Oxidative stress elicited by modifying the ceramide acyl chain length reduces the rate of clathrin-mediated endocytosis

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Summary: Altering the sphingolipid acyl chain length causes oxidative stress, which reduces transcription of Hsc70 via Sp1, resulting in reduced rates of clathrin-mediated endocytosis due to the inability of Hsc70 to efficiently uncoat clathrin-coated vesicles.
Abstract

Sphingolipids modulate clathrin-mediated endocytosis (CME) by altering biophysical properties of membranes. We now examine CME in astrocytes cultured from ceramide synthase 2 (CerS2) null mice, which have an altered sphingolipid acyl chain composition. The rate of endocytosis of low-density lipoprotein and transferrin, which are internalized via CME, was reduced in CerS2 null astrocytes, although the rate of caveolin-mediated endocytosis was unaltered. Levels of clathrin heavy chain were increased, which was due to decreased levels of Hsc70, a protein involved in clathrin uncoating. Hsc70 levels were decreased because of lower levels of binding of Sp1 to position -68 in the Hsc70 promoter. Levels of Sp1 were down-regulated due to oxidative stress, which was elevated 4-fold in CerS2 null astrocytes. Furthermore, induction of oxidative stress in wild type astrocytes decreased the rate of CME whereas amelioration of oxidative stress in CerS2 null astrocytes reversed the decrease. Our data are consistent with the notion that sphingolipids not only change membrane biophysical properties but altering their composition can result in downstream effects that indirectly impinge upon a number of cellular pathways, such as CME.
Introduction

The lipid composition of the plasma membrane plays a vital role in regulating clathrin-mediated endocytosis (CME) (Lauwers et al., 2016) and sphingolipids (SLs) are of particular importance in this regard (Cheng et al., 2006). It is perhaps not surprising that SLs play such a critical role in regulating CME, since SLs have distinct biophysical properties, and together with cholesterol, ceramide and sphingomyelin increase the order and reduce the fluidity of membranes leading to increased rigidity, tighter packing and formation of ceramide-enriched microdomains (Pinto et al., 2013). These microdomains enable amplification of signaling pathways by reorganizing the membrane and clustering specific signaling components (Zhang et al., 2009).

Most studies examining the role of SLs in CME have been performed using inhibitors of SL biosynthesis (Meyer et al., 2012) or by altering levels of specific SL classes, such as sphingomyelin (Shakor et al., 2011). We previously demonstrated that altering the SL acyl chain length also leads to changes in membrane biophysical properties (Silva et al., 2012), which may be one of the reasons that ceramide synthase 2 (CerS2) null mice, which are unable to synthesize very long-chain SLs (Pewzner-Jung et al., 2010a; Pewzner-Jung et al., 2010b), displays a variety of phenotypes related to ligand and receptor internalization (Ali et al., 2013; Park et al., 2012; Park et al., 2014). However, mechanistic information on how CME is regulated by the SL acyl chain composition is not available. We now analyze CME in astrocytes cultured from CerS2 null mice, and show that they contain elevated levels of reactive oxygen species (ROS), which triggers a reduction in the transcription of Hsc70, a protein involved in uncoating clathrin vesicles, and as a consequence, reduces the rate of CME but not of endocytosis via caveolin-mediated pathways. We suggest that, at least in astrocytes, CME is not directly regulated by the SL acyl chain composition, but rather via a down-stream pathway that is modulated by the oxidative state of the cell.
Results

Characterization of astrocytes from CerS2 null mice

In mammals, endocytosis of nutrients by astrocytes is important for normal neuronal function (Jiang and Chen, 2009). Astrocytes were isolated from wild type (WT) and CerS2 null mice. The cultures contained ~95% astrocytes and <0.1% microglia (using an anti-glial fibrillary acidic protein (GFAP) antibody to detect astrocytes and anti-CD68 for microglia) (Fig. 1A). As expected, in CerS2 null astrocytes, mRNA levels of CerS2 were undetectable and mRNA levels of the other five mammalian CerS (Levy and Futerman, 2010) were unaltered (Fig. 1B). Likewise, CerS2 activity was undetectable in CerS2 null astrocytes using C24:1-CoA as substrate, although a small amount of residual activity could be detected using C22-CoA (Fig. 1C), probably due to the partial overlapping substrate specificities of CerS4 and CerS2 (Riebeling et al., 2003). Analysis of the astrocyte SL composition was consistent with mRNA and CerS activity levels, inasmuch as C24:1-ceramide was barely detectable in CerS2 null astrocytes (as were levels of the more complex SLs, C24:1-SM and C24:1-glucosylceramide (not shown)), and C22:0-ceramide levels were reduced by ~35% compared to WT astrocytes (Fig. 1D). Total ceramide levels were essentially unaltered (Fig. 1D) due to increased levels of C16:0- and C18:0-ceramide, similar to changes in the SL profile in CerS2 null mouse liver (Pewzner-Jung et al., 2010b). However, unlike liver, levels of the long chain bases, sphinganine (3.9 ± 0.7 pmol/mg in WT and 5.6 ± 1.1 pmol/mg in CerS2 null) and sphingosine (86.4 ± 3.4 pmol/mg in WT and 75.2 ± 3.3 pmol/mg in CerS2 null) were essentially unaltered.

Reduced rates of CME in astrocytes from CerS2 null mice

We next examined the rate of CME in astrocytes using two fluorescently-labeled ligands (Keyel et al., 2006). Both 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Dil)-low density lipoprotein (Dil-LDL) and Cy5-transferrin (Cy5-Tf) were internalized at a
slower rate in CerS2 null astrocytes than in WT astrocytes (Figs. 2A, B). In contrast, the rate of caveolin-mediated endocytosis, measured using BODIPY-lactosylceramide (BODIPY-LacCer) (Singh et al., 2007), was unaltered (Fig. 2C). The differences in the kinetic parameters of uptake between Cy5-Tf (Fig. 2A) and Dil-LDL (Fig. 2B) is probably due to differences in the rate of recycling and sorting and between LDL and Tf receptors (Ghosh et al., 1994).

LDL receptor and Tf receptor levels were not changed (Fig. 3A), suggesting that the reason for the reduced rate of CME is related to one or other aspect of CME rather than reduced levels of the receptors that bind the ligands. Indeed, levels of the clathrin heavy chain were elevated 3.5-fold (Fig. 3A), and distinct large puncta of the clathrin heavy chain were observed in the cytoplasm of CerS2 null astrocytes (Fig. 3B), similar to that observed in mice which lack the clathrin-coated vesicle uncoating protein, auxilin-1 (Yim et al., 2010). In the case of CerS2 null mouse astrocytes, levels of auxilin-1 mRNA were unchanged (Fig. 3C), but levels of heat shock cognate 70 (Hsc70) mRNA were reduced by ~3-fold (Fig. 3C) and protein levels were reduced by 25 ± 5% (Fig. 3D). Hsc70 also plays a key role in the uncoating of clathrin-coated vesicles (Greene and Eisenberg, 1990).

**Transcriptional regulation of Hsc70 by specificity protein 1 transcription factor**

Since Hsc70 mRNA levels were reduced (Fig. 3C), we next examined levels of two transcription factors that regulate Hsc70 expression, namely specificity protein 1 (Sp1) (Wilke et al., 2000) and heat shock factor 1 (HSF-1) (Yasuhara et al., 2011). Levels of Sp1 were reduced by 35 ± 4% whereas levels of HSF-1 were unaltered (Fig. 4A); the reduction in Sp1 protein levels was not due to changes in its transcription (Fig. 4B). Two putative binding motifs for Sp1 are found in positions -174 and -68 from the transcription start site (Fig. 4C). Chromatin immunoprecipitation (ChIP) demonstrated that Sp1 binds to position -68, whereas no binding of Sp1 to position -174 could be detected (Fig. 4D). Sp1 binding to position -68
was significantly reduced in CerS2 null astrocytes (Fig. 4D). Levels of two other Sp1 targets, glycolipid transfer protein (Zou et al., 2011) and fatty acid synthase (Archer, 2011) were also reduced, but levels of two predicted non-targets of Sp1, GFAP and toll-like receptor 4, were unaltered (Fig. 4E). These results suggest that Hsc70 is transcriptionally regulated by Sp1 in CerS2 null astrocytes, and that additional cellular pathways regulated by Sp1 might also be affected in CerS2 null astrocytes.

CME is attenuated by the oxidative state of the cells

Sp1 is sensitive to its O-glycosylation (GlcNAc) state inasmuch as Sp1 hypo-glycosylation leads to its proteasomal degradation (Han and Kudlow, 1997). Reactive oxygen species (ROS) were shown to induce proteasome mediated reduction of Sp1 levels via reduction of Sp1 O-glycosylation (Kim et al., 2012), whereas N-acetyl cysteine (NAC) was shown to mitigate this effect (Hsin et al., 2010). Thus, we next measured ROS levels in CerS2 null astrocytes, which were elevated ~4-fold (Fig. 5A), along with a 60 ± 18% increase in levels of 3-nitrotyrosine (Fig. 5B). ROS overproduction in CerS2 null astrocytes is likely due to reduced activity of mitochondrial respiratory chain complex IV (COX) (Fig. 5C), similar to that observed in liver from CerS2 null mice (Zigdon et al., 2013). Based on these results, we suggest a pathway by which oxidative stress in CerS2 null astrocytes results in lower Sp1 levels and hence lower levels of Hsc70, which directly affects the rate of CME. This was tested by inducing ROS, using H₂O₂, in WT astrocytes, which resulted in a slower rate of uptake of Cy5-Tf, and by ameliorating ROS in CerS2 null astrocytes by NAC, which resulted in increased rates of Cy5-Tf uptake (Fig. 6A, B). Similar results were seen with Dil-LDL (Fig. 6C, D). Concomitantly, H₂O₂ treatment decreased Sp1 and Hsc70 levels in WT astrocytes to levels similar to non-treated CerS2 null astrocytes, while NAC treatment increased their levels in CerS2 null astrocytes to the same level as in non-treated WT
astrocytes (Fig. 6E). Neither ROS nor NAC affected LDL receptor or Tf receptor levels in WT or CerS2 null astrocytes (Fig. 6E).

Discussion

In the current study, we demonstrate a novel mechanism by which altering the SL acyl chain length can affect the rate of CME, which is apparently independent of changes in membrane biophysical properties. In this pathway, oxidative stress, caused by ROS overproduction via mitochondrial complex IV dysfunction, as observed in hepatocytes upon loss of very-long acyl chain ceramides and elevation of long-chain ceramides (Zigdon et al., 2013), results in down-regulation of the transcription factor, Sp1, which binds to the promoter region of Hsc70, a key player involved in uncoating the clathrin coat. As a result, clathrin aggregates in the cytoplasm, rendering it inaccessible for additional rounds of CME (Fig. 7). Disruption of another uncoating protein, auxilin-1 (Yim et al., 2010), results in a similar phenotype, as does knockdown of Hsc70 (Yu et al., 2014), whereas a complete knockout of Hsc70 in mice is embryonically lethal (Florin et al., 2004) and auxilin-1 knockout mice exhibit elevated clathrin heavy chain levels (Hirst et al., 2008) and reduced CME (Yim et al., 2010).

SLs can reside in lipid rafts (Simons and van Meer, 1988), platforms through which caveolae are formed and thereby uptake ligands through caveolin-mediated endocytosis (Mayor and Pagano, 2007). Although we observed significant changes in the SL profile of CerS2 null astrocytes, caveolin-mediated endocytosis was unaltered, presumably since total ceramide levels were unaltered in CerS2 null astrocytes.

We have previously observed a number of changes in receptor activation and/or internalization in hepatocytes. For instance, altering the SL acyl chain composition abrogates insulin receptor phosphorylation, but this effect is directly caused by changes in membrane
biophysical properties, with the insulin receptor unable to translocate into detergent-resistant membranes (Park et al., 2012). Likewise, palmitic acid was not internalized by CerS2 null hepatocytes, which was caused by the inability of CD36/FAT to translocate into detergent-resistant membranes prior to internalization (Park et al., 2014). The mechanism by which tumor necrosis factor α receptor-1 internalization was inhibited in CerS2 null hepatocytes was not established (Ali et al., 2013), but we suggested that this might be related to altered recruitment of adaptor proteins required for clathrin-coated pit formation. Finally, altering the SL acyl chain composition also affects intracellular trafficking of connexin 32 (Park et al., 2013), again due to altered biophysical properties. Thus, SLs have direct effects on receptor internalization and trafficking, but also indirect effects such as that reported in the current study, whereby dysfunction of mitochondrial complex IV affects ROS levels and concomitant changes in pathways affected by ROS, such as endocytosis (Parry et al., 2008; Poumay and Dupal, 1988). Whether similar changes occur upon acute changes in SL levels in signaling pathways (Morales et al., 2007) is currently unknown, but the current study highlights the need to pay attention to both direct effects of SLs on membrane biophysical properties and also on indirect effects caused by activation of down-stream pathways, such as defective clathrin uncoating caused by oxidative stress.
Materials and Methods

Astrocyte cultures - Cortical astrocytes from 2-4 day old WT and CerS2 null mice (Pewzner-Jung et al., 2010b) were isolated as described (Schildge et al., 2013). Astrocytes were grown to 95% confluency for 7-10 days, other cells were removed from the dishes by vigorous shaking (2 h, 200 rpm on an orbital shaker), and astrocytes were replated and grown to 95% confluency for up to 23 days. Cells were cultured in Dulbecco’s modified eagle’s medium (DMEM, Gibco™) containing 10% fetal calf serum (Gibco™) and 2% penicillin/streptomycin.

Immunohistochemistry - Astrocytes were cultured on 12 or 13 mm glass coverslips to a confluence of ~70%. Cells were fixed by incubating with 3% paraformaldehyde in phosphate buffered saline (PBS) at room temperature for 15 min, rinsed three times with PBS for 4 min and blocked with 4% bovine serum albumin for 90 min. Cells were then either co-incubated for 1 h with anti-GFAP (for astrocytes, 1:1,000, Bio-Rad, Hercules, California, USA) and anti-CD68 (for microglia, 1:1,000) antibodies, or with an anti-clathrin heavy chain (1:50, Cell Signalling, Bioke, Leiden, the Netherlands) antibody. Cells were washed three times with PBS for 4 min and incubated with a secondary fluorescently-labeled antibody (anti-rat, anti-mouse or anti rabbit; 1:250, Jackson Immunoresearch Labs, West Grove, Pennsylvania, USA) for 60 min. Cells were rinsed three times with PBS for 4 min, incubated for 10 min with Hoechst 33342 (1:5,000, Molecular probes, Eugene, Oregon, USA), and then rinsed three times with PBS for 4 min. Glass coverslips were mounted on slides using Gel Mount (Molecular Probes, Eugene, Oregon, USA) and analyzed by fluorescence microscopy using an Olympus IX 81 FluoView 1000 microscope (Olympus, Tokyo, Japan).

RNA extraction and polymerase chain reaction - Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. cDNA
synthesis was performed using a Verso cDNA kit (Thermo Scientific, Waltham, Massachusetts, USA). qPCR was performed as described (Vitner et al., 2016). The relative amounts of mRNA were calculated from the cyclic threshold values using hypoxanthine guanine phosphoribosyl transferase (HPRT) or TATA binding protein (TBP) for normalization. Primers are listed in Table 1.

_CerS assays_ - Cells were homogenized in 20 mM HEPES-KOH, pH 7.2, 25 mM KCl, 250 mM sucrose and 2 mM MgCl₂ containing a protease inhibitor cocktail (Sigma, St. Louise, Missouri, USA). Protein content was determined using the BCA reagent (Pierce, Rockford, Illinois, USA). Homogenates were incubated with 15 μM NBD-sphinganine (Tidhar et al., 2014) (Avanti Polar Lipids, Alabaster, Alabama, USA), 20 μM defatted bovine serum albumin (Sigma, St. Louis, Missouri, USA), and 50 μM C16–CoA (for CerS5/6), C18-CoA (for CerS1/4), C20-CoA (for CerS4) and C22-CoA or C24-CoA (for CerS2) (Avanti Polar Lipids, Alabaster, Alabama, USA) for 5-40 min at 37°C. Lipids were extracted and separated by thin layer chromatography using chloroform/methanol/2M NH₄OH (40:10:1, v/v/v) as the developing solvent. NBD-labeled lipids were visualized using a Typhoon 9410 variable mode imager and quantified by ImageQuantTL (GE Healthcare, Chalfont St Giles, UK).

_Lipid analysis_ - SL analysis by LC-ESI MS/MS was performed using a PE-Sciex API 3000 triple quadrupole mass spectrometer and an ABI 4000 quadrupole-linear ion trap mass spectrometer (Shaner et al., 2009; Sullards et al., 2011).

_Uptake of fluorescently-labeled ligands_ - WT and CerS2 null astrocytes were cultured in 96-well plates and grown overnight. Cells were incubated for various times with fluorescently-labeled ligands (Dil-LDL (1:100); BODIPY-LacCer (1:100); Life Technologies, Carlsbad, California, USA; Cy5-Tf (1:100), Jackson Immunoresearch Labs, West Grove, Pennsylvania, USA). Cells were washed three times with PBS to remove unbound ligand, and incubated for 4 min with 500 mM NaCl in 200 mM acetic acid, pH 3.5 to remove Dil-LDL or
to remove Cy5-Tf bound to the cell surface (Megias et al., 2000). For BODIPY-LacCer, back exchange was performed by incubating cells with 5% defatted bovine serum albumin for 10 min. In some cases, cells were incubated with 100 μM H$_2$O$_2$ or 100 μM NAC for 4 h prior to addition of the fluorescent ligand. Internalized fluorescent ligand was quantified using a plate reader at the following wavelengths: Dil-LDL; excitation 528 nm, emission, 590 nm; BODIPY-LacCer, excitation 485 nm, emission, 528nm; Cy5-transferrin, excitation 630 nm, emission 680 nm.

*Western blotting* - Western blotting (Zigdon et al., 2015) was performed with the following antibodies, diluted in PBS and 0.1% Tween 20: rabbit anti-clathrin heavy chain (1:1,000, Cell Signalling, Bioke, Leiden, the Netherlands), rabbit anti-LDL receptor (1:1,000), mouse anti-Hsc70 (1:1,000), rabbit anti-Sp1 (1:1,000), rabbit anti-3-nitrotyrosine 4 (1:1,000) obtained from Abcam (Cambridge, UK), rabbit anti-Tf receptor (1:1,000 Santa Cruz, Huissen, The Netherlands), mouse anti-GAPDH (1:5,000, Millipore, Billerica, USA). Densitometry was performed using ImageQuant software (Amersham Biosciences, Little Chalfont, UK).

*Transcription factor binding site analysis* - Transcription factor binding site analysis was performed using the Genomatix Genome Analyzer (GGA) MatInspector program (Cartharius et al., 2005), Matrix Library Version 9.0, searching with the V$SP1F$ matrix.

*Chromatin immune precipitation* - ChIP was performed as described (Tarcic et al., 2016). Cells were fixed in 0.1 vol of formaldehyde (11%) followed by 0.06 vol of 2.5 M glycine. Immunoprecipitation was performed using 3 μg of antibody in 0.1% Brij-35. DNA was purified using a QIAGEN PCR purification kit (QIAGEN Industries, Venlo, Netherlands) followed by real-time qRT-PCR.

*Reactive oxygen species* - ROS were measured using chloro-methyl 6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (cmH$_2$DCF-DA) (Invitrogen, Carlsbad, California,
USA). WT and CerS2 null astrocytes were incubated with 100 μM cmH$_2$DCF-DA or PBS for 30 min at 37°C and then washed 3 times with PBS. Conversion of cmH$_2$DCF-DA to cmDCF was determined using an Eclipse iCyt flow cytometer equipped with 488 nm solid state air cooled lasers, with 25 mW on the flow cell and with standard filter set-up; cmDCF was measured in the green channel with an excitation of 488 nm and emission of 525 ± 50 nm.

**Mitochondrial complex IV activity** - Activities of mitochondrial respiratory chain complex IV (COX) and citrate synthase (CS), a mitochondrial marker, were determined in cell homogenates as previously described (Saada et al., 2004). Measurements were performed using a double-beam spectrophotometer (UVIKON 930, Secomam, France).

**Confocal microscopy** - Confocal microscopy was performed using an Olympus IX 81 FluoView 1000 microscope and a UPLSAPO 60x objective. Images were processed and analyzed using FV-1000 software (Olympus, Tokyo, Japan).
**Author contributions**

GV designed and performed the experiments and wrote the manuscript. SBD performed the bioinformatics analyses for Hsc70 promoter analysis and helped with writing the manuscript. OT performed the ChIP assays. JD and AHM carried out the ESI-MS/MS analyses of SLs and AS assayed complex IV activity. YPJ generated CerS2 null mice and AHF supervised and funded the studies and wrote the manuscript.

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**Conflict interest**

The authors declare no conflict of interest.

**Abbreviations used**

CME, clathrin-mediated endocytosis; Tf, transferrin; LDL, low-density lipoprotein; SLs, sphingolipids; CerS2, ceramide synthase 2; WT, wild type; GFAP, glial fibrillary acidic protein; Hsc70, heat shock cognate 70; Sp1, specificity protein 1; ROS, reactive oxygen species; COX, mitochondrial respiratory chain complex IV; NAC, N-acetyl cysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline.
References


Table 1. Primers used in the current study.

F, forward; R, reverse.

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Figures

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Fig. 1. Characterization of CerS2 null astrocytes. (A) Astrocytes were purified and co-stained with anti-GFAP (green) and anti-CD68 antibodies (red). Nuclei were stained with Hoechst 33348 (blue). Scale bar, 40 μm. (B) mRNA levels of CerS. Values are means ± s.e.m., n≥3. ***, p<0.001. (C) CerS activity using different acyl-CoAs. Values are means ± s.e.m., n≥4. ***, p<0.001. (D) Mass spectrometry analysis of ceramide species. Values are means ± s.e.m., n=3 for CerS2 null, n=2 for WT. *, p<0.05; **, p<0.01; ***, p<0.001. The insert shows total ceramide levels. In panels B-E, WT data is black and CerS2 null is grey.
**Fig. 2. The rate of CME in CerS2 null astrocytes.** Cells were incubated with (A) Dil-LDL, (B) Cy5-Tf or (C) BODIPY-LacCer for various times and the amount of internalized ligand analyzed. Values are means ± s.e.m., n=3. *, p<0.05; **, p<0.01. WT is indicated by circles and CerS2 null by squares.
**Fig. 3. Dysregulated CME in CerS2 null astrocytes.** (A) Western blot of clathrin heavy chain (CHC), LDL receptor (LDL-R) and Tf receptor (Tf-R) in astrocytes. GAPDH was used as a loading control. The right-hand panel shows quantification of the Western blots. Values are means ± s.e.m. **, p<0.01. (B) Distribution of clathrin heavy chain determined by confocal microscopy. Clathrin heavy chain (*red*), Hoechst 33348 (*blue*). Arrows indicate large puncta of clathrin heavy chains in CerS2 null astrocytes. The image is representative of three
independent experiments. Scale bar, 10 μm. (C) mRNA levels of auxilin-1 and Hsc70. n=2. Values are means ± s.d. *, p<0.05. (D) Western blot of Hsc70. GAPDH was used as a loading control. The lower panel shows quantification of the Western blots. Values are means ± s.e.m., n=5. **, p<0.01.
Fig. 4. Sp1 regulates Hsc70 in CerS2 null astrocytes. (A) Western blot of Sp1 and HSF-1. GAPDH was used as a loading control. The right-hand panel shows quantification. Values are means ± s.e.m., n=4. *, p<0.05. (B) mRNA levels of Sp1. Values are means ± s.d., n=4. (C) Schematic representation of the promoter region of the mouse Hsc70 (Hspa8) gene. The first
exon is shown as a gray box. The transcription start site (TSS) and the two putative Sp1 binding sites are also indicated. Taken from mouse genome version GRCm38, chr9:40,801,066-40,801,348. (D) Chromatin IP analysis of the binding of Sp1 transcription factor to positions -68 and -174 in the Hsc70 promoter. HA antibody was used as a binding control for Sp1 binding. n=3, values are means ± s.e.m. *, p<0.05. (E) mRNA levels of fatty acid synthase, glycolipid transfer protein, toll-like receptor 4 (TLR4) and GFAP. Values are means ± s.e.m., n=3. *, p<0.05; ***, p<0.001.
Fig. 5. Increased oxidative stress in CerS2 null astrocytes. (A) FACS analysis of ROS levels in WT (green) and CerS2 null (red) astrocytes using cmH2DCF-DA. Individual samples contained at least 30,000 cells in each sample. The right-hand panel shows quantification. n=3. Values are means ± s.e.m. ***, p<0.001. (B) Western blot of 3-nitrotyrosine. GAPDH was used as a loading control. The right-hand panel shows quantification. n=4. Values are means ± s.e.m., **, p<0.01. (C) Activity of mitochondrial complex IV normalized to citrate synthase (CS) in WT and CerS2 null astrocytes. Values are means ± s.d., n=4. *** , p<0.001.
Fig. 6. Oxidative stress regulates CME through the Sp1-Hsc70 axis. Astrocytes were incubated with and without H2O2 (100 μM, 4 h) or NAC (100 μM, 4 h), followed by incubation with (A) Cy5-Tf (red) or (C) Dil-LDL (red) for the indicated times. Hoechst 33348 (blue). Scale bar, 10 μm. Rate of uptake of (B) Cy5-Tf or (D) Dil-LDL. Values are means ± s.e.m., n=3. *, p<0.05; **, p<0.01; ***, p<0.001. (E) Western blot of Sp1, Hsc70, Tf receptor and LDL receptor. GAPDH was used as a loading control. The right-hand panel shows quantification. Values are means ± s.e.m., n=3. *, p<0.05; **, p<0.01.
Fig. 7. Putative scheme of the effect of altering the SL acyl chain length on CME.

Altering the SL acyl chain length (red) can either lead to changes in membrane biophysical properties or to activation of downstream pathways (blue). In the current study (black), we demonstrate the activation of one such pathway, which eventually leads to a reduced rate of clathrin-mediated endocytosis.