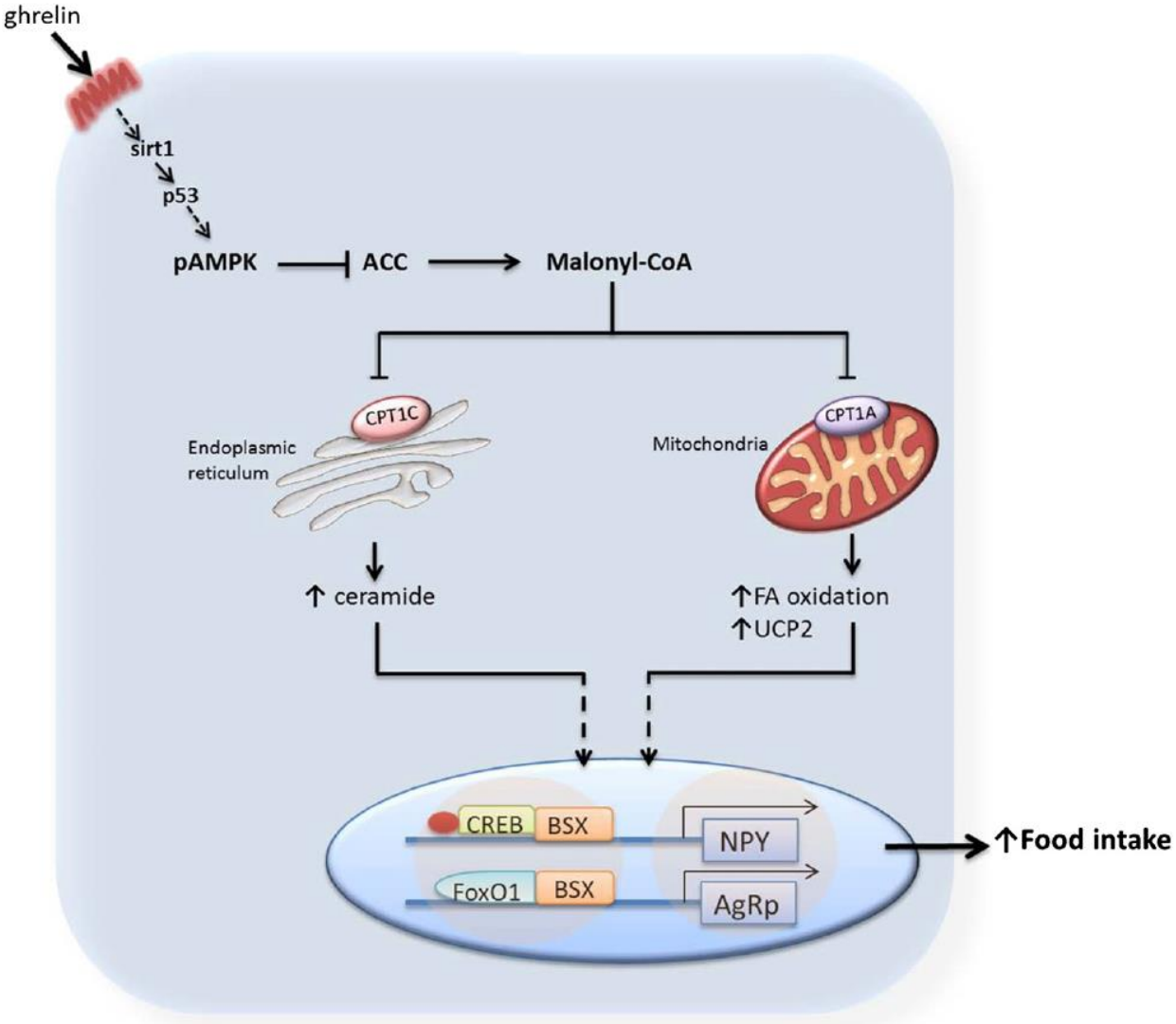

FIG. 6. Schematic diagram showing the involvement of CPT1C and ceramides in the hypothalamic ghrelin signaling pathway. Ghrelin stimulates the hypothalamic SIRT1/p53/AMPK axis, leading to decreased levels of hypothalamic malonyl-CoA, the physiological inhibitor of CPT1 enzymes. In the "classical" mechanism of ghrelin action, this effect promotes disinhibition of CPT1A, increased fatty acid (FA) oxidation, and altered reactive oxygen species levels. These metabolic changes ultimately activate the nuclear transcription machinery (pCREB, FoxO1, and brain-specific homeobox transcription factor [BSX]), increasing mRNA expression of *Agrp* and *Npy* genes. Here, we demonstrate the existence of a parallel downstream pathway involving CPT1C, a specific brain isoform located in the ER, which triggers a short-term increase in ceramide synthesis after ghrelin treatment. This new pathway is of physiological importance because the orexigenic action of ghrelin is totally blunted in CPT1C KO mice or in mice treated with an inhibitor of ceramide synthesis. The fact that central ceramide treatment induces food intake and triggers orexigenic neuropeptides expression in CPT1C KO mice, which have the canonical ghrelin signaling pathway activated during satiating conditions, but not in WT mice, indicates that both branches need to be activated for ghrelin to exert its orexigenic effect.

Here, we demonstrate that central ghrelin administration promotes a marked short-term increase in the MBH C18:0 ceramide concentration mediated by CPT1C, and that this increase in ceramide levels is necessary to induce hyperphagia and AgRP and NPY expression. In fact, inhibition of hypothalamic ceramide synthesis with myriocin negated the orexigenic action of ghrelin treatment and normalized AgRP and NPY expression in the ARC. Having shown that central inhibition of ceramide synthesis blocked the orexigenic action of ghrelin, we aimed to investigate whether ceramide treatment induced food intake in animals fed ad libitum. Our data show that central injection of ceramide increased the levels of AgRP and NPY and induced food intake in CPT1C KO mice, which had the ghrelin canonical pathway constitutively activated. Quite opposite, ceramide had no effect in fed WT mice, indicating that ceramide is necessary but not sufficient to induce food intake. Altogether, these results indicate that, besides the canonical SIRT1/p53/AMPK/ACC/CPT1A/UCP2 pathway, ghrelin-induced food intake is mediated by specific modulation of CPT1C and ceramide concentration in the MBH (Fig. 6). Therefore, the reduction of hypothalamic malonyl-CoA levels after ghrelin treatment (4) would activate two parallel routes, fatty acid oxidation–mediated by CPT1A and ceramide synthesis mediated by CPT1C, and both routes require triggering for ghrelin to exert its orexigenic effects. As described previously, blocking fatty acid oxidation by inhibition of CPT1A (4) or by deletion of

UCP2 (5) blunts the orexigenic effects of ghrelin. The evidence presented here demonstrates that the deletion of CPT1C or the inhibition of ceramide synthesis itself also blunts food intake after ghrelin administration, indicating that both parallel routes are required for an appropriate orexigenic response of ghrelin. Considering that ghrelin and leptin are conceptualized as the "yin and yang" in the hypothalamic regulation of feeding (26,27), our data about ghrelin and ceramide are in agreement with those of our previous report showing that the anorectic action of leptin is associated with decreased ceramide concentration and CPT1C function in the hypothalamus (15).

The cellular implications of our findings are multiple. First, this is the first evidence linking the effect of an orexigenic hormone, such as ghrelin, with a molecular mechanism involving the normal function of the ER, i.e., ceramide synthesis. In this regard, ceramides and their derivate sphingomyelin are one of the major lipids in plasmatic membranes of neurons, traditionally having been considered as structural lipids. However, current data are challenging that view, demonstrating that ceramides can act as signaling molecules in a bulk of processes, such as differentiation, proliferation, apoptosis, and neuronal plasticity, and can regulate the function of various kinases, phosphatases, deacetylases, and others (28). In this sense, ceramides could regulate the expression of orexigenic neuropeptides AgRP and NPY by modulating the activity of the transcription factors involved. An alternative hypothesis might be a mechanism involving hypothalamic ER stress. Ceramides are one of the most reactive lipid species at the peripheral level, and impaired ceramide content in pancreatic β-cells and hepatocytes induces lipotoxicity and subsequently ER stress (29–31). Taking into account that hypothalamic ER stress also has been recently proposed as a central mechanism modulating energy homeostasis and particularly leptin resistance (32–35), it would be reasonable to hypothesize that CPT1C, ceramide-induced lipotoxicity, and ER stress might play a role in the effects of ghrelin at the hypothalamic level.

In summary, our study shows that CPT1C and ceramides are part of a new hypothalamic mechanism mediating the action of ghrelin on feeding through increased *Agrp* and *Npy* gene expression. Our data also describe activation of hypothalamic CPT1C and ceramides as mediators of food intake, which is of potential importance for the understanding and treatment of obesity.

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S.R. and L.M. performed collection and assembly of data, data analysis, and interpretation. J.J. performed collection and assembly of data. P.C. performed collection and assembly of data, data analysis, and interpretation. M.P. performed collection of data. J.C. performed data interpretation. D.S. and F.G.H. performed data interpretation and acquired financial support. C.D. performed data interpretation. M.L. and N.C. were responsible for conception and design, data analysis and interpretation, and wrote the manuscript. N.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Carnitine palmitoyltransferase 1C deficiency causes motor impairment and hypoactivity.

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Research report

Carnitine palmitoyltransferase 1C deficiency causes motor impairment and hypoactivity

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HIGHLIGHTS

- CPT1C deficiency produces a progressive deterioration of motor function starting at a young ages.
- CPT1C deficiency causes incoordination and muscle weakness.
- CPT1C-deficient mice exhibit reduced locomotor activity during the exploration of new environments and during the dark phase of the day.
- CPT1C is involved in ceramide metabolism in the cerebellum, striatum, and motor cortex.
- CPT1C expression in the cerebellum, striatum and motor cortex is low after birth and increases progressively being maximum during weaning.

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ABSTRACT

Carnitine palmitoyltransferase 1c (CPT1C), a brain-specific protein localized in the endoplasmic reticulum of neurons, is expressed in almost all brain regions, but its only known functions to date are involved in the hypothalamic control of energy homeostasis and in hippocampus-dependent spatial learning. To identify other physiological and behavioral functions of this protein, we performed a battery of neurological tests on Cpt1c-deficient mice. The animals showed intact autonomic and sensory systems, but some motor disturbances were observed. A more detailed study of motor function revealed impaired coordination and gait, severe muscle weakness, and reduced daily locomotor activity. Analysis of motor function in these mice at ages of 6–24 weeks showed that motor disorders were already present in young animals and that impairment increased progressively with age. Analysis of CPT1C expression in different motor brain areas during development revealed that CPT1C levels were low from birth to postnatal day 10 and then rapidly increased peaking at postnatal day 21, which suggests that CPT1C plays a relevant role in motor function during and after weaning. As CPT1C is known to regulate ceramide levels, we measured these biolipids in different motor areas in adult mice. Cerebellar, striatum, and motor cortex extracts from Cpt1c knockout mice showed reduced levels of ceramide and its derivative sphingosine when compared to wild-type animals. Our results indicate that altered ceramide metabolism in motor brain areas induced by Cpt1c deficiency causes progressive motor dysfunction from a young age.

1. Introduction

Carnitine palmitoyltransferase 1c (CPT1C) is a brain-specific enzyme with negligible catalytic activity, unlike the liver (CPT1A) or muscle (CPT1B) isoforms [1–3]. CPT1 enzymes transfer 1 molecule of carnitine to long-chain acyl-CoA to form long-chain acyl-carnitine, facilitating the entrance of fatty acids into the mitochondria for beta-oxidation [4]. The molecular function of CPT1C in particular is intriguing for several reasons: it is the most abundant CPT1 isoform in the brain, it is located in the

Abbreviations: CPT1, carnitine palmitoyltransferase 1; ER, endoplasmic reticulum; KO, knockout.

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endoplasmic reticulum (ER) instead of the mitochondria, and it does not facilitate fatty acid oxidation [3]. Our group recently demonstrated that Cpt1c overexpression increases ceramide levels in cultured neurons while Cpt1c deficiency reduces them. In addition, we showed that dendritic spine maturation in Cpt1c-deficient neurons was impaired. Interestingly, ceramide treatment of Cpt1c knockout (KO) cultured neurons restored dendritic spine morphology, indicating that ceramide levels regulated by CPT1C play an important role in spinogenesis [5].

At the behavioral level, the involvement of CPT1C in the control of food intake and energy homeostasis has been clearly demonstrated. Cpt1c KO mice have a reduced food intake but are more sensitive to the harmful effects of a high fat diet and become obese and insulin resistant more easily, demonstrating the role of CPT1C in the hypothalamus [2,6]. In fact, CPT1C and ceramide have been shown to be involved in hypothalamic leptin and ghrelin signaling [7,8]. At the same time, we have recently shown that CPT1C is involved in spatial learning: Cpt1c KO mice require more time than wild-type (WT) mice to learn the position of a hidden platform in the Morris water maze test, a hippocampal-dependent task [5].

Although CPT1C is expressed in almost all brain regions, very few behavioral functions have been described in this protein. The present work demonstrates that CPT1C plays an important role in motor coordination, locomotor activity, and muscle strength.

2. Materials and methods

2.1. Animals

All mice used in this study were male. For each test 7–12 mice per genotype and age were used. Unless otherwise indicated, adult animals were tested at 11–14 weeks of age. In developmental experiments, the same group of animals was tested at different ages. The animals were generated and genotyped as described by other authors [5]. All behavioral testing was conducted by the same experimenters in an isolated room at the same time of day. The behavioral experimenters were blinded as to the genetic status of the animals. All animal procedures followed the guidelines of the European Community Directive (EU directive No. 86/609, EU decree 2001-486), met the National Institute of Health standards for use of laboratory animals (No. A5388-01), and were approved by the local ethics committee (CEEA-PRBB).

2.2. Neurological testing

The SmithKline Beecham Harwell Imperial College Royal London Hospital phenotype assessment (SHIRPA) primary screen, a comprehensive semiquantitative routine testing protocol, was used to identify and characterize phenotype impairments [9]. Assessment of each animal began by observing the undisturbed behavior of mice in a clear Perspex cylinder (height, 15 cm; diameter, 11 cm) to detect wild running or stereotypy. The mice were then transferred to an arena (56 cm × 34 cm), where their motor behavior and sensory function were observed. The animals underwent screening for vibrissae, corneal, and pinna response to an approaching cotton swab, visual acuity, auditory response (Preyer reflex), vestibular function (contact righting reflex and negative geotaxis), grip strength, and body tone. In the last part of the test, changes in excitability, aggression, general fear, vocalization and salivation, as well as piloerection were recorded to analyze autonomic function. In the touch escape test, the response of the animal to a finger stroke from above was recorded and scored as follows: 0 = no response; 1 = mild (escape response to firm stroke); 2 = moderate (rapid response to light stroke); 3 = vigorous (escape response to approach).

2.3. Rotarod test

A commercially available rotarod apparatus (Rotarod LE8500, Panlab SA, Barcelona, Spain) was used. The experimental design consisted of 2 consecutive trials of 1 min (Day 1) in which mice learned to remain on the rod at the minimum speed (4 rpm) followed by a second session (Day 2) in which 2 separate tasks were performed: In the first of these tasks, motor coordination and balance were evaluated by measuring the latency to fall off the rod in consecutive trials with increasingly faster fixed rotational speeds (4, 7, 10, 14, 19, 24, and 34 rpm). Animals were allowed to stay on the rod for a maximum period of 1 min per trial, with a resting period of 15 min allowed between trials. In the second task, the accelerating rod test, rotation speed was increased from 4 to 40 rpm and the latency to fall off the rod was recorded. Only 1 trial was performed by each animal at each rotational speed for each task.

2.4. Paw print test

The paw print test, designed to evaluate the walking pattern of mice, was adapted from the methods described in a previously published work [10]. The hind paws of the mice were coated with black, nontoxic waterproof ink. Animals were then placed at 1 end of a long and narrow tunnel (10 cm × 10 cm × 70 cm), which they spontaneously entered and partially or totally transversed. A clean sheet of white paper (length, 35.5 cm) was placed on the floor of the tunnel to record the paw prints. Footprints made at the beginning and at the end, representing initial and final movement respectively, were excluded from the analysis. Footprint patterns were analyzed from a minimum of 5 step cycles for each trial. Stride length was calculated as the average distance between 2 footprints of the same paw during forward locomotion.

2.5. Bar hang test

Neuromuscular strength was assessed using the wire hang test. A mouse was placed on a wire cage lid that was then gently waved in the air, causing it to grip the wire. The lid was then turned upside down approximately 15 cm above a surface of soft bedding material. Latency to fall or latency to use the hind limbs to climb up the bar was recorded with a 60-s cutoff time. The percentage of animals that fell and the percentage of animals that climbed up the bar were calculated.

2.6. Grip force test

The force exerted by the forelimbs was assessed as described by other authors [10]. The grasping ring was set up vertically, which caused the mouse to grasp it more consistently. The system was activated manually when the mouse held firmly to the grasping ring of a digital push-pull strain gauge (Grip Strength Meter, BIOSEB, Chaville, France). Each trial was repeated 3 times.

2.7. Locomotor activity test

Locomotor activity was evaluated using actimetry boxes (45 cm × 45 cm; IR Actimeter system, Panlab SA, Barcelona, Spain) contained in a soundproof cupboard. Backward and forward movements were monitored with a grid of infrared beams over a 24-h period, producing an index of locomotor activity based on the number of beam breaks in the grid.

2.8. Antibodies and Western blot analysis

Western blot analysis was performed as described in [11] with some modifications. Dissected brain regions were homogenized in 20 mM of Tris–HCl pH = 7.4, 150 mM of NaCl, 5 mM of EDTA, 1% Nonidet P-40 and the protease inhibitors PMSF, pepstatin and leupeptin. Tissue debris was eliminated by centrifugation at 4000 rpm for 10 min. 20 μg of protein extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Rabbit antibodies against the *c-ter* region of mouse CPT1C (amino acids 796–810) [3] were used at a 1:2000 dilution. The secondary antibody (anti-rabbit IgG, Jackson Laboratories) was used at a 1:5000 dilution. Blots were developed with the ECL Western blotting system from Amersham Biosciences.

2.9. Ceramide and sphingosine quantification

Ceramides and sphingosine were extracted and analyzed using an API 3000 (PE Sciex) liquid chromatography–electrospray ionization tandem mass spectrometer in positive ionization mode following the methods of other authors [5,12]. Concentrations were determined by multiple reaction monitoring (MRM) with N-heptadecanoyl-d-erythro-sphingosine (C17-ceramide) or deuterated sphingosine as internal standard (50 ng mL^{-1}). The method was linear over a range of 2–600 ng mL^{-1}.

2.10. Statistics

Data were expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA or by the Student's *t*-test. Performance in the rotarod test was compared using repeated measures ANOVA. Categorical variables were analyzed using a chi-square test and nonparametric variables were analyzed with the Mann–Whitney *U* test.

3. Results

3.1. Cpt1c KO mice show impaired coordination and reduced muscle strength

We examined Cpt1c KO mice using the protocol for the neurological semiquantitative test SHIRPA [9]. This simple observational test showed no significant differences between genotypes in terms

Table 1
SHIRPA test. Observational assessment of mice (n = 12).

	WT (n = 12)	KO (n = 12)
General health		
Body position	Sitting or standing	Sitting or standing
Breathing	Normal	Normal
Trembling	None	None
Trunk arching	Present (50%)	Present (50%)
Piloerection	None	None
Salivation	Normal	Normal
Sensory reflexes		
Visual placing	Upon vibrissae contact	Before vibrissae contact (18 mm)
Corneal reflex	Active single eye blink	Active single eye blink
Pinna reflex	Active retraction, moderate brisk flick	Active retraction, moderate brisk flick
Toe pinch	None	None
Righting reflex	Yes	Yes
Tail elevation	Horizontal extended	Horizontal extended
Preyer reflex	Yes	Yes
Grip response	Present	Present
Reaching	Before vibrissae contact (18 mm)	Before vibrissae contact (18 mm)
Emotional domain		
Irritability	Struggle during supine restraint	Struggle during supine restraint
Fear	None	None
Startle response	Slightly less than 1 cm	Slightly less than 1 cm
Transfer arousal	No freeze, immediate movement	No freeze, immediate movement
Touch escape	Moderate (rapid response to light stroke)	Mild (escape response to firm stroke)*
Aggression	None	None
Motor abilities		
Activity	Vigorous, rapid/dart movement	Casual scratch, groom, slow movement*
Negative geotaxis	Yes	Yes

The asterisk (*) indicates differences.

of general health, sensory reflexes, or autonomous function, however Cpt1c KO mice presented hypoactivity and delayed touch escape (chi-square test, $P < 0.05$) (Table 1).

In view of the data, we then performed a series of motor tests to analyze motor function in detail. The motor tests results for Cpt1c KO mice showed impairment in all parameters measured. Cpt1c KO mice presented a much shorter latency to fall in the rotarod test at fixed rotational speeds and in the accelerating test, indicating impaired motor coordination, and therefore disturbances in cerebellar function (repeated measures ANOVA test, $F[1,21] = 24.890$, $P = 0.000$; differences between genotypes were analyzed for statistical significance using the Student's t-test) (Fig. 1A and B).

When the walking pattern was examined by the paw print test, Cpt1c KO mice showed a significant reduction in stride length (one-way ANOVA test, $F[1,23] = 5.145$ $P = 0.033$) (Fig. 1C), which can be indicative of ataxia.

When muscular strength was measured using the bar hang test, latency to fall was shorter (one-way ANOVA test, $F[1,23] = 6.90$, $P = 0.015$) and the time required to climb up the bar using the hind limbs was greater for Cpt1c KO mice (one-way ANOVA test, $F[1,23] = 19.66$, $P = 0.000$) (Fig. 1E). The percentage of animals that fell was greater in Cpt1c KO mice (67%) than in WT mice (0%) (chi-square test, $P < 0.001$), and the percentage of animals that were able to use their hind limbs to climb up to the bar was lower in Cpt1c KO mice (33%) than in WT mice (100%) (chi-square test, $P < 0.05$). These results suggest that in addition to impaired coordination, Cpt1c KO mice have reduced muscle strength.

Finally, the grip strength test was performed to measure the vertical force of forelimbs. Cpt1c KO mice showed a significant reduction in forelimb vertical force when compared with WT mice (one-way ANOVA test, $F[1,23] = 19.63$, $P = 0.000$) (Fig. 1D), confirming the muscle weakness detected in the previous tests. All these results demonstrate that Cpt1c KO mice exhibit deficits in motor function, especially in coordination and strength skills.

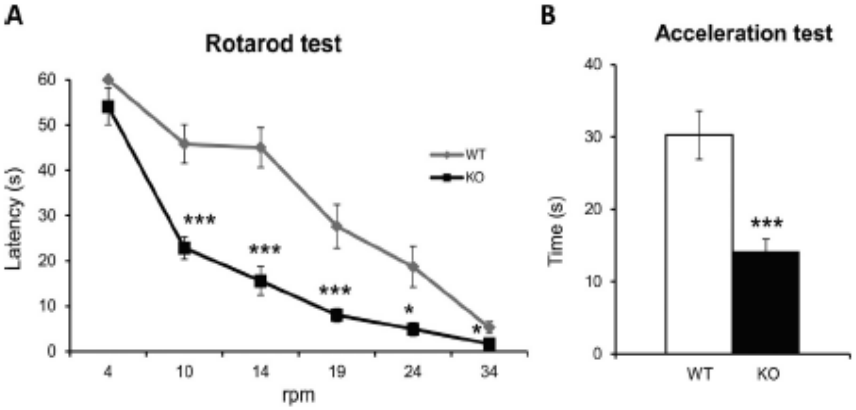
3.2. Cpt1c KO mice are hypoactive

To further analyze the motor phenotype, we performed a 24-h actimetry test (Fig. 2) to measure daily locomotor activity. Mice in this test were 14 weeks old. Results showed that locomotor activity was strongly reduced in Cpt1c KO mice throughout the circadian period. Hypoactivity affected the animals during the first 2 h after entering the new cage (exploratory activity), and during the dark period (feeding time). The Student's t-test was applied to measure differences between genotypes, at each specific hour. In sum, total locomotor activity was reduced to 70% (WT: $51.6 \pm 6.4 \times 10^3$ beam breaks; KO: $35.9 \pm 2.1 \times 10^3$ beam breaks; Student's t-test, $P < 0.01$).

3.3. Motor deficiencies appear in young animals and worsen progressively with age

To determine the age of onset of motor impairment, we performed the rotarod test, the bar hang test, and the 24-h actimetry test in mice aged 6–24 weeks. The same animals (7 WT mice and 7 KO mice) were used in all tests during development. As shown in Fig. 3, motor deficiencies were present in young Cpt1c KO mice, and increased gradually with age. The Mann–Whitney U test was applied to determine the statistical significance in the differences between genotypes for each age and behavioral test.

Coordination measured by the rotarod test was statistically impaired in Cpt1c KO mice at 7 weeks of age at high rotational speeds (≥19 rpm) (Mann–Whitney U test, $P < 0.05$ at 19 rmp, $P < 0.01$ at 24 and 34 rpm). With age, incoordination increased progressively and was even observed at 9 rpm speed in mice aged 11 weeks (Mann–Whitney U test, $P < 0.05$) (Fig 3A). In the accelerating rod, latency to fall off the rod was clearly reduced in Cpt1c KO mice at all ages analyzed (Mann–Whitney U test, $P < 0.05$) (Fig. 3B), indicating that coordination impairment is probably present at even younger ages.



E

	WT	KO
Latency to fall (s)	60±0	42±7**
Fall (%)	0	67***
Latency to use 1 hind limb (s)	6±3	43±7***
Use of 1 hind limb (%)	100	33*

Fig. 1. Motor function deficit in Cpt1c KO mice. (A) Rotarod test. Evaluation of performance during consecutive trials with increasing rotational speeds. (B) Accelerating rod test. Rotation speed was increased from 4 to 40 rpm during a single session of 1 min. (C) Paw print test. The distance between 2 steps using the same limb is measured over a distance of 20 cm. (D) Grip strength meter. Measurement of forelimb grip strength. The test was performed for 60 s. (E) Bar hang test. Latency to fall and to use the hindlimbs to climb up the bar was measured with a 60-s cutoff time. The percentage of animals that fell or used one hindlimb to climb up the bar is shown. Data are represented as mean ± SEM ($n = 12$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

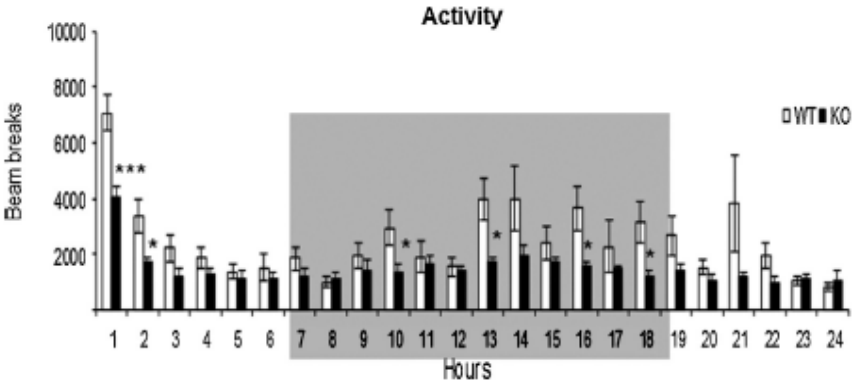


Fig. 2. Locomotor activity (actimetry) over a 24-h period. Locomotor activity in actimetry boxes measured per hour. The grey rectangle represents dark hours. Data are represented as mean ± SEM ($n = 12$). * $P < 0.05$; *** $P < 0.001$.

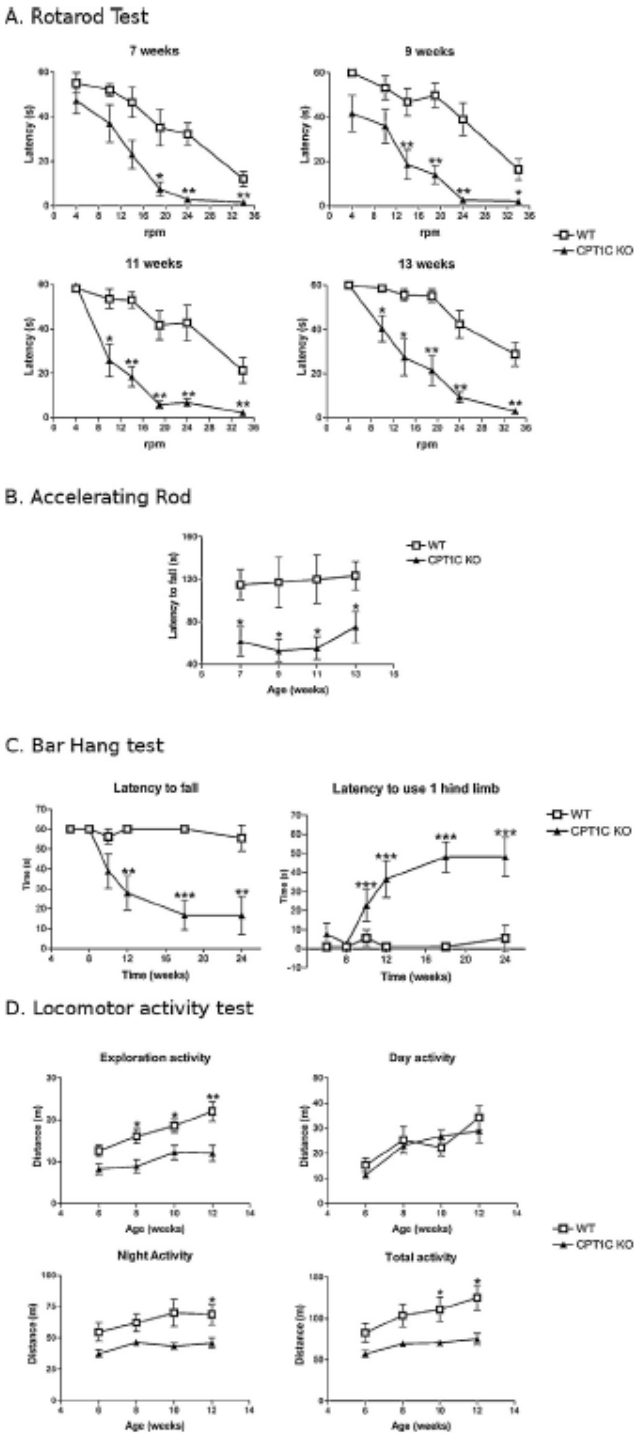


Fig. 3. Motor function at different ages. The rotarod test (A) and accelerating rod test (B) were performed at 7, 9, 11 and 13 weeks of age. The sessions on the rotarod lasted 1 min. In the accelerating rod the velocity increased from 4 to 40 rpm in 3 min. (C) Bar hang test. Latency to fall from the bar and latency to use 1 hindlimb to climb up the bar was measured at 6, 8, 10, 12, 18 and 24 weeks of age. (D) Locomotor activity during a 24-h cycle. Locomotor activity was measured at 6, 8, 10 and 12 weeks of age. Exploration activity (the first 2 h after entering the new cage), day activity (activity during the light phase), night activity (activity during the dark phase) and total activity (24 h) are shown. The same group of animals was used in all the tests. Data are represented as mean ± SEM ($n = 7$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

The bar hang test, which measures mainly muscle strength, revealed no differences between genotypes at an early age (6 weeks of life). However, a clear impaired ability to remain hanging on the bar or climb up over it was observed at 10 weeks of age, with impairment increasing progressively and peaking at 18 weeks of age (Mann–Whitney U test, $P < 0.001$) (Fig. 3C)

General locomotor activity was also measured at several ages (Fig. 3D). At 6 weeks of age, KO mice were slightly hypoactive but the differences were not statistically different. At 8 weeks of age the exploration activity (the first 2 h after entering a new cage) was reduced in KO mice (Mann–Whitney U test, $P < 0.05$) and differences between genotypes increased with age (Mann–Whitney U test, $P < 0.01$ at 12 weeks of age). Locomotor activity during the dark phase (night activity) was reduced in KO mice at the age of 12 weeks (Mann–Whitney U test, $P < 0.05$). Locomotor activity during the light phase (day activity) showed no differences between genotypes. Finally, total locomotor activity (24-h period) in Cpt1c KO mice was gradually reduced with age, showing statistically significant differences at 10 weeks of age (Mann–Whitney U test; $P < 0.05$).

3.4. CPT1C expression during development

We studied CPT1C expression in different brain regions during development and found that CPT1C protein levels in the three brain regions analyzed (cerebellum, striatum and motor cortex) were low from birth to postnatal day 10 (P10), at which point they increased gradually and peaked on postnatal day 21. For the statistical analysis, the data were considered to follow a normal distribution, and the Student's t-test was applied to compare CPT1C expression on each postnatal day with P10 values. In adulthood, CPT1C expression levels were substantially reduced in the striatum and cerebellum, but not in the motor cortex, where CPT1C expression remained elevated at 8 weeks of age (Fig. 4). All these results suggest that the main function of CPT1C occurs after weaning and that its absence causes a progressive deterioration of motor abilities from a young age to early adulthood.

3.5. Cpt1c KO mice have reduced levels of ceramide and sphingosine in the cerebellum, striatum, and motor cortex

As CPT1C is involved in the synthesis of ceramide in neurons [5], we decided to measure ceramide and sphingosine (a ceramide derivative) in different brain regions involved in motor function from Cpt1c KO and WT mice. We analyzed under *ad libitum* and fasting conditions based in the knowledge that levels of malonyl-CoA (the physiological inhibitor of CPT1 enzymes) in the brain are modified according to the energy status of the animals, with levels being high after feeding and reduced during fasting [13]. Fig. 5 shows that the levels of C18:0 ceramide, the most abundant ceramide in the brain [14], were reduced in Cpt1c KO mice in the cerebellum, motor cortex and striatum. This reduction was higher during fasting, when the levels of malonyl-CoA were diminished. A similar pattern was observed for sphingosine. The Student t-test was applied to compare genotypes for each feeding condition and to compare feeding conditions for each genotype. These results indicate that ceramide metabolism is impaired in the cerebellum, striatum, and motor cortex in Cpt1c KO mice, mainly during the fasting state.

4. Discussion

The brain specific isoform CPT1C was first described in 2002 [1]. Numerous studies have described its hypothalamic role in the regulation of food intake and energy homeostasis [1,6–8,15,16]. However, CPT1C is not only expressed in the hypothalamus but also

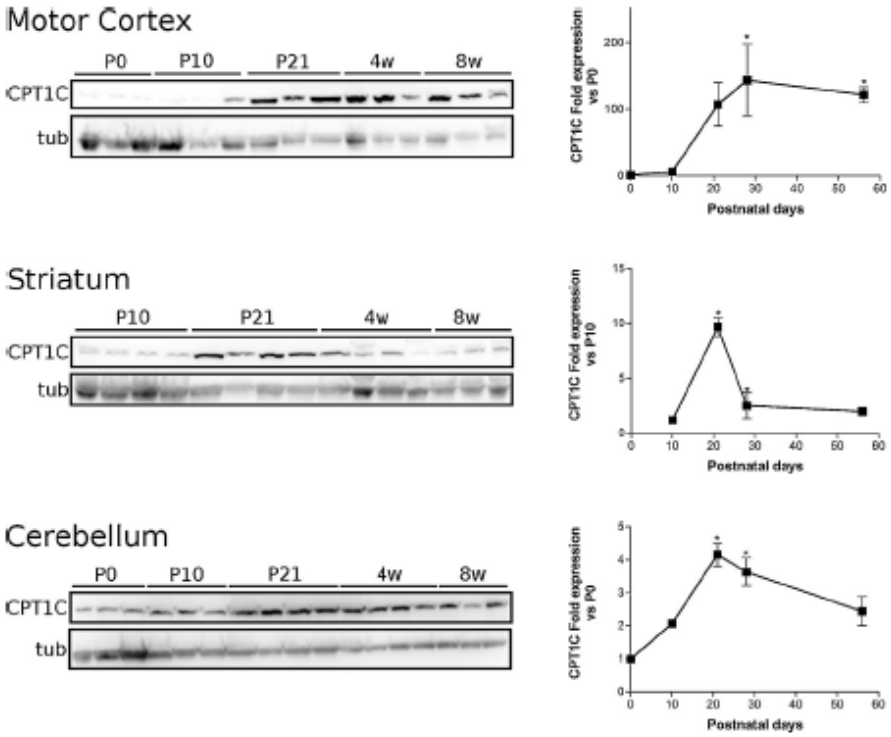


Fig. 4. CPT1C expression in the motor cortex, striatum and cerebellum during mouse development. CPT1C protein levels were measured using Western blot analysis at different postnatal days. CPT1C levels were normalized by the tubulin (tub) expression. Data are represented as mean ± SEM ($n = 4$). * $P < 0.05$.

throughout the brain, involving areas that include the hippocampus, cortex and cerebellum. [1]. Our group has recently shown that this enzyme is involved in spatial learning by regulating the maturation of dendritic spines in hippocampal neurons [5]. By studying the behavioral phenotype of Cpt1c-deficient mice, the present work extends the range of known functions in which this protein is involved. Cpt1c-deficient mice show clear motor deficits such as impaired coordination, imbalance, and muscle weakness. In addition, these mice show reduced locomotor activity during the dark period (feeding time) and during the exploration of a new cage. It is remarkable to note that the autonomous and sensory systems of these animals are not affected.

In our study, motor dysfunction in Cpt1c KO mice was observed at a young age (6 weeks) and increased progressively with age. Our finding show that a deficiency in CPT1C, a protein expressed mainly in neurons causes progressive impairment in neuronal function, suggesting that some kind of neurodegeneration is taking place. Incoordination, impaired balance and hypoactivity appear at earlier ages than muscle weakness, suggesting that neuronal deterioration develops in a specific timeframe that varies depending on the type of neurons.

Remarkably, CPT1C levels were found to increase greatly at postnatal day 21, the precise moment of weaning, in the 3 motor brain regions analyzed. This indicates that CPT1C expression is triggered by weaning, and that CPT1C function is relevant from postnatal day 21 to adulthood. These data allow us to hypothesize that motor deficits are probably inexistent before weaning and that the onset of motor disorders occurs at between 3 and 7 weeks of life.

An interesting finding of the study is that Cpt1c KO mice have reduced levels of ceramide and sphingosine in the cerebellum, striatum, and motor cortex. It has been previously demonstrated that CPT1C regulates the levels of ceramides in neurons (Cpt1c overexpression increases ceramide levels while Cpt1c deletion reduces them [5]), and therefore it is not unreasonable to conclude that CPT1C modulates ceramide levels in those brain regions, mainly at young ages and in the early adulthood

Some authors have described the role of ceramide in the development and survival of neurons. In fact, ceramide and its metabolite sphingosine have been reported to be lipidic factors necessary for cerebellar Purkinje cell survival and dendritic differentiation [17], and a reduction in ceramide synthesis in the brain causes cerebellar ataxia and Purkinje cell neurodegeneration [18]. At the same time, ceramide treatment of motoneurons prevents cell death through the inhibition of oxidative signals [19]. Other authors have described ceramide as a neuroprotector against oxidative insults [20]. Our group has also demonstrated that ceramide is necessary for adequate maturation of dendritic spines in hippocampal neurons [5]. Notably, alterations in both simple and complex sphingolipid composition also occur in the brains of patients with neurodegenerative diseases and in the aging brain [14,18]. Thus, it is not unreasonable to propose that altered ceramide levels in these motor brain areas are the cause of motor deficits.

Taking into account the alterations in energy homeostasis present in Cpt1c KO mice, however, it is possible that muscle weakness may also be a consequence of reduced fatty acid oxidation in muscles [21], a metabolic disturbance described in Cpt1c KO mice [1,6]. On the other hand, we cannot rule out that hypothalamic dysfunction in Cpt1c KO mice [2,6] is a contributing factor to reduced general locomotor activity.

In summary, our findings show that CPT1C deficiency results in the progressive impairment of motor function and daily locomotor activity, with onset occurring before the adult stage, when CPT1C expression in motor brain areas is high. In addition, ceramide levels in the cerebellum, striatum and motor cortex are significantly reduced in Cpt1c KO mice suggesting that CPT1C is involved in the control of ceramide levels in those brain regions, and that this biolipid plays a role in the degeneration of the motor phenotype. To date, no Cpt1c mutations have been described in any human disorder affecting motor function. Further studies will be needed

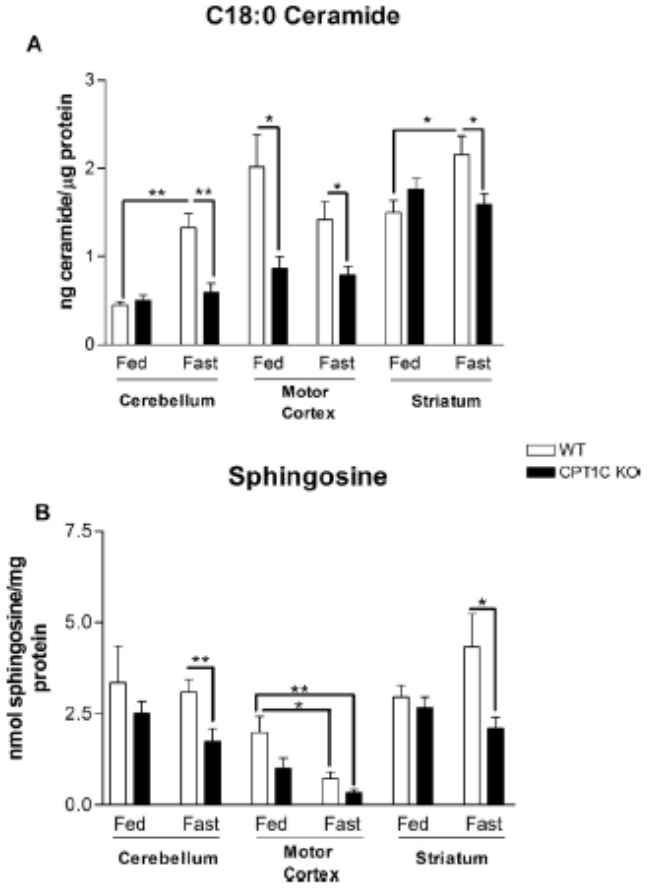


Fig. 5. Ceramide and sphingosine levels in the brain. Ceramide C18:0 (A) and sphingosine (B) levels were measured by LC-ESI-MS/MS under fed and fasting conditions in different brain areas in adult (8 weeks of age) WT and Cpt1c KO mice. Data is presented as mean ± SEM ($n = 10$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

to determine whether Cpt1c is mutated in patients suffering from idiopathic motor degeneration.

5. Conclusions

The present work demonstrates that Cpt1c deficiency, in addition to causing disturbances in peripheral energy metabolism [2,6] and impaired spatial learning [5], produces a progressive deterioration of motor function starting at a young ages and continuing into early adulthood, resulting in motor incoordination, muscle weakness, and hypoactivity. In addition, ceramide and sphingosine levels in the cerebellum, striatum, and motor cortex are lower in Cpt1c KO mice when compared to WT mice. Our results suggest that ceramide levels regulated by CPT1C play an important role in brain regions that control motor function.

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Ceramide levels regulated by carnitine palmitoyltransferase 1C control dendritic spine maturation and cognition.

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Ceramide Levels Regulated by Carnitine Palmitoyltransferase 1C Control Dendritic Spine Maturation and Cognition*

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Background: CPT1C is highly expressed in hippocampus, but its cellular and physiological function is unknown.
Results: CPT1C overexpression increases ceramide levels, and CPT1C deficiency impairs dendritic spine morphology and spatial learning.
Conclusion: Regulation of ceramide levels by CPT1C is necessary for proper spine maturation.
Significance: We describe a new function of CPT1C in cognition.

The brain-specific isoform carnitine palmitoyltransferase 1C (CPT1C) has been implicated in the hypothalamic regulation of food intake and energy homeostasis. Nevertheless, its molecular function is not completely understood, and its role in other brain areas is unknown. We demonstrate that CPT1C is expressed in pyramidal neurons of the hippocampus and is located in the endoplasmic reticulum throughout the neuron, even inside dendritic spines. We used molecular, cellular, and behavioral approaches to determine CPT1C function. First, we analyzed the implication of CPT1C in ceramide metabolism. CPT1C overexpression in primary hippocampal cultured neurons increased ceramide levels, whereas in CPT1C-deficient neurons, ceramide levels were diminished. Correspondingly, CPT1C knock-out (KO) mice showed reduced ceramide levels in the hippocampus. At the cellular level, CPT1C deficiency altered dendritic spine morphology by increasing immature filopodia and reducing mature mushroom and stubby spines. Total protrusion density and spine head area in mature spines were unaffected. Treatment of cultured neurons with exogenous ceramide reverted the KO phenotype, as did ectopic overexpression of CPT1C, indicating that CPT1C regulation of spine maturation is mediated by ceramide. To study the repercussions of the KO phenotype on cognition, we performed the hippocampus-dependent Morris water maze test on mice. Results show that CPT1C deficiency strongly impairs spatial learning. All of these results demonstrate that CPT1C regulates the levels of ceramide in the endoplasmic reticulum of hippocampal neurons, and this is a relevant mechanism for the correct maturation of dendritic spines and for proper spatial learning.

Carnitine palmitoyltransferase 1 (CPT1)[5] enzymes catalyze the conversion of long-chain acyl-CoA to acyl-carnitines, thus facilitating the transport of long-chain fatty acids across intracellular membranes. There are three isoforms: the liver isoform CPT1A (1), the muscle isoform CPT1B (2), and the brain-specific isoform CPT1C (3). CPT1A and CPT1B are localized in the outer mitochondrial membrane and are rate-limiting enzymes in fatty acid β-oxidation.

The main isoform in brain, CPT1C, highly differs from the two other isozymes. Its C-terminal region is longer than that of the other CPTs (3) and is located in the endoplasmic reticulum (ER) of cells rather than in mitochondria (4). It has low CPT1 activity (4), but it binds the CPT1 physiological inhibitor malonyl-CoA with the same affinity as CPT1A (5). Finally, CPT1C is only present in mammals and appears to stem from a relatively recent *cpt1a* gene duplication (3). The other isozymes are expressed in such organisms as fish, reptiles, amphibians, or insects. This suggests a specific role for CPT1C in more evolved brains.

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[5] The abbreviations used are: CPT1, carnitine palmitoyltransferase 1; ER, endoplasmic reticulum; MWM, Morris water maze; DIV, day(s) *in vitro*; AAV, adeno-associated virus; ANOVA, analysis of variance; EGFP, enhanced GFP.

At the physiological level, CPT1C contributes to the control of food intake and energy homeostasis (5, 6). Two independent groups developed a CPT1C knock-out (KO) mouse, and both lines showed decreased food intake with respect to wild-type (WT) animals. However, when fed a high fat diet, they were more susceptible to obesity and diabetes, presenting lower rates of peripheral fatty acid oxidation. All of these effects were attributed to the hypothalamic function of CPT1C because ectopic overexpression of CPT1C in hypothalamus protected mice from adverse weight gain caused by a high fat diet (7). Moreover, the involvement of CPT1C in energy homeostasis has also been confirmed in transgenic animals overexpressing CPT1C specifically in the brain (8). At the molecular level, in collaboration with the group of Dr. Gary Lopaschuk, we showed that CPT1C is involved in the anorectic action of leptin, by modulating ceramide synthesis in the arcuate nucleus of the hypothalamus (9).

Interestingly, recent findings in tumor cells showed a new, unexpected role of CPT1C in the metabolic transformations reported in tumor cell growth (10). The authors demonstrated that CPT1C is frequently expressed in human lung tumors and protects cancerous cells from death induced by glucose deprivation or hypoxia. The results suggest that CPT1C might provide unidentified fatty acid-derived products that would be beneficial for cell survival under metabolic stress.

However, despite these recent findings about CPT1C, little is known about its physiological role during brain development and function. The finding that CPT1C is highly expressed in hippocampus (3) prompted us to look after other brain CPT1C functions beyond the control of energy homeostasis. Our results show that CPT1C is located in the ER of hippocampal neurons and regulates the maturation of dendritic spines by increasing ceramide levels. At the behavioral level, we demonstrate for the first time that CPT1C is involved in spatial learning.

EXPERIMENTAL PROCEDURES

Construction of Targeting Vector and Generation of KO Mice—A construct was generated using the pPNT vector (11). After correct recombination, this vector caused a 2.9-kb genomic deletion, including exons 12–15. The targeting construct was electroporated into 129/SvEv embryonic stem cells (ESC) by the Centre de Biotecnologia Animal i Teràpia Gènica at the Universitat Autonoma de Barcelona. Two positive ESC clones were expanded and verified for correct recombination by PCR amplification and Southern blot analysis. $CPT1C^{+/-}$ cells were injected into C57BL/6J blastocyst. Chimeric mice displaying >50% coat color chimerism were bred with C57BL/6J females to generate F1 offspring. The sixth backcrossed generation was used in all of the experiments.

Animal Housing—In behavioral studies, only males at 10–14 weeks of age were tested ($n = 12$). All of the behavioral testing was conducted by the same experimenters, blinded as to the genetic status of animals, in an isolated room and at the same time of day. All animal procedures met the guidelines of European Community Directive 86/609/EEC (EU directive 86/609, EU decree 2001-486) and Standards for Use of Laboratory Animals A5388-01 (National Institutes of Health) and were approved by the local ethics committee.

Morris Water Maze (MWM) Test—To test hippocampus-dependent spatial cognition, the MWM test was used, as described elsewhere (12). The water maze consisted of a circular pool (diameter 1.20 m, height 0.5 m). A white escape platform (15-cm diameter, height 24 cm) was located 1 cm below the water surface in a fixed position (northeast quadrant, 22 cm away the wall). All of the trials were recorded and traced with an image tracking system (SMART, Panlab, Spain) connected to a video camera. Escape latencies, length of the swimming paths, and swimming speed for each animal and trial were monitored and computed.

Cell Cultures and Plasmid Transfection—Hippocampal cultured neurons were obtained and cultured as described elsewhere (13). For plasmid transfection, neurons were grown for 14 days *in vitro* (DIV), transfected using the Effecten kit (Qiagen), and analyzed at 15 DIV. After transfection, neurons were fixed with 4% paraformaldehyde and 4% sucrose in PBS. Samples were mounted using Gel/Mount anti-fading medium (Invitrogen).

Virus Development and Cell Culture Infection—Two adeno-associated virus (AAV) vectors, serotype 1, AAV1-GFP, AAV1-CPT1C were constructed to drive cell expression of GFP and CPT1C, respectively. Vector plasmids carried the transgene expression cassette, including the cytomegalovirus promoter, the cDNA sequence of GFP and the rat CPT1C (3), the woodchuck posttranscriptional regulatory element (accession number AY468 486) to enhance transcription (14), and the bovine growth hormone polyadenosine transcription termination signal (bGH poly(A)) (bases 2326–2533, GenBank™ accession number M57764). The expression cassette was flanked by two inverted terminal repeats derived from AAV serotype 2. AAV1 vectors were produced in insect cells using baculovirus (15). The vector preparations used in this study had titers of 1×10^{12} and 2.5×10^{12} genome copies/ml for AAV1-GFP and AAV1-CPT1C, respectively.

AAV1-CPT1C virus infection was performed at 7 DIV in cells cultured in 6-well plates. Medium was removed and kept apart to be reused later. 0.5 ml of neurobasal medium without B27 and containing 0.5 mM glutamine and AAV at a concentration of 100,000 viruses/cell was added to each well and left to stand for 2 h. Then 1.5 ml of the preconditioned medium kept apart was added and left to stand for a further 7 days. Then cells were removed for analysis of CPT1C expression and ceramide levels. Myriocin (Sigma) treatment was performed 8 h before cell recollection.

Immunodetection in Brain Sections and Cultured Cells—Coronal sections (30 μm) from adult mouse forebrains were incubated with primary antibodies against glial fibrillary acidic protein (1:500; Chemicon MAB360) and CPT1C (1:100) overnight at 4 °C, washed three times in PBS (0.1 M), and incubated for 2 h with secondary antibodies coupled to fluorochromes Alexa 488 (for green fluorescence) and Alexa 568 (for red fluorescence) at a dilution of 1:500. In cultured neurons, anti-calreticulin polyclonal antibody (BD Biosciences) was used at a dilution of 1:50 for 1 h at 37 °C, and for the red fluorescence, the secondary antibody goat anti-mouse Alexafluor 546 (Molecular

Probes) (1:500) was used. Sections and coverslips were mounted with Mowiol and observed using a Confocal Leica TCS SP2 (Leica Lasertechnik GmbH, Mannheim, Germany).

Image Analysis and Quantification of Dendrite Spine Density—Images were acquired using a digital camera (SpotRT, Diagnostic Instruments) attached to an epifluorescence microscope (Zeiss) equipped with a ×63 objective (Plan-Apochromat, Zeiss). All quantitative measurements were carried out using MetaMorph software (Molecular Devices). Approximately 100 dendrites from independent transfections were randomly selected for each construct to quantify the number of protrusions in proximal 50-mm sections of dendrites. Lengths of protrusions were determined by measuring the distance between the tip and the base.

Western Blot Analysis—Rabbit antibodies against the C-terminal region of mouse CPT1C and against CPT1A were as described elsewhere (4). Generally, 60 μg of protein extracts were subjected to SDS-PAGE. Dilutions of 1:500 and 1:1000 of anti-CPT1C and anti-CPT1A primary antibodies, respectively, were used. A 1:5000 dilution of secondary antibody was used. The blots were developed with the ECL Western blotting system (Amersham Biosciences).

Ceramide Quantification—Ceramides were extracted and analyzed via an LC-electrospray ionization-MS/MS system (API 3000 PE Sciex) in positive ionization as described elsewhere (16). Their concentrations were measured by MRM experiments using *N*-heptadecanoyl-D-*erythro*-sphingosine (C17-ceramide) as an internal standard (50 ng/ml). The method was linear over the range from 2 to 600 ng/ml.

Cell Feeding with Deuterated Serine—Hippocampal neurons at 14 DIV were treated with 4 mM DL-serine-d_7 (CDN Isotopes, Cluzau Infolab) for different times. Ceramides were extracted, and two C18:0 deuterated ceramides were identified with an orbitrap mass spectrometer (Thermo Scientific). These ceramides were subsequently quantified using LC-electrospray ionization-MS/MS (API 3000 PE Sciex). The most abundant analyte corresponded to ceramide C18:0-d_3, determined by a Q1 m/z = 569,567 and Q3 m/z = 267,287. Areas under the peak were measured and normalized with sample protein concentration.

Statistics—Data are expressed as means ± S.E. Statistical significance was determined by Student's t test for the difference between two normal groups, and the Mann-Whitney U test was used for non-normal distribution. One-way ANOVA with Bonferroni test for post hoc analysis was used for more than two groups. Performances in the MWM tests were compared using repeated measures ANOVA.

RESULTS

CPT1C Is Located throughout ER of Hippocampal Neurons, Even Penetrating into Dendritic Spines—It was previously described that CPT1C is highly expressed in the hippocampus (3). In order to determine the precise localization of the protein, we performed brain sections and incubated them with an anti-CPT1C antibody, kindly provided by the Wolfgang laboratory and previously used in the literature (4, 7). Fig. 1*A* clearly shows that CPT1C (in *green*) is expressed in pyramidal neurons of the hippocampus. Astrocytes were identified by glial fibrillary acidic protein (an astrocyte marker) antibody.

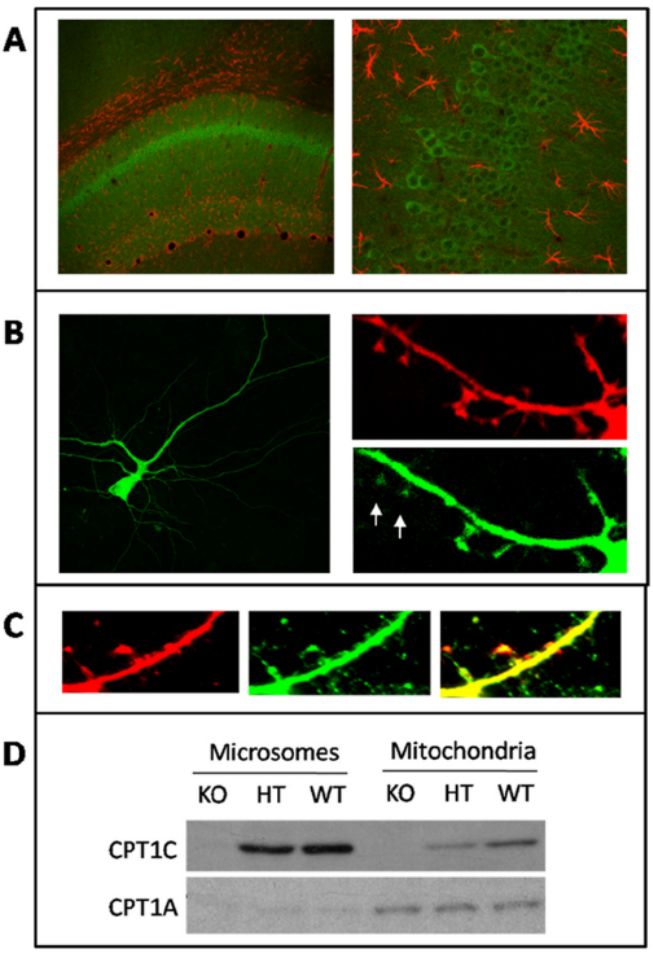

FIGURE 1. **CPT1C location in hippocampal neurons.** *A*, CPT1C is present in neurons of the hippocampus, mainly pyramidal cells. Brain sections were double-immunodetected with anti-CPT1C antibody (*green*) and anti-glial fibrillary acidic protein antibody (*red*). *B*, hippocampal cultured neurons were double-transfected with pCPT1C-EGFP and pDS-Red at 11 DIV and visualized at 15 DIV. Images show that CPT1C is present in neuronal body, dendritic shaft, and spines (marked with *arrows*). pDs-Red transfection was performed to display the outline of the neuron. *C*, hippocampal cultured neurons were transfected with pDS-ER-Red to stain the ER. At 15 DIV, cells were immunodetected with anti-CPT1C antibodies (*green*). The *merge image* (*yellow*) demonstrates that CPT1C is localized to the ER membrane. *D*, Western blot analysis of CPT1C and CPT1A proteins in isolated microsomes and mitochondria from hippocampus of WT, heterozygous (*HT*), and KO mice.

To analyze the detailed localization of CPT1C in hippocampal neurons, we performed neuronal primary cultures and transfected them with pCPT1C-EGFP, a plasmid that encodes CPT1C fused to the N-terminal region of green fluorescence protein (EGFP) (4). Fig. 1*B* shows that CPT1C is located throughout the neuron, in neuronal bodies and dendrites. Detailed photographs of dendrites demonstrate that CPT1C is present mainly in shafts but also in spines (marked with *arrows*). The same cultures were transfected with pDS-Red (Clontech) that encodes the *Discosoma* sp. red fluorescent protein in the cytosol to display the outline of the cell.

To confirm that subcellular localization of endogenous CPT1C was in the ER, we transfected the cultured neurons with pDs-ER-Red2 (Clontech), which stains the ER *red*, and immu-

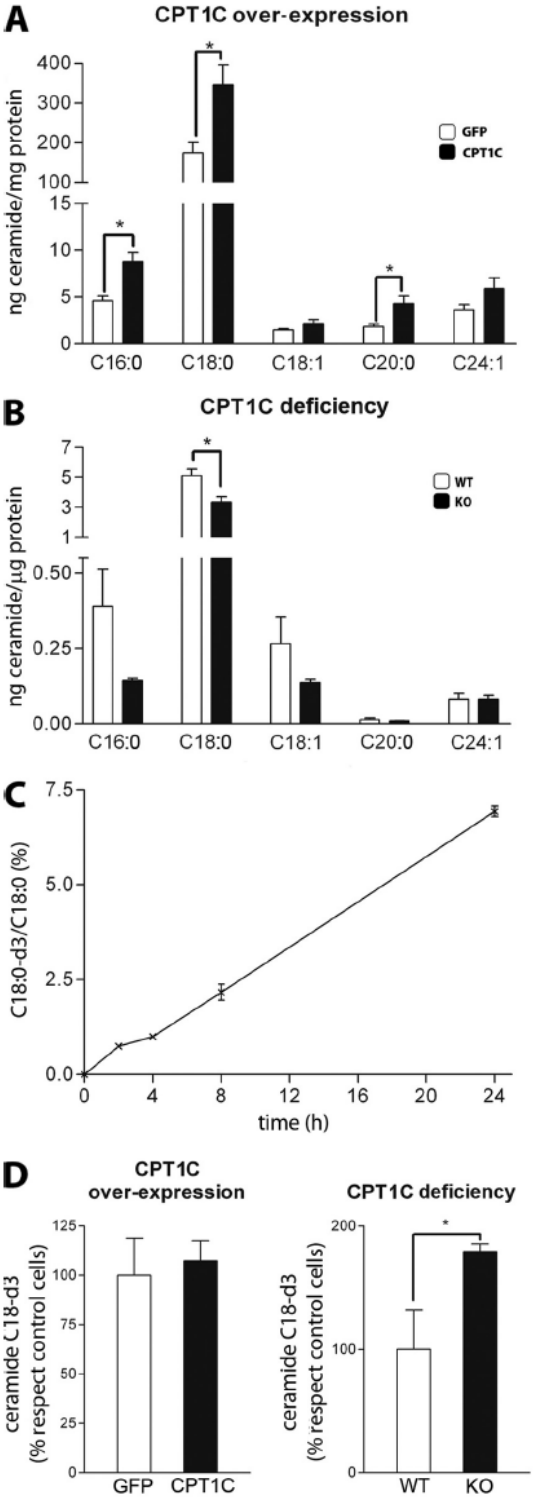

FIGURE 2. **Regulation of ceramide levels by CPT1C.** *A*, levels of ceramides in hippocampal neurons transduced with AAV1-GFP (as a control) or AAV1-CPT1C at 7 DIV. Cells were collected at 14 DIV. *B*, levels of ceramides in hippocampal neurons from WT and CPT1C KO mice. Cells were collected at 14 DIV. *C*, time course of incorporation of serine-d_7 into ceramide C18:0-d_3. Hippocampal cultured neurons from WT animals were treated with 4 mM serine-d_7 at DIV14. Ceramides C18:0 and C18:0-d_3 were analyzed at different times, and the percentage of incorporation is shown. *D*, effect of CPT1C overexpression and CPT1C deficiency on serine-d_7 incorporation into ceramide C18:0-d_3. Hippocampal cultured cells were transduced with AAV1-GFP (as a control) or AAV1-CPT1C at 7 DIV. Cells were treated with serine-d_7 at DIV 14 and collected after 2.5 h of treatment. The percentage of variation in ceramide C18:0-d_3 levels compared with the control cells is shown. *Error bars*, S.E.; $n = 6$; *, $p < 0.05$.

nodetected endogenous CPT1C with anti-CPT1C antibody (in *green*). Fig. 1*C* shows that CPT1C is localized in the ER of cultured hippocampal neurons. Finally, Western blot experiments were also performed with different cellular fractions from mouse brain. Anti-CPT1A antibodies were used as a marker for mitochondria. Samples were retrieved from WT, CPT1C KO, and heterozygous mice developed in our laboratory (described under "Experimental Procedures"). Fig. 1*D* shows that CPT1C is present mainly in the microsomal fraction and that CPT1A is present mainly in mitochondria, confirming that CPT1C localizes to the ER membrane of cells.

CPT1C Regulates Levels of Ceramide in Cultured Neurons—Our group has recently reported that CPT1C regulates ceramide synthesis in arcuate nucleus of the hypothalamus as part of the signaling pathway of leptin (9). We wanted to examine whether CPT1C was also regulating ceramide levels in hippocampal neurons. We overexpressed CPT1C in primary hippocampal neurons using AAV1-CPT1C viruses. A 4-fold increase in CPT1C protein levels resulted in a 2-fold increase in ceramide levels with respect to control cells (cells infected with AAV1-GFP) (Fig. 2*A*). CPT1C overexpression mainly increased saturated ceramides (C16:0, C18:0, and C20:0). Ceramide C18:0 was the most abundant one in the hippocampal cultures, being 20 times more concentrated than the rest. We also measured ceramide levels in hippocampal cultured neurons from CPT1C KO mice. As shown in Fig. 2*B*, C18:0 ceramide levels were lower in KO cells than in WT cells, confirming that CPT1C regulates ceramide levels in hippocampal neurons.

Because ceramide present in ER comes mainly from *de novo* synthesis, we examined whether CPT1C was activating this pathway. A 24-h pulse with 4 mM deuterated serine was first carried out in control neurons. The incorporation of deuterated serine into ceramide was linear during the first 24 h, at which point it reached a level of 7% (Fig. 2*C*). Because we were able to measure deuterated ceramide at the short time of 2 h, we decided to perform the next experiments at 2.5 h to minimize interference with other ceramide metabolic pathways. Fig. 2*D* shows that deuterated ceramide synthesis was not increased by CPT1C overexpression and was not decreased in CPT1C KO cells. These results clearly indicate that CPT1C does not activate the *de novo* synthesis of ceramide and suggest that CPT1C regulates ceramide levels by acting on another metabolic pathway. Surprisingly, the *de novo* synthesis of ceramide was even increased in CPT1C KO cells, suggesting that these neurons were activating this basal pathway to counterbalance the reduction in ceramide levels caused by CPT1C deficiency.

CPT1C KO Mice Have Reduced Ceramide Levels in Hippocampus—To examine whether CPT1C is involved in the regulation of hippocampal ceramide synthesis in adult mice, we measured ceramide levels in hippocampus from WT and CPT1C KO mice under *ad libitum* and fasted conditions. CPT1C KO mice showed lower ceramide levels in hippocampus than WT animals, mainly during fasting (Fig. 3). The most abundant ceramide found in the hippocampus was the C18:0

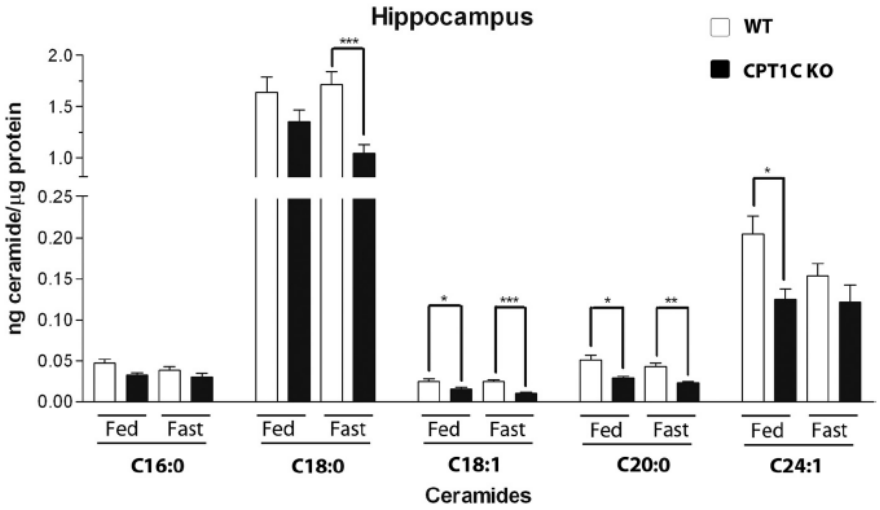

FIGURE 3. **Ceramide levels in hippocampus from *ad libitum* and fasted CPT1C KO and WT mice.** Fasted mice were deprived of food for 15 h. Different ceramide species were measured: ceramide C16:0, ceramide C18:0, ceramide C18:1, ceramide C20:0, and ceramide C24:1. *Error bars*, S.E. $n = 6$; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$, ANOVA test.

ceramide, being about 20 times more abundant than the rest (like in hippocampal cultures), which agrees with previous literature indicating that the major ceramide in brain neurons is C18:0 (17). Importantly, C18:0 ceramide levels were higher in WT than in KO mice during fasting, when it is known that the concentration of malonyl-CoA (the physiological inhibitor of CPT1 enzymes) is highly reduced (18), suggesting that CPT1C activity in the hippocampus is modulated by malonyl-CoA.

CPT1C Deficiency Increases Filopodia Density and Reduces Spine Maturation in Hippocampal Neurons—To examine the effects of CPT1C deficiency on dendritic spine density and morphology, we performed primary hippocampal cultures from CPT1C KO and WT mice, transfected the neurons with green fluorescent protein (GFP), and examined dendritic spines at 15 DIV. Neurons from CPT1C KO mice had the same protrusion density but larger protrusion length than WT neurons (Fig. 4, *A–C*). Morphological analysis revealed that CPT1C KO mice had a strong increase in filopodia number and a marked reduction of mature (mushroom and stubby) spines (Fig. 4, *D–G*). However, the spine head area in mature spines was the same in both genotypes (Fig. 4*H*). Overexpression of CPT1C on KO cultures reduced filopodia density and increased the percentage of mature spines to values similar to WT cultures (Fig. 5, *B* and *D*), confirming the requirement of CPT1C for efficient spine maturation.

Ceramide Treatment Rescues CPT1C KO Phenotype on Spine Morphology—To corroborate that the reduction in ceramide synthesis caused by CPT1C deletion is the cause of the spine phenotype, we set up a rescue experiment in which CPT1C KO hippocampal cultures were incubated with 1.5 μM soluble C-6 ceramide for 7 days (from DIV 8 to 15). The ceramide dose used (<3 μM) does not induce neuronal apoptosis in hippocampal cultures (19). Exogenous ceramide treatment reversed the CPT1C KO phenotype by decreasing immature filopodia and restoring mature spine density to normal levels (Fig. 5, *A* and *D*). These results indicate that CPT1C regulation of spine maturation is mediated by ceramide.

Finally, to confirm that a reduction in ceramide levels is the cause of impaired spinogenesis, we treated hippocampal cultured neurons with myriocin, an inhibitor of ceramide biosynthesis, which has been described to reduce ceramide levels in cultured neurons (20). As shown in Fig. 5, *C* and *D*, myriocin treatment increased the density of filopodia and reduced the percentage of mature spines, a phenotype that completely resembles that observed in CPT1C KO cells.

CPT1C KO Mice Have Impaired Spatial Learning—To examine the spine maturation defects on cognition, we performed the MWM test. This test is usually used to measure hippocampus-dependent spatial navigation learning in mice. In the MWM, CPT1C KO showed significantly higher escape latency (delayed learning) during the 10 sessions of the acquisition phase (Fig. 6, *A* and *B*). The learning curves were significantly different from those of WT mice (repeated measures ANOVA $F(1.22) = 6.726$, $p = 0.017$) in the absence of swimming speed alteration, indicating pure learning impairment, with poorer performance not associated with motor deficits (Fig. 6*C*). Moreover, in the cued session, where the platform is visible (*black flag*), the escape latency of CPT1C KO mice was similar to that of the WT (Fig. 6*A*).

To test visuospatial memory, the platform was removed, and the time spent in each quadrant was measured. No significant differences between genotypes were detected in the preference for the trained quadrant, indicating that once the platform position was learned, it was equally retained in CPT1C KO and WT mice (Fig. 6*D*); the CPT1C KO deficits seem to be limited to the learning phase.

In the reversal test (Fig. 6*E*), which evaluates the ability of the mice to learn a new platform position (cognitive flexibility), no significant differences were observed between genotypes in the percentage of time spent in the previously trained quadrant (northeast; repeated measures ANOVA, $F(1.22) = 0.086$, $p = 0.772$). However, KO mice spent less time in the new goal quadrant (southwest; repeated measures ANOVA, $F(1.22) = 8.676$,

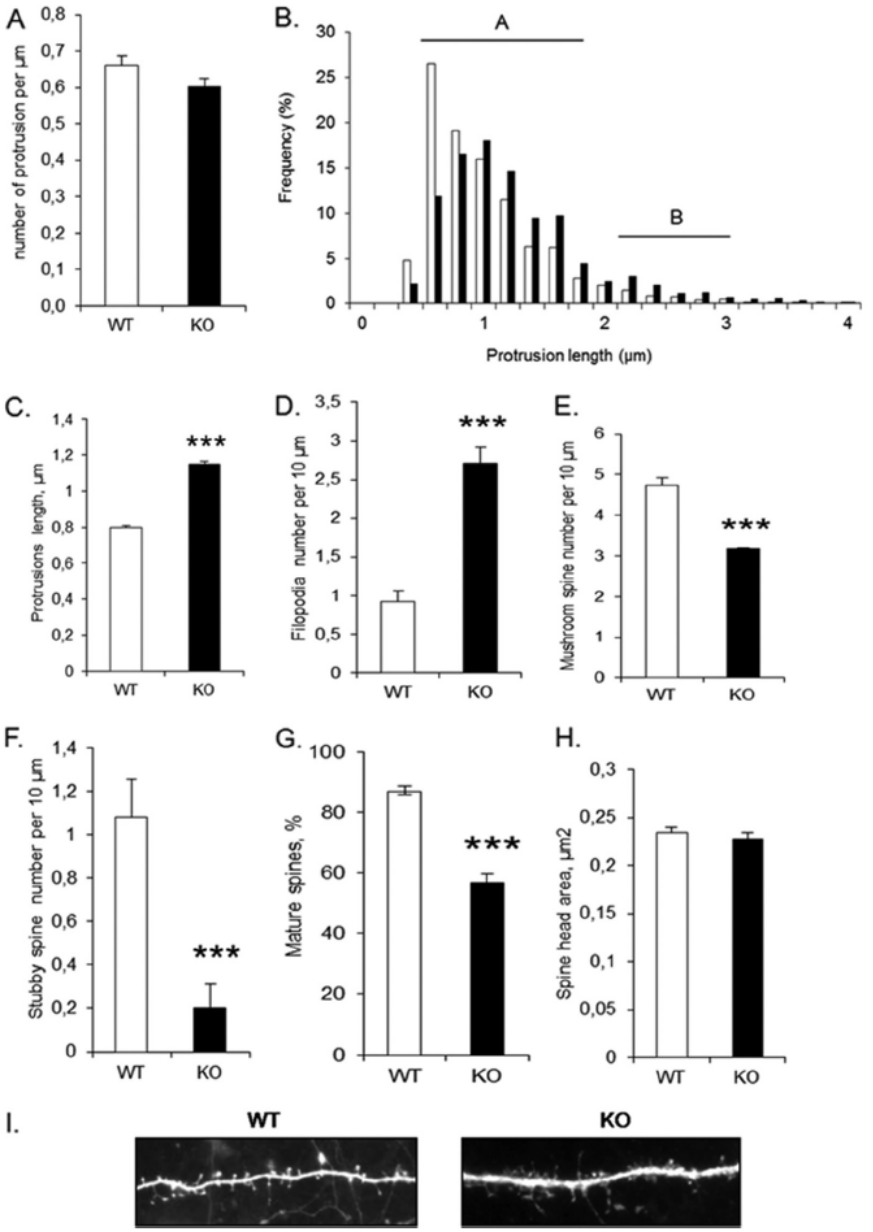

FIGURE 4. **Dendritic spine density and morphology from CPT1C KO and WT hippocampal neurons.** Hippocampal neurons were transfected (13 DIV) with pEGFP to visualize the outline of the cell. Protrusions were analyzed 2 days after transfection. Protrusion density (*A*) and protrusion length (*B* and *C*) were measured. Mature spines (*A*) and filopodia (*B*) are indicated. Spine morphology (*D–F*) was assayed by analysis of types of protrusions: filopodia (without head), mushroom (with head and neck), and stubby (with only head). *G*, percentage of mature spines (mushroom and stubby) relative to the total number of protrusions was also measured. *H*, spine head area was measured in mushroom and stubby spines. *I*, a representative image of dendritic spines from WT and CPT1C KO neurons. For the quantification of protrusion density, spine length, and morphology, ~100 dendrites from independent transfections were selected randomly. Student's *t* tests were used to assess statistical significance of the differences. *Error bars*, S.E.; ***, $p < 0.001$.

$p = 0.007$), thus supporting the hypothesis of a hippocampus-dependent learning deficit in CPT1C KO mice.

DISCUSSION

Dendritic spine formation begins in the embryo and continues into early postnatal life but also occurs in the adult organism, where it contributes significantly to learning and memory formation. We demonstrate that the brain isoform CPT1C is present in dendritic spines and regulates the levels of ceramide in neurons, which is key to the transformation of dendritic filopodia into mature spines. This is the first time that CPT1C or ceramide levels have been directly involved in spine morphogenesis. At the physiological level, we show for the first time that CPT1C is involved in spatial learning.

CPT1C Regulates Ceramide Levels in Neurons—One of the relevant contributions of this study is the confirmation that CPT1C increases the levels of ceramide. We had previously described it in the arcuate nucleus of hypothalamus (9), and we now demonstrate it in hippocampal cultured neurons. In consequence, it may be a general phenomenon in neurons. We do not know the molecular mechanism by which CPT1C increases ceramide levels, but our results clearly demonstrate that it does

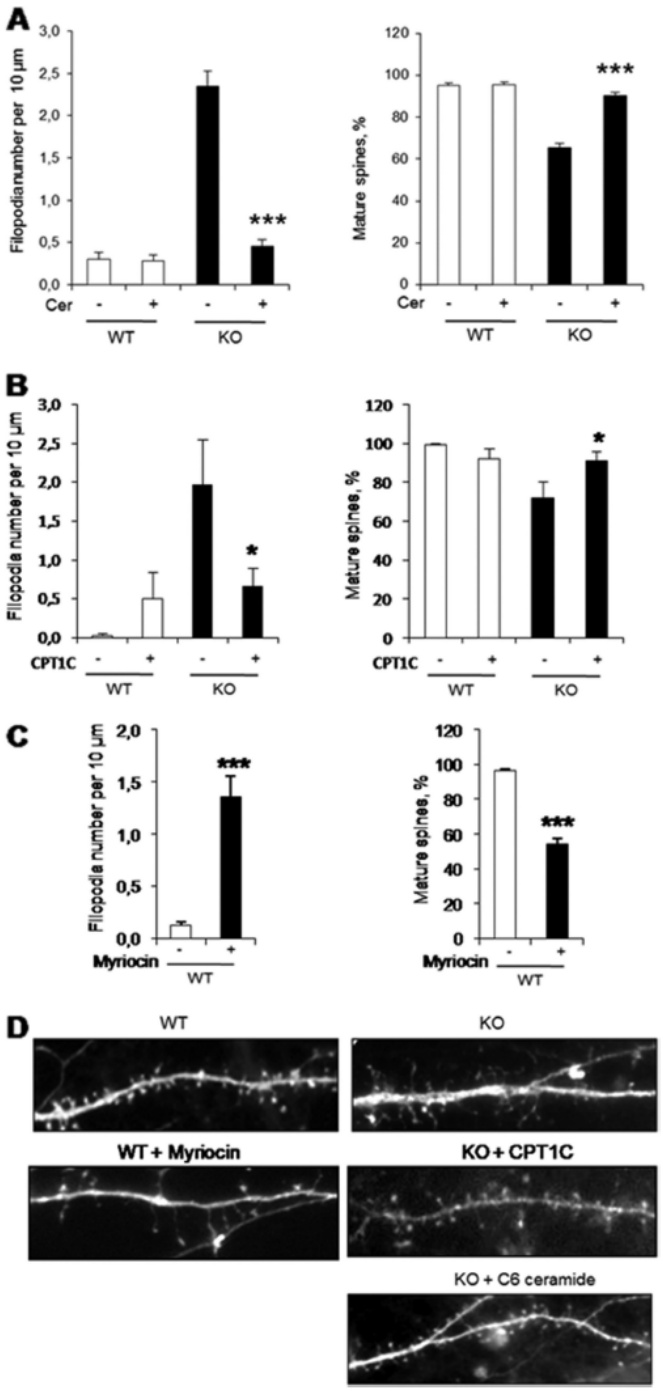

FIGURE 5. **Rescue of CPT1C KO phenotype on spine morphology by CPT1C expression or ceramide treatment.** *A*, hippocampal neurons treated with 1.5 μM C6-ceramide at 7 DIV and transfected with pEGFP (BD Biosciences) at 12 DIV, fixed, and analyzed for the morphology of dendritic protrusions at 15 DIV. *B*, hippocampal neurons were transfected with pIRES-CPT1C at 7 DIV and analyzed for spine morphology at 15 DIV. pIRES-CPT1C vector expresses both CPT1C and GFP proteins, which permits us to visualize in *green* the cells overexpressing CPT1C. *C*, hippocampal neurons at DIV9 were treated with 10 μM myriocin until 15 DIV. Cells were transfected with pEGFP at 12 DIV and analyzed for the morphology of dendritic protrusions at 15 DIV. *D*, a representative image showing dendritic spines from WT mice, KO mice, KO mice treated with C6-ceramide, KO mice transfected with pIRES-CPT1C, and WT mice treated with myriocin. For the quantification of spine morphology, ~100 dendrites from independent transfections were selected randomly. Student's *t* tests and ANOVA post hoc were used to assess statistical significance of the differences. *Error bars*, S.E.; *, $p < 0.05$; ***, $p < 0.001$.

not enhance *de novo* synthesis, as suggested previously (9). Although the *de novo* synthesis of ceramide is the main source of ceramide in ER, it can also be produced from the sphingosine pool (salvage pathway) or from the dephosphorylation of ceramide-1-phosphate. Therefore, CPT1C could be activating either of these two pathways. Another possibility is that CPT1C increases the levels of ceramide by inhibiting its elimination (by conversion to sphingosine, phosphorylation to ceramide-1-phosphate or incorporation into sphingomyelin). Further research is therefore required to determine the precise metabolic pathway in which CPT1C is involved.

Because CPT1C has low catalytic activity *in vitro* (4, 5), we hypothesize that CPT1C regulates the activity of this other enzyme involved in ceramide metabolism by protein-protein interaction. Therefore, under fasting conditions or reduction of malonyl-CoA levels, CPT1C might change its conformation and regulate this other enzyme, resulting in increased levels of ceramide.

CPT1C in Dendritic Spine Maturation—Our results implicate CPT1C in dendritic spine maturation. In absolute numbers, in cultured hippocampal neurons from CPT1C KO mice, the increase in filopodia corresponds with the decline in mature spine number, without altering the overall density of dendritic protrusions, which indicates that CPT1C is not necessary for the formation of new protrusions. However, it is necessary for the conversion of filopodia into mature spines. In addition, results show that the effect of CPT1C on dendritic spines is mediated by ceramide. The addition of ceramide to the cultured medium at low concentration reversed the CPT1C KO phenotype and induced spine maturation. A recent study demonstrates the presence of a new long-chain acyl-CoA synthetase (ACSL4) isoenzyme that localizes specifically in the ER of neurons. Its deficiency increases the percentage of filopodia and reduces the percentage of mature spines (21), in accordance with our results. This highlights the importance of fatty acid metabolism in spinogenesis and suggests that ACSL could provide the substrate necessary for ceramide synthesis in the ER of neurons.

There is only one study that correlates ceramide with the formation of dendritic spines (20). The authors report that coupled inhibition of cholesterol and ceramide synthesis causes alterations in the density and morphology of dendritic spines. Our work sheds light on the regulation of this process and identifies a role for CPT1C in the fine tuning of the modulation of ceramide synthesis, which is essential for the maturation of dendritic spines. The mechanism by which ceramides regulate spine maturation is unknown. However, ceramide binds to and regulates the activity of enzymes and signaling proteins, such as kinases, phosphatases, or membrane receptors (22). One example is protein phosphatase 1, which is activated by ceramide (23) and has been implicated in the conversion of filopodia into mature spines (24). In addition, ceramide is the building block of all cellular sphingolipids, which, in addition to cholesterol, are essential components of lipid rafts. These membrane microdomains are needed for the correct trafficking, anchorage, and activity of synaptic proteins and are preferred platforms for membrane-linked actin polymerization (25). All of these phenomena are necessary for synapse stability and matu-

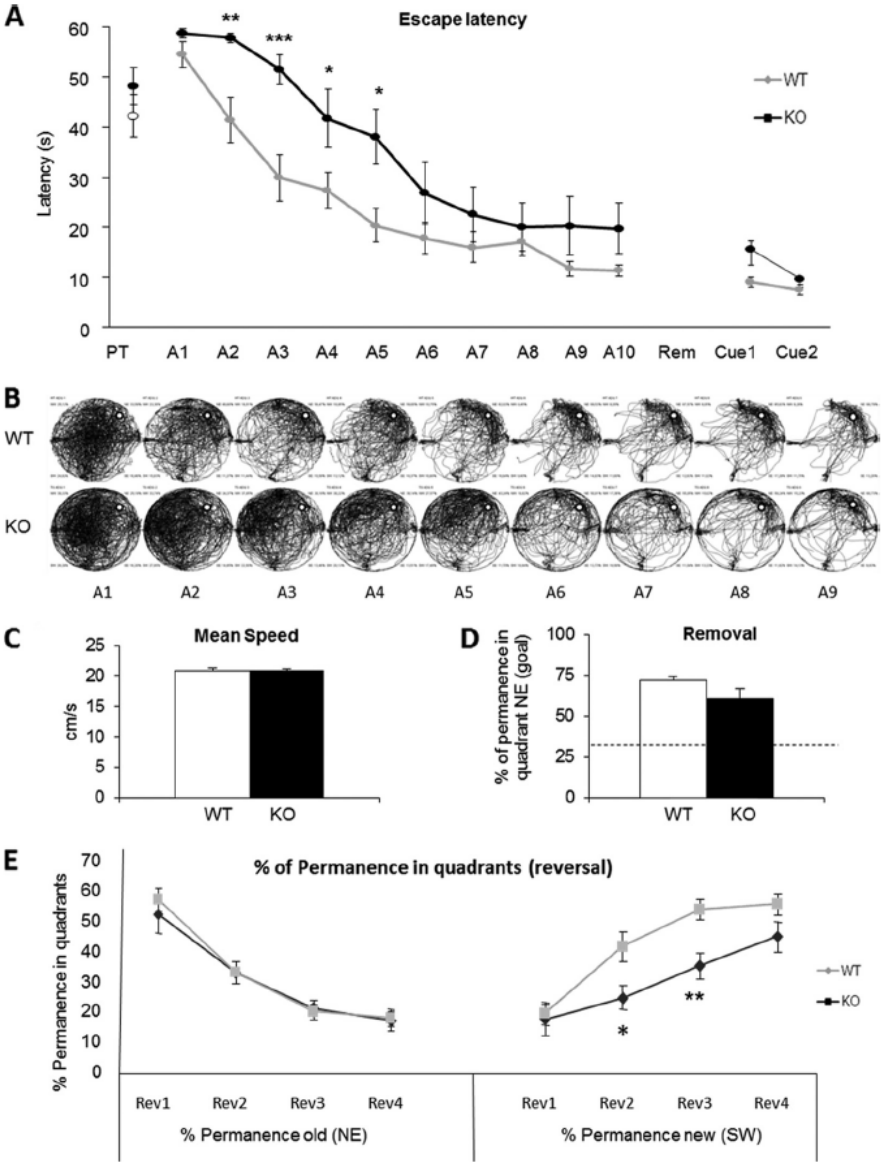

FIGURE 6. **Spatial learning and memory measured by MWM test.** *A*, MWM performance of CPT1C KO and WT mice during the learning sessions as latency (s) to find the platform along the acquisition phase (*A*), removal (*Rem*), and cued sessions (*Cue*). *PT*, pretraining. *B*, visual pathway traced by all animals. The white round platform is located in the northeast (*NE*) quadrant. *C*, mean swimming speed along acquisition sessions. *D*, percentage of time spent in the target quadrant (*NE*) during the removal session; *discontinuous lines* represent the chance level in this session. *E*, percentage of permanence in quadrants during the reversal (*Rev*) session. Data are represented as mean ± S.E. (*error bars*); *, $p < 0.05$; **, $p < 0.05$; ***, $p < 0.001$, ANOVA test.

ration of dendritic spines. Therefore, the diminished ceramide levels found in CPT1C KO mice could alter the regulation of specific proteins or alter the formation of lipid rafts needed for synapse consolidation and spine maturation.

Physiologic Relevance of CPT1C—CPT1C-deficient mice present spatial learning impairment, with a clear delay in the acquisition phase, although they eventually learn and remember the location of the platform. It is important to emphasize that CPT1C deficiency does not affect swimming velocity or motivation and that longer acquisition times correspond to learning deficiencies. On the other hand, memory and cognitive flexibility (ability to modify behavior in an increasingly demanding cognitive task) are not altered in CPT1C KO mice. This indicates that CPT1C deficiency affects the process of consolidating new information but not retention or extinction. This phenotype could be directly related with the impaired dendritic spine maturation found in CPT1C KO mice and with the intact spine head area of mature spines found in both genotypes. In cognitive sciences, it is accepted that spine volume changes regulate new memory acquisition by enlarging and stabilizing smaller spines, whereas the existing memory persistence depends on changing volumes of larger spines (26). In addition, in human patients and most animal models of mental retardation, dendritic spines tend to be abnormally small and immature.

Results from our work show that CPT1C has other physiological roles apart from the regulation of food intake and energy homeostasis. We demonstrate that the molecular function of CPT1C is the fine tuning regulation of ceramide levels in neurons, which is needed for spine maturation during brain devel-

opment. At the behavioral level, we demonstrate for the first time the involvement of CPT1C in learning, which opens the possibility that CPT1C mutations might be the cause of some human cognition disabilities of unknown etiology.

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