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CPT1C IS LOCALIZED IN ENDOPLASMIC RETICULUM OF NEURONS AND HAS CARNITINE PALMITOYLTRANSFERASE ACTIVITY

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SUMMARY

CPT1c is a carnitine palmitoyl transferase-1 isoform that is expressed only in the brain. The enzyme has recently been localized in neuron mitochondria. Although it has high sequence identity with the other two CPT1 isoenzymes (a,b), no CPT activity has been detected to date. Our results indicate that CPT1c is expressed in neurons but not in astrocytes of mouse brain sections. Over-expression of CPT1c fused to the green fluorescent protein in cultured cells demonstrates that CPT1c is localized in the endoplasmic reticulum (ER) rather than mitochondria, and that the N-terminal region of CPT1c is responsible for ER protein localization. Western blot experiments with cell fractions from adult mouse brain corroborate these results. In addition, over-expression studies demonstrate that CPT1c does not participate in mitochondrial fatty-acid oxidation, as would be expected from its sub-cellular localization.
In order to identify the substrate of CPT1c enzyme, rat cDNA was over-expressed in neuronal PC-12 cells and the levels of acylcarnitines were measured by HPLC-mass spectrometry. Palmitoyl carnitine was the only acylcarnitine to increase in transfected cells, which indicates that palmitoyl-CoA is the enzyme substrate and that CPT1c has CPT1 activity. Microsomal fractions of PC-12 and HEK293T cells over-expressing CPT1c protein showed a significant increase in CPT1 activity of 0.57 and 0.13, nmols·mg^{-1}·min^{-1} respectively, which is about 50 % higher than endogenous CPT1 activity. Kinetic studies demonstrate that CPT1c has similar affinity to CPT1a for both substrates, but 20-300 times lower catalytic efficiency.

INTRODUCTION

CPT1 (Carnitine palmitoyltransferase 1) catalyses the conversion of long-chain fatty acyl-CoAs into acylcarnitines, the first step in the transport of long chain fatty acids from the cytoplasm to the mitochondrial matrix, where they undergo the beta-oxidation. This reaction is not only central to the control of fatty acid oxidation, but it also determines the availability of long-chain acyl-CoA for other processes, notably the synthesis of complex lipids.

There are three different CPT1 isozymes: CPT1a (also called L-CPT1) encoded by *CPT1a*, CPT1b (also called M-CPT1) encoded by *CPT1b*, and the recently described CPT1c (also called CPT1-C) encoded by *CPT1c*. *CPT1a* and *CPT1b* have been extensively studied since they were cloned for the first time, in 1993 and 1995 respectively (1, 2). CPT1a is the most ubiquitous expressed isoform and is found not only in liver but also in pancreas, kidney, brain, blood, and embryonic tissues. CPT1b is expressed only in brown adipose tissue, muscle and heart. Both isozymes present significantly different kinetic and regulatory properties: CPT1a displays higher affinity for

its substrate carnitine and a lower affinity for the physiological inhibitor malonyl-CoA than the muscle isoform (3). In addition, the aminoacid residues that are critical for catalytic activity or malonyl-CoA sensitivity have been identified for both enzymes, and three-dimensional structures have been predicted based on the carnitine acetyl transferase, carnitine octanoyl transferase and carnitine palmitoyl transferase II crystals (4). CPT1a and CPT1b are localized in the outer mitochondrial membrane with the N- and C-terminus facing to the cytosolic side. Western blotting and activity characterization suggested that CPT1a was also localized in microsomes but expression studies with EGFP fused to the C-terminus of CPT1a showed that CPT1a is targeted only to mitochondria, and that previous detection of CPT1a in microsomes was probably derived from membrane contact sites between ER and mitochondria (5). CPT1a and CPT1b have a critical role in the heart, liver and pancreatic-β-cells, and are potential targets for the treatment of metabolic disorders, including diabetes and coronary heart disease.

Less is known about CPT1c. Although the protein sequence is highly similar to that of the other two isozymes, CPT1c expressed in yeast or HEK293T cells displays no catalytic activity with common acyl-CoA esters as substrates (6, 7). One explanation is that palmitoyl-CoA is not a substrate for CPT1c and that another brain-specific acyl-CoA might be its natural substrate. Expression studies indicate that CPT1c is localized exclusively in the CNS, with homogeneous distribution in all areas (hippocampus, cortex, hypothalamus, etc.). The pattern resembles that of FAS, acetyl-CoA carboxylase-alpha (enzymes related to biosynthesis) rather than CPT1a or ACC-beta (enzymes related to oxidation) (6, 8). The capacity of CPT1c to bind malonyl-CoA has been demonstrated, and it has been suggested that CPT1c regulates malonyl-CoA availability in the brain cell.

It has recently been reported that knock-out mice for CPT1c ingest less food and have a lower body weight when fed a standard diet. When these animals are fed high-fat chow, body weight increases more than control animals and they become resistant to insulin, suggesting that CPT1c is involved in energy homeostasis and control of body weight (7). Moreover, ectopic over-expression of CPT1c by stereotactic hypothalamic injection of a CPT1c adenoviral vector protects mice from adverse weight gain caused by high-fat diet (9).

In this paper we report that CPT1c is localised in neurons but not in astrocytes of adult brain. We also demonstrate that CPT1c is localised in the ER of the cells and not in mitochondria, and that CPT1c shows carnitine palmitoyl transferase activity.

EXPERIMENTAL PROCEDURES

Culture of PC-12, SHSY5Y, Fibroblasts and HEK293T cells

The human neuroblastoma cell line, SHSY5Y, the human embryonic kidney-derived cell line, HEK293T, and human fibroblasts cells were grown at 37ºC in the presence of 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium) with high glucose containing 2mM glutamine, 10% foetal calf serum, penicillin (100 units/ml) and streptomycin (100µg/ml). Medium for PC-12 cells was DMEM with high glucose containing 2mM glutamine, sodium pyruvate, 5% foetal calf serum, 10% horse serum, penicillin (100units/ml) and streptomycin (100µg/ml).

Cells cultured in 24-wells plates were transfected with 0.8µg of plasmid (purified with the Qiagen Maxi Prep Kit) using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's protocol. Transfection efficiency was about 30%-50%.

Plasmid constructions:

pCPT1c-EGFP and pCPT1a-EGFP:

rat CPT1c cDNA was obtained by RT-PCR performed with 2 µg of total rat brain RNA. The 2700 bp fragment amplified was cloned in pBluescript and sequenced. pEGFP-N3 vector (from Clontech, BD Biosciences) was used to clone the coding region of *CPT1c* or *CPT1a*, to create pCPT1c-EGFP and pCPT1a-EGFP respectively. pCPT1c-EGFP and pCPT1a-EGFP plasmids encode CPT1c and CPT1a proteins fused to the N- terminal region of EGFP (green fluorescence protein), respectively.

Chimera constructions:

pCPT1ac-EGFP:

460bp of the 5' coding sequence of rat *CPT1a* gene was PCR amplified with primers which created a *Hin*dIII site and an *Hpa*I site at the ends of the amplified fragment. This PCR product was cloned into a pCPT1c-EGFP plasmid previously digested by *Hin*dIII and *Hpa*I (which deleted the 460 bp of the 5' terminus of *CPT1c* coding sequence). The resulting plasmid encodes a fused protein constituted by the N terminus and the two transmembrane domains of CPT1a, the catalytic domain of CPT1c, and EGFP.

pCPTca-EGFP:

A segment of the first 462bp of rat *CPT1c* gene was PCR amplified with two primers that created a *Hin*dIII site a *PpuM*I site at the ends of the amplified fragment. This PCR product was digested and cloned into a pCPT1a-EGFP plasmid, previously digested by *Hin*dIII and *PpuM*I (which deleted the 460 bp of the 5' terminus of *CPT1c* coding sequence). The resulting vector contained the N terminus and the two transmembrane domains of CPT1c and the catalytic domain of CPT1a fused to EGFP.

pIRES-CPT1a and pIRES-CPT1c

The coding region of rat *CPT1a* and *CPT1c* were cloned in vector pIRES2-EGFP (Clontech, BD Biosciences), which permits both the gene of interest and the EGFP gene to be translated from a single bicistronic mRNA.

Co-localization studies in brain sections

For co-localization studies we performed combined in situ hybridization / immunocytochemistry or double immunofluorescence, using standard protocols.

For combined *in situ* hybridization, coronal sections (30µm) from adult mouse forebrains were used. Processed sections were hybridized overnight at 56ºC, with *cpt1c* riboprobes (full rat cDNA) labelled with digoxigenin-d-UTP (Roche) at a concentration of 500 ng/ml. After stringent washing, sections were incubated at 4ºC overnight with an anti-DIG antibody (1/2000) conjugated to alkaline phosphatase (Roche) and developed with BCIP/NBT substrate (Invitrogen). Tissue sections were mounted on gelatinized slides with Mowiol. Those sections which were hybridized with control sense riboprobes did not give any hybridization signal.

After *in situ* hybridization, some slices were collected and processed by inmunofluorescence. The primary antibody was mouse anti-NeuN (1:75) (Chemicon). The secondary antibody was biotinylated (Vector Laboratories, Inc., Burlingame, CA, USA). The streptavidin-horseradish peroxidase complex was from Amersham. Sections were developed with 0.03% diaminobenzidine and 0.003% hydrogen peroxide, mounted onto slides, dehydrated and coverslipped with DPX.

In double immunofluorescence experiments, sections obtained as indicated above were incubated with primary antibodies against GFAP (1/500, Chemicon MAB360) and CPT1c (1/100) overnight at 4 ºC in the same blocking solution. The sections were washed three times in PBS 0.1 M and incubated for two hours with secondary antibodies coupled to fluorochromes Alexa 488 (for green fluorescence) and Alexa 568 (for red fluorescence) at a dilution 1/500. Sections were mounted with Mowiol and observed using a Confocal Leica TCS SP2 (Leica Lasertechnik GmbH, Mannheim Germany). Images were saved in Tiff format and analyzed using Adobe Photoshop 3.0 Software.

Co-localization studies in culture cells

Cultured cells were grown on lysine-treated coverslips in 24-well plates. Co-localization studies were performed 48 hours after transfection with plasmids containing *CPT1c* or *CPT1a* fused to the 5'end of *EGFP*. To visualize the ER, cells were washed twice in PBS (10mM), fixed with 3% paraformaldehyde in 100mM phosphate buffer and 60mM sucrose for 15 minutes at room temperature, and then washed twice in PBS. Cells were permeabilized with 1% (w/v) of Triton X-100 in PBS and 20mM glycine for 10 minutes at room temperature and then washed twice in PBS. Non-specific binding of antibody was blocked by incubation with 1% (w/v) BSA in PBS with glycine 20mM at room temperature for 30 minutes. Cells were then incubated with mouse anti-calnexin polyclonal antibody (BD Biosciences) [1:50 in 1% (w/v) BSA/PBS/20mM glycine/0.2% Triton X-100] for 1 hour at 37ºC. After

washing twice in PBS/20mM glycine, cells were incubated with goat anti-mouse Alexafluor 546 (Molecular Probes) [1:500 in 1% (w/v) BSA/PBS/20mM glycine/0.2% Triton X-100] for 1 hour at 37ºC, and then washed twice in PBS. Coverslips were mounted on glass slides with Mowiol. Mitochondria were visualized by incubating cells with 500nM Mito Tracker Orange CM-H2TMRos (Molecular Probes) in complete medium for 30 minutes, followed by 30 minutes in complete medium without Mito Tracker, after which they were fixed as mentioned above.
Fluorescent staining patterns were visualized in a fluorescent microscope (Leica). The captured images were processed using Adobe Photoshop 5.0 software.

RNA extraction and Real-Time PCR conditions
RNA was extracted from cells by the Trizol method (Invitrogen) and quantified spectrophotometrically. 2 µg of total RNA was incubated with DNase and reverse transcribed by Superscript III (Invitrogen) following manufacturer's conditions. 2 µl of RT reaction was used in the Real-Time PCR amplification with Taqman and primers designed by Applied Biosystems, following the manufacturer's conditions. 18S expression assay was used to normalise the samples.

Lipid extraction
Cells were washed in cold PBS buffer and gently collected with a pipette. They were then centrifuged at 700x*g* for 5 min at 4ºC and washed in PBS. 20 µl of samples was taken for Bradford protein assay. After that, 200 µl of 0.2 M NaCl was added to the pellet and the mixture was immediately frozen in liquid N_2. To separate aqueous and lipid phases, 750 µl of Folch reagent (chloroform:methanol, 2:1) and 50 µl of 0.1 M KOH were added and, after vigorous vortex-mixing, the phases were separated by 15 min centrifugation at 2000x*g* at 4ºC. The top aqueous phase was removed and the lipid phase was washed in 200 µl of methanol/water/chloroform (48:47:3). After vortex-mixing, centrifugation was performed at 700x*g* for 5 min at 4ºC and the lower phase (lipid extract) was dried.

Quantification of acyl-carnitines by HPLC
Acylcarnitines were analyzed via an LC-ESI-MS/MS System (API 3000 PE Sciex) in positive ionization mode as described in (10). Quantification was done through multiple reaction monitoring (MRM) experiments using the isotope dilution method with deuterated palmitoyl carnitine as internal standard (200 $ng \cdot mL^{-1}$). 10 µl of sample was injected in the LC-ESI-MS/MS system. MRM transitions were as follows: 400.4/85.2 for quantification of palmitoylcarnitine, 4001.4/341.4 for confirmation of palmitoylcarnitine and 403.4/85.2 for quantification of d_2-palmitoylcarnitine. The method was linear over the range from 2 to 2000 $ng \cdot mL^{-1.}$ The limit of detection (LOD) and the limit of quantification (LOQ) were 0.14 $ng \cdot mL^{-1}$ (0.35 $nmol \cdot L^{-1}$) and 0.48 $ng \cdot mL^{-1}$ (1.2 $nmol \cdot L^{-1}$) respectively (in standard solutions).

Microsome purification
Cells were recovered by centrifugation at 1200x*g* for 5 min at 4ºC, washed in 1.5 ml PBS, and re-suspended in 2 ml of lysis buffer (250 mM sucrose, 10 mM Tris pH 7.4, 1 mM EDTA, supplemented with 1 mM PMSF, 0.5 mM benzamidine, 10 ng/ ml leupeptin and 100 ng/ml pepstatin). Cells were disrupted by Douncer homogenization (30 pulses with loose pestle and 30 pulses with tight pestle). Homogenates were centrifuged at 2000x*g* for 3 min at 4ºC to remove cell debris. This crude extracted was further centrifuged at 10,000x*g* for 30 min at 4ºC to remove the mitochondrial fraction. Supernatant was centrifuged at 10,000x*g* for 1 h at 4ºC to sediment the microsomal fraction. Pellets were immediately used in the carnitine palmitoyltransferase activity assay.

CPT1 activity
Radiometric method: Carnitine acyltransferase activity was determined by the radiometric method as previously described (11). The substrates were palmitoyl-CoA and L-(methyl-^{3}H) carnitine. Enzyme activity was assayed for 4 min at 30ºC in a total volume of 200 µL. The protein sample, 40 µl (20 µg), was pre-incubated for 1 min, and then 160 µl of the reaction mixture was added. The final concentrations were 105 mM Tris- HCl (pH 7.2), 2 mM KCN, 15 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 250 µM reduced glutathione, 50

μM palmitoyl-CoA, 400 μM L-(methyl-3H) carnitine (0.3 μCi) and 0.1% defatted bovine albumin. Reactions were stopped by the addition of 200 μl HCl 1.2 N, and the product acyl-L-(methyl-^{3}H) carnitine was extracted with water-saturated n-butanol. Values were estimated by analyzing the data from three experiments performed in triplicate. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as standard.
Chromatographic method: the same previously procedure was followed except that all carnitine used was cold (not radioactive). In addition, acylcarnitines extracted with water-saturated n-butanol were analyzed by an LC-ESI-MS/MS system, as described above.

Western blot experiments
A polyclonal rabbit antibody against the last fifteen residues (796 to 810) of mouse CPT1c was developed following the indications in (7), by Sigma-Genosys. The specificity of the antibody was determined by Elisa and Western blot experiments. For CPT1a detection, a polyclonal antibody against amino acids 317-430 of rat-CPT1a (12) was used. Generally, 60 μg of protein extracts were subjected to SDS-PAGE. A 1:2000 dilution of anti-CPT1c was used as primary antibody. The secondary antibody was used at 1:5000 dilution. The blots were developed with the ECL Western blotting system from Amersham Biosciences.

Palmitate oxidation
Palmitate oxidation to CO_2 and acid-soluble products were measured in PC-12 cells 48 h after transfection. On the day of the assay, cells were washed in KRBH 0.1% BSA, preincubated at 37ºC for 30 min in KRBH 1% BSA and washed again. Cells were incubated for 2 h at 37ºC with fresh KRBH containing 2.5 mM glucose, 0.8 mM carnitine, 0.25 mM palmitate and 1μCi/mL [1-^{14}C]palmitate bound to 1% BSA. Oxidation measurements were performed as previously described (13).

RESULTS

CPT1c cell type localization

In order to identify the types of brain cell in which *CPT1c* is expressed, co-localization studies with NeuN (a nuclear neuronal marker), or GFAP (glial fibrillary acidic protein, an astrocyte marker) antibodies were performed in adult mouse brain sections. Fig 1 shows co-labelling of *CPT1c* mRNA, as revealed by *in situ* hybridization studies (ISH), with NeuN. This pattern confirms that *CPT1c* is expressed mainly in neurons. In addition, no co-localization was detected between CPT1c and GFAP (double immunohistochemistry) (Fig 1d), indicating that CPT1c is not present in brain astrocytes.

CPT1c sub-cellular localization

CPT1c is localized in endoplasmic reticulum of cultured cells

In order to study the intracellular localization of CPT1c, fibroblasts were transiently transfected with pCPT1a-EGFP or pCPT1c-EGFP, which encode CPT1a or CPT1c proteins, respectively, fused at their C-terminal end to EGFP. 48-52 hours after transfection, the fluorescent pattern shown by CPT1a-EGFP (which was expressed in a punctuate manner) was different from that of CPT1c-EGFP (which was expressed in a reticular manner). Co-localization studies were performed with mitotracker, a potential-sensitive dye which accumulates in mitochondria, and with anti-calnexin, an ER integral protein. In some experiments cells were co-transfected with pDsRed2-ER (Clontech, Takara BioEurope, SAS), a subcellular localization vector that stains the ER red. Fig. 2 clearly shows that CPT1c is localised in the ER membrane, but not in mitochondria (Fig. 2). In contrast, CPT1a is localised in mitochondria, as previously described in other cells (5). The slight co-localization of CPT1a with the product of pDsRed2-ER may be due to the contacts between the ER membrane and the mitochondrial outer membrane, labeled as MAM (mitochondrial-associated membranes). In order to assess whether either isoform is localised in peroxisomes,

other organelles implicated in fatty acid oxidation, co-localization studies were performed with anti-PMP70, a peroxisomal membrane protein. No major co-localization was observed between PMP70 and CPT1c or CPT1a. The slight co-localization of CPT1c with PM70 may be due to a residual localization of this protein in peroxisomes (Fig 3). The same experiments were performed with SH-SY5Y cells, PC-12 cells and HEK293T cells with same results.

CPT1c is localised in microsomal fraction of adult mouse brain

To eliminate the possibility that over-expression experiments in cultured cells could modify the sub-cellular localization of CPT1c, we performed Western blot experiments with different cellular fractions of some adult mouse tissues. CPT1c was only present in brain tissue and absent in any other tissues analyzed (Fig 4). In addition, CPT1c was localized in the microsomal fraction of brain (Fig 4). Only some levels of CPT1c protein were present in brain mitochondria, probably by residual contamination from microsomes. The same membranes, once de-hybridized, were used with CPT1a antibodies, as a positive control for mitochondria. CPT1a was present at high levels in mitochondria from liver and kidney, and some residual levels were found in the microsomal fraction of all tissues examined.

The N-terminal region of the protein is responsible for CPT1c specific subcellular localization

We aimed to test whether the amino-terminal end of CPT1c was responsible for the ER localization. We made new chimeric plasmid constructions in which 460 bp of the 5' end of *CPT1a* gene (which encodes the two trans-membrane domains and the mitochondrial import signal described by the Prip-Buus group (14) was replaced by the 5' end of *CPT1c*, and *viceversa* (see scheme in Fig. 5). The recombinant plasmids were called pCPT1ca-EGFP and pCPT1ac-EGFP, respectively. SY-SHSY cells transiently transfected with those constructions showed that CPT1ca-EGFP was localized in ER, and that CPT1ac-EGFP was localized in mitochondria, indicating that exchange of amino-terminal ends between the two CPT1 isoforms swapped the intracellular localization of recombinant chimeric proteins (Fig 5). These results demonstrate that the amino-terminal end of CPT1c lacks the mitochondrial import signal present in CPT1a and contains a putative microsomal targeting signal responsible for ER localization.

CPT1c does not participate in fatty acid oxidation

To examine whether CPT1c participates in mitochondrial fatty acid oxidation, we measured increases in CO_2 in PC-12 cells over-expressing CPT1c. As expected by its sub-cellular localization, CPT1c did not increase fatty acid oxidation, while CPT1a did it (see table 1).

CPT1c substrate identification

In order to identify the substrate of CPT1c, we over-expressed the enzyme in PC-12 cells and attempted to identify any increased acyl carnitine species present in the lipid cell extract, 48 hours after transient transfection. PC-12 cells were easily transfected with Lipofectamine (Invitrogen) or Metafecten (Biontex, Germany) with transfection efficiencies of about 40-70 % of total PC-12 cells, as measured by the fluorescence in a cell-counter FACS Scan. PC-12 cells were transfected with pIRES-CPT1c, pIRES-CPT1a, or empty pIRES. Western blot experiments showed a 5-10 fold increase in CPT1c and CPT1a levels in transfected cells. The lipid fraction of transfected cells was extracted, and the levels of acylcarnitines were measured. To quantify acylcarnitines, we used a new HPLC-MS/MS method where no derivatization or ionic-pair chromatography is needed (10). Precursor ion scan of *m/z* 85 experiment allows the identification of all acylcarnitines present in the sample. Areas below chromatographic peaks (chromatograms acquired in MRM mode) were measured for all acylcarnitines detected. Fig 6 shows relative areas from chromatographic peaks present in over-expressing cells compared to control (cells transfected with empty expression vector). Cells transfected with pIRES-CPT1c showed an increase of more than two-fold in

palmitoylcarnitine levels (Fig. 6). No other acyl-carnitine was significantly increased. Cells transfected with pIRES-CPT1a (positive control) showed a five-fold increase in palmitoylcarnitine levels and a 2-3 fold increase in other long-chain acylcarnitines. The Wilcoxon statistic test (a non parametric test for two paired samples) between CPT1c transfected cells and control cells indicated that only palmitoylcarnitine levels increased significantly in CPT1c transfected cells. These results indicate that CPT1c has carnitine palmitoyl transferase activity and that palmitoyl-CoA is a substrate for the CPT1c isoenzyme.

CPT1c activity

Once palmitoyl-CoA had been identified as a CPT1c substrate, we compared CPT1 activity in isolated microsomal fractions of PC-12 and HEK293T cells transfected with pIRES-CPT1c with the activity in fractions transfected with empty pIRES vector. CPT1c was over-expressed more than ten fold and the protein was found mainly in the microsomal fraction (Fig 7a). Western-blot membrane was reprobed with mouse anti-CPT1a antibodies to determine the residual CPT1a protein present in the microsomal fraction of PC-12 cells (Fig 7b), which is responsible for the endogenous activity in microsomes of control cells. The same antibodies could not be used in HEK293T cells because they do not recognize the human CPT1a protein. Palmitoyl carnitine formed in the CPT1 assay was measured by the same HPLC-MS/MS method used to identify the substrate (10). Microsomes from CPT1c-transfected cells showed a 50% increase in CPT1 activity compared to control cells (endogenous activity) (Table 2). Km and V_{max} values for both substrates were calculated (Fig 8 and Table 3). Km values were similar to those of CPT1a (25), while V_{max} values were 66 times lower than those of CPT1a (25). For example, CPT1c catalytic efficiencies for palmitoyl-CoA and carnitine were 320 and 25 times lower, respectively, than those of CPT1a.

CPT1 sensitivity to malonyl-CoA was not measured in cultured transfected cells because CPT1c activity was too low and the microsomal fraction always retained residual CPT1a activity that masked any inhibitory effect of malonyl-CoA.

DISCUSSION

The presence of a third CPT1 isoform, CPT1c, in the mammalian brain is intriguing. It might show specific expression patterns, cellular localization or biochemical properties that would make it different enough from the other two isoforms to explain its occurrence. The data we report here on the peculiarities of CPT1c may provide clues to its cellular function.

CPT1c is expressed only in the mammalian brain. The other CPT1 isoforms are expressed in other tissues and are present in other organisms like birds, fishes, reptiles, amphibians or insects. This suggests that CPT1c has a specific function in more evolved brains. Price *et al*., (6) showed that CPT1c was expressed in all regions of brain, in a similar pattern to that shown by neurons. Dai *et al*., have recently demonstrated that CPT1c is localised to neurons of the CNS (9). Our results confirm these findings and demonstrate that CPT1c is not expressed in astrocytes, suggesting that CPT1c function is specific to neurons.

The statement that CPT1c is localized in mitochondria stems from observation of CPT1c protein in mitochondrial fraction of cells (6), and from co-localization studies with mitotracker in GT1-7 hypothalamic cells (9). In the first study (6), CPT1c was also found in the microsomal fraction, as revealed by Western blot experiments, although the authors attributed this to contamination problems in cellular fractioning process. In the second study (9) the authors conclude that CPT1c co-localizes with mitotracker, although the images did not show perfect matching and co-localization studies were not performed with any ER marker. In contrast, subcellular localization studies performed by our group in cultured cells and also in adult brain clearly demonstrate that CPT1c is localized in the ER, not in mitochondria. These results indicate that CPT1c has a different metabolic function than CPT1a or CPT1b, which is other than facilitating the import of long chain fatty acid into mitochondria or peroxisomes to undergo beta-

oxidation, as demonstrated in palmitate oxidation experiments. Localization of CPT1c in the ER implicates it in a biosynthetic rather than a catabolic pathway.

Intracellular localization experiments with chimeric proteins indicate that the N-terminal region of CPT1c, which includes the two transmembrane domains, is responsible for ER specific localization. These results complement previous studies in CPT1a protein (14). Prip-Buus and colleagues demonstrate that a region just downstream the second transmembrane domain (residues 123-147) is important for mitochondrial transport of CPT1a. Aminoacid sequence comparison between CPT1a and CPT1c demonstrates that the putative mitochondrial transport sequence is partially altered in CPT1c, with fewer positively charged amino acids (one charged residue versus four). In addition, the second transmembrane domain is longer in CPT1c than in the other two isoforms, which may enable it to sort proteins to the ER rather than to mitochondrial outer membrane (15).

Previous studies (6,7) had shown that CPT1c had no enzyme activity in yeast or HEK293T cells with palmitoyl-CoA or other acyl-CoA molecules as substrate. This indicated that the CPT1c substrate could be a rare acyl-CoA specific to the brain. We thus attempted to measure variations in all acylcarnitine levels in neural cells over-expressing CPT1c. We found that palmitoylcarnitine was the only product that was increased, indicating that pamitoyl CoA was the preferred acyl-CoA substrate for CPT1c. Activity measurements in microsomal fractions from PC-12 and HKE293T cells confirmed that CPT1c has carnitine palmitoyltransferase activity. The failure of other authors (6,7) to detect CPT1c activity has two possible explanations: 1) they used mitochondrial fractions instead of microsomal; and 2) they used a radiometric assay instead of a chromatographic method. The HPLC-MS/MS method produces reliable and accurate measurements of palmitoyl carnitine concentrations in biological samples with a sensitivity limit of 0.48 ng/ml, which corresponds to a specific activity of 0.0045 $nmols \cdot mg^{-1} \cdot min^{-1}$ in our CPT1 assay conditions (10). The limit of sensitivity of the radiometric assay, calculated as the standard deviation of ten blank points with a signal-to-noise ratio of 3, corresponds to specific activity of 0.4 $nmols \cdot mg^{-1} \cdot min^{-1}$. This indicates that the chromatographic method is 100 times more sensitive than the radiometric, as described elsewhere (10). Recently, other authors have also measured CPT1 activity by a tandem mass spectrometry method because of its accuracy and sensitivity (16).

CPT1c has 100 times lower specific activity than CPT1a and CPT1b. One explanation is that CPT1c participates in a biosynthetic pathway, facilitating the constant transport of palmitate across the ER membrane, rather than in a highly active catabolic pathway such as fatty-acid oxidation. Another explanation is that CPT1c acts as a metabolic sensor. CPT1c may have low activity in standard or optimal conditions (assay conditions), but its activity increases in certain situations (stress, presence of signal molecules, etc).

Lane and co-workers conclude that hypothalamic CPT1c has a role in energy homeostasis and the control of food ingestion (7). In addition to this localised function, the wide distribution of the protein in the brain suggests a more general, ubiquitous function, perhaps related with the equilibrium between acyl-CoA pools in the cytosol and the ER lumen. Although it is not known whether CPT2 is present in ER of neurons, we hypothesise that CPT1c facilitates the entry of palmitoyl-CoA to the ER lumen. It is been reported that palmitoyl-CoA cannot cross the ER membrane, although palmitoylcarnitine can (17-20). CPT1a or CPT1b, probably localized in mitochondria-endoplasmic reticulum connections (MAM) (21) may facilitate the entry of palmitoyl-CoA to the reticulum. In the brain, however, fatty acids are not usually oxidized, and levels of CPT1a or CPT1b are low or nonexistent. Thus the occurrence of a specific CPT1c localized in the ER membrane may ensure the entry of palmitoyl-CoA to the lumen of ER. Another possibility is that CPT1c modulates the palmitoyl-CoA pool associated with the ER, thus regulating the synthesis of ceramide and sphingolipids, which are important for signal transduction, modification of neuronal membranes, and brain plasticity (22-24).

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Figure legends

Fig 1: Co-localization studies of CPT1c mRNA with NeuN and GFAP proteins in brain sections. Brain sections were processed using ISH with *CPT1c* antisense riboprobe (a) or immunocytochemistry with NeuN primary antibodies and biotinylated secondary antibodies (b) or both methods (c). Mouse adult brain sections were processed by double immunocytochemistry with CPT1c antibodies (green stain) and GFAP (molecular marker of astrocytes, red stain) (d).

Fig 2. Co-localization studies of CPT1c in mitochondria and ER. Fibroblasts were transfected with pCPT1c-EGFP (panels A to F) or pCPT1a-EGFP (panels G to L) and incubated with anti-calnexin as primary antibody (panel B) or stained by mitotracker (panels E and K) or co-transfected with pDsRed2-ER (panel H). Images were taken by confocal microscopy with a filter to see green emission, red emission, or the merged image (panels C, F, I and L).

Fig. 3. Co-localization studies of CPT1c in perixosomes. HEK293T cells transfected by pCPT1c-EGFP or pCPT1a-EGFP were incubated with anti-PMP70 as primary antibody. Images were taken by confocal microscopy with a filter to see green emission, red emission, or the merged image.

Fig 4. Western-blot of CPT1c in mitochondrial and ER cell fractions from different tissues of adult mouse. 60 μg of protein cell fraction were run in each line. The same membranes were incubated with anti-CPT1c and anti-CPT1a antibodies.

Fig 5: Sub-cellular localization of fused proteins CPT1a-EGFP, CPT1c-EGFP, CPT1ca-EGFP and CPT1ac-EGFP in cultured cells. Up: Schematic representation of fusion proteins. *CPT1a* coding region is represented in white, *CPT1c* coding region in black, and *EGFP* coding region in grey. Down: SH-SY5Y human neuroblastoma cells were transfected with recombinant plasmids. 48 hours after transfection cells were visualized in a fluorescent microscope using a 100x objective. CPT1a-EGFP and CPT1ac-EGPF have mitochondrial localization (punctuate pattern). CPT1c-EGFP and CPT1ca-EGFP present a ER localization (reticular pattern).

Fig 6: Relative levels of different acyl-carnitines in PC12 cells. PC-12 cells were transfected with empty pIRES vector (control cells), or pIRES-CPT1c (white columns) or pIRES-CPT1a (black columns). 48 hours after transfection lipid extracts were obtained and acyl-carnitines determined by HPLC- mass spectrum chromatography. Y-axis represents the area below the chromatographic peak compared to control cells. These values represent the mean of three independent experiments except for palmitoyl-carnitine, in which represents the mean of six independent experiments. *p < 0.05 vs. control cells. The amount of pamitoylcarnitine, myristoylcarnitine and arachidonoylcarnitine in control cells were 0.5 ± 0.2, 0.2 ± 0.1 and 0.02 ± 0.01 nmols/mg respectively. For oleoylcarnitine and linoleoylcarnitine, only the chromatographic peak was measured.

Fig 7. Western blot of transfected PC-12 and HEK293T cells. Cells were transfected with pIRES-CPT1c (C), or empty pIRES (Ø). 40 μg of microsomes (mc) or mitochondria (mt) were run in each lane of SDS-acrylamide gel. **A**. anti-ratCPT1c antibody**. B**. Anti rat-CPT1a antibody.

Fig 8. Kinetic analysis of CPT1c over-expressed in PC-12 cells. 20 μg of microsomes were incubated at increasing concentrations of carnitine (A) and palmitoyl-CoA (B), and CPT1 activity was measured.

Table 1: Palmitate oxidation in PC-12 cells over-expressing CPT1c. 48 h after transfection of cultured cells with pIRES-CPT1c, pIRES-CPT1a or empty pIRES, cells were incubated for 2 h with [1-^{14}C]palmitate. Palmitate oxidation to CO_2 was determined. Data are presented as the mean ± the standard error of three independent experiments. Data for CPT1a are significantly different from control cells ($p < 0.05$)

	[^{14}C]CO_2 production (nmol / mg / h)
Empty pIRES	6.1 ± 0.9
pIRES- CPT1c	5.7 ± 0.7
pIRES-CPT1a	9.2 ± 2.1

Table 2: Carnitine palmitoyl transferase activity (nmols palmitoyl carnitine / mg / min) in PC-12 and HEK293T cells. Cells were transfected with pIRES-CPT1c or empty vector pIRES (control cells). 48h after transfection, cells were collected and 40 μg of microsomal fraction were assayed for CPT1 activity. The palmitoyl carnitine formed in the assay was determined by HPLC-mass chromatography. Activity is presented as the mean ± the standard error. Wilcoxon test for non-parametric paired samples was used. n = number of experiments. Absolute and percentage (%) increases in CPT1c activity are compared to control cells.

Cells	Plasmid transfection	n	Activity (nmols palmitoyl-carnitine / mg / min)	p	Absolute increase	% increase
PC12	Control	7	1.37 ± 0.81			
	CPT1c	7	1.94 ± 0.96	<0.05	0.57	41.6
293	Control	9	0.22 ± 0.11			
	CPT1c	9	0.35 ± 0.18	<0.05	0.13	59

Table 3: Kinetic parameters of CPT1c over-expressed in PC-12 cells. Microsomes of PC-12 cells over-expressing CPT1c were assayed for activity with different palmitoyl- CoA and carnitine concentrations to calculate Km and Vmax values for both substrates. The results are the mean ± standard deviation of three experiments. CPT1a kinetic parameters were obtained from a previous article (25)

Isoform	CPT1c	CPT1a
Km palmitoyl-CoA (μM)	25.35 ± 7.77	4.9 ± 0.3
Vmax palmitoyl-CoA (nmols/min/mg)	0.095 ± 0.012	6.3 ± 0.4
Catalytic efficiency palmitoyl-CoA (Vmax/Km)	0.004	1.28
Km carnitine (μM)	58.53 ± 21.31	127.0 ± 4.5
Vmax carnitine (nmols/min/mg)	0.090 ± 0.010	6.6 ± 0.8
Catalytic efficiency carnitine (Vmax/Km)	0.002	0.05

Figure 1

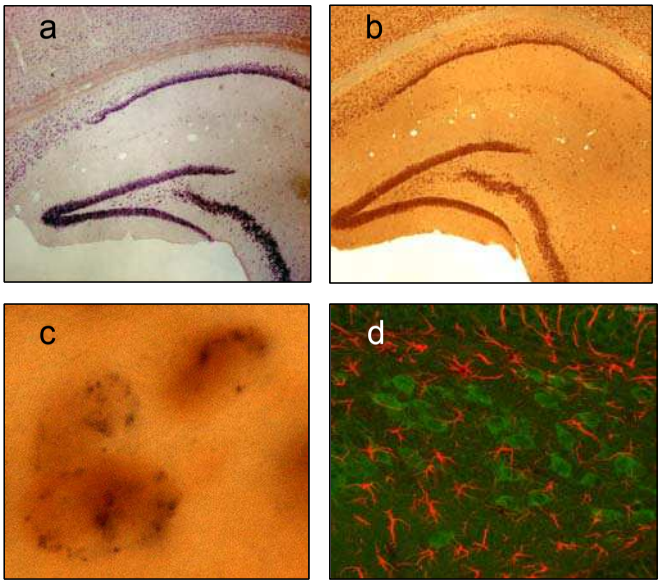


Figure 2

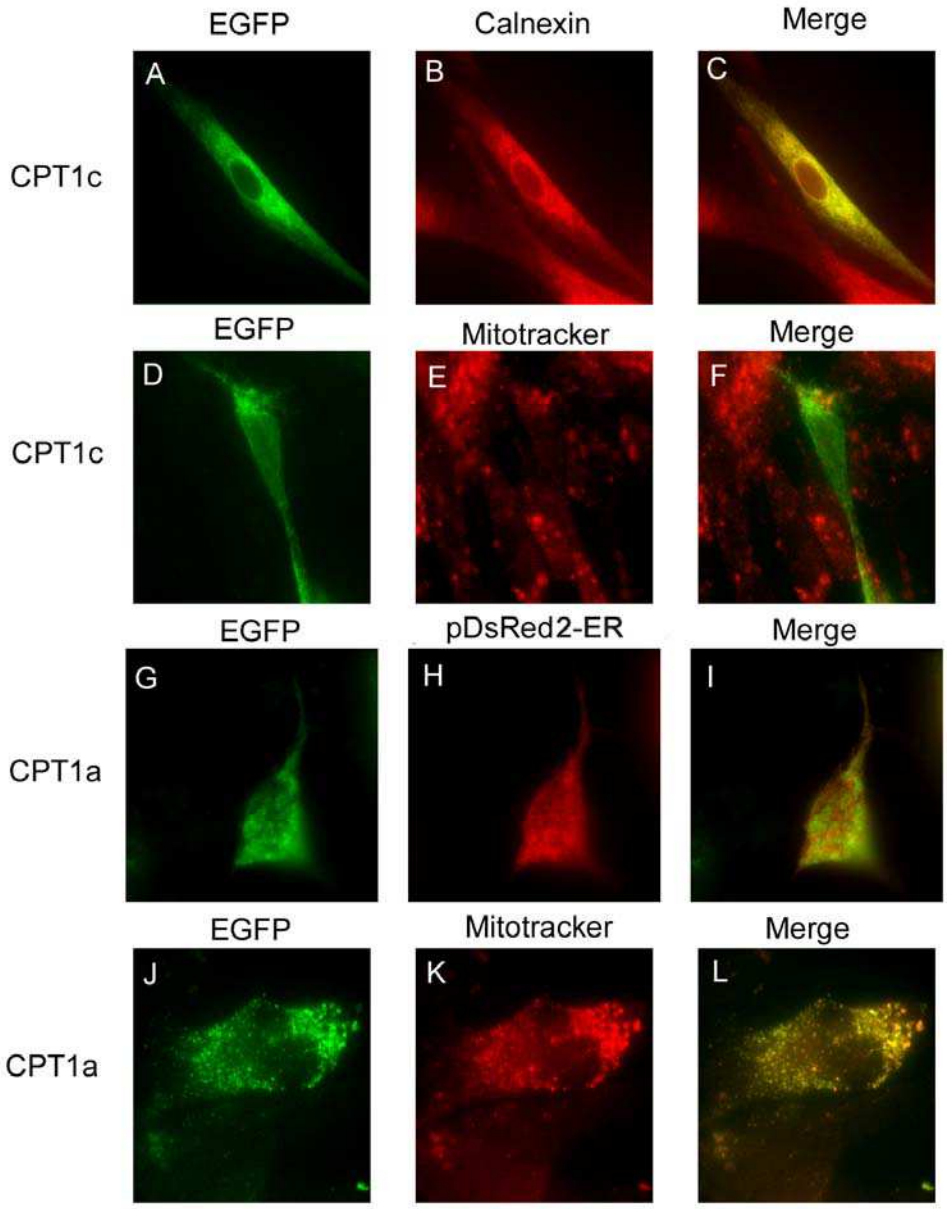

EGFP
Calnexin
Merge
A
B
C
CPT1c
EGFP
Mitotracker
Merge
D
E
F
CPT1c
EGFP
pDsRed2-ER
Merge
G
H
I
CPT1a
EGFP
Mitotracker
Merge
J
K
L
CPT1a

Figure 3

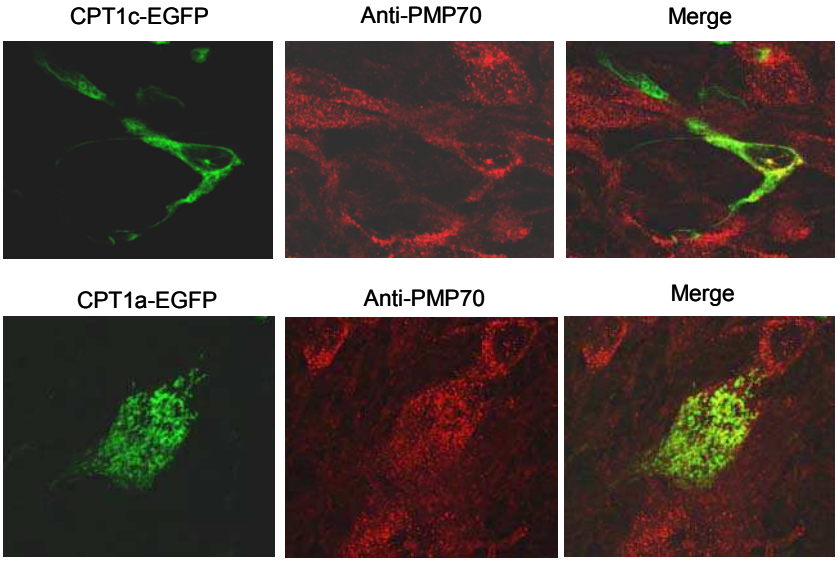


Figure 4

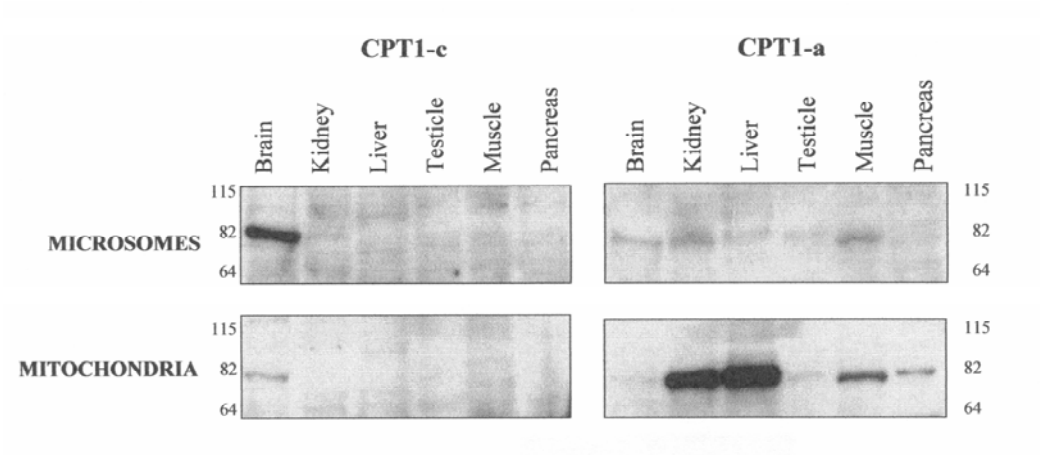
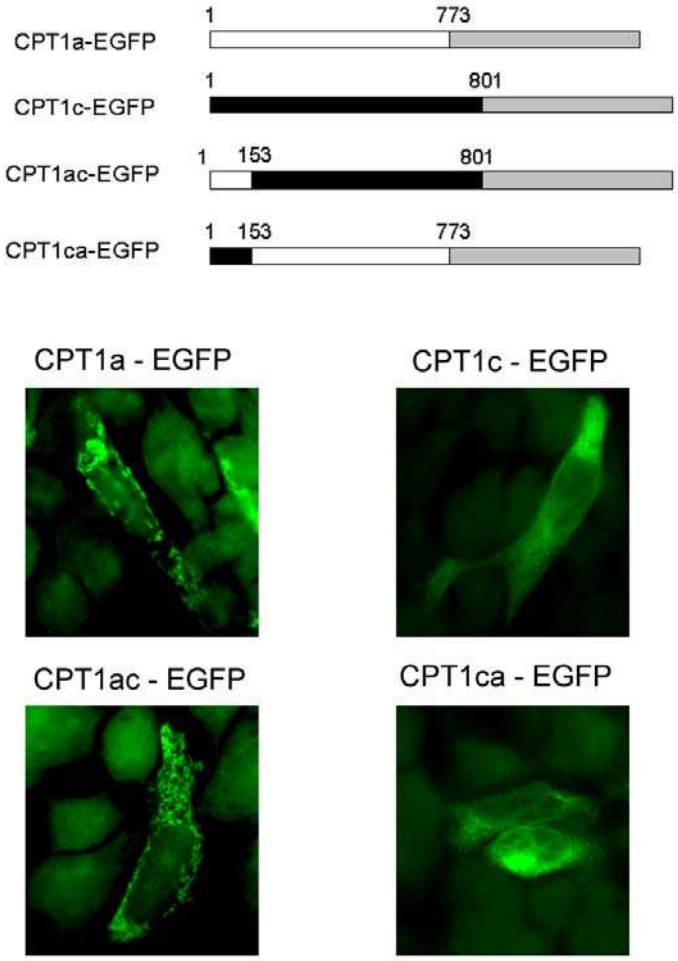

CPT1-c
CPT1-a
Brain
Kidney
Liver
Testicle
Muscle
Pancreas
MICROSOMES
MITOCHONDRIA
115
82
64

Figure 5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773
CPT1c-EGFP
1
801
CPT1ac-EGFP
1
153
801
CPT1ca-EGFP
1
153
773
CPT1a - EGFP
CPT1c - EGFP
CPT1ac - EGFP
CPT1ca - EGFP

Figure 6

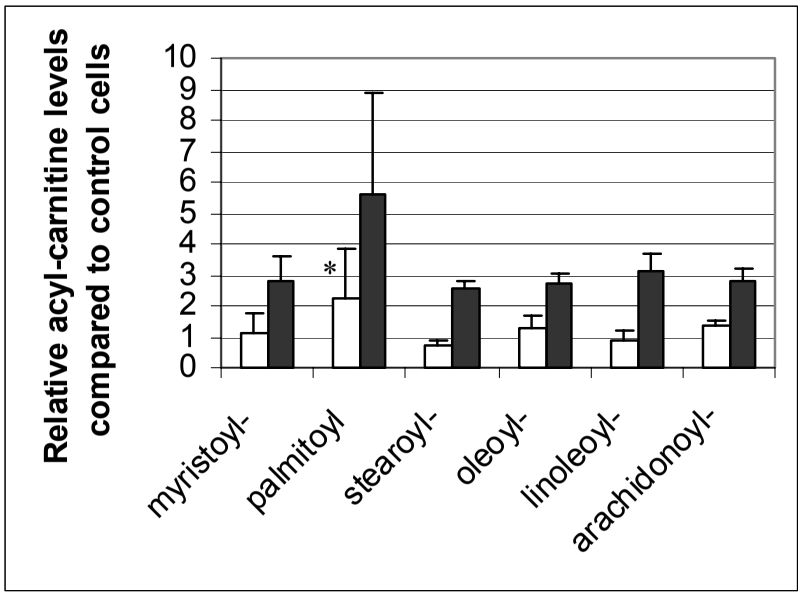

Relative acyl-carnitine levels compared to control cells
10
9
8
7
6
5
4
3
2
1
0
*
myristoyl-
palmitoyl
stearoyl-
oleoyl-
linoleoyl-
arachidonoyl-

Figure 7

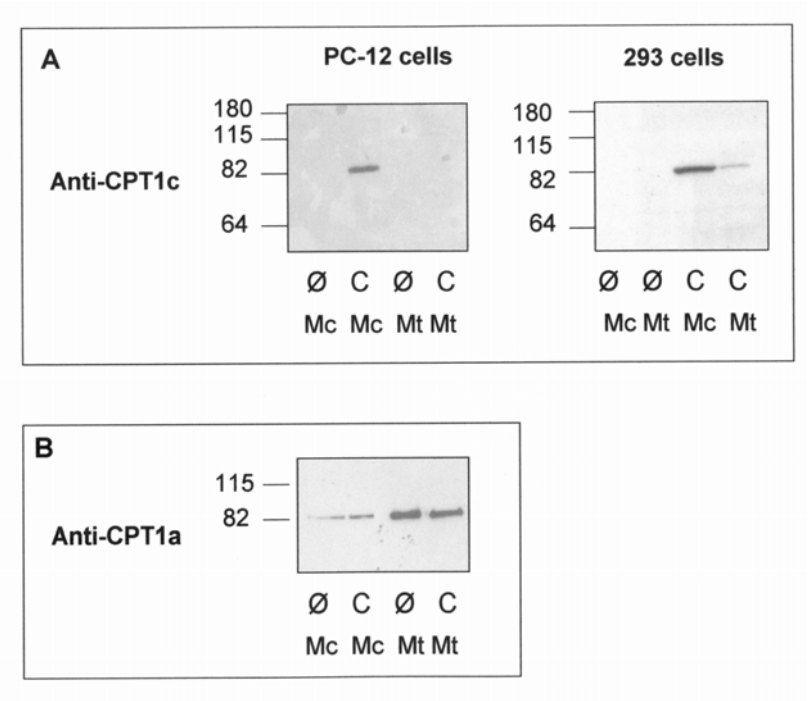
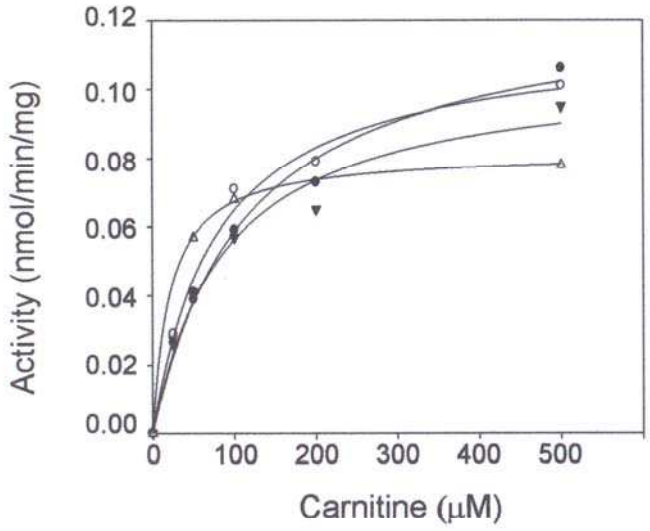
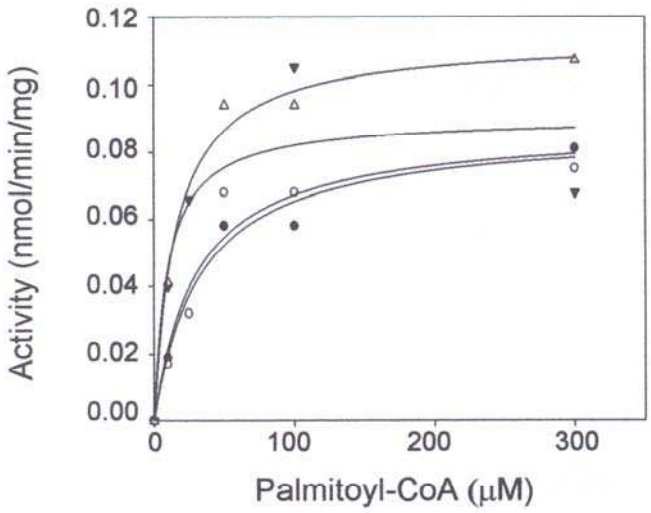

A
PC-12 cells
293 cells
Anti-CPT1c
180
115
82
64
Ø C Ø C
Mc Mc Mt Mt
180
115
82
64
Ø Ø C C
Mc Mt Mc Mt
B
Anti-CPT1a
115
82
Ø C Ø C
Mc Mc Mt Mt

Figure 8


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Activity (nmol/min/mg)
0
100
200
300
400
500
Carnitine (μM)


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Activity (nmol/min/mg)
0
100
200
300
Palmitoyl-CoA (μM)

CPT1C is localized in endoplasmic reticulum of neurons and has carnitine palmitoyltransferase activity

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