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Trans fatty acids enhance amyloidogenic processing of the Alzheimer amyloid precursor protein (APP)☆

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Abstract

Hydrogenation of oils and diary products of ruminant animals leads to an increasing amount of *trans* fatty acids in the human diet. *Trans* fatty acids are incorporated in several lipids and accumulate in the membrane of cells. Here we systematically investigate whether the regulated intramembrane proteolysis of the amyloid precursor protein (APP) is affected by *trans* fatty acids compared to the *cis* conformation. Our experiments clearly show that *trans* fatty acids compared to *cis* fatty acids increase amyloidogenic and decrease nonamyloidogenic processing of APP, resulting in an increased production of amyloid beta (Aβ) peptides, main components of senile plaques, which are a characteristic neuropathological hallmark for Alzheimer's disease (AD). Moreover, our results show that oligomerization and aggregation of Aβ are increased by *trans* fatty acids. The mechanisms identified by this *in vitro* study suggest that the intake of *trans* fatty acids potentially increases the AD risk or causes an earlier onset of the disease.

Keywords: Alzheimer's disease; *Cis*/*trans* fatty acids; Nutrition; Amyloid precursor protein (APP) processing; Lipids; Amyloid beta (Aβ)

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that currently affects over 35 million people worldwide. One of the pathological hallmarks of AD is extracellular senile plaques composed of aggregated amyloid-beta (Aβ) peptides, which are released by sequential processing of the amyloid precursor protein (APP) [1–4]. For the generation of Aβ, APP is processed in the amyloidogenic pathway by β- and γ-secretases. β-Secretase BACE1 [5] cleaves APP within the extracellular/luminal domain, generating a soluble fragment of APP (sAPPβ) and a 99-amino-acid-long membrane-bound C-terminal fragment, termed β-CTF or C99. C99 is subsequently cleaved by γ-secretase, a protein complex consisting of at least four proteins, presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin, anterior pharynx-defective 1 (APH1a and APH1b) and presenilin enhancer 2 (PEN2), to release Aβ [6]. PS1 and PS2 represent the catalytic subunits of the γ-secretase complex; however, the other components are essential to form an active γ-secretase complex [7–9]. Beside amyloidogenic processing by β- and γ-secretases, APP can be cleaved in a nonamyloidogenic pathway by α-secretases [10,11]. α-Secretases were identified as members of the ADAM family (*a* *d*isintegrin *a*nd *m*etalloproteinase) and cleave APP within the Aβ domain, thus precluding the formation of Aβ [11–13]. Processing by α-secretases generates soluble α-secreted APP (sAPPα) and a C-terminal fragment C83, which is cleaved by the γ-secretase complex to generate p3 [14,15]. Both amyloidogenic and nonamyloidogenic processing of APP belong to the mechanism of regulated intramembrane proteolysis (RIP) [16]. RIP follows a two-step pattern: An initial cut outside the membrane is followed by a second cut inside the membrane, which is performed by secretases that, unlike others, can function inside the lipid environment of cellular membranes. As the γ-secretase complex is responsible for the second cut of APP-RIP, lipids might play a crucial role in the processing of APP. Indeed, we and others found that lipid composition of cellular membranes influences proteolytic processing of APP [17–21].

So far, research has focused mainly on cholesterol, an important component of cellular membranes. Cholesterol, which can either be endogenously synthesized or taken up with the diet, has been shown

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to increase Aβ generation [22–25]. Beside cholesterol, an increasing amount of *trans* fatty acids (FAs) recently appeared in the human diet [26]. It has been reported that *trans* FAs nowadays contribute 4%–12% of the total dietary fat intake in the population of the United States, corresponding to 13.3 g of *trans* fats per person per day [27,28]. *Trans* fats have been linked so far to coronary heart disease and arteriosclerosis [29,30]; however, their influence on neurodegenerative disorders is poorly understood. Epidemiological studies concerning the consumption of *trans* FAs and the relative risk of developing AD revealed diverse results. Morris et al. found that individuals with a high dietary intake of *trans* FAs have an elevated relative risk of developing AD [31]. In contrast, no significant relationship between *trans* FAs consumption and AD was observed in the Rotterdam study [32]. Furthermore, Naqvi et al. recently reported that higher intake of *trans* FAs is not associated with cognitive decline in women [33], whereas high *trans* FAs intake was found to increase cognitive decline in women with type 2 diabetes [34] and among older persons with no history of diabetes at the beginning of the epidemiological study [35].

In the present study, we elucidate the effect of *trans* FAs on the processing of APP and their impact on AD.

2. Methods and materials

2.1. Cell culture

SH-SY5Y wild-type cells were cultivated in Dulbecco's modified Eagle's medium (Sigma, Taufkirchen, Germany) supplemented with 10% fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany) and, for APP695- and C99-transfected SH-SY5Y cells, additionally with Hygromycin B (400 μg/ml) (PAN Biotech, Aidenbach, Germany). C99 represents the C-terminal fragment of β-secretase cleaved APP; the corresponding construct for the generation of C99 overexpressing SH-SY5Y cells is described [36]. Oleic acid, elaidic acid, linoleic acid and conjugated linoleic acid (CLA) (Cayman, Ann Harbor, USA) were solved in ethanol p.a. (Sigma, Taufkirchen, Germany).

For incubation, FAs were added to culture medium with 1% FCS in a final concentration of 50 μM for SH-SY5Y cells and 25 μM for cortical neurons; final content of ethanol was 8.5 mM for SH-SY5Y cells and 4.25 mM for cortical neurons, which equate to 0.5‰ (v/v) and 0.25‰ (v/v). The medium was sonicated for 5 min using a Bandelin Sonopuls sonicator (Bandelin, Berlin, Germany) with an intensity of 50% before being added to the cells. Incubation was carried out for 5 days, and the medium with FAs was changed every 12 h.

2.2. Preparation of cortical neurons

Cortical neurons were prepared from mice (E14). Briefly, brains of mouse embryos were trypsinized for 15 min at 37°C and washed five times with Hanks balanced salt solution (HBSS) buffer. Afterwards, brains were homogenized in 1 ml HBSS using a glass pipette. Cell suspension was seeded onto cell culture plates (Sarstedt, Nümbrecht, Germany) coated with poly-L-lysine (Sigma, Taufkirchen, Germany) and cultivated for 7 days at 37°C and 7% CO_2 before starting FA incubation.

2.3. Antibodies

Aβ analysis was performed as described before [37]. For the detection of ADAM10, cell lysates were separated on 10%–20% Tricine gels (Anamed, Groβ-Bieberau, Germany). For the detection of BACE1, nicastrin, sAPPα, α-CTF and β-CTF, we used 10%–20% Tricine gels (Invitrogen, Darmstadt, Germany). Western blot (WB) analysis was performed with anti-ADAM10 (ab47993) (1:2000, Merck, Darmstadt, Germany), anti-BACE1 (B0806) (1:1000, Sigma, Taufkirchen, Germany) and anti-nicastrin (N1660) (1:1500, Sigma, Taufkirchen, Germany). As secondary antibody, we used anti-rabbit (1:5000) (Promega, Mannheim, Germany). Densitometric quantification was done using Image Gauge V3.45 software.

2.4. Immunoprecipitation

Secreted Aβ, intracellular Aβ and β-CTF were immunoprecipitated with 20 μl protein G-Sepharose (Sigma, Taufkirchen, Germany) and W02 antibody (5 μg/ml; Millipore, Billerica, MA, USA). For immunoprecipitation of α-CTF, we used 20 μl protein A-Sepharose (Sigma, Taufkirchen, Germany) and antibody 2214 (5 μg/ml; produced at the Centre for Molecular Biology, University Heidelberg, Germany; published in Ref. [38]). All samples were adjusted to the same protein amount compared to the corresponding control. The immunoprecipitates were separated using 10%–20% Tricine Gels (Invitrogen, Darmstadt, Germany). WB analysis of Aβ, β-CTF and intracellular Aβ was performed according to Ida et al. [37]. For detection of sAPPα and α-CTF, we used W02 (1 μg/ml) and antibody 2214 (1:5000), respectively. As secondary antibodies, anti-rabbit (1:5000) (Promega, Mannheim, Germany) and anti-mouse (P0260) (1:10000, Dako, Hamburg, Germany) were used. Image Gauge V3.45 software was used for densitometric quantification.

2.5. Determination of β- and γ-secretase activity

Detection of β- and γ-secretase activity was performed as described before [22]. In brief, cells and brains (B6 mice brains) were washed three times with ice-cold phosphate-buffered saline, scraped off in sucrose buffer (10 mM Tris/HCl pH7.4 including 1 mM EDTA and 200 mM sucrose) and homogenized using a PotterS (Braun, Melsungen, Germany) at maximum speed (25 strokes) on ice. Protein amount was adjusted according to Smith et al. [39]. Samples were centrifuged at 900 rcf for 10 min at 4°C, and the obtained postnuclear fractions were incubated with FAs (100 μM) for 30 min at 37°C. Postnuclear fractions were ultracentrifuged at 55 000 rpm for 75 min at 4°C. Purified membranes were resuspended using cannulae with decreasing diameter in sucrose buffer. After adding 10 μM fluorogenic γ-secretase substrate [40] (Calbiochem, Darmstadt, Germany) or 20 μM β-secretase substrate IV [41] (Calbiochem, Darmstadt, Germany), fluorescence was measured continuously at an excitation wavelength of 355±10 nm and an emission wavelength of 440±10 nm for γ-secretase or 345±5 nm/500±2.5 nm for β-secretase at 37°C under light exclusion using a Safire^2 Fluorometer (Tecan, Crailsheim, Germany).

2.6. Quantitative real-time experiments

TRIzol reagent (Invitrogen, Karlsruhe, Germany) was used to extract total RNA according to manufacturer's protocols. Two micrograms total RNA was then reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany), and quantitative real-time polymerase chain reaction (RT-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). All results were normalized to β-actin gene expression, and changes detected in gene expression were calculated using $2^{-(\Delta\Delta Ct)}$ method [42]. Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). The following human primer sequences were used:

ADAM10: 5′-GCA AAC TGA AAC CTG GGA AA-3′ and 5′-TTC CTT CCC TTG CAC AGT CT-3′; APH1a: 5′-CAG CCA TTA TCC TGC TCC AT-3′ and 5′-GGA ATG TCA GTC CCG ATG TC-3′; APH1b: 5′-GTG TCA GCC CAG ACC TTC AT-3′ and 5′-CAG GCA GAG TTT CAG GCT TC-3′; BACE1: 5′-AAT ACC TGC GGT GGA AGA TG-3′ and 5′-GCC CTC CAT GAT AAC AGC TC-3′; NCSTN: 5′-CTG TAC GGA ACC AGG TGG AG-3′ and 5′-GAG AGG CTG GGA CTG ATT TG-3′; PS1: 5′-CTC AAT TCT GAA TGC TGC CA-3′ and 5′-GGC ATG GAT GAC CTT ATA GCA-3′; PS2: 5′-GAT CAG CGT CAT CGT GGT TA-3′ and 5′-GGA ACA GCA GCA TCA GTG AA-3′; PEN2: 5′-CAT CTT CTG GTT CTT CCG AGA G-3′ and 5′-AGA AGA GGA AGC CCA CAG C-3′; β- Actin: 5′-CTT CCT GGG CAT GGA GTC-3′ and 5′-AGC ACT GTG TTG GCG TAC AG-3′.

2.7. Fluorescence Activated Cell Sorting (FACS) analysis

For the detection of total APP, confluent grown cells were washed, decollated, fixed and stained with antibody A8967 [43] (1:200; Sigma, Taufkirchen, Germany) and Ab6717 [44] (1:200; Abcam, Cambridge, UK). To determine intracellular APP levels, cells have been trypsinized before fixation, and APP was detected as described above. FACS analysis was performed with FACSCantoII (Becton Dickinson, Heidelberg, Germany) and FACSDiva 6.1 software. To evaluate the extracellular APP, total and intracellular distribution of APP was subtracted.

2.8. Electron microscopy

For measuring Aβ aggregates in the presence of FAs, 10 μM Aβ42 (W.M. Keck Biotechnology, New Haven, CT, USA) was incubated with 100 μM FAs at 37°C for 24 h. Seven microliters was transferred onto a copper grid (G220T7, Agar Scientific Limited, Stansted, UK) and incubated for 5 min before washing twice with ddH_2O (Sigma, Taufkirchen, Germany) and once with 1% uranyl acetate (Merck, Darmstadt, Germany) for 1 min. The dried grid was analyzed using the transmission electron microscope Tecnai Biotwin 120 (Fei, Hillsboo, OR, USA).

2.9. Aβ aggregation

To measure Aβ aggregation, we used a thioflavin-T-based assay in accordance to Dahse et al. [45]. Briefly, we incubated 50 μM Aβ40 (generous gift of B. Penke, Szeged, Ungary) and 20 μM thioflavin T (Sigma, Taufkirchen, Germany) in 100 μl total volume per well on a black 96-well plate (Corning, Lowell, MA, USA). Thioflavin T assay was measured for at least 6 h in intervals of 10 min using a Safire^2 Fluorometer (Tecan, Crailsheim, Germany) at 37°C with an excitation wavelength at 450±5 nm and fluorescence detection at 482±5 nm.

2.10. 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 $***P \leq .001$.

3. Results

3.1. Effect of trans FAs on APP processing

In order to analyze the effect of *trans* FAs on APP processing, we selected four typical FAs, the monounsaturated FA oleic acid, which reflects the *cis* conformation of FA 18:1 and the corresponding *trans* conformation of FA 18:1 (elaidic acid). In addition to oleic and elaidic acid, we examined the polyunsaturated FA 18:2 in its *cis* conformation (linoleic acid) and the CLA, which represents the *trans* conformation of FA 18:2. As a cellular system, we used the neuroblastoma cell line SH-SY5Y, transfected either with APP695 wild-type (APP695) or with the truncated APP construct C99 [46]. In contrast to APP695 transfected cells, where β- and γ-secretase activities are essential for Aβ generation, C99 transfected cells only need γ-secretase activity for the release of Aβ. Secreted Aβ levels were significantly increased in both APP695 and C99 transfected cells incubated with the *trans* FA elaidic acid compared to cells treated with oleic acid, the corresponding *cis* conformation of elaidic acid (Fig. 1A). Increased levels of Aβ were also detected for intracellular Aβ in SH-SY5Y cells stably expressing APP695. The C-terminal fragment α-CTF and sAPPα, both products of α-secretase processing of APP, were slightly but significantly decreased when cells were treated with the *trans* FA elaidic acid compared to oleic acid (Fig. 1A). β-CTF levels were not significantly decreased in cells incubated with elaidic acid. Increased levels of secreted Aβ and intracellular Aβ were also observed for the polyunsaturated *trans* FA CLA in comparison to the *cis* conformation of FA 18:2, linoleic acid (Fig. 1B). Secreted Aβ levels were elevated in APP695 as well as in C99 transfected SH-SY5Y cells. In line with decreased production of α-CTF and sAPPα in cells treated with elaidic acid, α-CTF and sAPPα levels were reduced when cells were treated with the *trans* FA CLA. In contrast to elaidic acid where the reduction of β-CTF was not significant, β-CTF was significantly decreased in the presence of CLA (Fig. 1B). Since we observed increased levels of Aβ

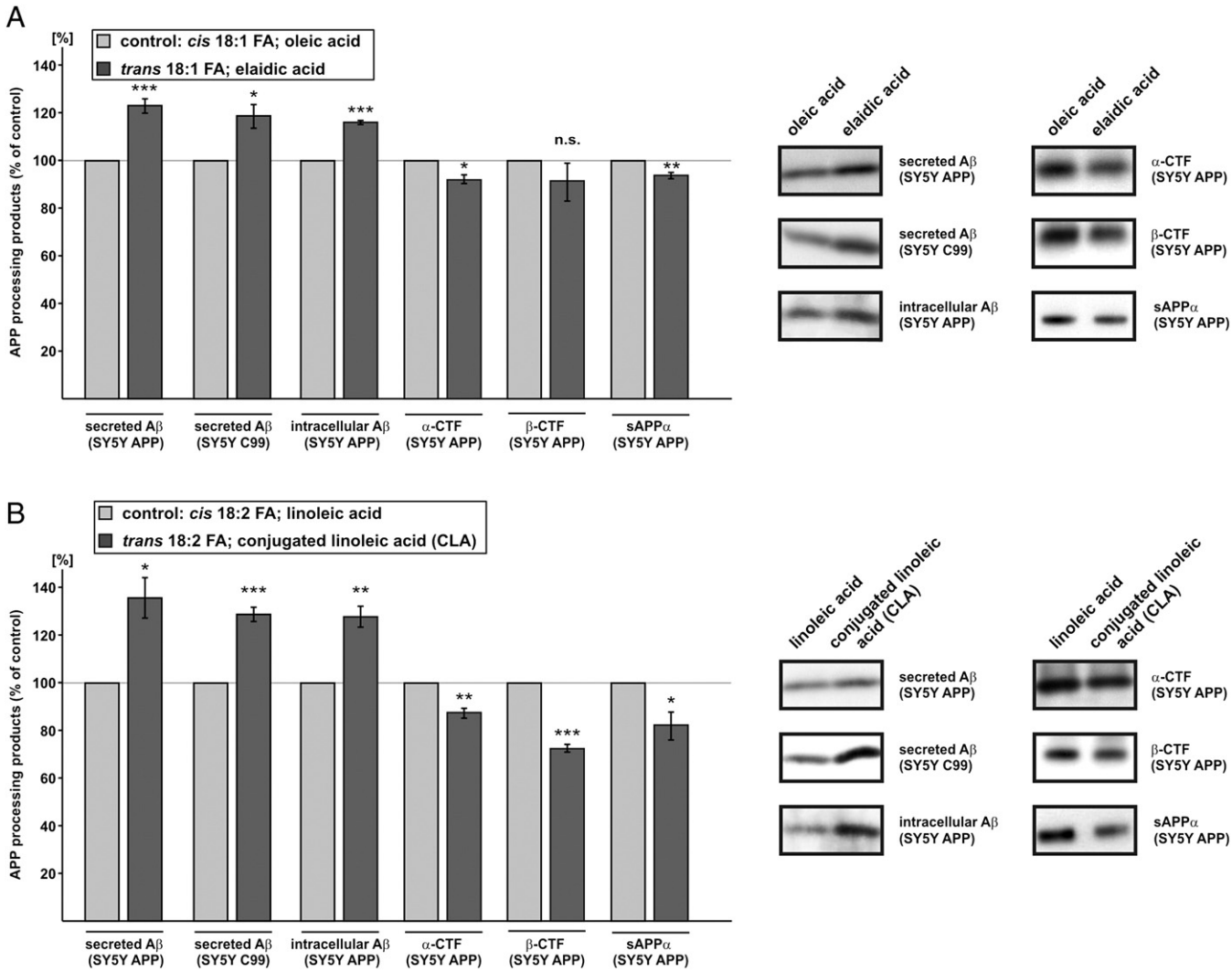

Fig. 1. Effect of *trans* FA on APP processing products. Human neuroblastoma SH-SY5Y cells (SY5Y) were incubated with *trans* FA elaidic acid compared to the *cis* conformation oleic acid (A) and the *trans* FA CLA compared to linoleic acid (B). SH-SY5Y cells stably transfected with either APP695 (SY5Y APP) or C99, representing the β-cleaved C-terminal APP fragment (SY5Y C99), were used for the detection of secreted Aβ. Intracellular Aβ was determined for SY5Y APP expressing cells. The C-terminal fragments generated by α-secretase processing (α-CTF) and β-secretase processing (β-CTF) and α-secreted APP (sAPPα) were analyzed for SY5Y APP overexpressing cells. APP processing products were examined by WB analysis. Representative WBs of the described APP processing products are shown. All quantified data represent an average of at least three independent experiments. Error bars represent standard deviation of the mean. Asterisks show the statistical significance ($*P \leq .05$, $**P \leq .01$ and $***P \leq .001$, n.s.=not significant).

and decreased levels of α-CTF and sAPPα for both *trans* FAs elaidic acid and CLA, we further evaluated whether *trans* FAs increase the amyloidogenic processing of APP and decrease the nonamyloidogenic processing of APP.

3.2. β-Secretase activity is elevated in the presence of trans FA

To examine whether *trans* FAs increase β-secretase activity, we first incubated purified membranes of SH-SY5Y wild-type (wt) cells with *trans* FAs and measured β-secretase activity directly with a fluorescent β-secretase assay [22]. β-Secretase activity of purified membranes was increased in presence of the *trans* FA elaidic acid compared to oleic acid (Fig. 2A), indicating a direct effect of *trans* FAs on β-secretase activity. To examine a potential direct effect of *trans* FAs on β-secretase activity *ex vivo*, we purified membranes of mouse brains. In agreement with our results obtained with SH-SY5Y wt cells, purified membranes of mouse brains show in the presence of elaidic acid a similar increased β-secretase activity (Fig. 2A). Additionally, we confirmed the observed effect in living cells; therefore, we cultured SH-SY5Y wt cells in the presence of *trans* FAs, and afterwards, membranes were purified. A similar effect on β-secretase activity was detected when SH-SY5Y wt cells were cultured in the presence of elaidic acid compared to oleic acid (Fig. 2A). Mouse cortical neurons incubated in cell culture with elaidic acid also revealed increased, but not significant, β-secretase activity in purified membranes of treated cells (Fig. 2A). The same set of experiments was performed for linoleic acid and CLA. β-Secretase activity was elevated in purified membranes of SH-SY5Y wt cells and mouse brains in the presence of CLA; the effect strength was slightly increased compared to the incubation with elaidic acid (Fig. 2B). When CLA was incubated on living cells and membranes were purified afterwards, β-secretase activity was significantly increased for both SH-SY5Y wt cells and mouse cortical neurons (Fig. 2B).

3.3. Trans FAs increase γ-secretase activity

As described above, we observed an increase in secreted Aβ levels in C99 transfected cells, indicating that γ-secretase activity is elevated in the presence of *trans* FAs. This indication could be evaluated by measuring γ-secretase activity directly in purified membranes. γ-Secretase activity was increased in purified membranes of SH-SY5Y wt cells and mouse brains in the presence of elaidic acid compared to oleic acid (Fig. 3A). Elevated levels of γ-secretase activity were also observed in SH-SY5Y wt cells and mouse cortical neurons incubated in cell culture with elaidic acid (Fig. 3A). Increased γ-secretase activity was also observed for CLA and in line with measured β-secretase activity, the magnitude of the γ-secretase effect was stronger as observed for the *trans* FA elaidic acid (Fig. 3B).

3.4. RT-PCR analysis of ADAM10, BACE1 and the components of the γ-secretase complex

We observed increased β- and γ-secretase activity when purified membranes were incubated with *trans* FAs, arguing for a direct effect of *trans* FAs on secretase activities. Nevertheless, additional mechanisms might be present for increased β- and γ-secretase activities. One of these mechanisms could be altered gene expression. To evaluate this question, we performed RT-PCR analysis of cells incubated with the *trans* FA elaidic acid. As control, we incubated cells with oleic acid, representing the *cis* conformation of FA 18:1. mRNA levels were significantly increased for the γ-secretase components PS1, PS2, APH1a, APH1b and nicastrin in cells incubated with elaidic acid (Fig. 4A). Gene transcription of PEN2, a further protein of the γ-secretase complex, was not significantly increased. RT-PCR analysis of BACE1 also showed increased levels of BACE1 mRNA, indicating that increased secretase activities are also caused by elevated gene transcription. As shown above, levels of sAPPα and α-CTF were significantly decreased, suggesting decreased α-secretase processing of APP in the presence of *trans* FAs. In order to examine whether reduced α-secretase processing might be caused by reduced gene transcription, we also performed RT-PCR analysis of ADAM10. In line with these findings, mRNA levels of ADAM10 were decreased in cells incubated with elaidic acid compared to cells incubated with oleic acid (Fig. 4A). The polyunsaturated *trans* FA CLA also increased gene expression of the γ-secretase components (Fig. 4B). In the case of CLA, all γ-secretase components were significantly increased. In line with the results obtained for the monounsaturated *trans* FA elaidic acid, mRNA level of BACE1 was elevated, whereas gene transcription of ADAM10 was decreased (Fig. 4B), indicating that

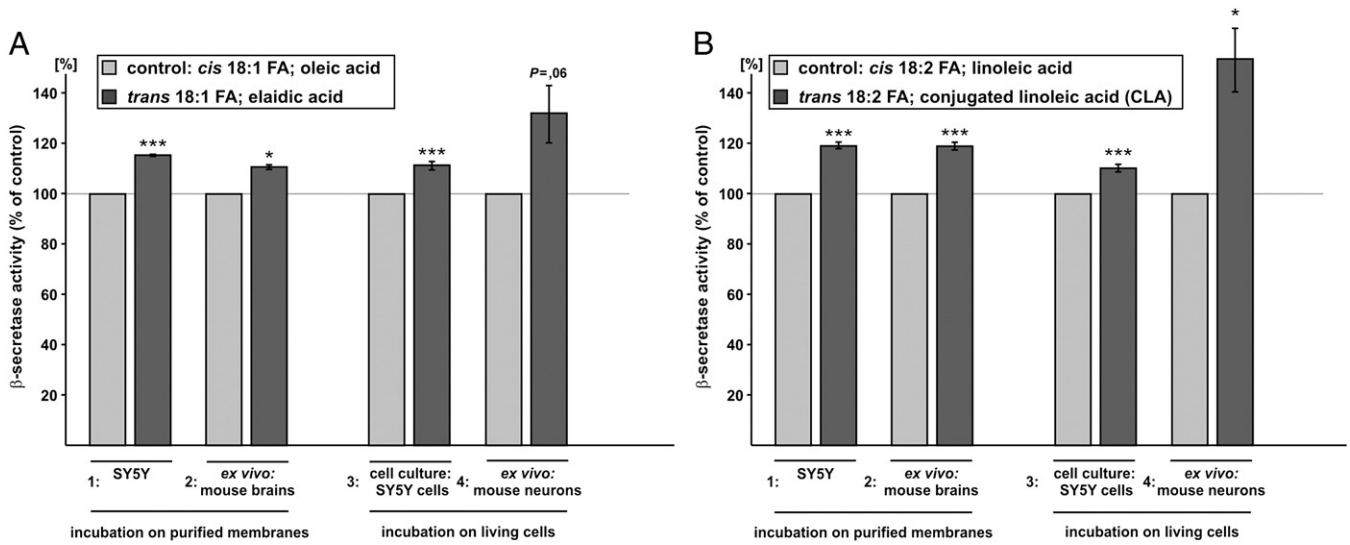

Fig. 2. Effect of the *trans* FAs elaidic acid (A) and CLA (B) on β-secretase activity compared to the corresponding *cis* conformation. *Trans* FAs incubated on purified membranes of SH-SY5Y wt cells (SY5Y) resulted in a direct increase in β-secretase activity (SY5Y, 1). Similar results were obtained with membranes isolated from mouse brains (*ex vivo* mouse brains, 2). SY5Y cells incubated with *trans* FAs (cell culture SY5Y cells, 3) and mouse cortical neurons (*ex vivo* mouse neurons, 4) also revealed increased β-secretase activity. All quantified data represent an average of at least three independent experiments. Illustration and statistical significance as described for Fig. 1.

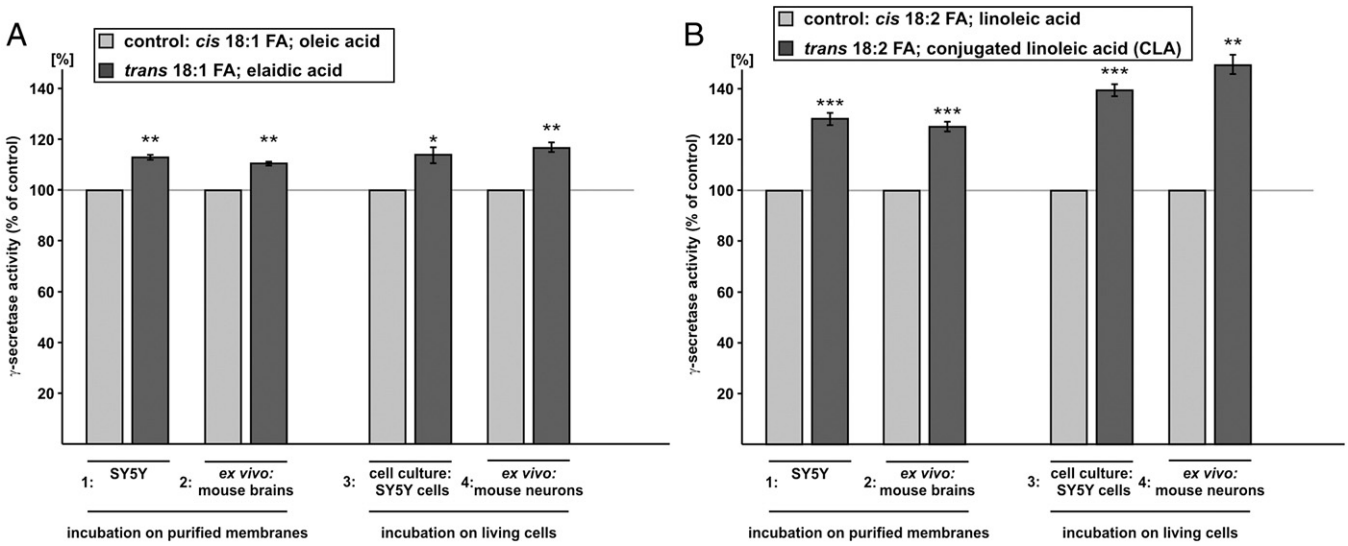

Fig. 3. Determination of γ-secretase activity in the presence of *trans* FAs. (A) Elaidic acid compared to oleic acid. (B) CLA compared to linoleic acid. γ-Secretase activity was determined using a fluorescent assay; *trans* FAs were incubated directly on purified membranes of SY5Y cells (1) and membranes isolated from mouse brains (2). An increase in γ-secretase activity was also determined when SY5Y cells (3) or mouse cortical neurons (4) were cultured in the presence of *trans* FAs. Purified membranes of incubated cells were subjected to the γ-secretase assay. All quantified data represent an average of at least three independent experiments. Illustration and statistical significance as described for Fig. 1.

trans FAs increase gene expression of the proteins involved in the amyloidogenic processing, whereas ADAM10 gene expression, necessary for nonamyloidogenic processing of APP, is decreased.

3.5. Protein level of ADAM10, nicastrin and BACE1 in the presence of trans FA

Altered gene transcription of the secretases involved in proteolytic processing of APP should result in altered protein levels. To analyze if protein levels are changed in the presence of *trans* FAs, we performed WB analysis of ADAM10, nicastrin and BACE1. ADAM10 is discussed to be the predominant α-secretase in SH-SY5Y cells [47]; nicastrin promotes maturation and trafficking of the γ-secretase components, finally resulting in a catalytically active γ-secretase complex [48–50]; and BACE1 represents the main β-secretase activity in neuronal cells [51].

ADAM10 protein levels were significantly decreased in the presence of the *trans* FAs elaidic acid and CLA compared to the corresponding *cis* FAs oleic acid and linoleic acid (Fig. 5 A and B). Both *trans* FAs also increased the protein level of nicastrin; however, the effect on nicastrin protein level was not as pronounced as observed for gene transcription. BACE1 protein level was not significantly elevated for the *trans* FA elaidic acid compared to oleic acid (Fig. 5A). For CLA, the protein level of BACE1 was significantly elevated (Fig. 5B).

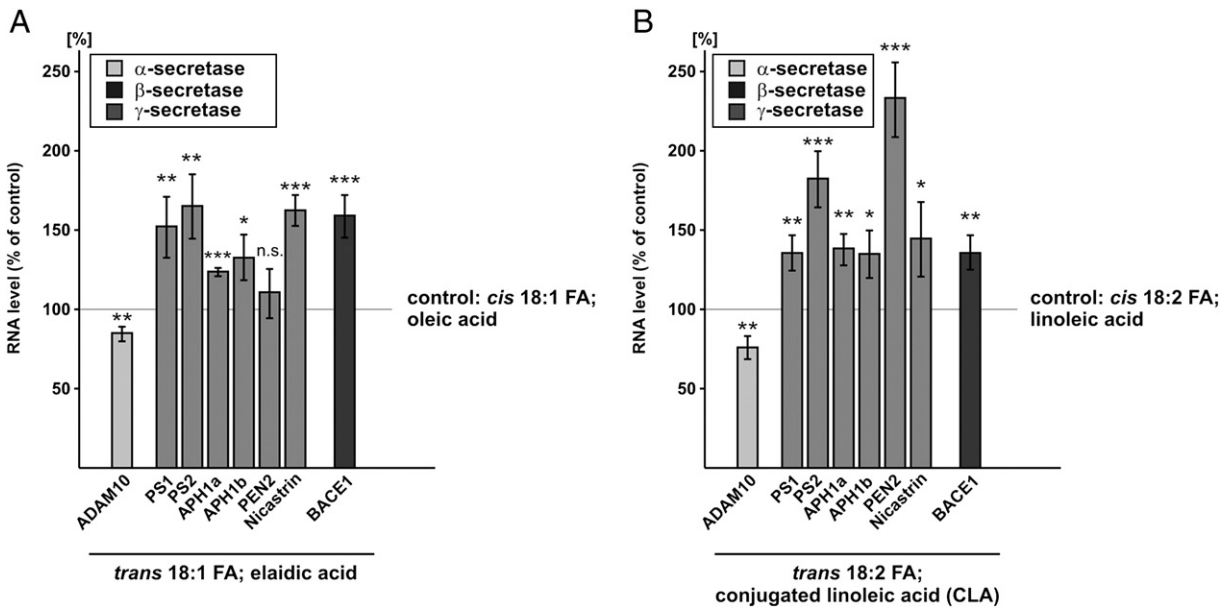

Fig. 4. Expression of ADAM10, BACE1 and components of the γ-secretase complex in the presence of *trans* FAs. mRNA levels were determined via RT-PCR. (A) Influence of *trans* FA elaidic acid compared to *cis* conformation (oleic acid) on gene transcription. (B) *Trans* FA CLA compared to linoleic acid. ADAM10 (light gray bar) is proposed to be the major α-secretase in neuronal cells. The components of the γ-secretase complex (PS1, PS2, APH1A, APH1B, PEN2 and nicastrin) are presented in gray bars. BACE1, which is the main β-secretase in neurons is shown in dark gray bars. All quantified data represent an average of at least three independent experiments. Illustration and statistical significance as described for Fig. 1.

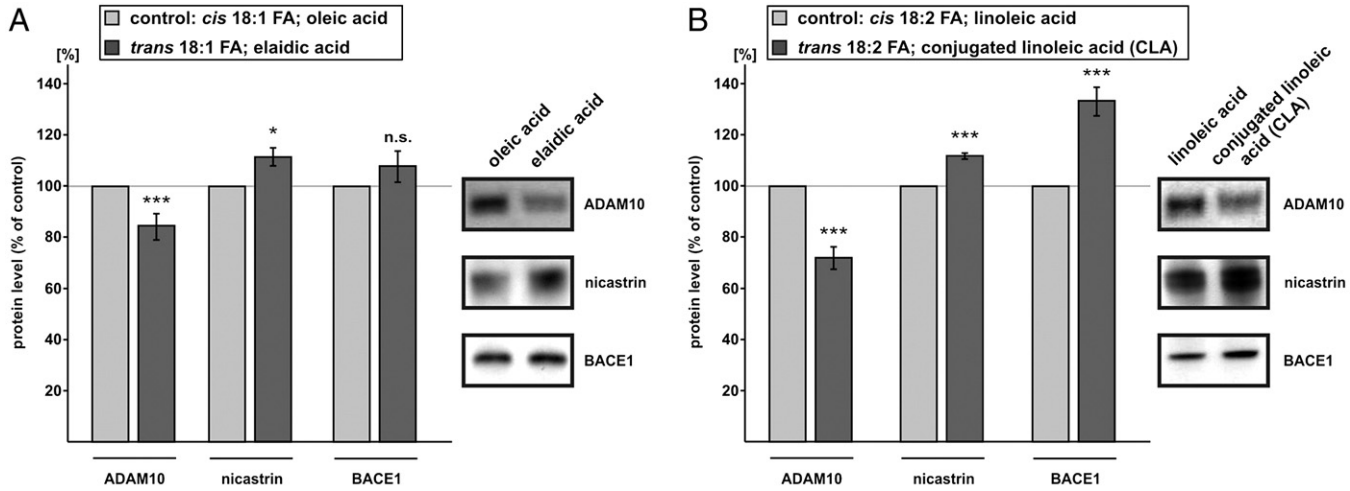

Fig. 5. Protein levels of ADAM10, nicastrin and BACE1 in the presence of *trans* FAs compared to *cis* conformation. Protein levels of SH-SY5Y wt cells incubated with (A) oleic acid and elaidic acid and (B) linoleic acid and CLA were evaluated by WB analysis. In accordance to the decreased ADAM10 mRNA levels, the protein levels of ADAM10 were decreased in the presence of *trans* FAs. Nicastrin was exemplarily chosen to analyze the proteins of the γ-secretase complex. Nicastrin protein level was increased in the presence of both *trans* FAs. BACE1 protein levels were slightly but not significantly increased in the presence of elaidic acid; incubation of CLA resulted in a statistically significant increase of BACE1. Representative WBs are shown. All quantified data represent an average of at least three independent experiments. Illustration and statistical significance as described for Fig. 1.

3.6. Trans FA influence APP transport to the cell surface

In the presence of the *trans* FAs elaidic acid and CLA, we found an accumulation of intracellular APP (Fig. 6A). There are two potential explanations for increased intracellular APP levels. Either *trans* FAs increase gene expression of APP or *trans* FAs decrease APP transport to the cell surface, thus resulting in an accumulation of intracellular APP. To elucidate both mechanisms, we first analyzed APP gene expression via RT-PCR analysis. Indeed, *trans* FAs increased the expression of APP (Fig. 6B). To investigate if *trans* FAs might also influence APP transport to the plasma membrane, we performed FACS analysis of SH-SY5Y cells incubated with *trans* FAs elaidic acid and CLA and the corresponding *cis* FAs oleic acid and linoleic acid. In the presence of *trans* FAs, the amount of APP detected at the cell surface was decreased (Fig. 6C), indicating impaired APP transport through the secretory pathway. Increased intracellular levels of APP are therefore a consequence of elevated APP gene expression and impaired transport of APP to the plasma membrane.

3.7. Aβ aggregation is elevated in cells exposed to trans FA

Additionally, we analyzed if *trans* FAs alter Aβ aggregation. For this approach, we first performed electron microscopy (EM) studies in cells treated with *trans* FAs. The amount of Aβ aggregates was higher for both *trans* FAs elaidic acid and CLA than that observed for the *cis* FAs oleic acid and linoleic acid (Fig. 7A and B). To confirm these results, we analyzed Aβ aggregation using a fluorescent thioflavin-T-based assay. Increased Aβ aggregation was

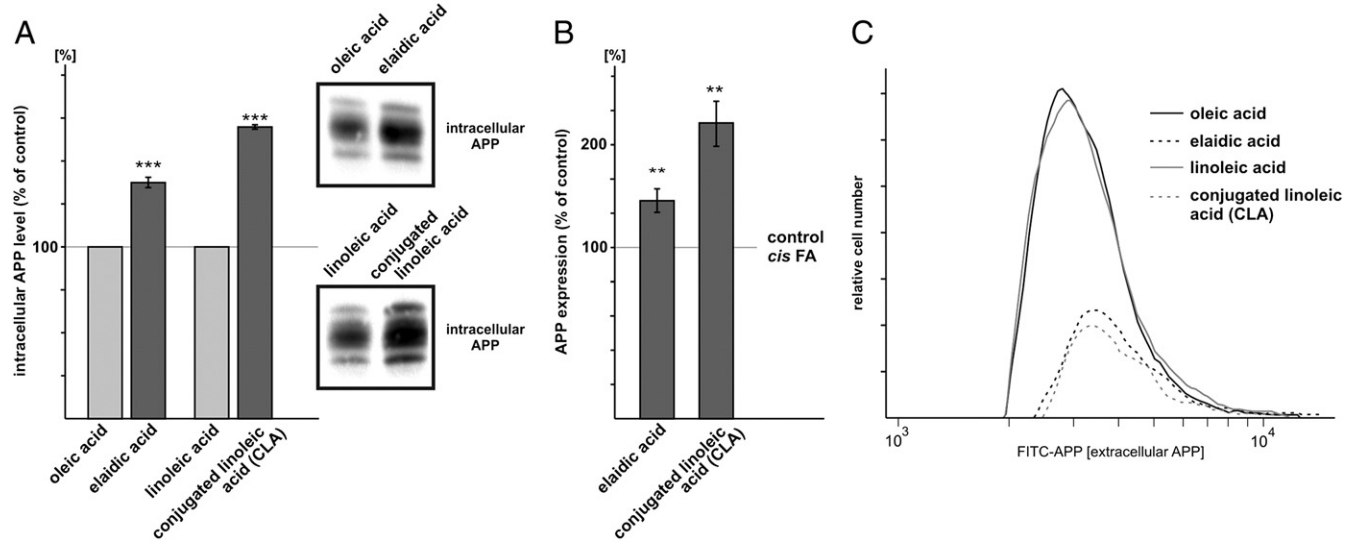

Fig. 6. Effect of *trans* FAs on intracellular APP level and APP sorting. SH-SY5Y wt cells were incubated with *trans* FAs. For the detection of intracellular APP, WB analysis was performed (A). In the presence of both *trans* FAs elaidic acid and CLA, an increase of intracellular APP was observed. Representative WBs are shown. (B) Analysis of APP gene transcription in the presence of *trans* FAs. RT-PCR analysis was performed to evaluate APP mRNA level. Elaidic acid and CLA showed an increase in APP expression. (C) FACS analysis of cell surface localized APP in the presence of *trans* FAs: Elaidic acid and CLA reduce cell surface localized APP. All quantified data represent an average of at least three independent experiments. Illustration and statistical significance as described for Fig. 1.

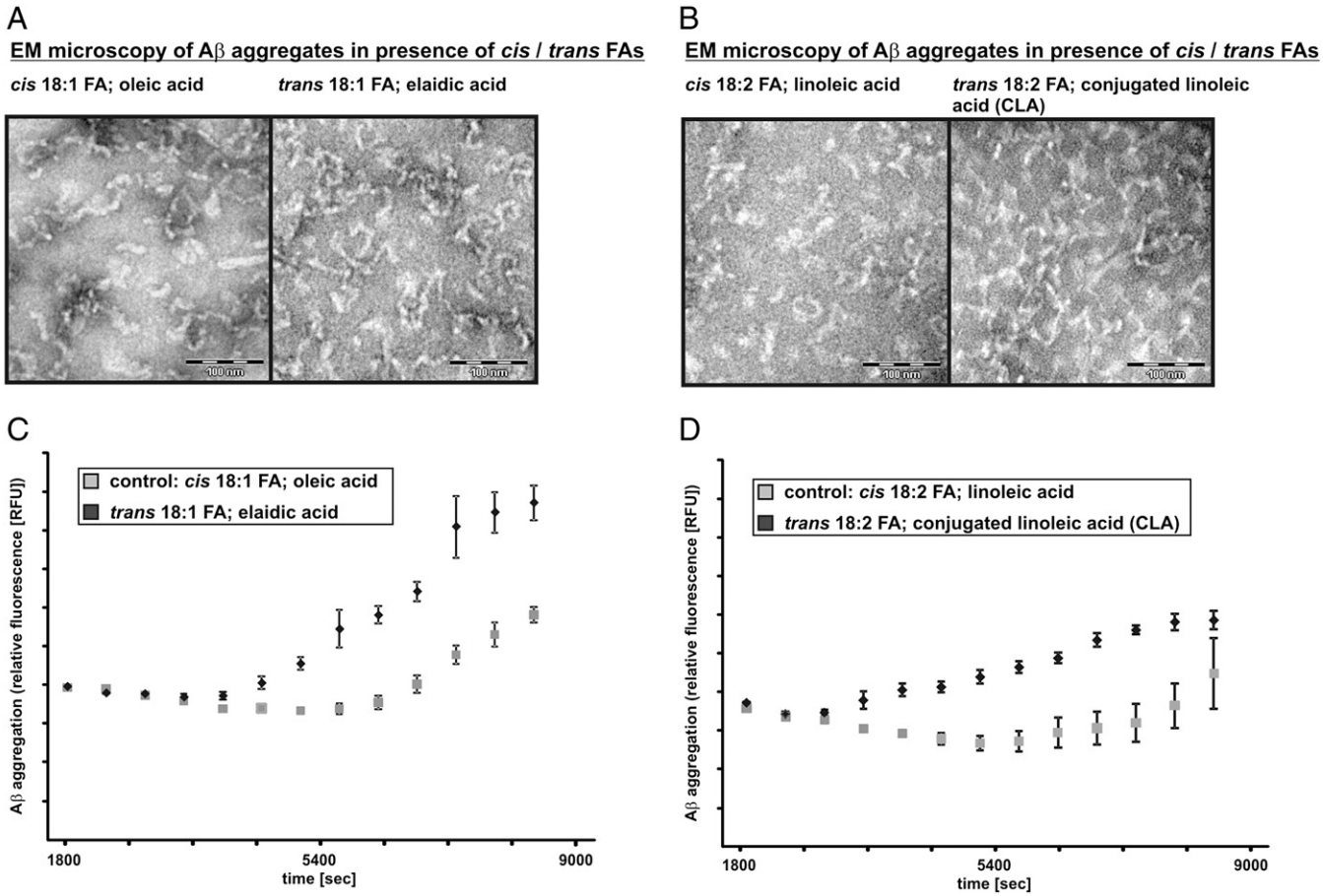

Fig. 7. Effect of *trans* FAs on Aβ aggregation. Synthetic Aβ40 was incubated for 24h with t*rans* FAs elaidic acid (A) and CLA (B) compared to the *cis* conformation, and Aβ aggregation was analyzed via transmission EM. Representative EM pictures are shown. In the presence of *trans* FAs, more aggregates and oligomers occurred. For quantitative analysis, a thioflavin T assay was used to monitor the aggregation in the presence of *trans* FAs elaidic acid (C) and CLA (D). In accordance with the EM analysis, oligomerization and aggregation were increased in the presence of *trans* FAs. All quantified data represent an average of at least three independent experiments. Illustration and statistical significance as described for Fig. 1.

detected in cells incubated with the *trans* FAs elaidic acid and CLA (Fig. 7C and D).

4. Discussion

Trans FAs are present in animal as well as vegetable food products. *Trans* FAs naturally occur in small amounts in meat, milk and dairy products like cheese, butter and cream as a result of bacterial fermentation in ruminant animals. However, most of the *trans* FAs in the human diet are artificial. Since the first successful hydrogenation of oils was reported, there has been a steady increase in the amount of *trans* FAs appearing in the human diet [26]. *Trans* FAs are produced from *cis* forms of unsaturated FAs during the hydrogenation of vegetable oil, a process used to transform oil from a liquid to a semisolid or solid state. These fats are further used for the production of, e.g., margarines, shortening, bakery products and deep frying fat. For that reason, *trans* FAs are also present in various bakery products like cakes, biscuits and chocolates and foods prepared with deep-frying fat, e.g., French fries.

FAs can be incorporated into phospholipids, and once incorporated into membrane phospholipids, *trans* FAs may alter membrane properties like membrane fluidity, thus affecting biochemical processes and cellular functions [52–57]. As all APP processing secretases are transmembrane proteins that cleave APP within or close to the transmembrane domain of APP (Fig. 8A), we elucidated if *trans* FAs change RIP of APP. It has been reported that lipid components of cellular membranes, like cholesterol, sphingomyelin and gangliosides, alter Aβ production [17–21]. A systematic investigation of *trans* FAs on proteolytic processing of APP and their impact on AD is still missing. Taking into consideration that *trans* FAs contrary to *cis* FAs may accumulate in the body over time [58] and that there is an increasing amount of *trans* FAs in the human diet, *trans* FAs might play a crucial role in the development of AD. A potential impact of *trans* FAs in the development of AD is supported by an epidemiological study in which 815 participants aged 65 years and older who were unaffected by AD at the beginning of the study completed a food-frequency questionnaire and were clinically evaluated for several years [31]. Individuals who ate approximately 4.8 g/day of *trans* FAs showed a fivefold higher relative risk to develop AD than participants consuming approximately 1.8 g/day of *trans* FAs after multivariable adjustment for other fats [31]. However, Engelhart et al. found no significant relationship between *trans* FAs dietary intake and the potential risk to develop AD [32] by continuously monitoring 5395 subjects with normal condition at baseline for incident dementia, who underwent complete dietary assessment by a semiquantitative food-frequency questionnaire. Similar diverse conclusions are so far present from epidemiological studies on the relationship between *trans* FAs and cognitive decline. Whereas Morris et al. describe that the dietary intake of *trans* FAs increases cognitive decline among older persons [35], especially when consumed in synergy with copper [59], it has been recently reported that higher consumption of *trans* FAs is not associated with cognitive decline in

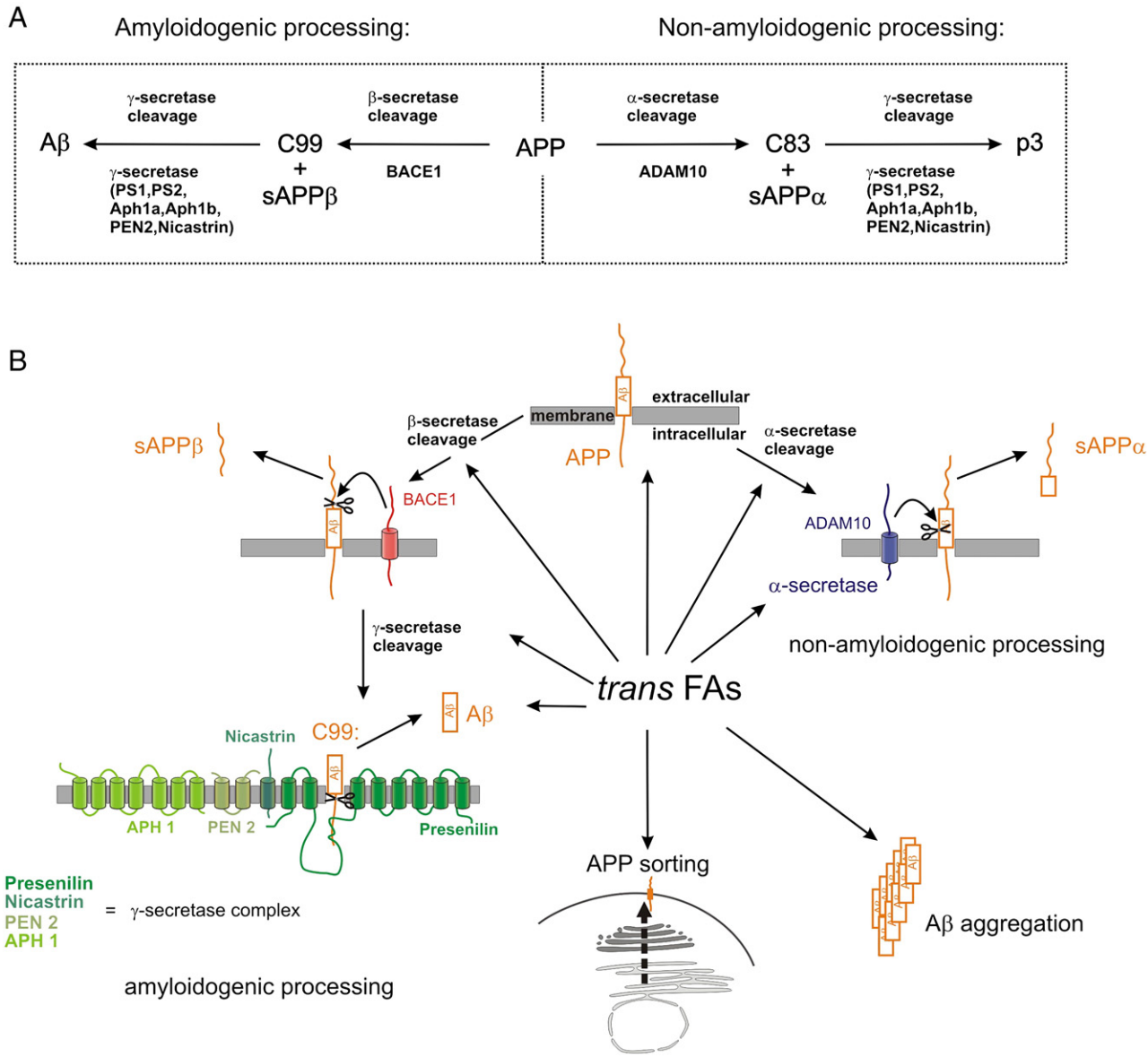

Fig. 8. Model of the pleiotropic effects of *trans* FAs on APP processing. (A) The APP can be cleaved in an amyloidogenic and a nonamyloidogenic pathway. For amyloidogenic processing of APP, APP is first cleaved within its extracellular/luminal domain by β-secretase BACE1, generating soluble β-secreted APP (sAPPβ) and a C-terminal membrane-bound fragment, C99. C99 is further cleaved by γ-secretase, a protein complex consisting of at least four proteins: PS1 or PS2, nicastrin, Aph1a or Aph1b, and PEN2. The initial cut by β-secretase BACE1 generates the N-terminus of Aβ, whereas the final cut of C99 by γ-secretase generates the C-terminus of Aβ, thus releasing Aβ peptides. In the nonamyloidogenic pathway, APP is first cleaved by α-secretases, identified as members of the ADAM family. Processing by α-secretase generates sAPPα and a C-terminal membrane-bound fragment, C83, which is further cleaved by the γ-secretase complex to generate the peptide p3. The α-secretase cleaves APP within the Aβ domain, thus precluding the formation of Aβ peptides. (B) *Trans* FAs directly increase the secretase activities of β- and γ-secretase and increase the levels of the secretase proteins. In contrast, the nonamyloidogenic pathway is decreased as ADAM10 levels are decreased, resulting in a decreased production of sAPPα. Beside the increased production of Aβ, *trans* FAs increase the oligomerization an aggregation of Aβ and lead to an accumulation of intracellular APP, whereas APP on the plasma membrane decreases, suggesting that, additionally, the transport of APP is impaired in the presence of *trans* FAs.

women [33]. However, controversial findings might be a result of differences between study parameters used like observation times, age or number of participants, or methods of cognitive testing.

Our studies on the proteolytic processing of APP revealed that *trans* FAs increase amyloidogenic processing and decrease the nonamyloidogenic pathway of APP processing.

Trans FAs act on both pathways via a pleiotropic mechanism (Fig. 8B). Nonamyloidogenic processing was decreased in the presence of *trans* FAs due to decreased expression of the α-secretase ADAM10 and resulting ADAM10 protein levels. However, our results indicate that α-secretase processing of APP is also influenced by altered APP protein transport to the plasma membrane. FACS analysis of cells incubated with *trans* FAs showed reduced levels of APP at the cell surface. In agreement with reduced APP levels on the cell surface, we detected increased intracellular APP. This finding can be explained by reduced transport of APP to the cell surface. Decreased APP transport to the plasma membrane results in a reduction in APP α-secretase processing because nonamyloidogenic processing of APP primarily takes place at the cell surface [60–62]. Reduced levels of APP at the cell surface and increased intracellular APP levels could therefore be the result of rapid proteolytic processing of APP at the cell surface once APP has reached the plasma membrane. In contrast to the nonamyloidogenic APP processing, the amyloidogenic pathway involving β- and γ-secretases is elevated in the presence of *trans* FAs. β- and γ-secretase processing is elevated by increased expression of BACE1 and the components of the γ-secretase complex (PS1, PS2,

nicastrin, APH1a and PEN2), which could be confirmed with the detection of increased BACE1 and nicastrin protein levels in cells incubated with *trans* FAs. Interestingly it has been reported that an elevated APP processing results in an increased APP and BACE expression [63]. In line with these findings, we observed increased APP, BACE and γ-secretase expression in the presence of *trans* FAs. This might therefore be a consequence of the direct effect of *trans* FAs on β- and γ-secretase activities as shown for purified membranes of SH-SY5Y wt cells and purified membranes of mice brains. Both mechanisms increased expression of β- and γ-secretase as well as a direct effect of *trans* FAs on the catalytic activity of these secretases, increase Aβ generation. SH-SY5Y cells, either stably expressing APP695 or C99 showed an elevated secreted Aβ level. Increased β-secretase processing of APP should result in elevated levels of β-CTF. The analysis of β-CTF revealed decreased levels of β-CTF in the presence of *trans* FAs. This, on first sight, contradictory result can be explained by the fact that the generated β-cleaved C-terminal fragments are rapidly degraded by γ-secretase, which itself is elevated in the presence of *trans* FAs. As described for α-secretase processing, APP sorting is affected by *trans* FAs, which might contribute to elevated amyloidogenic processing of APP. Compared to α-secretase activity, β- and γ-secretase activities have been published to be also present in intracellular compartments, e.g., the endoplasmic reticulum and the Golgi apparatus [64–67]. Reduced or retarded APP protein transport to the cell surface in the presence of *trans* FAs could lead to an increased intracellular amyloidogenic processing of APP, which is in line with elevated intracellular Aβ levels observed for *trans* FAs. Beside the clear fact that amyloidogenic processing is elevated in the presence of *trans* FAs, we analyzed if Aβ aggregation is also increased in the presence of *trans* FAs. Indeed, more Aβ aggregates could be detected in the presence of *trans* FAs, indicating that *trans* FAs not only influence amyloidogenic processing of APP but also elevate Aβ oligomerization and aggregation, which are essential for the formation of senile amyloid plaques. Although the observed effect strengths are moderate and in the range of 10% to 50%, they apparently act synergistically to increase the amount of Aβ. Beside our findings that *trans* FAs directly affect the amyloidogenic processing of APP and therefore increase Aβ production, it has been reported that *trans* FAs raise the total cholesterol to high-density lipoprotein ratio [68–72], whereas the brain docosahexaenoic acid levels are decreased [73]. Several studies underlined that increased cholesterol is one of the major AD risk factors, whilst DHA has been reported to be beneficial and protective [22,23,74–76]. In line with these findings, it has been proposed that diets with a high *trans* fat content might be associated with accelerated cognitive decline [59]. Especially considering life-long exposure, *trans* FAs might therefore increase the AD risk or cause an earlier disease onset.

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