Research Article

Intracellular APP Domain Regulates Serine-Palmitoyl-CoA Transferase Expression and Is Affected in Alzheimer's Disease

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Lipids play an important role as risk or protective factors in Alzheimer's disease (AD), a disease biochemically characterized by the accumulation of amyloid beta peptides (Aβ), released by proteolytic processing of the amyloid precursor protein (APP). Changes in sphingolipid metabolism have been associated to the development of AD. The key enzyme in sphingolipid *de novo* synthesis is serine-palmitoyl-CoA transferase (SPT). In the present study we identified a new physiological function of APP in sphingolipid synthesis. The APP intracellular domain (AICD) was found to decrease the expression of the SPT subunit SPTLC2, the catalytic subunit of the SPT heterodimer, resulting in that decreased SPT activity. AICD function was dependent on Fe65 and SPTLC2 levels are increased in APP knock-in mice missing a functional AICD domain. SPTLC2 levels are also increased in familial and sporadic AD *postmortem* brains, suggesting that SPT is involved in AD pathology.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the most common cause of dementia in the elderly, clinically characterized by a progressive loss of memory. Pathological hallmarks for AD are the presence of amyloid plaques, composed of amyloid beta peptides (Aβ), and neurofibrillary tangles, which consist of hyperphosphorylated tau proteins [1–3]. Aβ peptides are released by sequential processing of the amyloid precursor protein (APP), a large type-I transmembrane protein, by β- and γ-secretases. The β-secretase BACE1 generates the N-terminus of Aβ and a C-terminal stub of 99 amino acids

(aa), which is further cleaved by γ-secretase to release Aβ and the intracellular domain of APP (AICD) [4–6]. The γ-secretase represents a protein complex of at least four proteins in which the presenilins constitute the active centre of the protease [7–10]. APP, BACE1 as well as Presenilin 1 (PS1), Presenilin 2 (PS2) and the other components of the γ-secretase complex are all transmembrane proteins, pointing towards a role of lipids, especially the lipid composition of cellular membranes, in the development of AD. Several lipids, including cholesterol and the sphingolipids sphingomyelin and ganglioside GM1, have been shown to influence the generation of Aβ [11–13] and a deregulation of sphingolipid metabolism was recently connected to AD

[12, 14]. The first step involved in sphingolipid *de novo* synthesis is the condensation of serine and palmitoyl-CoA to generate 3-dehydrosphinganine, catalyzed by the enzyme serine-palmitoyl transferase (SPT), which is suggested to be the rate-limiting enzyme in sphingolipid synthesis (Figure 1) [15]. 3-Dehydrosphinganine is further transformed to dihydroceramide, which is then desaturated to form ceramide, the simplest sphingolipid. Ceramide can be converted to sphingomyelin, sphingosine or various glycosphingolipids, which are ubiquitous constituents of membrane lipids and which are involved in various cellular events, including signal transduction, proliferation, differentiation, apoptosis and the maintenance of neuronal tissues and cells [16–19]. Furthermore, sphingolipids along with cholesterol have been shown to be required for the formation of detergent-resistant membrane microdomains, also called rafts, which are discussed to be the membrane microdomains where amyloidogenic processing of APP preferentially occurs [20–24].

2. Materials and Methods

2.1. Cell Culture. SH-SY5Y, MEF PS1r, MEF PS1/2−/−, MEF APPwt, MEF APP/APLP2−/− and MEF carrying PS1 familial Alzheimer's Disease mutations (E280A, A285V, T354I) cells were cultivated in DMEM (Sigma, Taufkirchen, Germany), 10% FCS (PAN Biotech, Aidenbach, Germany). For PS1 or PS-FAD/pCDNA3.1 retransfected MEF PS1/2−/− cells additional Zeocin (300 μg/mL) (Invitrogen, Karlsruhe, Germany) and for SH-SY5Y-FE65 Knock-down cells additional HygromycinB (400 μg/mL) (PAN Biotech, Aidenbach, Germany) was used.

2.2. Human and Murine Brain Material. Human FAD, SAD and corresponding control brain samples were obtained from Brain-Net (for details see Tables 1 and 2 in Supplementary Materials available online at doi:10.4061/2011/695413). Age- (+/− 3 months) and gender-matched APP−/− mice brains and APP△CT15 mice brains and corresponding controls have been described previously and at least 3 mice brains of different mice were analysed [25].

2.3. Determination of Peptides Effects. To determine the effect of Aβ40 (10 ng/mL) and Aβ42 (1 ng/mL) (B. Penke, Szeged, Hungary) or AICD (sequence in 1-letter code: KMQQNGYENPTYKFFEQMQN) (2 μM) (Genscript Corporation, Piscataway, USA) synthetic peptides were incubated for 6 days in cell culture. Detection of intracellular Aβ was performed as described previously [26].

2.4. Knock-Down Experiments. According to the manufacturers protocol we used the SureSilencing shRNA Plasmid (SABioscience, Frederick, USA). The following insert sequences were used to generate the Fe65 knock-down: 5′-TCC CTG GAC CAC TCT AAA CTT-3′; 5′-CAA CCC AGG GAT CAA GTG TTT-3′; 5′-AAG GCT TTG AGG ATG GAG AAT-3′; 5′-TGT CCA CAC GTT TGC ATT CAT-3′. As

control the following sequence was used: 5′-GGA ATC TCA TTC GAT GCA TAC-3′.

2.5. Quantitative Real-Time PCR Experiments. Total RNA was extracted from cells or tissue using TRIzol reagent (Invitrogen, Karlsruhe, Germany), according to manufacturers' protocols. 2 μg total RNA were reverse-transcribed using High Capacity cDNA Reverse Transcription Kits, and quantitative real-time PCR analysis was carried out using Fast SYBR Green Master Mix on 7500 Fast Real Time PCR System (7500 Fast System SDS Software 1.3.1.; Applied Biosystems, Darmstadt, Germany). Changes in gene expression were calculated using 2-($\Delta\Delta$Ct) method [27]. Results were normalized to β-actin. The following primer sequences were used: **murine**: Sptlc1: 5′-GCA GGA GCG TTC TGA TCT TA-3′ and 5′-CCG GAC ACG ATG TTG TAG TT-3′; Sptlc2: 5′-AAG TGC CAC CAT GCA ACA GA-3′ and 5′-TTG GCT CCA GGC ACA CTA CA-3′; β-Actin: 5′-CCT AGG CAC CAG GGT GTG AT-3′ and 5′-TCT CCA TGT CGT CCC AGT TG′; **human**: Sptlc2: 5′-TAT GGA GCT GGA GTG TGC AG-3′ and 5′-GAA TTC GTT GCA AAT CCC AT-3′; β-Actin: 5′-CTT CCT GGG CAT GGA GTC-3′ and 5′-AGC ACT GTG TTG GCG TAC AG-3′.

2.6. Lipid Extraction. A modified Bligh and Dyer [28] method was used to extract lipids to measure SPT activity as described below. After stopping the reaction by adding 3,75 mL CHCl$_3$: MeOH : HCl (1 : 2 : 0,06), mixture was vortexed for 1 h at room temperature (RT). Then 1,25 mL CHCl$_3$ was added and vortexed again for 1 h at RT. After adding 1,25 mL CHCl$_3$ and 1,25 mL H$_2$O, samples were vortexed for another 10 min before centrifugation at 5000 rpm for 10 min. The phase containing lipids was transferred to another glass tube and evaporated under nitrogen-flow at 30°C. 1 mL H$_2$O was added to evaporated lipids before another 3,75 mL of CHCl$_3$: MeOH : HCl (1 : 2 : 0,06) was added. The extraction cycle described here was repeated one time and after final evaporation under nitrogen-flow at 30°C lipids were dissolved in 100 μL CHCl$_3$.

2.7. Protein Determination. Protein determination was carried out according to Smith et al. [29]. Briefly, we used 20 μL of bovine serum albumin (Sigma, Taufkirchen, Germany) for the standard curve in a concentration range of 0,1–1,1 μg/μL. 0,5–2 μL of each sample was loaded onto a 96-well plate (BD, Heidelberg, Germany) in triplicates. 200 μL of buffer (4% CuSO$_4$: BCA-solution (Sigma, Taufkirchen, Germany) (1 : 39)) was added to each well, and assay plate was incubated for 15 min at 37°C and for another 15 min at RT. Absorbance was determined at a wavelength of 550 nm using a MultiscanEX (Thermo Fisher Scientific, Schwerte, and Germany).

2.8. Determination of SPT Activity. For analysis of SPT enzyme activity cells are harvested into 500 μL buffer A containing 100 mM HEPES (Sigma, Taufkirchen, and Germany) and 50 μM pyridoxal phosphate (Sigma, Taufkirchen,

L-serine
+
palmitoyl-
CoA

Serine-palmitoyl-
transferase (SPT)

CoA-SH
CO_2

3-dehydro-
sphinganine

NADPH + H$^+$ 3-Dehydro-
sphinganine-
reductase
NADP$^+$

Sphinganine

Acyl-CoA   Ceramide
synthase
CoA-SH

Dihydro-
ceramide

"desaturase"

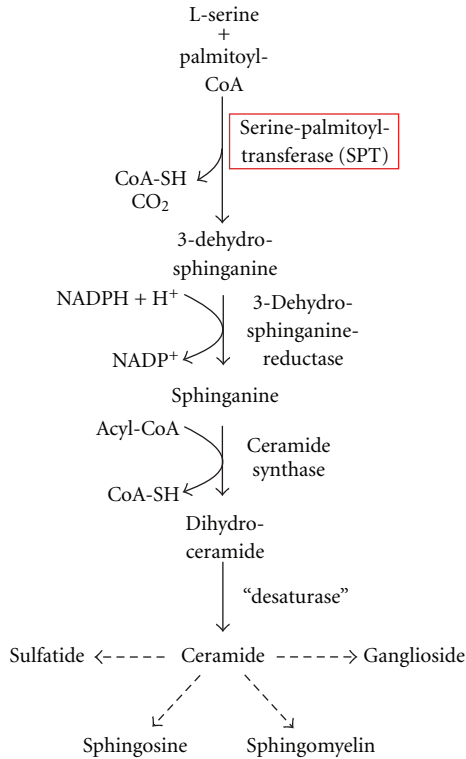Sulfatide ⟵ - - - - Ceramide - - - - ⟶ Ganglioside

Sphingosine   Sphingomyelin

Figure 1: Biosynthetic pathway of sphingolipid *de novo* synthesis.

Germany) supplemented with complete protease inhibitor, and protein levels are adjusted to protein amount of 2,5 mg/mL. The reaction is started by adding 400 μL of buffer B containing 1 mM palmitoyl-CoA (Larodan AB, Malmö, Sweden) and 10 μCi ^{14}C-L-serine (Perkin Elmer, Rodgau-Jügesheim, Germany) at 37°C in glass tubes. The reaction is stopped after 0, 2, 4, 8, 16, 32, and 64 min by transferring 500 μl of the reaction mixture in glass tubes containing 3,75 mL CHCl$_3$: MeOH : HCl (1 : 2 : 0,06). Lipid extraction was carried out as described above. To measure the radioactivity of the individual sample, 2 mL of scintillation liquid was added to each samples and radioactivity was determined in a scintillation counter (Perkin Elmer, Rodgau-Jügesheim, Germany).

2.9. Statistical Analysis. All quantified data represent an average of at least three independent experiments. Error bars represent standard deviation of the mean. Statistical significance was determined by two-tailed Student's *t*-test; significance was set at $^*P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$, n.d. = not detectable.

3. Results

3.1. Altered SPT Activity and SPTLC2 Expression in PS1/2- and APP/APLP2-Deficient Cells. To analyze the influence of APP and APP cleavage products on sphingolipid biosynthesis, we used mouse embryonic fibroblasts (MEFs) devoid of the catalytic components of the γ-secretase complex, PS1 and PS2 (MEF PS1/2−/−) [30, 31], and MEF devoid of

the PS substrate APP and the APP-like protein APLP2 (MEF APP/APLP2−/−). The common feature of both cell lines is the lack of Aβ peptides and of AICD. The analysis of the activity of the key enzyme for the regulation of sphingolipid levels in cells revealed that the SPT activity was significantly increased in MEF PS1/2−/− and MEF APP/APLP2−/− cells (Figures 2(a) and 2(b)) compared to the corresponding control cells. In order to examine if increased SPT activity is caused by an elevated SPT gene expression, we performed real-time PCR (RT-PCR) analysis of the corresponding cell lines. Mammalian SPT is a heterodimer of two subunits, the 53 kDa subunit long chain base 1 (SPTLC1 or LCB1) and the 63 kDa subunit long chain base 2 (SPTLC2 or LCB2) [32, 33]. Gene expression of the subunit SPTLC1 was not altered in PS1/2−/− cells compared to control cells, whereas gene transcription of the subunit SPTLC2 was significantly increased in PS1/2-deficient cells (Figure 2(c)). Interestingly, SPTLC2 is considered to be responsible for the catalytic activity of SPT [32, 34], indicating that the deficiency of PS1/2 influences the expression of the catalytic subunit of SPT. A similar result was obtained for APP/APLP2−/− compared to wt cells; gene expression of SPTLC1 was unchanged, whereas SPTLC2 gene transcription was significantly increased in APP/APLP2-deficient cells (Figure 2(d)), suggesting that not PS itself, but at least one of the cleavage products of APP regulates SPT gene transcription.

3.2. AICD Regulates SPTLC2 Expression. AICD is discussed to regulate gene transcription by a mechanism comparable to the function of the Notch intracellular domain, which is also released by γ-secretase activity, in gene expression [35–37]. To elucidate the effect of AICD on SPTLC2 gene transcription, we analyzed APP knock-in mouse embryonic fibroblasts deficient of full-length APP, expressing an APP construct, that lacks the last 15 aa from the C-terminus (MEF APP△CT15) and hence a functional AICD domain [25], compared to wt cells. Importantly, the deleted 15 aa include the presumably critical YENPTY motif of APP to which adaptor proteins like Fe65 and X11 are proposed to bind through their phosphotyrosine-binding domains and which are responsible for nuclear targeting of AICD [35, 38, 39]. Indeed, RT-PCR analysis of MEF APP△CT15 cells showed strongly increased gene expression of the SPT subunit SPTLC2 (Figure 3(a)), indicating that the presence of a functional AICD domain decreases SPTLC2 expression. In accordance with increased SPTLC2 expression, SPT activity was significantly increased in MEF APP△CT15 cells (Figure 3(a)). To exclude that altered Aβ production, which might be caused by the truncated APP construct APP△CT15 [40, 41], would be responsible for increased SPTLC2 expression in MEF APP△CT15 cells, we incubated MEF APP△CT15 cells with a synthetic AICD peptide, corresponding to the last 20 aa of the C-terminus of APP. APP△CT15 cells, incubated with solvent control only, showed in comparison to APP△CT15 cells incubated with the AICD peptide, increased SPTLC2 expression, emphasizing that AICD decreases SPTLC2 gene transcription (Figure 3(b)). Incubation with Aβ peptides and
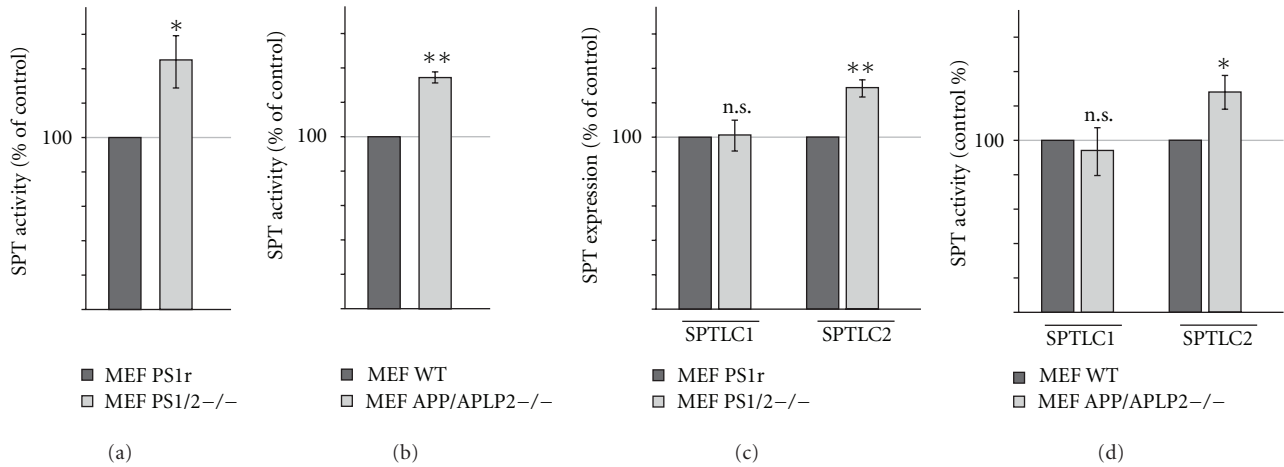
FIGURE 2: SPT activity and expression in PS1/2- and APP/APLP2-deficient cells. (a) SPT activity in mouse embryonic fibroblasts devoid of PS1 and PS2 (MEF PS1/2−/−) and MEF PS1/2−/− retransfected with PS1 (MEF PS1r). (b) SPT activity in mouse embryonic fibroblasts lacking APP and the APP-like protein APLP2 (MEF APP/APLP2−/−) and corresponding wild-type cells (MEF WT). (c) RT-PCR analysis of SPTLC1 and SPTLC2 expression, the two subunits of SPT, in MEF PS1/2−/− and MEF PS1r. (d) SPTLC1 and SPTLC2 expression in MEF APP/APLP2−/− and MEF WT.
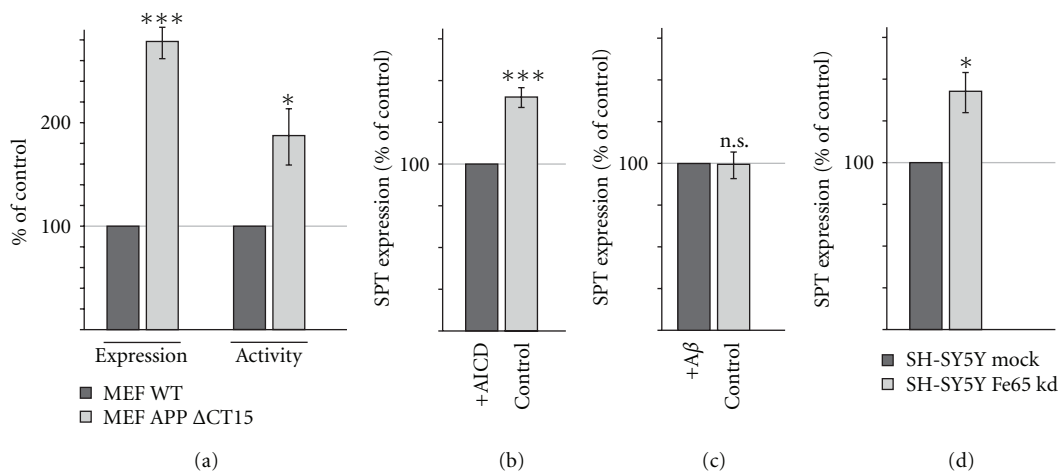

FIGURE 3: SPTLC2 expression is reduced in the presence of functional AICD. (a) Mouse embryonic fibroblasts expressing an APP construct lacking the last 15 amino acids (aa) and therefore a functional AICD domain (MEF APP△CT15) show increased SPT expression and activity compared to control fibroblasts (MEF WT). (b) MEF APP△CT15 cells incubated with functional AICD peptide show compared to MEF APP△CT15 cells incubated with solvent control decreased SPTLC2 expression. (c) MEF APP△CT15 cells incubated with Aβ peptides and solvent control showed no difference in SPTLC2 expression. (d) SPTLC2 expression in Fe65 knock-down SH-SY5Y cells is increased.

solvent control showed no differences in SPTLC2 expression (Figure 3(c)), demonstrating that Aβ peptides do not contribute to the regulation of SPTLC2 gene transcription. The uptake of the peptides was confirmed by incubating APP/APLP2−/− MEFs with Aβ peptide. Only in incubated cells intracellular Aβ could be detected by western blot analysis whereas the unincubated knockout cells showed no intracellular Aβ (supplemental Figure 1). To further evaluate the role of AICD in regulating gene expression of SPTLC2, we generated Fe65 knock-down human neuroblastoma SH-SY5Y cells. RT-PCR of Fe65 showed that Fe65 expression was reduced to 42%. As expected, the Fe65 knock-down cells had increased SPTLC2 expression (Figure 3(d)), further emphasizing a physiological role of AICD

in the regulation of SPT expression. Supporting the *in vivo* relevance of these findings, brains of APP knock-out mice (APP−/−) as well as brains of mice expressing the APP△CT15 construct had significantly increased SPTLC2 expression (Figures 4(a) and 4(b)). Taken together these results indicate that AICD regulates cellular SPTLC2 gene transcription *in vivo* and that this regulation is dependent on adaptor proteins like Fe65.

3.3. *Analysis of SPTLC2 Expression in FAD*. In order to evaluate a potential role of this AICD-mediated regulation of SPT in AD, we investigated whether familial forms of Alzheimer's disease (FAD) show changes in sphingolipid
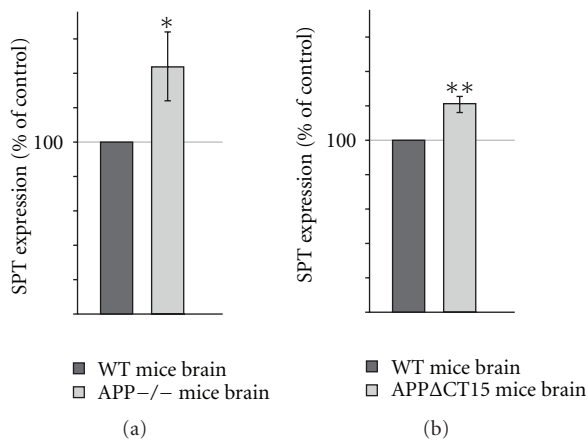
FIGURE 4: SPTLC2 expression in APP−/− and APP△CT15 mice brains. (a) SPTLC2 expression in APP-deficient mice brains (APP−/−) compared to wild-type (wt) mice brains. (b) SPTLC2 expression in APP△CT15 mice brains is increased compared to wt mice brains.

de novo synthesis. For this, MEF cells were generated that express familial PS1 mutations known to cause early onset Alzheimer's disease (EOAD) [42]. MEF PS1/2−/− cells were retransfected with three PS1-FAD mutations, E280A, A285V and T354I and wild-type PS1 (MEF PS1r). All FAD cell lines were PS expression level matched to the control cells. In accordance to the literature we found that analysed PS-FAD mutations result in a decreased total γ-secretase activity (data not shown) and therefore affecting AICD production [43–48]. RT-PCR analysis revealed that SPTLC2 expression was significantly increased in PS1 E280A, PS1 A285V, and PS1 T354I cells, supporting a potential role of SPT in AD (Figure 5(a)). Although the analysis of *postmortem* brain samples allows to draw only limited conclusions regarding the molecular mechanism, it should be noted that SPTLC2 expression was increased in familial AD *postmortem* brains, caused by the mutations I143T, L174R and L286V compared to age- and gender-matched controls (+/− 10 years). Confirming this result, analysis of *postmortem* brain samples of 40 sporadic AD brains compared to age- and gender-matched control brains showed that in 24 cases SPTLC2 expression was increased whereas in 16 cases SPTLC2 was decreased (Figures 5(b) and 5(c)). Although the mean difference between the sporadic AD cases compared to control brain samples reached significant levels (mean = 1,52; $P < .01$; SEM = 16%) it appears that in familial AD mutations the phenotype of increased SPTLC2 levels has a higher penetrance.

Although this finding emphasizes a potential role of SPT in AD and underlines our findings made by different cell culture and mouse models, it should be pointed out that in principal no molecular insights should be drawn by analyzing human *postmortem* material. Therefore and to avoid potential overinterpretation of these results obtained by *postmortem* brains we decided not to analyze the AICD levels.

4. Discussion

Sphingolipids play important roles in biological processes like regulation of cell growth and signal transduction and represent ubiquitous constituents of membrane lipids in eukaryotes [18, 49–51]. Serine-palmitoyl transferase (SPT) is the rate limiting enzyme that catalyzes the first step of *de novo* biosynthesis of sphingolipids, finally resulting in the synthesis of the three main types of complex sphingolipids: sphingomyelins, glycosphingolipids, and gangliosides [15]. Alterations in sphingolipid metabolism are discussed to contribute to the development of AD. Brains of AD patients show altered ganglioside level [52], elevated ceramide and sphingosine levels and reduced sphingomyelin levels [14]. Furthermore, gangliosides and sphingomyelin have been shown to influence $A\beta$ generation [12, 13]. Sphingolipids along with cholesterol have been shown to be enriched in detergent-resistant membrane microdomains, also called rafts [53–55]. Interestingly, β- and γ-secretases are discussed to be present in rafts [20–24]. Ganglioside GM1 is present in rafts, increases $A\beta$ generation and has been found to bind to $A\beta$ [13, 56, 57]. Notably GM1-$A\beta$ is favourably generated in the ganglioside-enriched, raft-like microdomains and exerts neurotoxic effects and might act as a seed for $A\beta$ aggregation in amyloid plaques [56, 58].

Although it is well established that a deregulation of sphingolipid metabolism is present in AD, the underlying cellular mechanism that causes changes in sphingolipid metabolism is poorly understood. It is known that $A\beta$ increases neutral and acidic sphingomyelinase activity [12, 14] and that expression of acidic sphingomyelinase is elevated in brains of AD patients [14]. In the present study we identified SPT, the rate limiting enzyme in sphingolipid biosynthesis, to be regulated by APP processing and to be affected in AD. The first indication of increased SPT activity in AD was obtained by the use of PS1/2- and APP/APLP2-deficient cell lines, which showed increased SPT activity. The elevated SPT activity is caused by increased expression of the SPT subunit SPTLC2, which represents the catalytic subunit of the SPT heterodimer [32, 34]. Because PS- and APP/APLP2-deficient cells are both devoid of $A\beta$ and AICD peptides, we analyzed whether these peptides are responsible for altered SPTLC2 expression. Analysis of mouse embryonic fibroblasts expressing an APP construct that lacks a functional AICD domain identified AICD as the molecular mediator of decreased SPTLC2 gene transcription. This result was further substantiated by the incubation of MEF APP△CT15 cells with AICD, resulting in decreased SPTLC2 expression in presence of AICD. By partially rescuing the altered SPTLC2 expression with an AICD peptide incubation, potential artefacts which could be caused by clonal heterogeneity of MEFs could be ruled out. Fe65 is an important protein that binds to the YENPTY motif in the APP C-terminus and is essential for nuclear transport of AICD [35, 38, 39]. Indeed, Fe65 knock-down increased SPTLC2 expression, which taken together with the above results clearly identifies AICD as a regulator of SPT transcription. AICD was controversially discussed to be involved in the regulation of gene transcription [35, 38, 39].
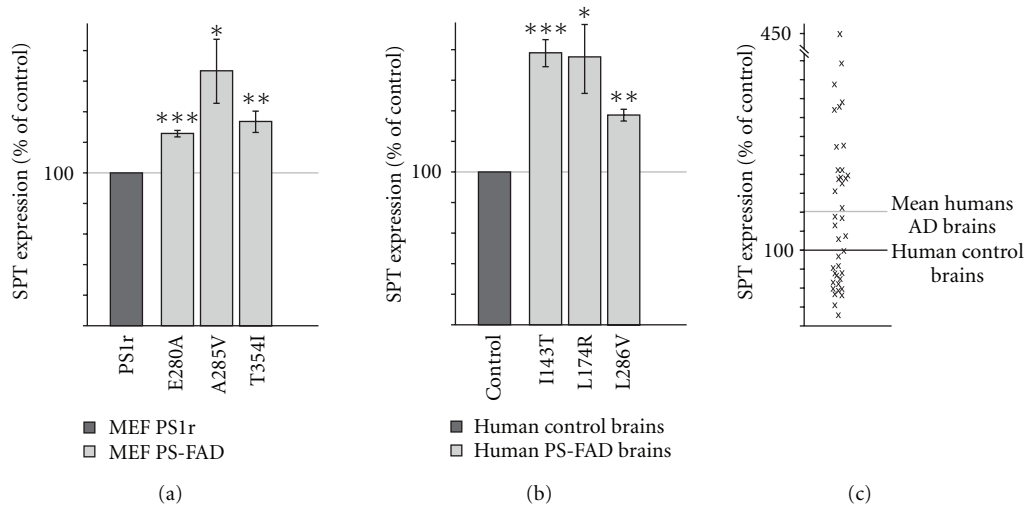
FIGURE 5: SPT expression in Alzheimer's disease. (a) Mouse embryonic fibroblasts devoid of PS1 and PS2 were retransfected with the familial PS1 mutations PS1 E280A, PS1 A285V, PS1 T354I (MEF PS-FAD), and PS1 wild-type (MEF PS1r), respectively. All PS1 mutations are known to cause early onset AD and show increased expression of SPTLC2. (b) Human *postmortem* PS-FAD brains, caused by the mutations I143T, L174R and L286V show increased SPTLC2 expression compared to age- and gender-matched control brains (+/− 10 years). (c) Analysis of SPTLC2 expression in 40 sporadic AD human *postmortem* brains compared to age- and gender-matched control brains (+/− 10 years). Pairwise normalization with the respective age- and gender-matched controls.

However, increasing evidence exists that AICD regulates the expression of multiple genes similar to the function of the Notch intracellular domain. For example, expression of APP, β-secretase BACE1, neprilysin, EGF-receptor, LRP1 and glycogen-synthase-kinase-3β (GSK-3β) has been shown to be regulated by AICD [35, 59–62]. Recently, two further genes were identified, patched homolog 1 (PTCH1) and transient receptor potential cation channel subfamily C member 5 (TRPC5) [63]. The identification of SPTLC2 expression to be regulated by AICD also contributes to our understanding of altered sphingolipid levels in AD. SPTLC2 expression was increased in cells expressing PS mutations known to cause EOAD and in human PS-FAD *postmortem* brains, supporting the relevance of altered SPT expression and activity in the development of AD. Taking into consideration that elevated SPT expression results in increased *de novo* synthesis of sphingolipids, major components of lipid rafts, one might speculate that increased SPTLC2 expression exerts its toxic effect by increased Aβ generation in lipid raft microdomains of the membrane, known to be involved in the amyloidogenic processing of APP. Nevertheless further experiments have to be done to clarify the question whether the observed change in SPTLC2 levels in the human sporadic and familiar AD brains are cause or consequence of Alzheimer's disease.

5. Conclusions

In conclusion, our results demonstrate that APP processing downregulates SPT expression, the rate limiting enzyme in sphingolipid *de novo* synthesis by an AICD/Fe65-mediated mechanism and that SPT expression is affected in AD.

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