PAPER DE LA PROTEÏNA CPT1C EN LA SÍNTESI DE CERAMIDES I EN LA FUNCIÓ MOTORA

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UNIVERSITAT INTERNACIONAL DE CATALUNYA

Departament de Ciències Bàsiques, Àrea de Farmacologia,
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Memòria de la tesi doctoral presentada per Jordi Jacas i Mateu per a optar al grau de Doctor per la Universitat Internacional de Catalunya.

Treball realitzat al Departament de Ciències Bàsiques de la Universitat Internacional de Catalunya, sota la direcció de la doctora Núria Casals Farré.

Sant Cugat del Vallès, 2015.

Dra. Núria Casals Farré
Directora de tesi

Jordi Jacas i Mateu
Doctorand

“És quan dormo que hi veig clar”

J.V. Foix

“El viatge és la recompensa”

Steve Jobs

íNDEX

ABREVIATURES

AA: Aminoàcid/s

ACC: Acetil-CoA carboxilasa

ADN: Àcid desoxiribonucleic

ADNc: Àcid desoxiribonucleic complementari

AICAR: 5-Aminoimidazol-4-carboxamida ribonucleòtid

AMP: Adenosina monofosfat

AMPK: Proteïna cinasa activada per AMP

Arc: Nucli arcuat (hipotàlem)

ATP: Adenosina trifosfat

AgRP: *Agouti-related protein*

ARN: Àcid ribonucleic

ARNm: ARN missatger

Bsx: *brain-specific homeobox protein*

CACT: Carnitina:acilcarnitina translocasa

CDasa: Ceramidasa

CerS: Ceramida Sintasa

CERT: Transportador de ceramida reticle-golgi

CoA: Coenzim A

cPLA$_2$: Fosfolipasa A2 citosòlica

CPT: Carnitina Palmitoiltransferasa

L-CPT1: Isoforma de fetge de CPT1; CPT1A

M-CPT1: Isoforma de múscul de CPT1; CPT1B

CART: *Cocaine and amphetamine regulated transcript*

CAT: Carnitina acetiltransferasa

CrAT: CAT

COT: Carnitina octanoiltransferasa

CrOT: COT

CREB: *cAMP response element-binding*

FAS: *Fatty acid synthase*, sintasa d'àcid gras

FAO: *Fatty acid oxidation*, oxidació d'àcids grassos

FoxO1: *Forkhead box protein O1*

G6pc: Glucosa-6-fosfat, subunitat catalítica

HPLC-ESI-MS/MS: *High performance liquid chromatography-electrospray inonization-mass spectrometer*, Cromatografia líquida d'alt rendiment-ionització amb electroesprai-espectròmetre de masses

ICV: Intracerebroventricular

Kcal: quilocaloria, unitat d'energia calorífica emprada en dietètica

KO: Knock-out

LC-CoA: *Long chain fatty acid coenzyme A*, àcid gras de cadena llarga coenzim A

LCAS: *Long-chain acyl-CoA synthetase*, sintetasa d'acil-CoA de cadena llarga

MAM: Membranes associades a mitocondris

MBH: Hipotàlem mediobasal

MCD: Malonil-CoA decarboxilasa

NPY: Neuropèptid Y

nSMasa: Esfingomielinasa neutra

Pck1: Fosfoenolpiruvat carboxicinasa 1

PHGDH: 3-fosfoglicerat dehidrogenasa

POMC: Proopiomelanocortina

p53: Proteïna 53

RE: Reticle endoplasmàtic

S1P: Esfingosina 1-fosfat

shRNA: *small hairpin RNA*

siRNA: *small interfering RNA*

SMasa: Esfingomielinasa

SMS: Esfingomielina sintasa

TAG: Triacilglicèrids

UCP2: *Uncoupling protein 2*

UGCG: Ceramida Glucosiltransferasa

VLDL: *Very-low density lipoproteins*, lipoproteïnes de molta baixa densitat

WT: *Wild type*, forma salvatge

INTRODUCCIÓ

1. Carnitina Aciltransferases

La β-oxidació dels àcids grassos al mitocondri és una de les principals fonts per a l'obtenció d'energia per a les cèl·lules. Per a que ocorri, cal que els àcids grassos siguin transportats a la matriu mitocondrial; aquesta funció és duta a terme per enzims amb activitat aciltransferasa (1). Diversos enzims pertanyen a aquesta família, segons la grandària de l'àcid gras emprat com a substrat:

- La carnitina acetiltransferasa (CrAT o CAT) utilitza acetil-CoA com a substrat (2).
- La carnitina octanoiltransferasa (CrOT o COT) exerceix el transport d'àcids grassos d'entre 8 i 10 carbonis entre els peroxisomes i els mitocondris (3).
- Les carnitines palmitoiltransferases (CPT) 1 i 2 exerceixen el transport d'àcids grassos de cadena llarga (LC-CoA, de l'acrònim en anglès) d'entre 16 i 20 carbonis (1).

1.1. El sistema CPT

El sistema de les carnitines palmitoiltransferases (CPT) permet l'entrada de LC-CoA a la matriu mitocondrial per a la posterior β-oxidació d'aquests mitjançant transesterificacions consecutives (Figura 1), involucrant diversos enzims (4). En aquest procés, l'enzim responsable de la síntesi de LC-CoA (LCAS) activa l'àcid palmític, obtenint palmitoil-CoA. La membrana mitocondrial no és permeable als acil-CoA, i és ara quan el sistema CPT exerceix la seva funció. En el primer pas, palmitoil-CoA és metabolitzat a palmitoilcarnitina per la CPT1, localitzada en la membrana externa mitocondrial. Aleshores la palmitoilcarnitina és transportada a l'interior del mitocondri en una reacció d'intercanvi catalitzada per la carnitina:acilcarnitina translocasa (CACT). Dins la matriu mitocondrial, palmitoil-CoA és recuperat per CPT2, i la β-oxidació pot començar (5,6).

La reacció catalitzada per CPT1 és un pas clau per a la regulació del fluxe de LC-CoA cap a la β-oxidació. Aquesta regulació l'exerceix malonil-CoA, un producte intermedi en la biosíntesi de LC-CoA (7). Aquesta reacció no només és clau per al control de l'oxidació de LC-CoA, sinó que a més determina la disponibilitat de LC-CoA per a d'altres processos, sobretot en la síntesi de lípids complexes.

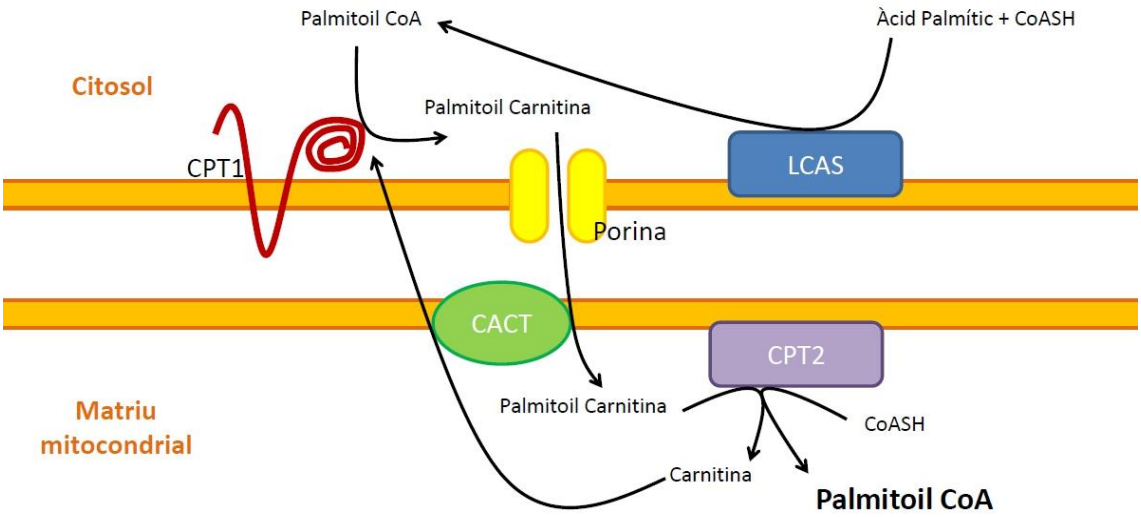

Figura 1. Mecanisme de transport de palmitoil-CoA des del citosol a la matriu mitocondrial exercit pel sistema CPT. Dades extretes de Kerner J *et al*., Biochim Biophys Acta, 2000. Gràfic: Jordi Jacas.

En mamífers, la família d'enzims CPT1 està composta per 3 isoformes diferents:

- CPT1A o isoforma de fetge (L-CPT1) és la que mostra una expressió més ubiqua d'entre totes les isoformes. Es pot trobar en mitocondri de fetge, pàncrees, ronyó, pulmó, melsa, intestí, cervell i ovari (8-10).
- CPT1B o isoforma de múscul (M-CPT1) fou identificada en mitocondri de múscul esquelètic i cardíac, teixit adipós i testicles (11,12). Malgrat que no s'ha aprofundit extensament en el paper de CPT1B en el cervell de vertebrats, diversos autors han iniciat la descripció de la seva presència i funció en hipotàlem de ratolí (13,14), així com en cervell de gall (15).
- CPT1C o isoforma de cervell fou identificada més recentment en el reticle endoplasmàtic de cervell així com de testicle (16,17). Algunes de les funcions i el mecanisme pel qual aquesta proteïna les exerceix són encara una incògnita.

1.2. CPT1A i CPT1B

CPT1A i CPT1B han estat a bastament estudiades des de que foren clonades per primera vegada. La similitud dels residus d'aminoàcids és alta (62%), tot i que ambdós enzims presentin diferències significatives en cinètica i propietats reguladores: CPT1A mostra més afinitat pel seu substrat carnitina però menys

per al seu inhibidor fisiològic malonil-CoA que no pas CPT1B (5,10,12) (Taula 1). El fet de que CPT1A i CPT1B mostrin diferències en la sensibilitat per al seu inhibidor reversible probablement explica la millor regulació de l'oxidació d'àcids grassos en cor i múscul esquelètic, en comparació amb el fetge.

Característica	CPT1A	CPT1B
Pes	≈88 kDa	≈88 kDa
IC_{50} Malonil-CoA	≈2,5 µM	≈0,03 µM
K_m Carnitina	=30 µM	≈500 µM
Expressió tissular		
Fetge	++++	-
Múscul esquelètic	(+)	++++
Cor	+	+++
Ronyó	++++	(+)
Pulmó	++++	(+)
Melsa	++++	-
Intestí	++++	-
Pàncrees	++++	-
Teixit adipós marró	(+)	++++
Teixit adipós blanc	(+)	+++
Ovari	++++	(+)
Testicle	(+)	++++
Fibroblasts	++++	-
Deficiència descrita en humans?	sí	no

Taula 1. Visió general dels enzims mitocondrials CPT1. (+) indica que se'n troben traces en comparació amb l'isoforma alternativa; - indica que no s'ha pogut detectar. Taula editada a partir d'allò publicat per McGarry JD i Brown NF, Eur J Biochem, 1997.

1.3. Regulació fisiològica de CPT1A i CPT1B en fetge i múscul

CPT1 està estretament regulada pel seu inhibidor fisiològic malonil-CoA, i és per això que aquest enzim és el més important des d'un punt de vista fisiològic en el procés de regulació de l'oxidació de LC-CoA (7). Aquest procés permet a la cèl·lula senyalitzar la disponibilitat relativa de lípids i carbohidrats que

s'empraran com a combustible en fetge, cor, múscul esquelètic i cèl·lules β del pàncrees (18) (Figura 2).

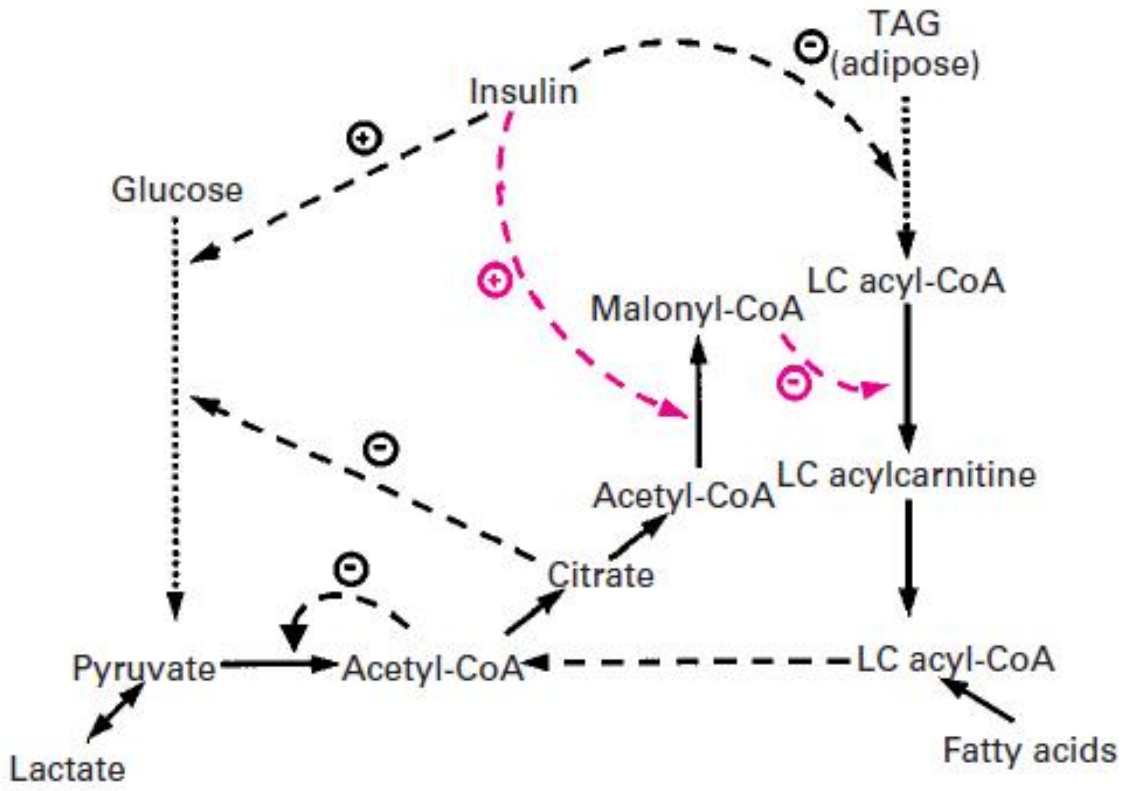

Figura 2. Malonil-CoA juga un paper essencial en la regulació de l'activitat de CPT1A i B segons la disponibilitat energètica de l'organisme, de forma coordinada amb insulina. Extret de Zammit VA *et al*., Biochem J, 1999.

En fetge, malonil-CoA actua com a metabòlit clau que assegura que l'oxidació i la síntesi d'àcids grassos no es dugui a terme de forma simultània. Així doncs, en presència de glucosa postprandial (nivells elevats d'insulina) la lipogènesi hepàtica roman activa, la concentració de malonil-CoA augmenta, CPT1 esdevé inactiva i els LC-CoA sintetitzats són esterificats en triacilglicèrids (TAG), formant lipoproteïnes de molta baixa densitat (VLDL). Les VLDL són aleshores transportades al teixit adipós per a emmagatzemament (10). Per altra banda, en situació de dejú (davallada dels nivells d'insulina) la glicòlisi es veu disminuïda, els nivells de malonil-CoA cauen i la síntesi d'àcids grassos s'atura. Aleshores, CPT1 s'activa i els àcids grassos lliures (no esterificats) són β-oxidats, esdevenint la producció de cossos cetònics (1,7).

En el cas de teixits que no exerceixen cap paper en la lipogènesi, com el cor o el múscul esquelètic, malonil-CoA actua fonamentalment com a molècula

intermediària de senyalització; els seus nivells fluctuen segons s'estigui en situació de sacietat o dejú, com succeeix al fetge (19). Per tant, les variacions en els nivells de malonil-CoA permet regular la capacitat oxidativa de LC-CoA del múscul (20). Durant el dejú, els nivells de malonil-CoA disminueixen i CPT1 veu incrementada la seva activitat, permetent l'entrada de LC-CoA a la matriu mitocondrial i augmentant la taxa oxidativa en múscul. Per altra banda, en situació de sacietat l'activitat de CPT1 es veu inhibida per l'augment dels nivells de malonil-CoA, i conseqüentment la taxa de la β-oxidació pateix una disminució.

Una de les peculiaritats del múscul és que expressa l'enzim acetil-CoA carboxilasa (ACC), que sintetitza malonil-CoA, però no expressa, o ho fa molt lleument, la sintasa d'àcid gras (FAS), que té com a substrat malonil-CoA. Per tant, aquest teixit necessita una via diferent per a poder disminuir els nivells de malonil-CoA; aquesta regulació és exercida per l'enzim malonil-CoA decarboxilasa (MCD), que s'expressa en abundància en múscul (7).

2. CPT1C: una nova isoforma localitzada al cervell de mamífers

Un estudi publicat el 2001 per Abu-Elheiga i col·laboradors (21) concloïa que en individus mancats d'ACC els nivells de malonil-CoA fisiològics davallaven i que el seu comportament alimentari derivava en hiperfàgia. Aquestes conclusions entraven en contradicció amb el fet que en humans deficients en CPT1A, aparentment els seus hàbits alimentaris no es veien significativament alterats (22), la qual cosa conduïa a pensar que en la regulació hipotalàmica de la ingesta alimentària, malonil-CoA realitzava la seva funció mitjançant una proteïna específica.

Fou a partir d'aquesta premissa que Price i col·laboradors (16) decidiren fer una cerca en bases de dades d'ADN i proteïnes intentant cercar ADNc i gens encara no descrits amb similituds a CPT1A. El resultat fou la troballa de CPT1C, la qual tenia una longitud extra de gairebé 30 aminoàcids (aa) respecte les isoformes de fetge i múscul (Figura 3).

L'estudi sobre la semblança i identitat de CPT1C respecte les altres isoformes conclou que CPT1C guarda major conservació respecte CPT1A que no pas CPT1B (23) (Taula 2).

CPT1	A vs B	A vs C	A vs B
% identitat	62,3	52,4	50,6
% semblança	77,7	69,6	66,3

Taula 2. Composició de la seqüència aminoàcida d'entre les tres isoformes de CPT1. La identitat i semblança foren determinades *in silico*. CPT1C mostra una major semblança amb CPT1A que no pas amb CPT1B. Elaborat a partir de la informació publicada per Wolfgang MJ *et al*., Proc Natl Acad Sci, 2006.

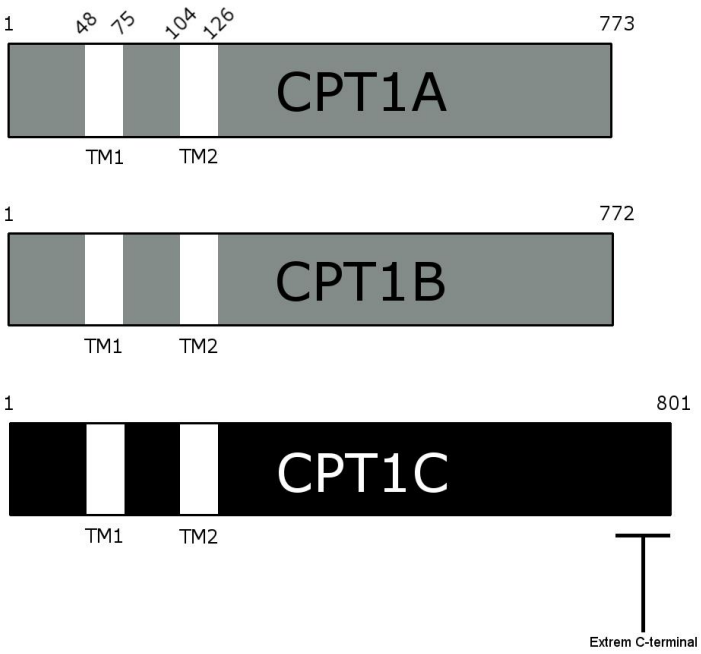

Figura 3. Representació esquemàtica de CPT1A, CPT1B i CPT1C de rata (Rn) mostrant l'extrem C-terminal extès de CPT1C. Els dominis transmembrana (TM1 i TM2) així com les posicions dels aminoàcids de l'inici i el final també hi són representats.

2.1. Distribució tissular

Des del seu descobriment, la comunitat científica ha tingut clar que CPT1C exerceix la seva funció principal al cervell, doncs és en aquest òrgan on s'ha demostrat repetidament la seva presència, concretament en les neurones. De forma menys precisa, també es pot afirmar que CPT1C s'expressa en testicle, ovari, intestí prim i còlon, tot i que únicament s'hagin pogut aïllar traces d'ARN (16,17,24).

Més recentment també s'ha descrit l'expressió de CPT1C en teixits tumorals de pulmó, els quals pateixen una transformació metabòlica que els permet sobreviure malgrat les condicions d'estrès metabòlic. És precisament l'expressió a l'alça de CPT1C el que permet a aquestes cèl·lules sobreviure malgrat la reducció de nutrients o en condicions d'hipòxia. De fet, quan CPT1C és deplecionada de la cèl·lula tumoral mitjançant shRNA la seva supervivència es veu compromesa i el seu creixement es veu disminuït (25).

2.2. Localització subcel·lular

El fet de que CPT1C disposi de dos segments transmembrana així com una alta homologia amb les isoformes A i B feia suposar que la seva localització subcel·lular seria el mitocondri. Malgrat que Price i col·laboradors determinessin que CPT1C es localitzava indistintament en la fracció mitocondrial com en la microsomal (16), Dai i col·laboradors establiren mitjançant immunofluorescència en microscopia confocal que CPT1C es localitza en mitocondri (24).

L'estudi fet per Sierra i col·laboradors (17) establí finalment que CPT1C era una proteïna que s'expressava fonamentalment en reticle endoplasmàtic (RE) gràcies a l'extrem N-terminal, que enlloc de tenir una senyal d'importació a mitocondri –com sí té CPT1A i B- té una senyal d'importació a RE (Figura 4).

De fet, les imatges obtingudes per Dai i col·laboradors el 2007 són de baixa qualitat i no permeten concloure que CPT1C es localitza únicament en mitocondri. Una possible explicació a aquests resultats tan divergents és que en realitat CPT1C té una distribució microsomal, però degut als contactes mitocondri-RE –coneguts com a membranes associades a mitocondris (MAM)- és possible que part de la CPT1C expressada en la cèl·lula tingui també un perfil d'expressió mitocondrial. Això explicaria que CPT1C pugui co-localitzar tant amb calnexina com amb el marcador mitocondrial MitoTracker.

2.3. Activitat enzimàtica

Un altre conflicte obert arran de la caracterització de CPT1C és sobre si té o no activitat palmitoiltransferasa. Els primers estudis realitzats determinaren que malgrat malonil-CoA té gran afinitat tant per CPT1C com per CPT1A (Figura 5), la capacitat catalítica mesurada en CPT1C mitjançant assajos radiomètrics és nul·la (16,23). Una vegada més, Sierra i col·laboradors van més enllà i determinen que CPT1C té una lleu activitat catalítica amb palmitoil-CoA com a substrat (17) mitjançant cromatografia líquida en tàndem amb un espectròmetre de masses (HPLC-ESI-MS/MS), una tècnica molt més sensible a petites variacions respecte als assajos radiomètrics (Figura 6). Per tant, una explicació

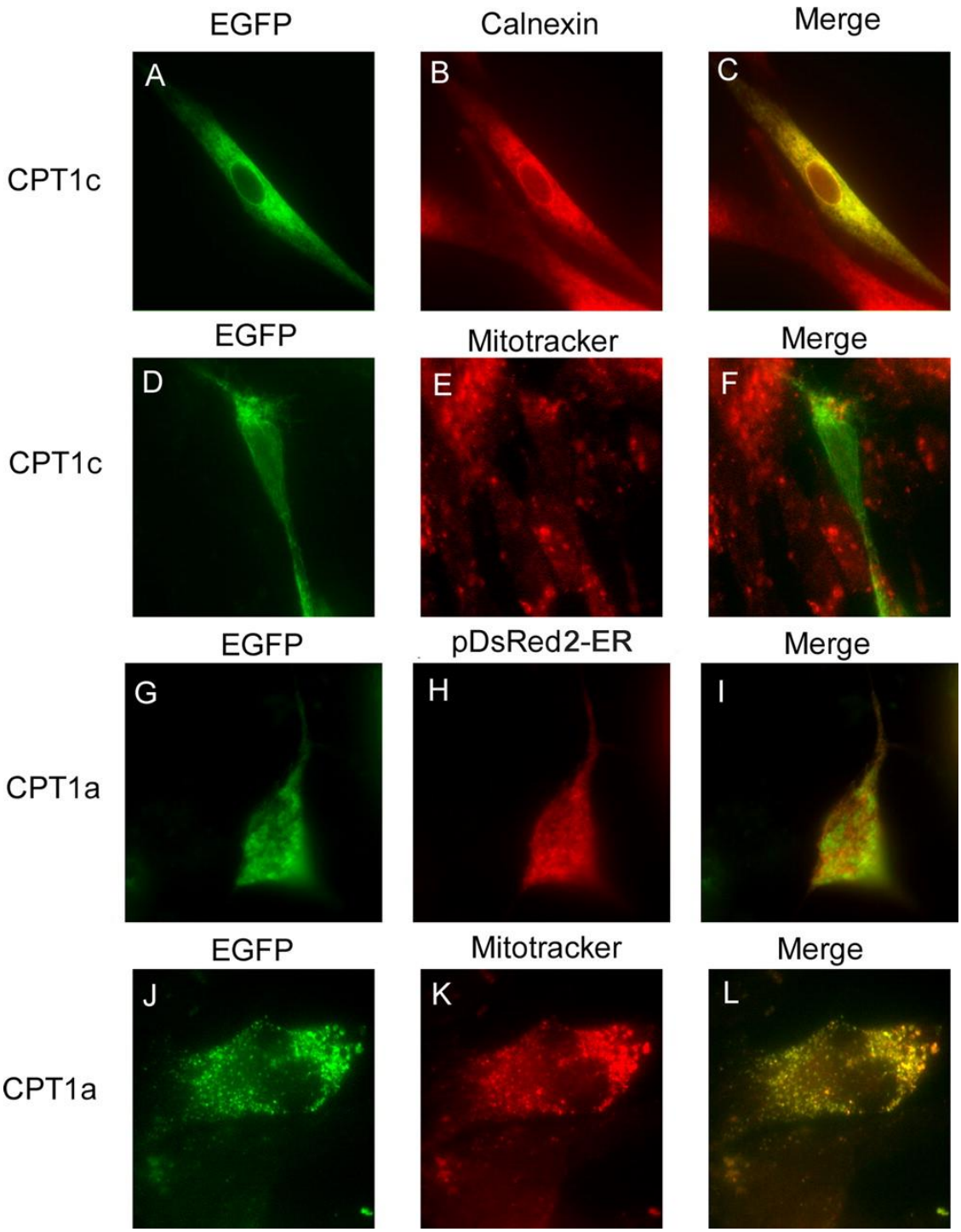

Figura 4. Estudi de colocalització de CPT1C. Imatges preses per microscopia confocal permeteren demostrar, a diferència del que fins aleshores estava descrit, que CPT1C es localitza preferentment a RE, sense excloure la possibilitat de que es pugui aïllar també en MAM. Extret de Sierra AY *et al*., J Biol Chem, 2008.

a que els primers autors fossin incapaços de detectar activitat catalítica en CPT1C podria ser que el seu mètode no fos prou sensible per detectar cap mena d'activitat en la proteïna en estudi.

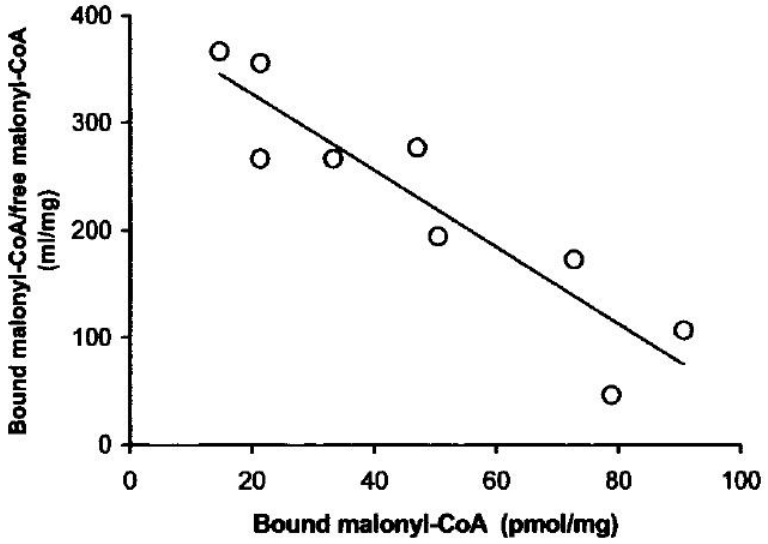

Figura 5. Afinitat de CPT1C per malonil-CoA. Diversos assajos d'unió de malonil-CoA a CPT1C, com el que es mostra en la imatge, han permès demostrar l'alta afinitat que es tenen. Extret de Price NT *et al*., Genomics, 2002.

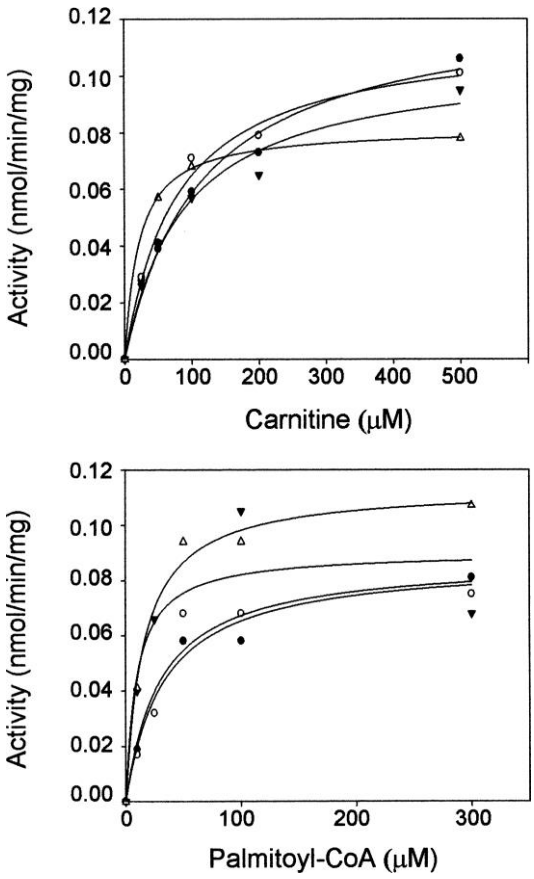

Figura 6. Assaig d'activitat de CPT1C. L'ús de tècniques més sensibles –HPLC-ESI-MS/MS- que les emprades fins aleshores permeteren demostrar, l'any 2008, que CPT1C té una lleu activitat CPT. Extret de Sierra AY *et al*., J Biol Chem, 2008.

Un estudi més recent als comentats suggereix que CPT1 disposa d'un domini regulador de l'activitat, anomenat domini N degut a que es troba a l'extrem N-terminal de la proteïna (26). Segons les condicions fisiològiques de la cèl·lula, la conformació d'aquest domini pot ser Nα (inactiva) o Nβ (activa) (Figura 7). Si bé CPT1A pot alternar les dues conformacions, CPT1C roman en conformació Nα de forma permanent, la qual cosa podria explicar la baixa activitat catalítica que mostra.

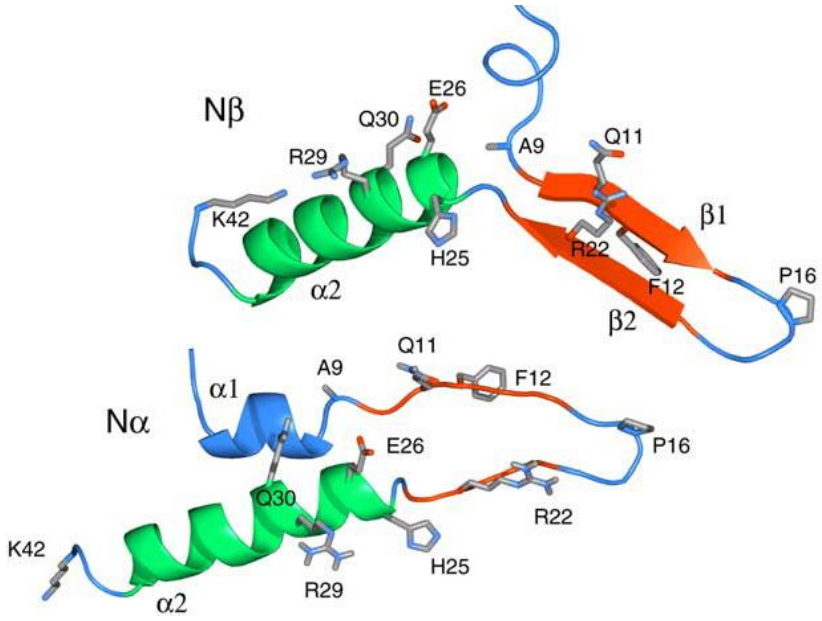

Figura 7. Models dels estats Nα i Nβ del domini regulador de CPT1A. Els aminoàcids que es troben substituïts en CPT1C es mostren representats en la imatge. Extret de Samanta S *et al*., Biopolymers, 2014.

3. Descripció de CPT1C *in vivo*

3.1. Fenotip de ratolins CPT1C-KO

Des del seu descobriment, diversos grups han aconseguit desenvolupar ratolins *knock-out* (KO) per a CPT1C (CPT1C-KO) per a l'estudi del seu fenotip. L'any 2006, Wolfgang i col·laboradors foren pioners i aconseguiren descriure diverses característiques del fenotip dels ratolins CPT1C-KO que serien clau per a l'inici de la recerca de la funció d'aquesta proteïna. Es veié que els animals mancats de CPT1C tenien un pes un 10% inferior respecte als individus salvatges o *wild type* (WT) així com una ingesta un 25% per sota respecte als animals WT i heterozigots després d'un dejú de 4 hores (Figura 8).

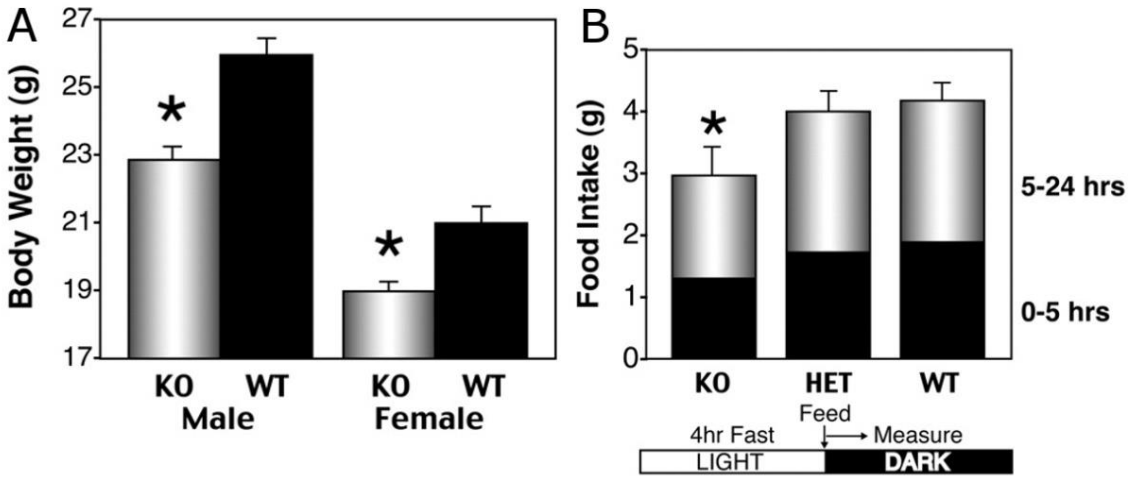

Figura 8. Els animals CPT1C-KO tenen alterat el pes corporal i la ingesta alimentària. En dieta normal, els ratolins CPT1C-KO tenen un pes corporal inferior als WT, així com una ingesta d'aliments un 25% inferior després d'un dejú de 4 hores. Extret de Wolfgang MJ *et al*., Proc Natl Acad Sci, 2006.

Pel que fa al control del pes corporal, tant els animals WT com els CPT1C-KO mostren un guany proporcionalment igual quan se'ls administra una dieta que conté un 10% de greix (Figura 9). En canvi, en dietes amb alt contingut gras (45%), els animals CPT1C-KO guanyen més pes proporcionalment que els WT a partir de 5 setmanes de dieta hipercalòrica i mostren una clara resistència a la insulina (23).

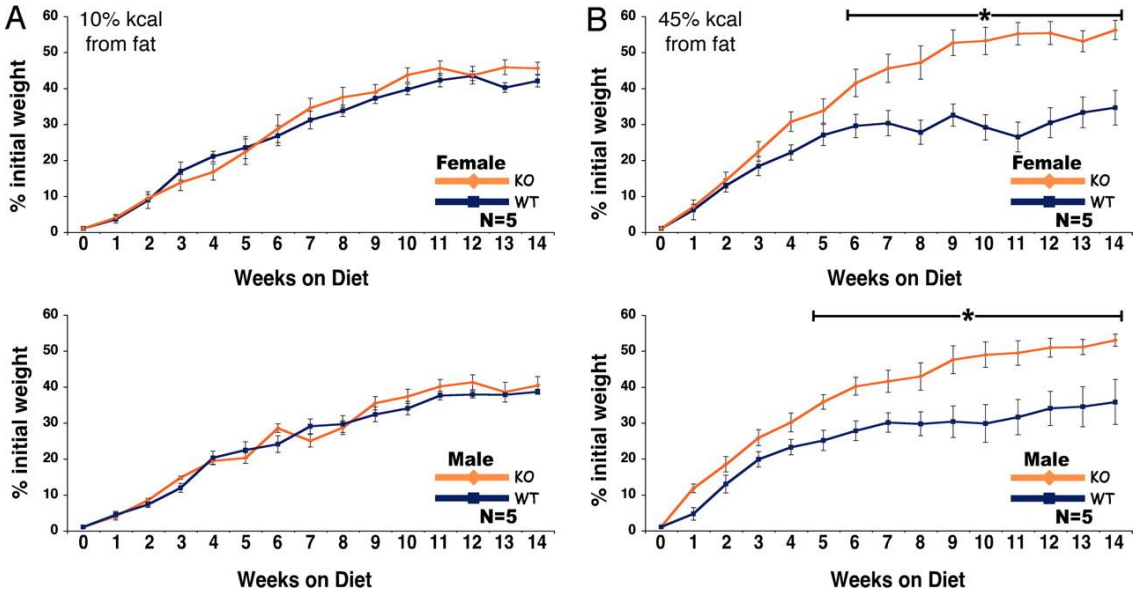

Figura 9. Els ratolins CPT1C-KO són més sensibles que WT a una dieta grassa. Mentre que en dieta amb un 10% de kcal provinents de greix els dos genotips d'animal guanyen proporcionalment el mateix pes, en dieta amb el 45% de kcal provinent de greix els animals CPT1C-KO guanyen proporcionalment més pes que els WT. Extret de Wolfgang MJ *et al*., Proc Natl Acad Sci, 2006.

Per altra banda, es demostrà que CPT1C exerceix un paper protector en situació de dieta hipercalòrica (60% de contingut en greix). En aquestes condicions, ratolins que sobreexpressaven CPT1C en l'hipotàlem menjaven la mateixa quantitat d'aliment que els animals control però el guany de pes fou un 25% inferior (Figura 10) (24). Aquest resultat i l'anterior, juntament amb el fet que CPT1C pot unir de forma intensa malonil-CoA (16,23) així com que els individus amb CPT1A mutada no veien alterats els seus hàbits alimentaris (22) es proposà que malonil-CoA exercia les seves funcions de regulació de la ingesta mitjançant CPT1C.

En aquestes mateixes condicions, també s'estudià com afectava a nivell perifèric la manca de CPT1C en ratolins. Així, després de 8 setmanes de dieta grassa, aquests animals mostraven un fenotip de resistència a la insulina en que la gluconeogènesi es trobava augmentada i la captació de glucosa disminuïda, principalment en fetge, teixit adipós i múscul; aquest fet coincidia amb que els gens *G6pc* i *Pck1*, reguladors de la glucosa hepàtica, es trobaven regulats a l'alça. Per altra banda, a l'observar què succeïa amb els LC-CoA, es comprovà que foren esterificats en forma de TAG, fet que concordava amb una

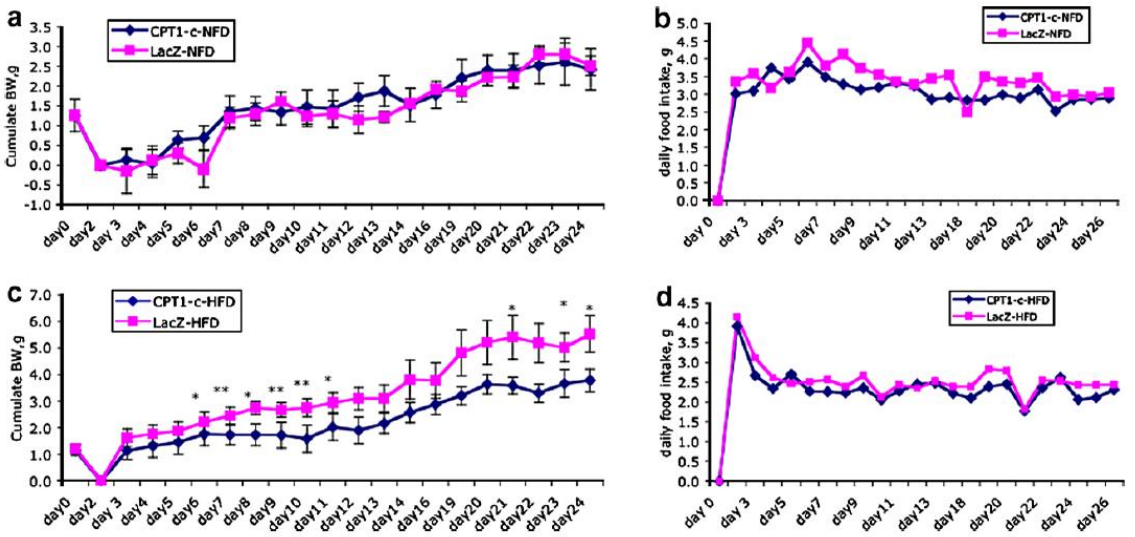

Figura 10. CPT1C exerceix un paper protector en dieta hipercalòrica. Quan CPT1C és sobreexpressada en hipotàlem de ratolí, aquests animals mostraven un increment de pes significativament inferior en dieta hipercalòrica (c,d; 60% de kcal provinents de greix) respecte a dieta normal (a,b; 10% de kcal provinents de greix). La ingesta era igual en cadascuna de les condicions. Extret de Dai Y *et al*., Biochem Biophys Res Commun, 2007.

davallada dels nivells de ARNm en fetge i múscul de diversos enzims implicats en l'oxidació de LC-CoA, incloent CPT1A i CPT1B (13).

A partir de les dades obtingudes de l'estudi del fenotip dels ratolins CPT1C-KO, es començà a indagar sobre la responsabilitat de CPT1C en el control del metabolisme perifèric. Com a punt de partida, es pogué comprovar que ni els nivells de malonil-CoA ni la resta d'enzims implicats en el metabolisme d'acils-CoA (CPT/CAT/COT) estaven alterats al cervell (27), a diferència de fetge i múscul. Amb això, es suggerí que enlloc d'exercir un paper directe sobre el metabolisme d'àcids grassos, CPT1C podia tenir un paper més aviat regulador mitjançant malonil-CoA. Aquesta conclusió, a més, concorda amb el fet de que els nivells de malonil-CoA estan regulats mitjançant AMPK, el sensor energètic cel·lular de la ràtio [ATP]/[AMP] (Figura 11).

3.2. Paper de CPT1C en la regulació de la ingesta

Malonil-CoA és un àcid gras intermedi implicat en la senyalització d'ingesta i sacietat que fluctua proporcionalment amb els neuropèptids orexigènics NPY i AgRP (de forma indirecta), així com els anorexigènics CART i POMC (de forma directa). També en hipotàlem, malonil-CoA fluctua conjuntament amb glucosa i AMPK, cosa que no succeeix en d'altres teixits del cervell, com el còrtex (28).

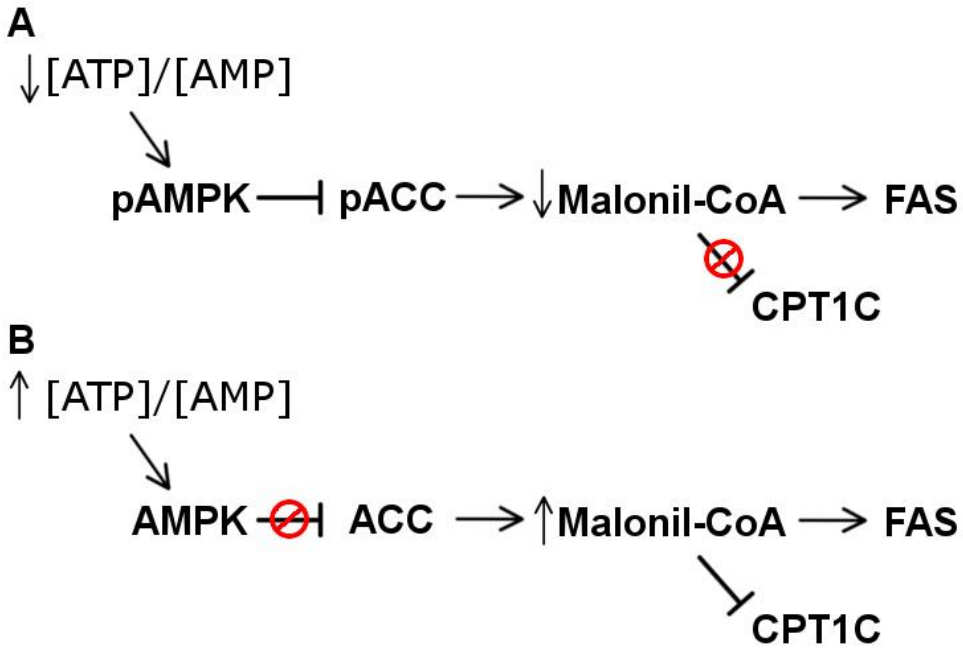

Figura 11. CPT1C, un sensor energètic? Degut a la seva escassa activitat enzimàtica, diversos autors han proposat que CPT1C en realitat actua com a sensor energètic integrat en la via AMPK/ACC/FAS de l'hipotàlem, éssent sensible als nivells de malonil-CoA i mostrant-se activa en condicions de dejú (A) o inactiva en condicions de sacietat (B). Esquema: Jordi Jacas.

Prenent malonil-CoA com a una de les molècules claus en la regulació hipotalàmica de la ingesta i sacietat, ha estat possible relacionar la seva funció sobre CPT1C a partir dels estímuls exercits per les hormones leptina (postprandial) i ghrelina (preprandial) en el control de la ingesta. En situació de sacietat leptina i malonil-CoA es troben elevats, i en situació de dejú ghrelina es troba elevada i malonil-CoA disminuït; quan CPT1C es veu sobreexpressada en el nucli arcuat (Arc) de rata, els efectes desencadenats per la infusió arcual de leptina o cerulenina (inhibidor de la FAS que augmenta malonil-CoA) són parcialment contrarestats, apreciant-se augments significatius en la ingesta d'aliment i pes corporal respecte els animals control (29). Aquest resultat dugué a la conclusió de que efectivament malonil-CoA exerceix la seva funció a través de CPT1C (Figura 12).

Per intentar resoldre l'enigma sobre el mecanisme d'acció de CPT1C, s'hipotetitzà que el lligam entre leptina, malonil-CoA i CPT1C per una banda, i els neuropèptids reguladors de la ingesta/sacietat per l'altra, podrien ser les ceramides. El motiu és que la síntesi *de novo* de les ceramides es duu a terme al RE a partir de la fusió d'una molècula de L-serina i palmitoil-CoA (30). Com

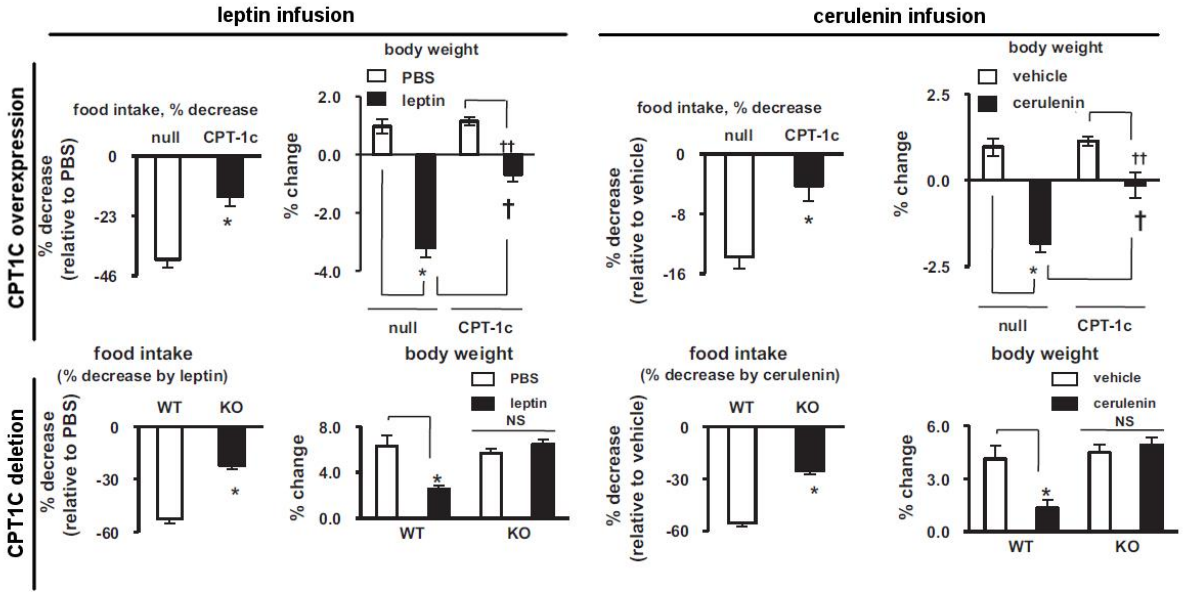

Figura 12. Efectes de leptina i malonil-CoA sobre CPT1C. CPT1C intervé en el procés de senyalització de sacietat iniciada per leptina mitjançant malonil-CoA. Tant quan CPT1C és sobreexpressada (panells superiors) com quan és deleccionada (panells inferiors) la funció de malonil-CoA es veu alterada ja sigui en ingesta alimentària com en pes corporal. Composició a partir de Gao S *et al*., Proc Natl Acad Sci, 2011.

que CPT1C es localitza al RE i uneix preferentment palmitoil-CoA, es proposà que les ceramides podrien tenir alguna funció de nexe entre CPT1C i els neuropèptids.

En aquest context es pogué comprovar que els nivells de ceramides en Arc variaven en funció de si CPT1C s'expressava o no; en cas de sobreexpressió, els nivells de ceramides augmentaven respecte al seu control, i quan CPT1C era delecionada, els nivells de ceramides davallaven. La leptina exercia un efecte semblant sobre les ceramides que el que succeïa en CPT1C-KO, el qual es podia revertir sobreexpressant CPT1C. Amb aquestes dades s'intentà demostrar que les ceramides estan implicades en la regulació hipotalàmica de la ingesta revertint els efectes de la leptina amb un anàleg de ceramida: la C6-ceramida. Així, mentre la leptina redueix la ingesta alimentària, el pes corporal, NPY i *Bsx*, la infusió en Arc de C6-ceramida aconsegueix revertir-ne els efectes. Per contra, quan la síntesi *de novo* de ceramides era interrompuda per miriocina, l'efecte resultant era semblant al d'una situació de sacietat (29).

3.3. CPT1C i càncer

Durant el desenvolupament d'un tumor en un organisme, les cèl·lules que conformen el teixit afectat pateixen una transformació metabòlica que es tradueix en un augment de la glicòlisi i de l'oxidació d'àcids grassos tot

controlant la despesa energètica, conferint a les cèl·lules tumorals una major eficiència metabòlica. Durant l'estudi de tràns crits activats per p53 mitjançant un garbellat d'ADNc per *microarray* es veié que CPT1C estava regulada a l'alça, i ràpidament es plantejà quin paper podria tenir en el metabolisme i la supervivència tumoral (25).

Un estudi desenvolupat per Zaugg i col·laboradors en la línia cel·lular MCF7, model de recerca en càncer, revelà que aquestes cèl·lules són capaces d'expressar CPT1C i que per tant eren potencialment vàlides per a l'estudi del paper de CPT1C en càncer. Aquest estudi revelà que malgrat CPT1A i CPT1B no es veuen alterades a nivell transcripcional, les cèl·lules canceroses mostren una regulació a l'alça en l'oxidació d'àcids grassos (FAO); de fet, la FAO fluctua de forma directament proporcional com ho fa CPT1C, evidenciant que aquestes cèl·lules tenen més resistència a l'escassetat energètica ja que la poden suplir amb l'oxidació d'àcids grassos i produir l'ATP que per altres vies no poden aconseguir. De fet, es revelà que en cèl·lules tumorals pAMPK era capaç de regular a l'alça CPT1C segons la disponibilitat energètica (+/- glucosa) així com la pròpia activació d'AMPK (+/- metformina) (25,32).

Per altra banda, i possiblement gràcies a la capacitat de les cèl·lules tumorals de suplir les mancances energètiques mitjançant la β-oxidació d'àcids grassos, la presència de CPT1C confereix als teixits cancerígens una clara resistència a sirolimus o rapamicina, un immunosupressor emprat també com a medicament en la lluita contra el càncer ja que frena la proliferació cel·lular i el creixement de tumors tot inhibint mTOR (25,32).

Zaugg i col·laboradors també varen estudiar com afectava l'estrès cel·lular en la supervivència i en l'expressió de CPT1C. Així, en situació d'hipòxia (0,2% d'O_2) les cèl·lules tumorals sofreixen una regulació a l'alça en l'expressió de CPT1C que els confereix una taxa de supervivència significativament superior respecte aquelles tractades amb CPT1C shRNA. En aquestes mateixes condicions però en situació d'hipoglucèmia, CPT1C també es trobava regulada a l'alça i mostrava una supervivència significativa en situació d'hipoglucèmia o en tractament amb 2-deoxiglucosa (25).

Per últim, i reprenent les causes que varen portar a relacionar CPT1C i càncer, s'ha volgut conèixer quin paper juga p53. En embrions E12,5 de ratolí C57BL/6

es pogué comprovar que en condicions de radiacions ionitzants la CPT1C era regulada a l'alça en el cervell d'espècimens $p53^{+/-}$, però no es veia cap canvi significatiu en els que eren $p53^{-/-}$. Igualment, en cultiu de fibroblasts d'embrions E13,5 de ratolí C57BL/6 s'experimentava un augment significatiu de CPT1C en condicions d'hipòxia per al tipus salvatge de p53. En canvi, quan p53 era delecionada, CPT1C no responia i en condicions d'hipòxia els seus nivells no variaven respecte al control de normòxia. També es pogué demostrar que la regulació a l'alça de CPT1C exercida per pAMPK és depenent de p53 (33).

Amb aquesta troballa es pogué descriure l'expressió de CPT1C almenys en tumors de pulmó, pit, cervell i en sarcomes (32). De fet, es plantejà la possibilitat de que els àcids grassos juguessin un paper clau en la disponibilitat energètica dels tumors, com ho és la glucosa, erigint CPT1C com a potencial diana terapèutica a tenir en consideració per al tractament i cura del càncer, doncs la seva supressió augmenta la supervivència dels individus malalts tot suprimint el desenvolupament de tumors (33).

4. Esfingolípids

En un dels estudis enfocats a esbrinar si CPT1C tenia activitat palmitoiltransferasa es suggeria la possibilitat de que aquesta proteïna exercís, d'alguna forma, un paper en la regulació dels nivells dels esfingolípids, concretament en la síntesi de ceramides. Degut a que les ceramides són resultat de la condensació d'una molècula de L-serina i de palmitoil-CoA a l'inici de la síntesi *de novo* al RE, es plantejà que possiblement CPT1C pogués tenir alguna funció en aquest procés, com ara de sensor o proveïdor de palmitoil-CoA (17).

A partir d'aquest plantejament es varen desenvolupar dos estudis anteriorment exposats en els quals es demostrava la implicació de les ceramides en la regulació dels processos bioquímics relacionats amb la ingesta. Aquests estudis foren capaços de relacionar ceramides amb regulació de la ingesta mitjançant uns efectes anorexigènics i orexigènics mai descrits, desenvolupats a través de NPY, AgRP, Bsx, pCREB i FoxO1 en resposta a estímuls desencadenats per leptina o ghrelina (29,31).

4.1. Síntesi d'esfingolípids

El procés de síntesi *de novo* dels esfingolípids es desenvolupa al RE per la condensació d'una molècula de L-serina amb una de palmitoil-CoA mitjançant l'enzim serina-palmitoiltransferasa (SPT), que exerceix de pas limitant en

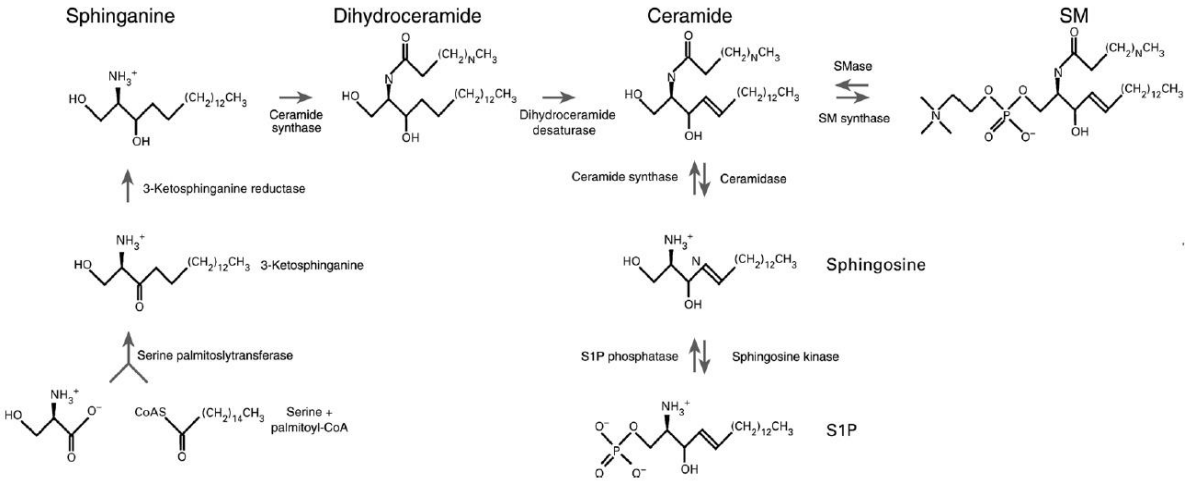

Figura 13. Esquema del metabolisme dels esfingolípids. La síntesi *de novo* dels esfingolípids, que es duu a terme al RE, té com a punt final les ceramides, que juguen un rol central o *hub* en la síntesi i degradació de la resta d'esfingolípids. Extret de Kolesnick R, J Clin Invest, 2002.

aquest procés, tot donant 3-cetoesfinganina. Després d'un pas de reducció s'obté esfinganina, en la qual intervé la ceramida sintasa (CerS), que incorpora un acil-CoA de tamany variable, segons la isoforma que intervingui. Per últim s'obté la ceramida, un cop superat un procés de desaturació (Figura 13) (30).

El metabolisme de ceramides, en general, també pot succeir per dues vies diferents a la *de novo*: a partir de la síntesi o degradació d'esfingomielina mitjançant l'esfingomielina sintasa (SMS) o l'esfingomielinasa (SMasa), respectivament; també a partir de la seva degradació a esfingosina mitjançant ceramidasa (CDasa) o la condensació d'esfingosina i un acil-CoA mitjançant CerS. Altres vies provenen dels glicoesfingolípids i de ceramida-1-fosfat (30).

En el procés de metabolisme de ceramides, que juguen un rol central o de *hub* en el metabolisme general dels esfingolípids, intervenen les anteriorment esmentades CerS; fins a l'actualitat se'n coneixen 6 isoformes ubicades al RE i als mitocondris, i la seva distribució tissular determina l'abundància de les diferents espècies de ceramida que existeixen (Figura 14). En general, l'isoforma CerS1 és la més abundant en cervell, la qual condueix a nivells significativament superiors de C18:0-ceramida respecte de la resta d'espècies (34).

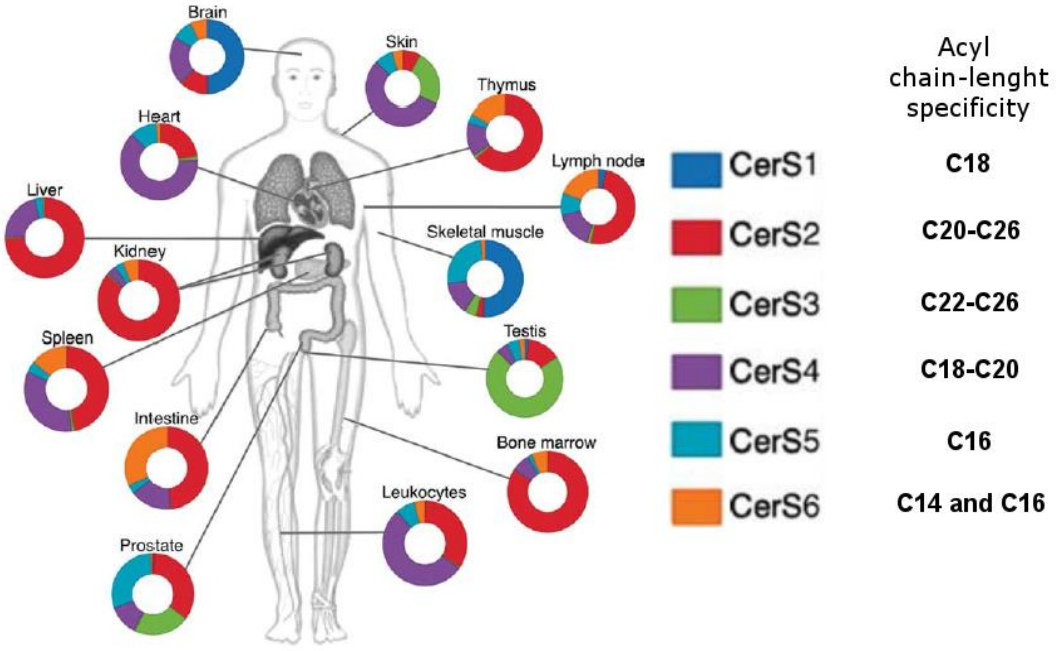

Figura 14. Distribució tissular de CerS i espècies sintetitzades. Segons el teixit que s'estigui estudiant, serà possible trobar una composició variable de CerS que per consegüent sintetitzarà una espècie determinada de ceramida segons l'afinitat que tingui per un àcid gras concret. Composició feta a partir de dades publicades per Levy M i Futerman AH, IUBMB Life, 2010.

A dia d'avui encara roman per a descriure de forma més precisa el mecanisme que regula l'activitat de CerS així com de la resta d'enzim implicats en el metabolisme de ceramides.

4.2. Funcions generals dels esfingolípids

Avui en dia hi ha disponible una extensa literatura que mira de descriure les funcions de les desenes d'espècies d'esfingolípids així com dels enzims que les fabriquen i de les proteïnes que tenen com a diana efectora. De totes aquestes publicacions els Drs. Charles Chalfant i Maurizio del Poeta han sabut editar una important obra recopilatòria del paper senyalitzador dels esfingolípids, que ha comptat amb la participació de diversos autors (35).

4.2.1. Ceramida

Com s'ha comentat anteriorment, les ceramides exerceixen un rol central en el metabolisme dels esfingolípids degut a que a partir d'aquestes molècules se'n poden sintetitzar els seus derivats. De forma general, es pot afirmar que les ceramides juguen un paper destacat en la resposta a estímuls que condueixen a l'apoptosi ja que la seva síntesi es veu incrementada a l'alça quan es produeix un estímul estressant per a la cèl·lula (35). En aquest cas, cal destacar l'acumulació de ceramida en mitocondri, que acaba afectant a la cadena respiratòria per a provocar la mort cel·lular (36).

Per altra banda, i mitjançant la concentració cel·lular de serina disponible per a la síntesi *de novo*, es pot controlar el transport de nutrients de la cèl·lula així com l'organització del citoesquelet, el cicle cel·lular i la transcripció d'ARN. També mitjançant un altre precursor de ceramida en la síntesi *de novo*, el palmitat, és possible regular la sensibilitat a insulina i el síndrome metabòlic.

Per últim, caldria destacar la importància que exerceixen les ceramides en determinar la fluïdibilitat de la membrana plasmàtica juntament amb esfingomielina i glicoesfingolípids, que acaben conformant les anomenades basses lipídiques o *lipid raft* en anglès. En aquest cas, la cèl·lula és capaç de regular les interaccions lípid-lípid tot alterant els nivells de ceramides dels *lipid raft*, permetent per exemple la invaginació de la membrana plasmàtica. També són capaces de controlar les interaccions entre la membrana plasmàtica i les proteïnes de membrana, així com la interacció d'aquestes proteïnes amb els

seus lligands, controlant per tant el desencadenament de la transducció de senyal (35).

4.2.2. Esfingomielina

Sintetitzada a partir de ceramida mitjançant la SMS, incorpora una molècula de fosfocolina cedida per la fosfatidilcolina. La seva síntesi està compartimentalitzada a l'aparell de Golgi, als lisosomes i a la membrana plasmàtica; és en aquesta última regió cel·lular on l'esfingomielina és més abundant, doncs la seva presència permet donar l'estructura idònia a la membrana plasmàtica ja que és capaç d'interaccionar i unir molècules de colesterol (30).

L'esfingomielina es pot degradar a ceramida per a poder respondre davant d'estímuls externs com ara radiacions o ROS (condueixen a apoptosi) així com citoquines com ara TNF-α (migració i adhesió cel·lular). Aquesta resposta l'exerceixen les SMases, enzims que produeixen ceramida a partir d'esfingomielina (30).

4.2.3. Glucosilceramida i glicoesfingolípids

La glucosilceramida és sintetitzada a l'aparell de Golgi a partir de la ceramida obtinguda sobretot per la via *de novo* i és el primer pas per a la síntesi de glicoesfingolípids més complexes, dels quals cal destacar-ne els gangliòsids ja que són abundants a la membrana plasmàtica de les neurones (37). Són imprescindibles per al desenvolupament embrionari doncs els individus KO per a Ceramida Glucosiltransferasa (UGCG) són inviables.

En general, els glicoesfingolípids forment part dels *lípid raft* els quals permeten la generació i organització de dominis en la membrana plasmàtica. També cal destacar-ne el seu paper de suport pel que fa a la protecció de l'epidermis, ja que proporciona impermeabilitat, així com la seva intervenció en la mielinització de les cèl·lules de Purkinje (30,35). Per últim, cal destacar que aquestes molècules faciliten l'aprenentatge i memòria en rates (38).

4.2.4. Esfingosina 1-fosfat

L'esfingosina 1-fosfat (S1P) es genera per la degradació de ceramida mitjançant la ceramidasa, que dóna esfingosina, que a la vegada és fosforilada

per una cinasa. En general, es pot afirmar que S1P genera proliferació, migració i supervivència, així com inflamació i angiogènesi (30). Desenvolupa la seva funció principalment a l'espai extracel·lular un cop és secretada per a acabar unint-se a receptors (S1Pr) acoblats a proteïnes G.

La seva producció i secreció es produeix en resposta a estímuls com ara factors de creixement i de citoquines, principalment.

4.2.5. Ceramida 1-fosfat

Generada per ceramida cinasa, aquesta molècula també està implicada en la regulació de la supervivència, el creixement cel·lular i la resposta inflamatòria. Se'n pot destacar que actua com a lligand de $cPLA_2$ per a potenciar la producció d'àcid araquidònic i potenciar la síntesi de prostaglandines. També és capaç d'inhibir la SMasa àcida, tot disminuint la generació de ceramida i prevenint l'apoptosi (35).

HIPÒTESI

Degut al rol central que exerceixen les ceramides en diverses situacions en animals mancats de CPT1C, es planteja el següent:

- CPT1C és capaç de regular la síntesi de ceramides, possiblement per la via *de novo*.
- L'alteració a la baixa en els nivells de ceramides en diferents regions motores del cervell causa alteracions en la funció motora de ratolins CPT1C-KO.

OBJECTIUS

Síntesi d'esfingolípids

- Determinar si hi ha diferències en els nivells de ceramides en diverses regions cerebrals de ratolins CPT1C-KO en situació *ad-libitum* i en dejú, comparats amb els mateixos grups de ratolins salvatges (WT).
- Estudiar si CPT1C participa en la síntesi *de novo* de ceramides en cultius primaris de neurones hipocampals.
- Determinar la resposta hipotalàmica a l'estímul de l'hormona ghrelina determinant els nivells de ceramides, en ratolins WT i CPT1C-KO.

Funció motora

- Estudiar la funció motora de ratolins WT i CPT1C-KO de diverses edats per a poder conèixer possibles dèficits derivats de l'absència de CPT1C.
- Mesurar els nivells d'expressió de CPT1C en diverses regions cerebrals de ratolí durant el desenvolupament postnatal i fins a l'edat adulta.

PUBLICACIONS

Ceramide levels regulated by Carnitine Palmitoyltransferase 1C control dendritic spine maduration and cognition

Carasco P, Sahún I, McDonald J, Ramírez S, Jacas J, Gratacós E, Sierra AY, Serra D, Herrero L, Acker-Palmer A, Hegardt FG, Dierssen M, Casals N.

J Biol CHem. 2012 Jun 15;287(25):21224-32. Doi: 10.1074/jbc.M111.337493. Epub 2012 Apr 26.

Aportacions: figures 2 i 3.

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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.

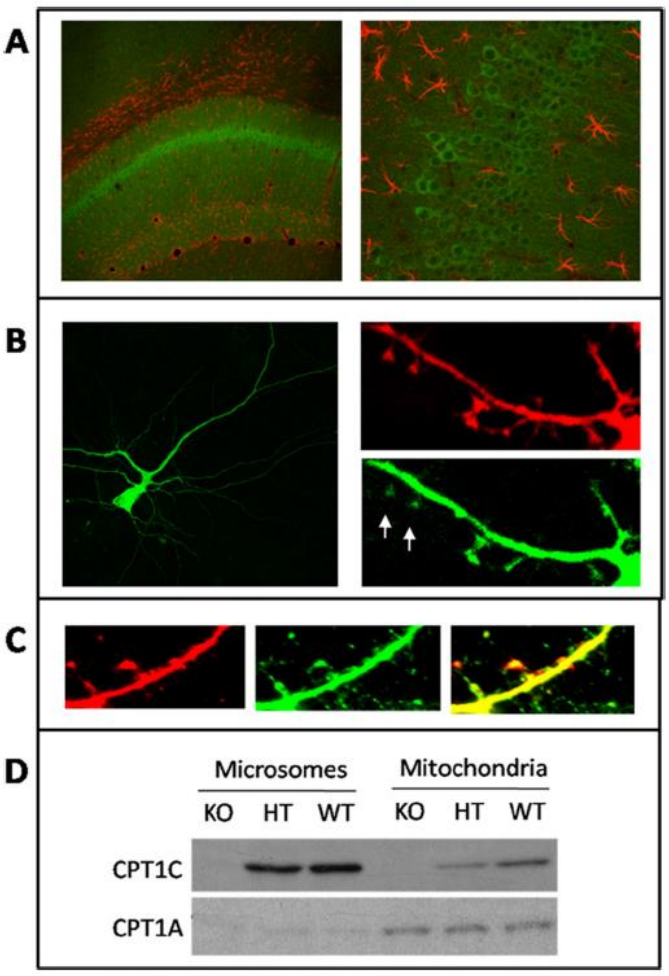

FIGURE 1. **CPT1C location in hippocampal neurons.** *A*, CPT1C is present in neurons of the hippocampus, mainly pyramidal cells. Brain sections were double-immunodetected with anti-CPT1C antibody (*green*) and anti-glial fibrillary acidic protein antibody (*red*). *B*, hippocampal cultured neurons were 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-

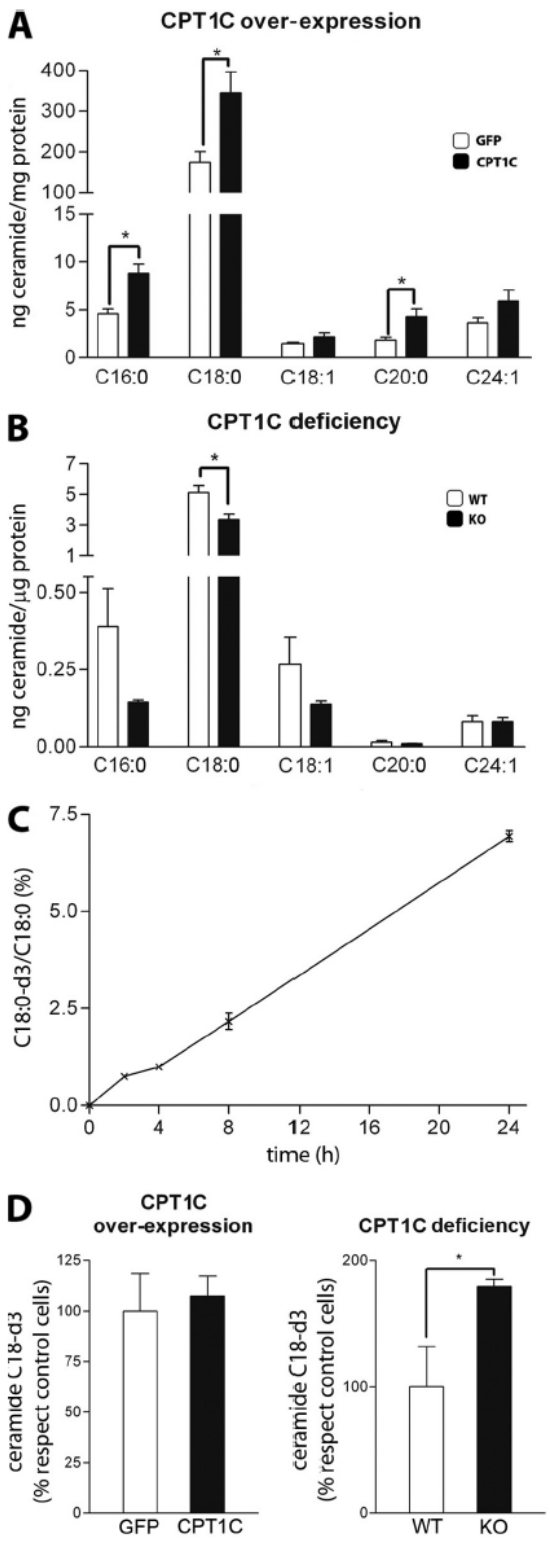

FIGURE 2. **Regulation of ceramide levels by CPT1C.** *A*, levels of ceramides in hippocampal neurons transduced with AAV1-GFP (as a control) or AAV1-CPT1C at 7 DIV. Cells were collected at 14 DIV. *B*, levels of ceramides in hippocampal neurons from WT and CPT1C KO mice. Cells were collected at 14 DIV. *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

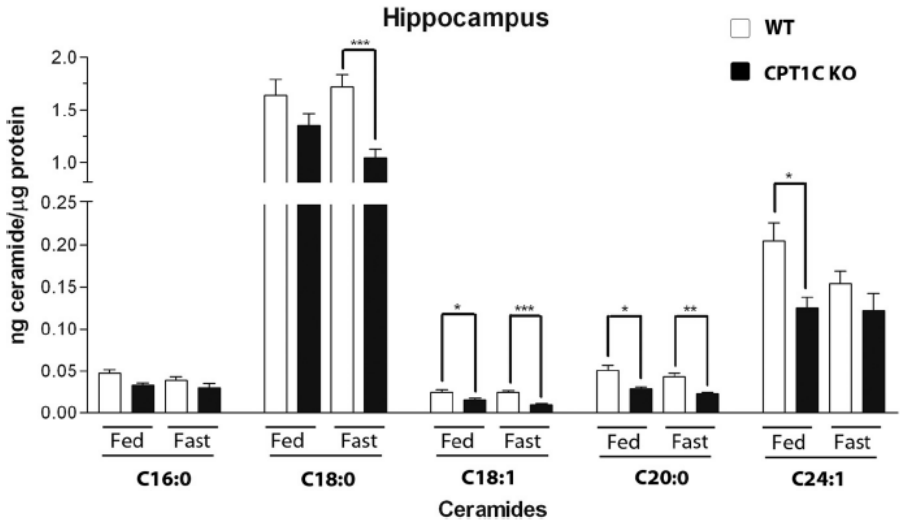

FIGURE 3. **Ceramide levels in hippocampus from *ad libitum* and fasted CPT1C KO and WT mice.** Fasted mice were deprived of food for 15 h. Different ceramide species were measured: ceramide C16:0, ceramide C18:0, ceramide C18:1, ceramide C20:0, and ceramide C24:1. *Error bars*, S.E. $n = 6$; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$, 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, $F(1.22) = 8.676$,

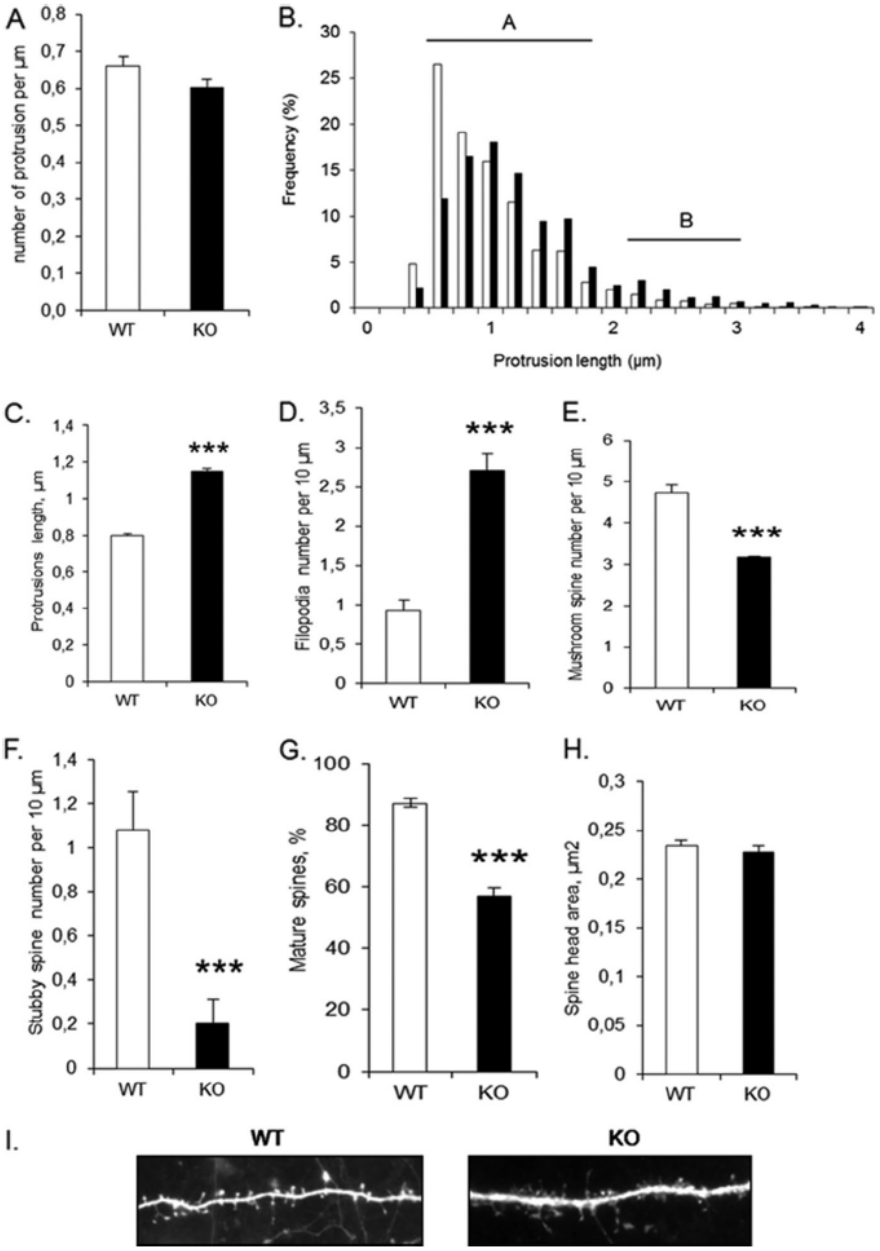

FIGURE 4. **Dendritic spine density and morphology from CPT1C KO and WT hippocampal neurons.** Hippocampal neurons were transfected (13 DIV) with pEGFP to visualize the outline of the cell. Protrusions were analyzed 2 days after transfection. Protrusion density (*A*) and protrusion length (*B* and *C*) 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

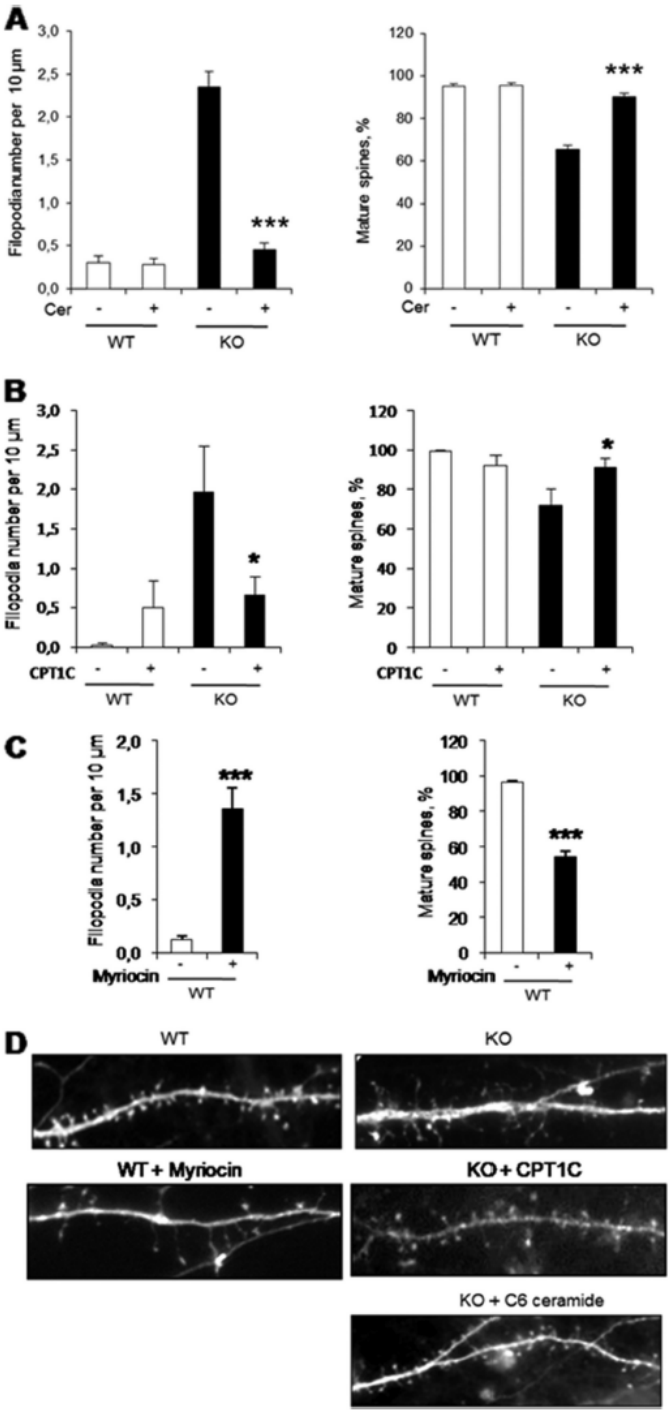

FIGURE 5. **Rescue of CPT1C KO phenotype on spine morphology by CPT1C expression or ceramide treatment.** *A*, hippocampal neurons treated with 1.5 μM C6-ceramide at 7 DIV and transfected with pEGFP (BD Biosciences) at 12 DIV, fixed, and analyzed for the morphology of dendritic protrusions at 15 DIV. *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 over-expressing 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-

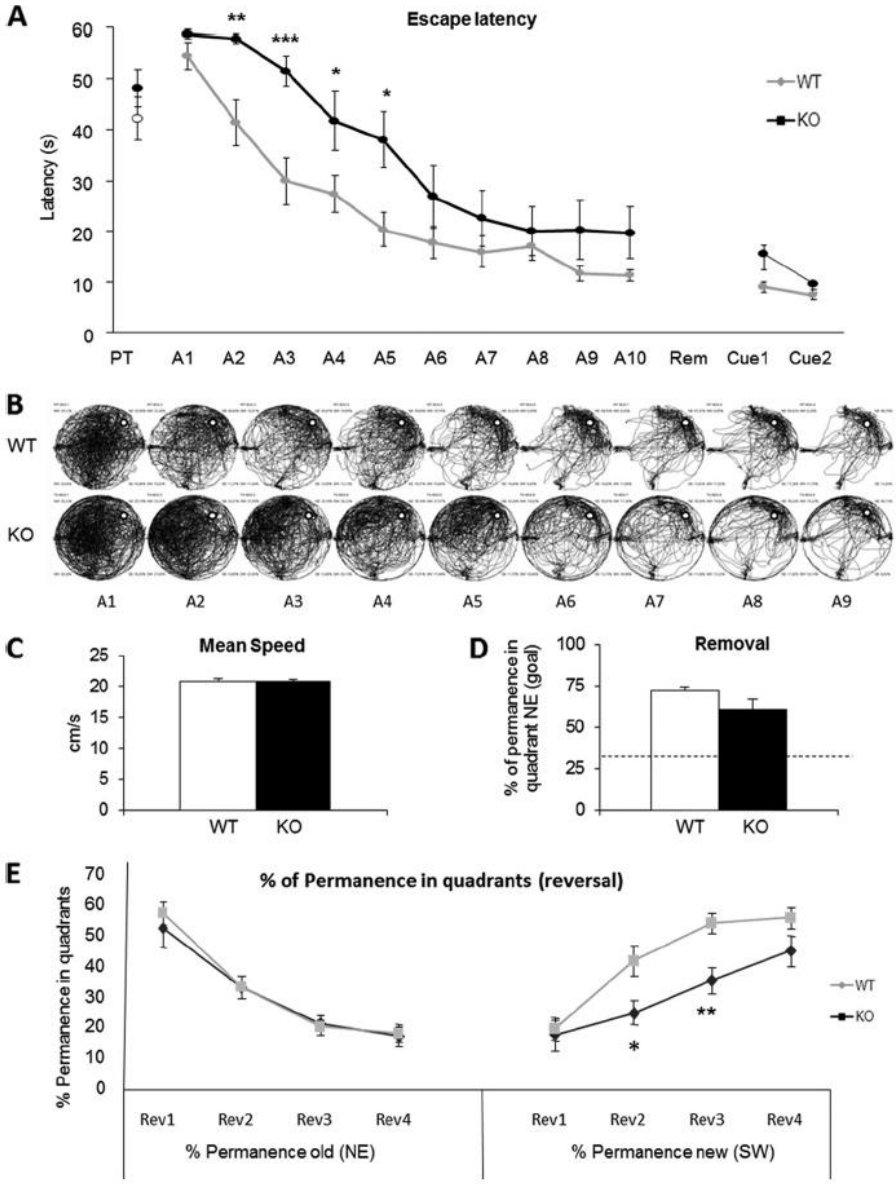

FIGURE 6. **Spatial learning and memory measured by MWM test.** *A*, MWM performance of CPT1C KO and WT mice during the learning sessions as latency (s) to find the platform along the acquisition phase (*A*), removal (*Rem*), and cued sessions (*Cue*). *PT*, pretraining. *B*, visual pathway traced by 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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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.

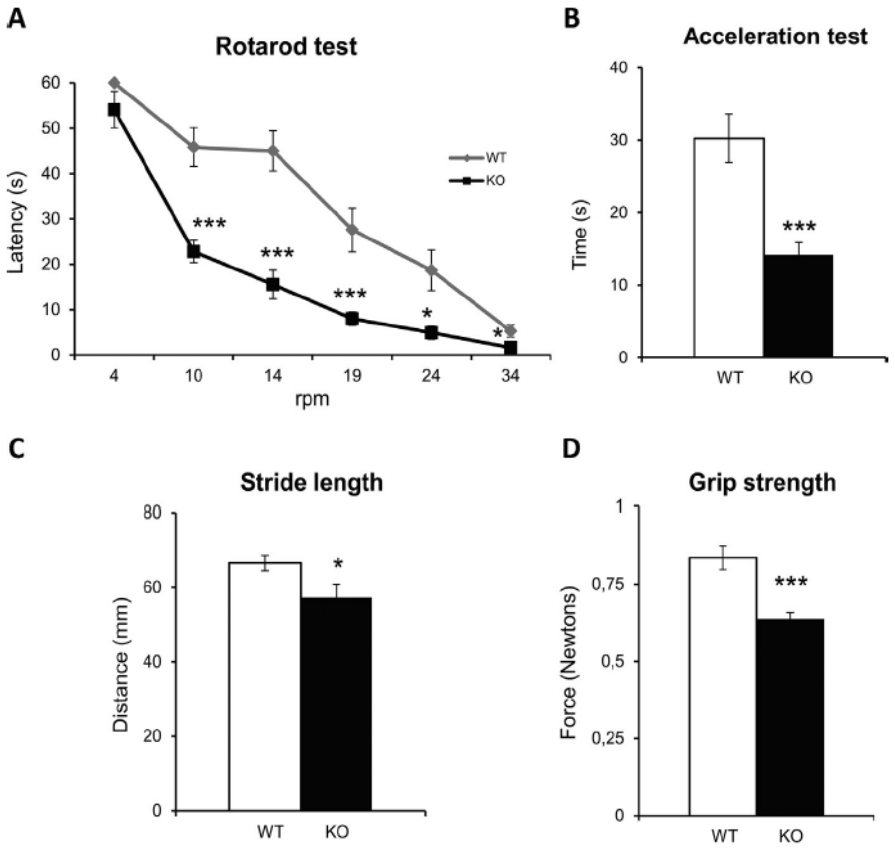
3.2. *Cpt1c KO mice are hypoactive*

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 ($\geq$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 ($n = 12$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

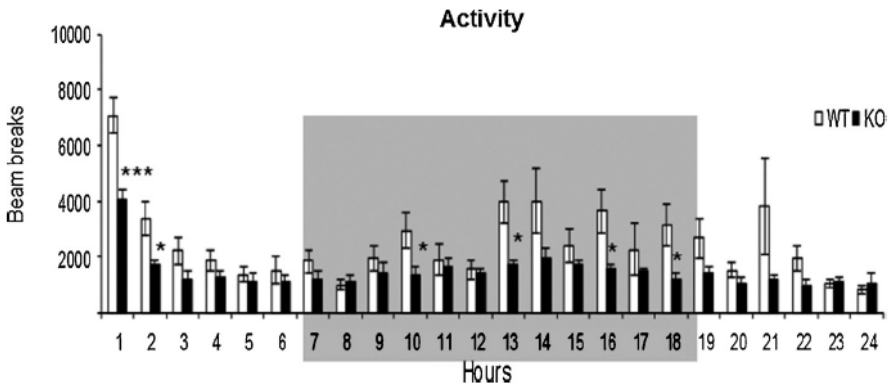


Fig. 2. Locomotor activity (actimetry) over a 24-h period. Locomotor activity in actimetry boxes measured per hour. The grey rectangle represents dark hours. Data are represented as mean ± SEM ($n = 12$). $^{*}P < 0.05$; $^{***}P < 0.001$.

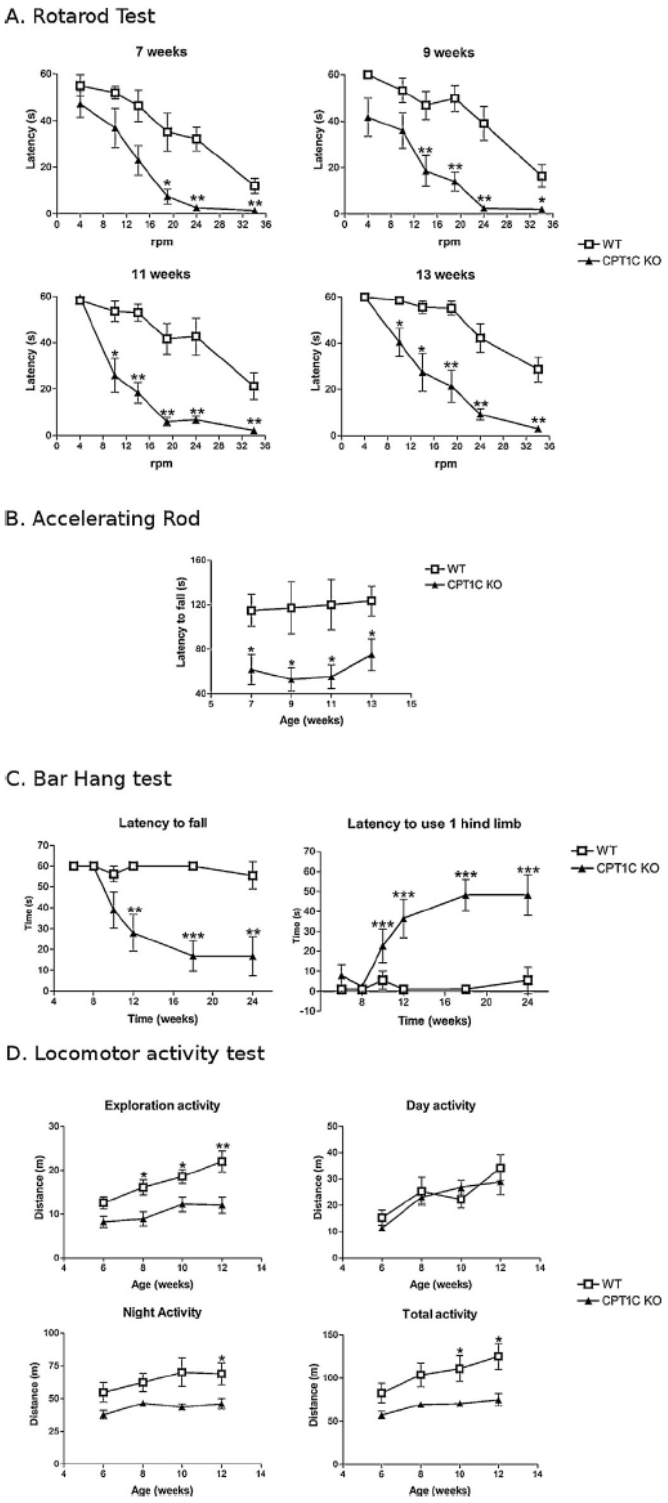


Fig. 3. Motor function at different ages. The rotarod test (A) and accelerating rod test (B) were performed at 7, 9, 11 and 13 weeks of age. The sessions on the rotarod lasted 1 min. In the accelerating rod the velocity increased from 4 to 40 rpm in 3 min. (C) Bar hang test. Latency to fall from the 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

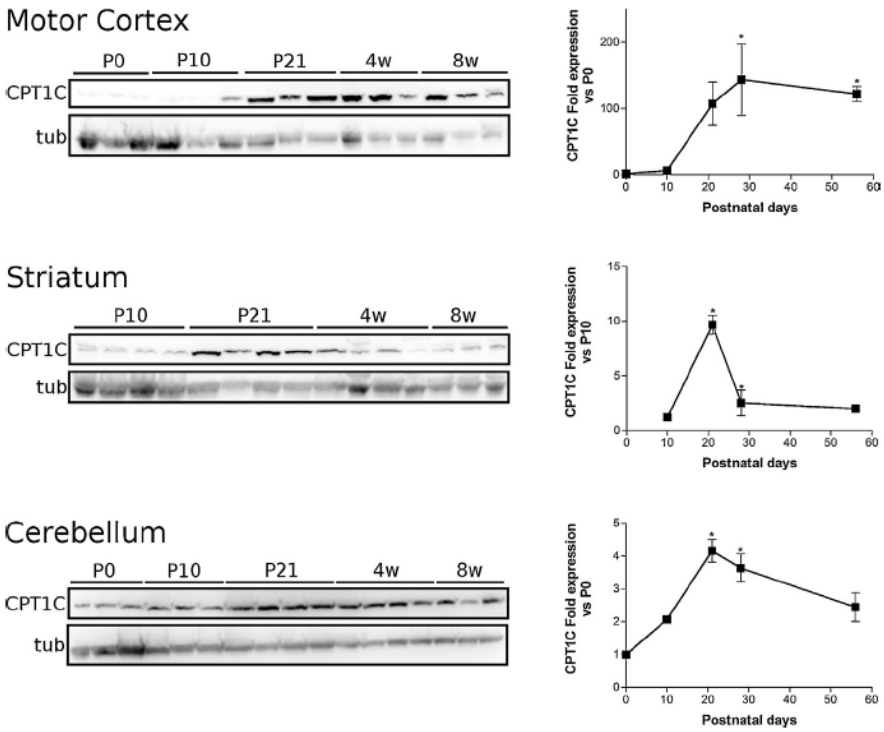


Fig. 4. CPT1C expression in the motor cortex, striatum and cerebellum during mouse development. CPT1C protein levels were measured using Western blot analysis at different postnatal days. CPT1C levels were normalized by the tubulin (tub) expression. Data are represented as mean ± SEM ($n = 4$). *$P 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

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

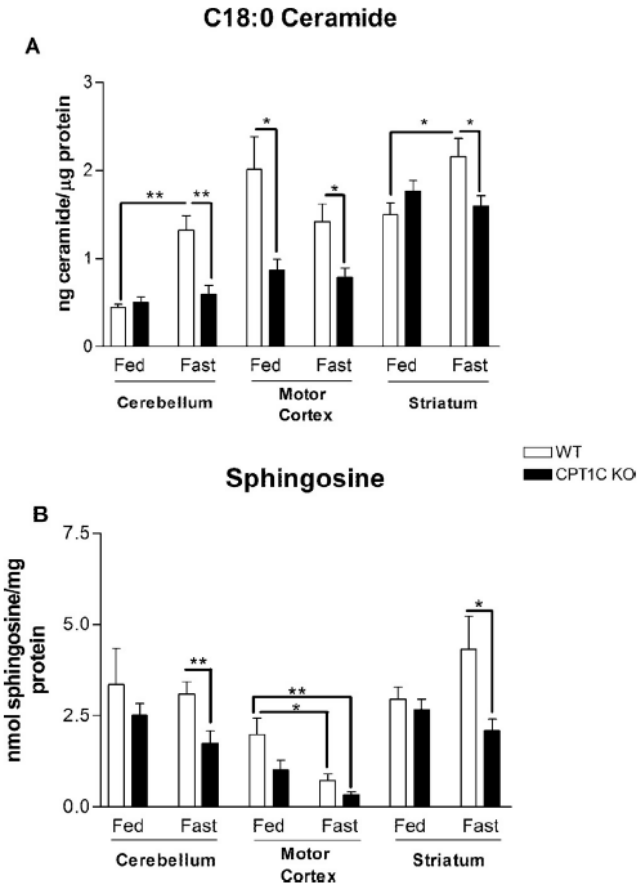


Fig. 5. Ceramide and sphingosine levels in the brain. Ceramide C18:0 (A) and sphingosine (B) levels were measured by LC-ESI-MS/MS under fed and fasting conditions in different brain areas in adult (8 weeks of age) WT and Cpt1c KO mice. Data is presented as mean ± SEM ($n = 10$). $^{*}P < 0.05$; $^{**}P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

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

Ramírez S, Martins L, Jacas J, Carasco P, Pozo M, Clotet J, Serra D, Hegardt FG, Diéguez C, López M, Casals N.

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Aportacions: figura 3.

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

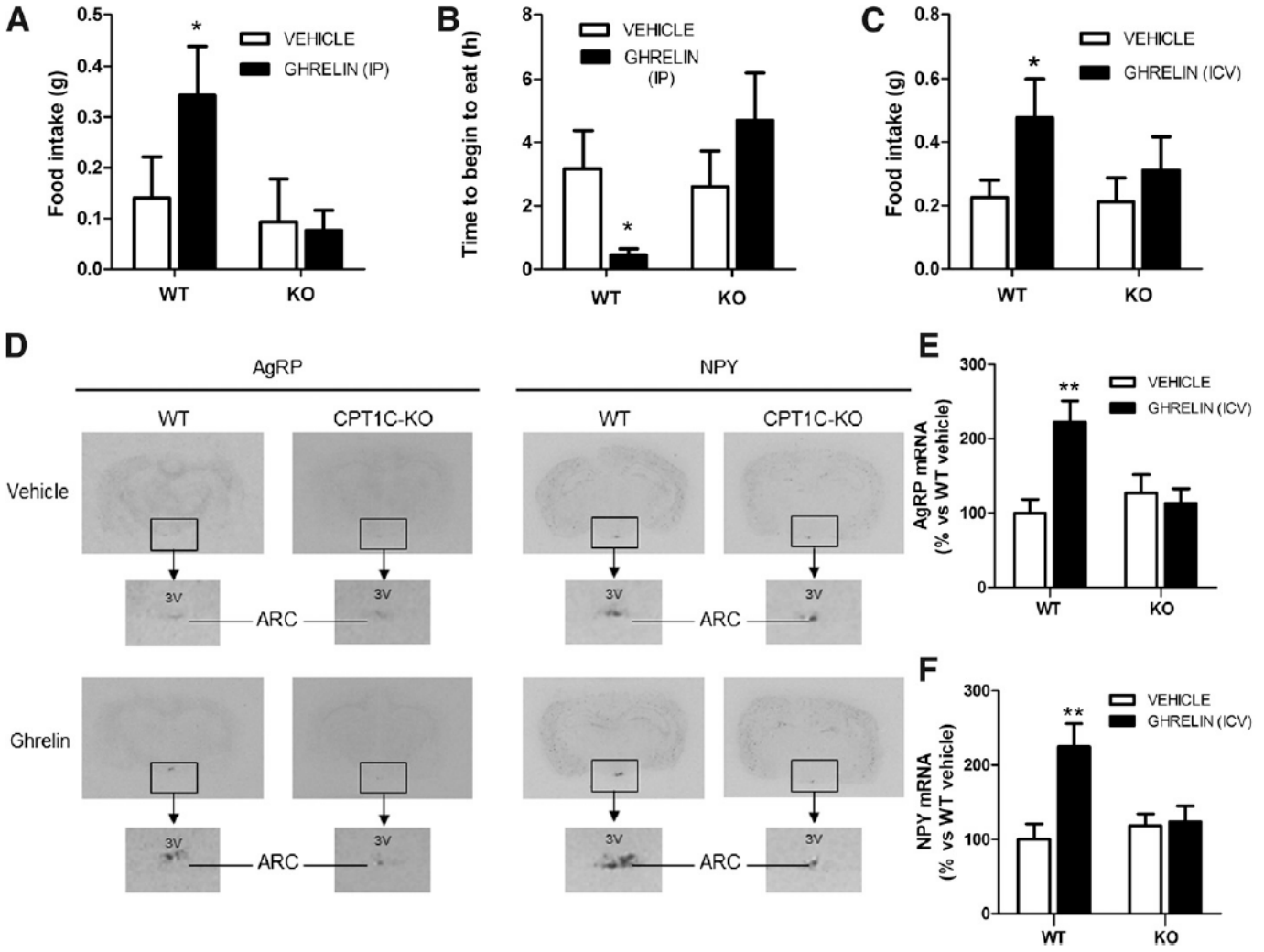

FIG. 1. Ghrelin does not induce orexigenic effects in CPT1C KO mice. *A*: The 2-h food intake in WT and CPT1C KO mice treated IP with vehicle (white bars) or with 10 μg ghrelin (black bars). *B*: Time to begin to eat after IP injection of vehicle (white bars) or 10 μg ghrelin (black bars). *C*: The 2-h 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

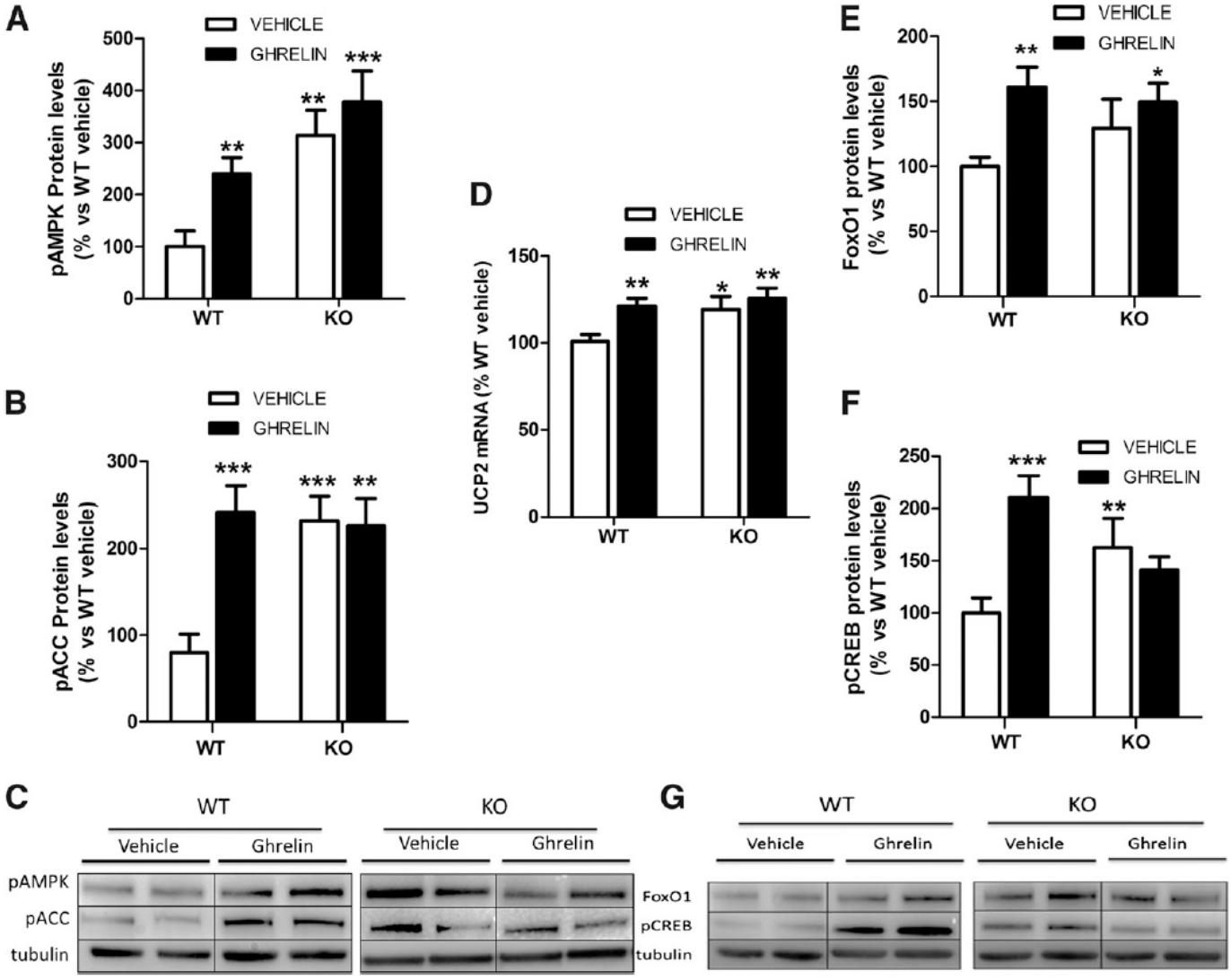

FIG. 2. The ghrelin signaling pathway in WT and CPT1C KO mice. Hypothalamic protein levels of pAMPK (*A* and *C*) and pACC (*B* and *C*), MBH mRNA levels of UCP2 measured by real-time PCR (*D*), and the hypothalamic protein levels of FoxO1 (*E* and *G*) and pCREB (*F* and *G*) in WT and CPT1C KO mice 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

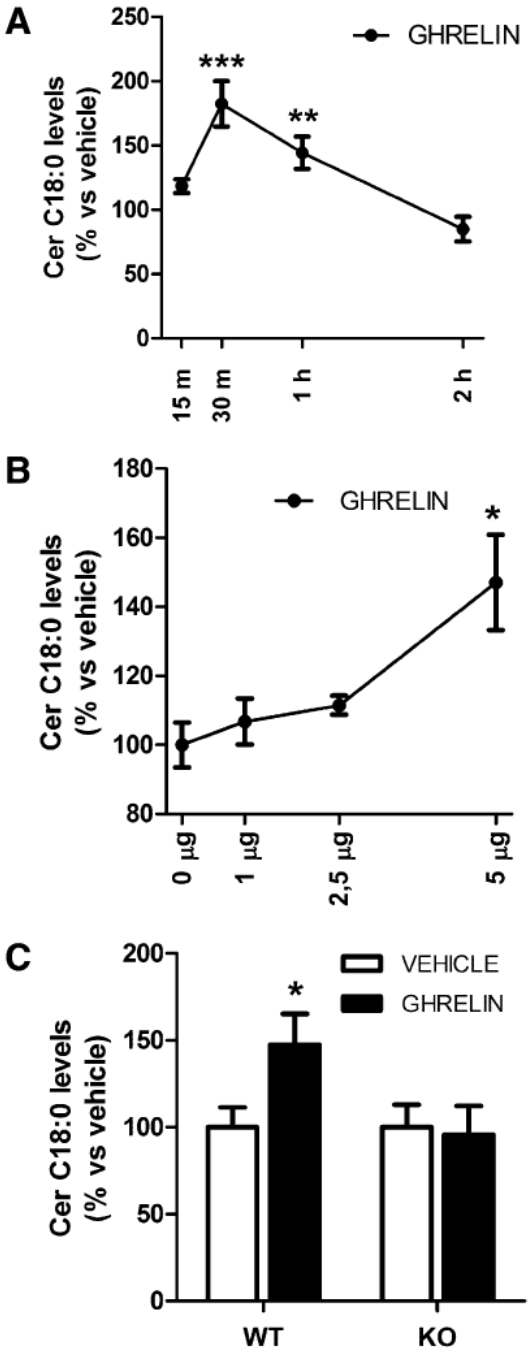

FIG. 3. MBH ceramide levels in response to ghrelin. ***A***: **Time course of MBH C18:0 ceramide levels in WT mice after ghrelin administration (ICV, 5 μg). Percentage of respective increase in vehicle-treated mice is represented.** ***B***: **MBH C18:0 ceramide levels in WT after 30 min of ICV administration 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.**

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

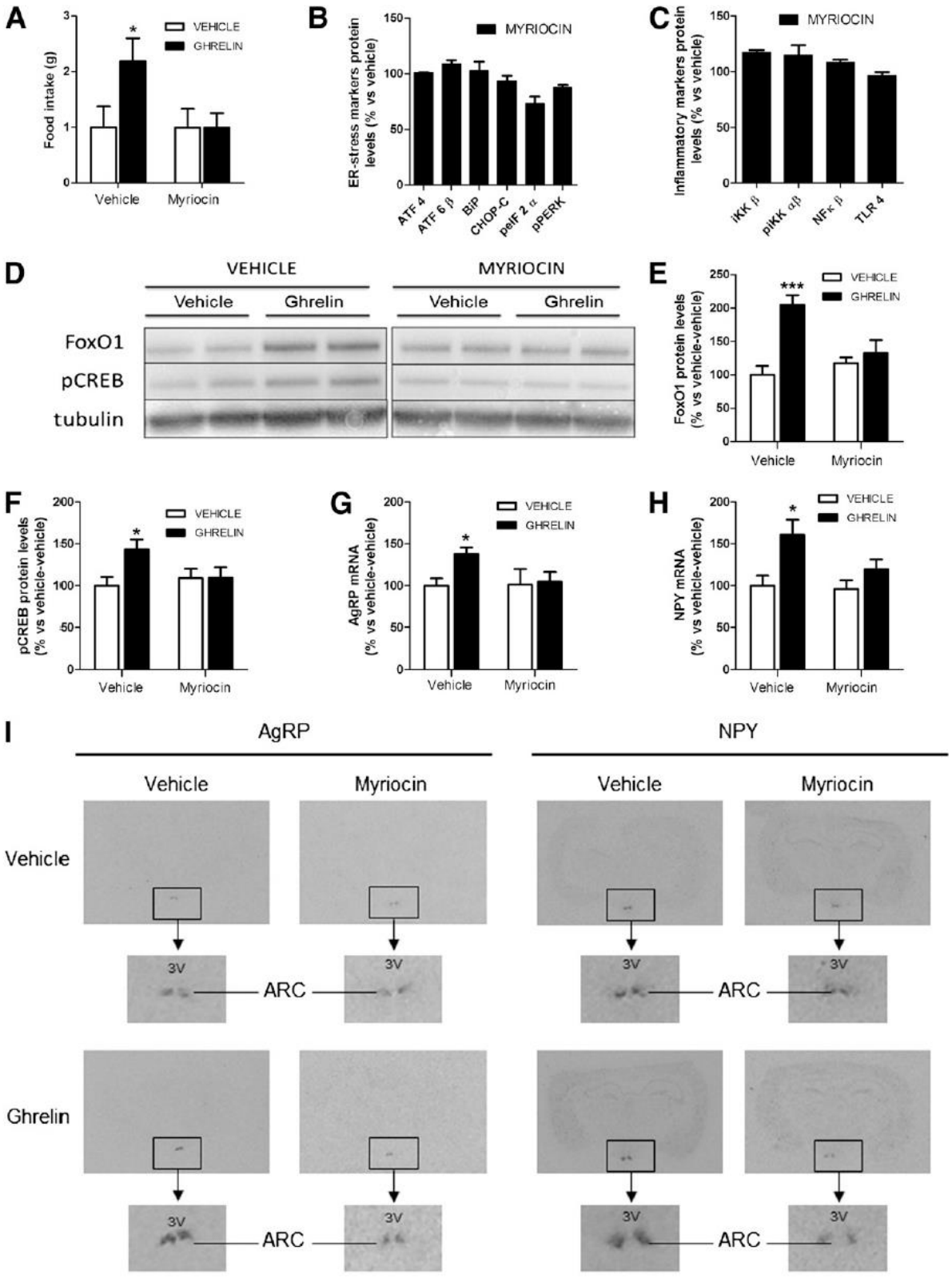

FIG. 4. Myriocin injection blocks the orexigenic effect of ghrelin. WT mice were pretreated with ICV vehicle or 4 μg ICV myriocin 1 h before ICV administration of vehicle (white bars) or 5 μg ICV administration of ghrelin (black bars). *A*: Food intake after 2 h of ghrelin injection. ICV injection of 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.

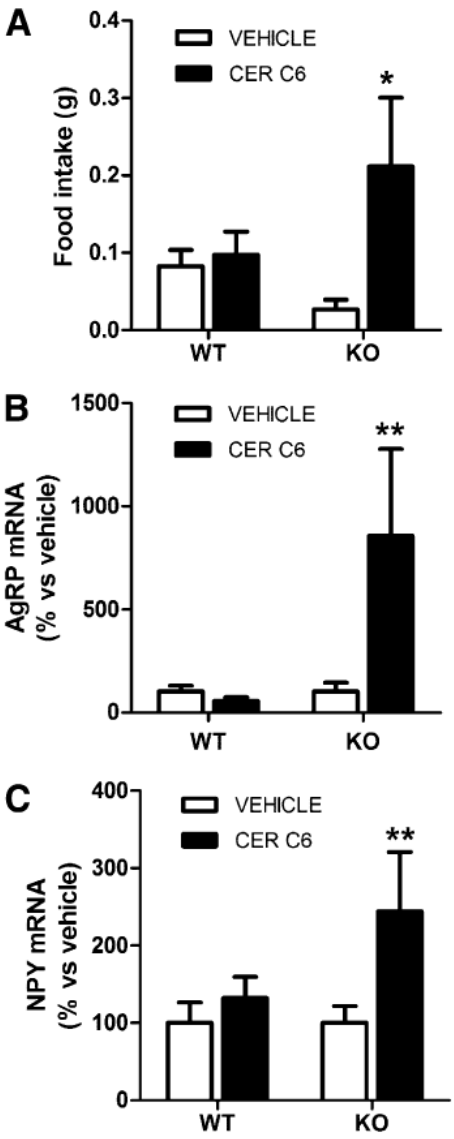

FIG. 5. Ceramide induces food intake in CPT1C KO mice. WT and CPT1C KO mice were treated with ICV vehicle (white bars) or 2.5 μg ICV ceramide C6:0 (black bars) at the beginning of the light cycle. Food intake (*A*) was measured 3 h after the injection. AgRP (*B*) and NPY (*C*) mRNA levels in MBH were 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.

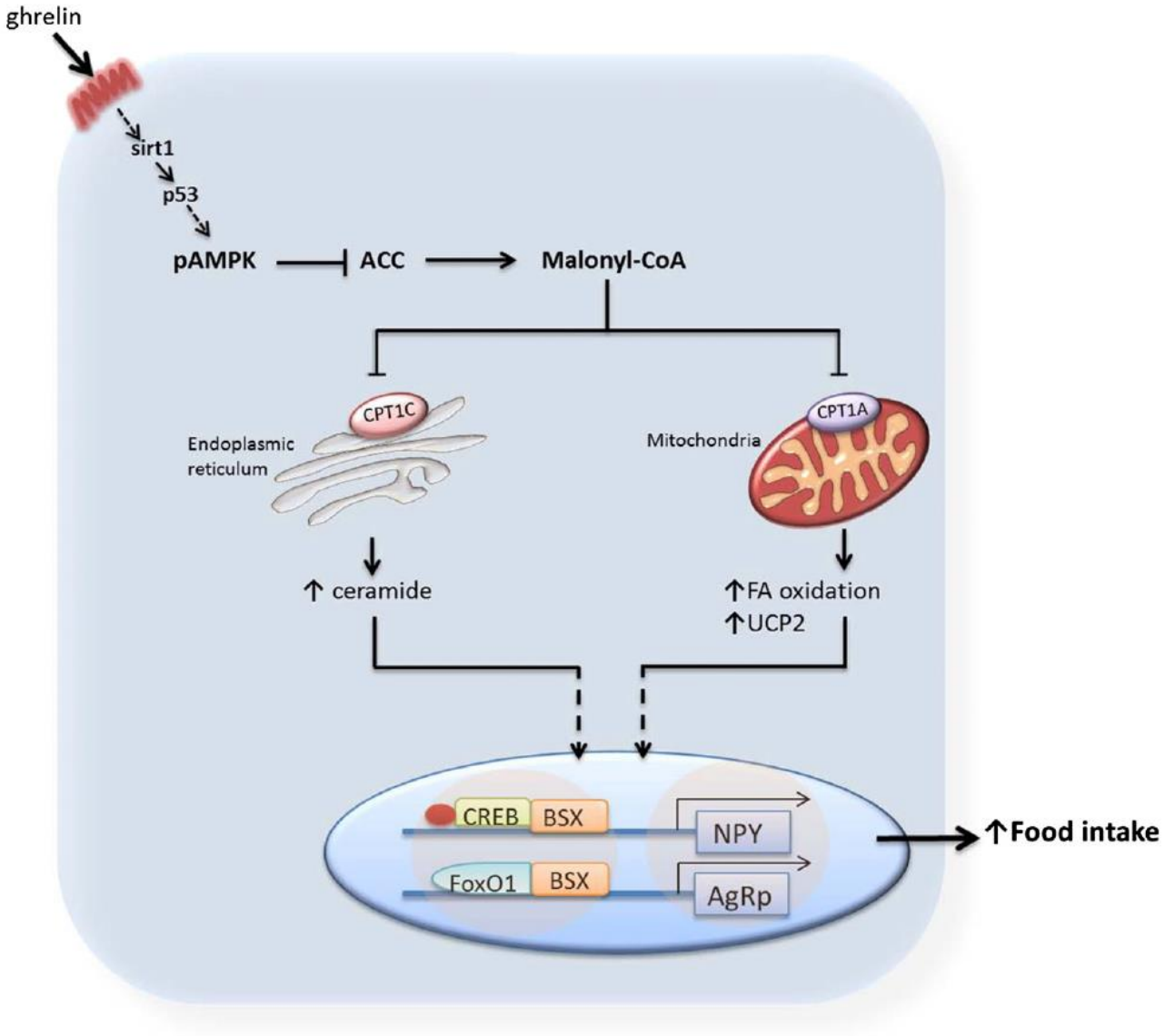

FIG. 6. Schematic diagram showing the involvement of CPT1C and ceramides in the hypothalamic ghrelin signaling pathway. Ghrelin stimulates the hypothalamic SIRT1/p53/AMPK axis, leading to decreased levels of hypothalamic malonyl-CoA, the physiological inhibitor of CPT1 enzymes. In the "classical" mechanism 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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No potential conflicts of interest relevant to this article were reported.

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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DISCUSSIÓ I CONCLUSIONS

1. CPT1C regula els nivells neuronals de ceramides

Una de les conclusions d'aquest estudi és que CPT1C és capaç de regular, d'alguna manera, els nivells de ceramides en particular i segurament els dels esfingolípids en general, per una via metabòlica encara desconeguda. Aquest fet ja fou descrit anteriorment (29) en nucli arcuat de rata, durant l'estudi de l'efecte de la leptina en el control central de la ingesta, i ara es demostra en hipocamp, cerebel, còrtex motor i nucli estriat de ratolí, intentant explicar les alteracions en la gènesi i maduració d'espines dendrítiques així com en les conseqüències motores que se'n deriven. També s'ha vist que en cultius primaris hipocampals de ratolí que la deficiència de CPT1C causa una davallada en els nivells de ceramides i la sobreexpressió d'aquesta proteïna en provoca un augment, comparat amb WT.

Així s'enceta una nova línia d'investigació per a determinar de quina manera CPT1C és capaç de regular el metabolisme dels esfingolípids, ja sigui de forma directa o indirecta. El fet de que els cultius hipocampals CPT1C-KO mostrin uns nivells davallats de ceramides respecte els WT, però que en canvi, la síntesi *de novo* es trobi regulada a l'alça, segurament s'explica pel propi disseny de l'experiment i per les propietats de l'enzim SPT. Ha estat a bastament demostrat la capacitat de SPT per a incrementar la seva activitat enzimàtica quan les cèl·lules en estudi han estat incubades amb concentracions creixents de serina en el medi de cultiu així com en presència de palmitat (39-41). En experiments duts a terme pel nostre grup en el marc d'aquesta tesi doctoral i que encara no han estat publicats es va voler comprovar com afectava a cultius hipocampals WT i CPT1C-KO la incubació amb 4 mM de [H^2]L-serina-d7 a la síntesi *de novo* de ceramides a temps curts (3h) i llargs (24h). Com en el primer dels articles defensats en aquesta tesi, els cultius de neurones CPT1C-KO mostra una concentració més elevada de ceramida C18:0 deuterada respecte a WT a les 3 hores, mentre que a les 24 hores aquestes diferències desapareixen (Figura 15).

Malgrat que en el primer article defensat en aquesta tesi es conclou que CPT1C no intervé en la síntesi *de novo* de ceramides i que l'increment observat és degut a un mecanisme compensatori independent de CPT1C, està acceptat

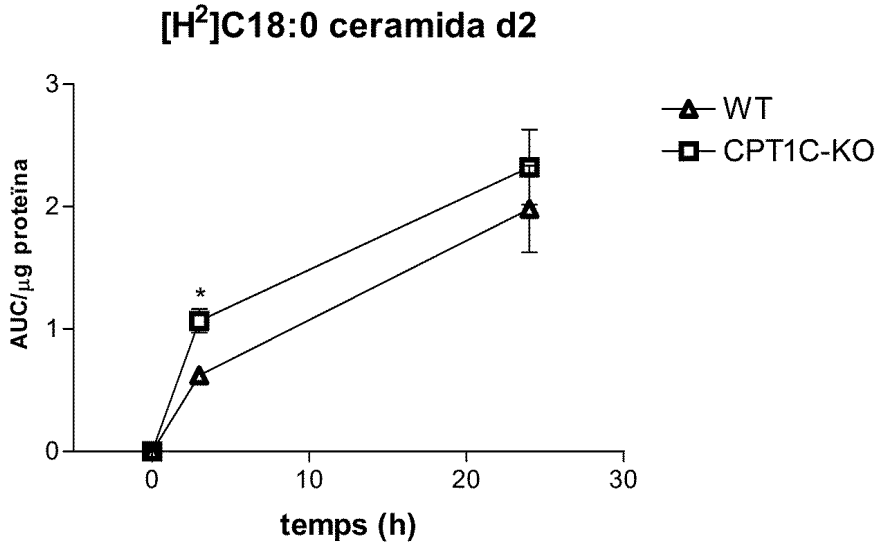

Figura 15. Estudi de la síntesi *de novo* de ceramides. Estudi de la incorporació de [H^2]L-serina-d7 (4 mM) a [H^2]C18 ceramida-d2 en cultius primaris hipocampals de ratolí WT i CPT1C-KO al llarg del temps.

que és precisament aquesta via la que s'encarrega de regular els nivells cel·lulars de ceramides, i que la resta de vies metabòliques dels esfingolípids actuen sobretot en resposta a estímuls que rep la cèl·lula per a desencadenar una acció concreta mediada per ceramides i els seus derivats (apoptosi, migració i adhesió cel·lular, etcètera) (30).

De tot això se'n pot concloure que les neurones hipocampals CPT1C-KO tenen plenament funcional el mecanisme de síntesi *de novo* de ceramides, doncs després de 24 hores d'incubació amb serina deuterada els nivells de ceramides són equivalents a les cèl·lules hipocampals WT, i per tant sí que sembla que CPT1C pot estar afectant la síntesi *de novo* de ceramides.

En un estudi de metabolòmica es va determinar que els ratolins CPT1C-KO disposen de nivells inferiors de palmitat al cervell respecte als WT (42), malgrat que aquestes diferències no són significatives. Es planteja, per tant, que aquesta petita diferència en la concentració de palmitat sigui suficient per afectar el rendiment de SPT i de retruc els nivells totals de ceramides, fent que siguin inferiors en ratolins CPT1C-KO. Havent d'aclarir aquest plantejament, també caldria establir de quina forma intervé CPT1C en aquest procés: d'una banda, podria actuar de forma directa com a sensor o fins i tot com a proveïdor

de palmitoil-CoA de SPT; per l'altra, intervindria de forma indirecta en la síntesi *de novo* de ceramides alterant la disponibilitat de palmitat de les neurones.

És conegut que malgrat la serina no és un aminoàcid essencial, les neurones no la poden sintetitzar i l'obtenen dels astròcits (43), els quals la sintetitzen a partir de 3-fosfoglicerat, un metabòlit derivat de la glicòlisi (44-46). En l'esmentat estudi de metabolòmica de cervell sencer de ratolins WT i CPT1C-KO també determina nivells de serina i de 3-fosfoglicerat. Aquest estudi revela que, malgrat no són prou significatius, els nivells de glucosa i 3-fosfoglicerat són inferiors en el cervell de ratolins CPT1C-KO respecte als WT. Aquest fet fa que hom esperi que els ratolins CPT1C-KO tinguin uns nivells davallats de serina respecte WT, ja que els seus precursors es troben disminuïts. Res més lluny de la realitat, els ratolins WT i CPT1C-KO tenen nivells equivalents de serina al cervell. Quina explicació se'n podria treure? Possiblement el disseny de l'experiment hi té a veure, doncs al cap i a la fi s'està estudiant una barreja heterogènia de cèl·lules del cervell, sense poder compartimentalitzar la concentració de serina en astròcits o hipocamp, per exemple. Per tant, caldrien estudis més concrets i segons el tipus cel·lular per determinar si realment hi ha una manca de 3-fosfoglicerat en astròcits que afecti la síntesi de serina, que acabaria afectant al rendiment de SPT i per tant a la síntesi *de novo* de ceramides.

La conclusió que se'n pot extreure de tot plegat és que per una via que encara roman determinar, CPT1C regula els nivells de ceramides de les neurones, molt probablement per la via *de novo*. El fenotip de les neurones CPT1C-KO es pot induir mitjançant miriocina, un inhibidor de SPT, tal i com queda demostrat en el primer treball que es defensa en aquesta tesi doctoral, dut a terme conjuntament amb la Dra. Patricia Carrasco. Per altra banda, els nivells de ceramides en neurones CPT1C-KO es poden restaurar a nivells basals dels cultius WT mitjançant serina deuterada incorporada externament al medi de cultiu.

2. Els ratolins CPT1C-KO tenen regulada a la baixa la resposta a ghrelina mediada per ceramides

En un l'estudi destinat a conèixer la relació entre l'efecte orexigènic de l'hormona ghrelina i CPT1C, es va analitzar de quina forma resultaven alterats els nivells de ceramides del MBH quan s'administrava ghrelina via ICV.

En el marc d'aquesta tesi doctoral es conclou que la senyal hormonal de ghrelina desencadena un augment transitori en la síntesi de ceramides en el MBH dels ratolins WT, obtenint el pic màxim d'augment als 30 minuts postadministració. Contrariament, els ratolins CPT1C-KO no mostraven cap resposta orxigènica al tractament amb ghrelina, i presentaven nivells de ceramides al MBH equivalents als obtinguts amb l'administració de vehicle. Aquesta es la primera vegada que es demostra que la variació dels nivells de ceramides al MBH regulen la ingesta i que aquests canvis son mediats per la proteïna CPT1C.

Aquest experiment es dugué a terme en el context de l'estudi realitzat per la Dra. Sara Ramírez i Luís Martins per demostrar el paper de CPT1C en el control de la ingesta. Per acabar de determinar que efectivament les ceramides eren capaces d'exercir un paper clau en la regulació hipotalàmica de la ingesta, estudiaren la senyalització desencadenada per la infusió intracerebroventricular (ICV) de ghrelina en Arc que desencadenà un augment de la ingesta alimentària acompanyada d'una regulació a l'alça dels neuropèptids NPY i AgRP en animals WT, cosa que no succeïa en CPT1C-KO. Curiosament, en canvi, els animals CPT1C-KO tenien regulats a l'alça els nivells basals de pAMPK, pACC, UCP2 i pCREB, i de forma no significativa, també FoxO1. Aquest resultat suggerí que la ruta de senyalització canònica de la ghrelina era activada fins i tot en animals CPT1C-KO, tot i que la manca de CPT1C bloquejava la inducció dels neuropèptids orexigènics i la ingesta alimentària

Considerant que: 1) la infusió de ghrelina ICV en l'hipotàlem mediobasal (MBH) causa un augment dels nivells de ceramides en ratolins WT però no en CPT1C-KO, i 2) l'inhibidor de la síntesi *de novo* de ceramides, miriocina, reverteix la regulació a l'alça en resposta a ghrelina de FoxO1, pCREB, AgRP i NPY en animals WT; s'investigà si la injecció ICV de ceramida era capaç de induir la

ingesta d'aliments en animals saciats. . El resultat fou que en animals CPT1C-KO, els quals tenen la via senyalitzada per ghrelina regulada a l'alça respecte a WT, quan se'ls administra C6-ceramida via ICV, presenten un augment en la ingesta alimentària així com els neuropèptids NPY i AgRP. Aquest resultat demostra que les ceramides són capaces de recuperar patrons alimentaris en aquells animals que tenen la senyalització de la ghrelina activada, com succeeix en animals CPT1C-KO; en canvi, aquesta resposta no és observable en animals WT, ja que la senyalització de ghrelina es troba inactiva.

Amb aquest resultat es considerà la possibilitat de que la ghrelina exerceixi la seva funció hipotalàmica a través de dues vies paral·leles, una mitocondrial regulada per CPT1A i una altre en el reticle endoplasmàtic regulada per CPT1C, éssent les dues vies imprescindibles per a què finalment s'expressin NPY i AgRP i es desencadeni la resposta al dejú .

3. Implicació de CPT1C en les deficiències motores i el desenvolupament

De la implicació de CPT1C en el metabolisme d'esfingolípids, caldria destacar com evolucionen els nivells de ceramides al llarg de l'envelliment i quina relació guarden aquestes variacions amb el fet que en els teixits estudiats CPT1C tingui un pic màxim d'expressió als 21 dies de vida, just quan es produeix el deslletament de les ventrades. En un estudi dut a terme durant aquesta tesi doctoral i que no ha estat publicat, es van mesurar mitjançant HPLC-ESI-MS/MS els nivells de ceramides de diverses regions cerebrals en ratolins WT i CPT1C-KO dejunats i saciats a les 4 setmanes d'edat. En aquest estudi es va veure que les diferències en els nivells de ceramides entre WT i CPT1C-KO eren mínimes o inexistents. Possiblement, a mesura que l'envelliment es produeix, les diferències entre WT i CPT1C-KO van apareixent, éssent especialment evidents en situació de dejú.

Aquesta situació probablement guardi relació amb el deteriorament progressiu que els ratolins CPT1C-KO mostren al llarg de l'envelliment. Aquest deteriorament progressiu és evident en els tests de coordinació motora, de força i d'activitat duts a terme en el segon article defensat en aquesta tesi doctoral.

En el *Bar Hang test* (mesura de la força de les potes davanteres i coordinació de les darreres) es veu l'empitjorament partint d'unes capacitats de força en edats joves de CPT1C-KO equivalents a les de ratolins WT i acabant amb la incapacitat de mantindre's subjecte a la barra. De forma similar succeeix amb les proves d'activitat i coordinació (Rotarod), tot i que en aquests casos ja es veuen diferències des d'edats joves però que van empitjorant amb l'envelliment. Això vol dir que en general els animals CPT1C-KO parteixen amb unes condicions motores parcialment desavantatjoses respecte WT que van empitjorant durant el desenvolupament possiblement degut als baixos nivells de ceramides i la proliferació d'espines dendrítiques immadures.

Alguns autors apunten possibles papers de les ceramides en el desenvolupament i supervivència de les neurones. Se sap que són lípids necessaris per a la diferenciació de les dendrites (47) i que una davallada dels

seus nivells basals en cervell provoca atàxia cerebral i neurodegeneració de les cèl·lules de Purkinje (48). De fet, una deficiència funcional en l'enzim 3-fosfoglicerat deshidrogenasa (PHGDH) causa un dèficit de L-serina, que al ser precursora en la síntesi *de novo* de ceramides, acaba provocant una davallada dels nivells dels esfingolípids (49), d'entre els quals caldria destacar els gangliòsids, sintetitzats a partir de glucosilceramida, l'absència dels quals podria tenir un paper important en la manca de maduresa de les espines neuronals (50). També s'ha pogut observar que en embrions deficients en PHGDH (moren en E13,5) hi ha dificultat en la generació de neurites en les neurones de la corda espinal (51), malgrat no es concreta el paper que hi juguen els esfingolípids en general i les gangliòsids en particular. Per últim, es defensa la possibilitat de que les ceramides protegeixin les motoneurones de la mort cel·lular produïda per senyals oxidatives (52,53).

Per altra banda, i tenint en compte el procés de formació i maduració d'espines dendrítiques, cabria la possibilitat que alteracions en la disponibilitat de colesterol i esfingolípids pugui acabar afectant a l'actina que al cap i a la fi acaba mantenint les espines formades (54). Amb això s'amplia el ventall de proteïnes implicades en la maduració i manteniment de les espines dendrítiques que es veuen alterades davant una manca d'esfingolípids.

Per tot plegat, seria assenyat afirmar que: els ratolins mancats de CPT1C tenen uns nivells de ceramides inferior als ratolins WT; que aquesta davallada de ceramides podria comprometre la disponibilitat d'altres esfingolípids que tenen un paper clau en la maduració i el desenvolupament del cervell; que aquesta maduració i desenvolupament deficients afecten negativament a la funció i coordinació motora.

4. Més enllà del RE: CPT1C i ceramides al mitocondri

Malgrat que la síntesi *de novo* de ceramides sempre s'ha associat indiscutiblement al RE, des de ja fa força anys s'està estudiant quin paper podrien desenvolupar els esfingolípids al mitocondri. Uns últims resultats obtinguts durant aquesta tesi doctoral, centrats en l'estudi dels nivells de ceramida en RE i mitocondri de cervells de ratolins WT i CPT1C-KO adults en situació de sacietat mostren com les diferències entre els dos genotips són força superiors en mitocondri que no pas en homogenat (Figura 16). El RE no mostra diferències.

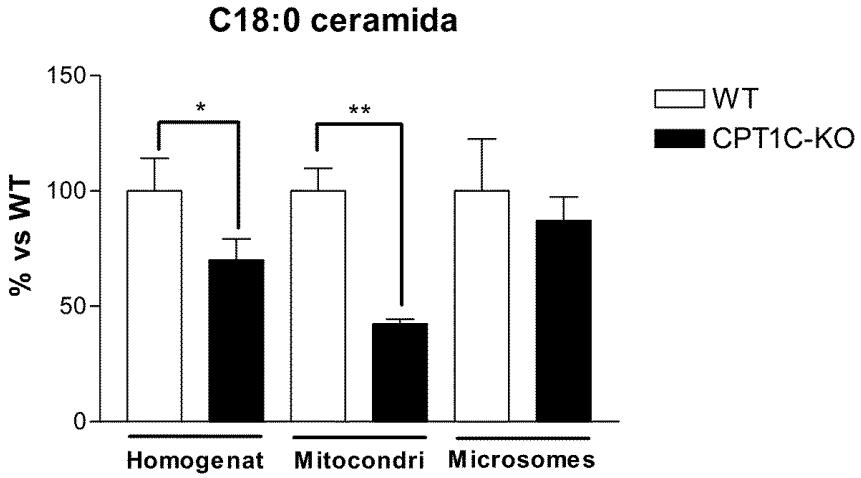

Figura 16. Mesura de ceramides en orgànuls cel·lulars de cervell de ratolí. Els cervells foren extrets de ratolins WT i CPT1C-KO adults, i els orgànuls foren obtinguts mitjançant centrifugacions successives. La ceramida fou determinada mitjançant HPLC-ESI-MS/MS.

Malgrat que l'origen de les ceramides que es troben al mitocondri és encara motiu de discussió, hi ha cert consens sobre la seva funció en aquest orgànul. Recentment s'ha pogut demostrar, per una banda, que el mitocondri disposa dels enzims necessaris per a la síntesi de ceramides, ja sigui mitjançant CerS (55) o activitat ceramidasa (CDasa) reversa (56) amb la finalitat d'intervenir en la senyalització de l'apoptosi; per altra banda, també es defensa la possibilitat de que les ceramides accedeixin al mitocondri a través de les MAM en forma de canals de ceramides, que alhora permeten el trànsit d'altres proteïnes (57).

El fet de que els ratolins CPT1C-KO disposin de nivells inferiors de ceramides en mitocondri respecte al genotip WT hauria de ser objecte d'estudi, concretament pel que fa a la resposta a senyals que condueixen a l'apoptosi.

Estem davant d'una possible alteració de la funció mitocondrial que podria anar des de la regulació de l'apoptosi a deficiències en la permeabilització de les membranes externa i interna, doncs està descrit que en part depèn de les ceramides (57).

Aquest estudi sobre els nivells de ceramides en RE i mitocondri fou dut a terme degut a la constància de que CPT1C, a més de trobar-se al RE, també és probable de que es trobi a les MAM, com ho demostra el Western Blot dut a terme contra diverses proteïnes i marcadors en mostres de mitocondri, RE i MAM (Figura 17). Aquest resultat, obtingut pel nostre grup en el marc d'aquesta tesi doctoral i que encara no ha estat publicat, mostra CPT1C col·localitzant amb proteïnes de reticle i mitocondri que també es troben en MAM. Les mostres es van obtenir de cervells de ratolins C57BL/6J mascles seguint un procés de separació per gradient de Percoll mitjançant centrifugacions successives a partir d'un protocol ja existent adaptat a mostres cerebrals (58).

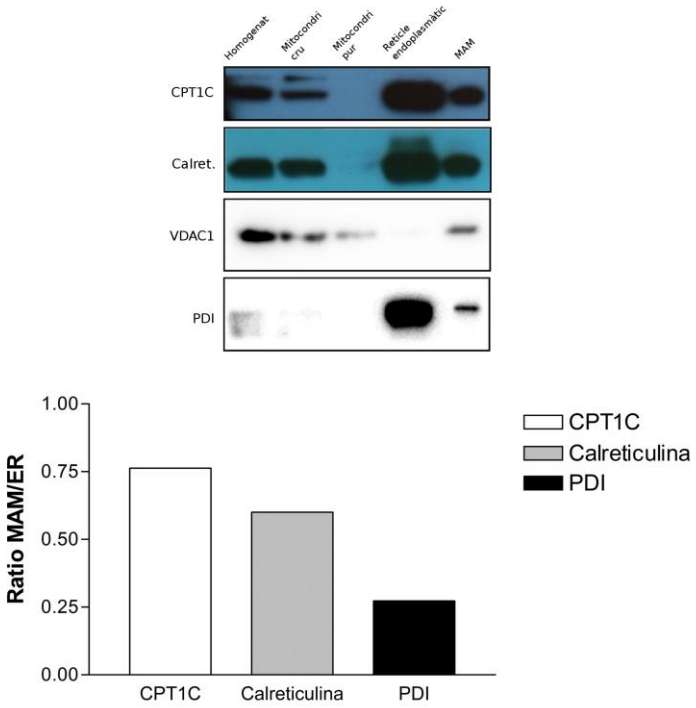

Figura 17. Presència de CPT1C en MAM. Després d'obtenir els orgànuls de cervells de ratolins WT per centrifugació en gradent mitjançant percoll, es va determinar la presència de diversos marcadors en els orgànuls: Clareticulina (marcador de RE i MAM), VDAC1 (marcador de mitocondri i MAM) i PDI (marcador de RE).

La troballa de CPT1C en MAM és un fet mancat d'estudis més extensos per a poder confirmar aquest extrem, i per tant, seria pertinent confirmar aquest resultat amb una immunocitoquímica. Malgrat tot, sembla prou evident que CPT1C es troba en MAM i caldria esbrinar si hi desenvolupa alguna funció.

Aquest descobriment permet diverses elocucions, com ara que CPT1C podria interaccionar amb CPT1A o fins i tot formar part dels trímers que aquesta isoforma mitocondrial constitueix a la membrana externa del mitocondri (59), participar en els complexes proteínics formats per CPT1A, ACSL i VDAC en la membrana externa mitocondrial per al transport de lípids (60) o bé facilitar el transport d'esfingolípids entre MAM i mitocondri, com s'ha demostrat que existeix (57).

Sigui com sigui, la participació de CPT1C en la funció mitocondrial és un camp del tot inexplorat en l'actualitat i les evidències semblen indicar que hi podria jugar un paper potencialment significatiu.

CONCLUSIONS

1. CPT1C regula els nivells neuronals de ceramides.
2. L'aminoàcid serina restaura els nivells basals de ceramides en cultius hipocampals de neurones de ratolí CPT1C-KO.
3. Els nivells mitocondrials de ceramides del cervell de ratolins es troben regulats a la baixa en absència de CPT1C.
4. La senyal hormonal de ghrelina desencadena un augment transitori en la síntesi de ceramides en el MBH de ratolins WT. Els ratolins CPT1C-KO no presenten cap resposta.
5. Ratolins CPT1C-KO manifesten alteracions en activitat, força i coordinació motora, respecte a WT.
6. En neurones de ratolí, CPT1C es localitza en RE i MAM.

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