



The interaction of beta-amyloid protein with cellular membranes stimulates its own production

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ABSTRACT

Gradual changes in steady-state levels of beta amyloid peptides (A β) in brain are considered an initial step in the amyloid cascade hypothesis of Alzheimer's disease. A β is a product of the secretase cleavage of amyloid precursor protein (APP). There is evidence that the membrane lipid environment may modulate secretase activity and alters its function. Cleavage of APP strongly depends on membrane properties. Since A β perturbs cell membrane fluidity, the cell membrane may be the location where the neurotoxic cascade of A β is initiated. Therefore, we tested effects of oligomeric A β on membrane fluidity of whole living cells, the impact of exogenous and cellular A β on the processing of APP and the role of GM-1 ganglioside. We present evidence that oligoA β _(1–40) stimulates the amyloidogenic processing of APP by reducing membrane fluidity and complexing with GM-1 ganglioside. This dynamic action of A β may start a vicious circle, where endogenous A β stimulates its own production. Based on our novel findings, we propose that oligoA β _(1–40) accelerates the proteolytic cleavage of APP by decreasing membrane fluidity.

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1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder. A neuropathological hallmark of AD is amyloid plaques [1], which contain beta-amyloid peptides (A β). Amphipathic A β tends towards self-aggregation and accumulation, which initiates a cascade that triggers complex pathological reactions eventually leading to neuronal dysfunction and cell death [2–4]. A β aggregation is a concentration-dependent phenomenon, which is initiated via a seeded polymerization reaction [5]. Monomeric A β initially forms poorly characterized nuclei that assemble into larger aggregates. The nucleation of A β is followed by oligomer and protofibril formation, which ultimately leads to insoluble amyloid fibril assembly [3]. Current research suggests that oligomeric forms of A β may play a major role in AD pathophysiology [3, 4]. Soluble amyloid oligomers (oligoA β) bind specifically to neurons, disrupt dendritic spines [6] and inhibit hippocampal long-term potentiation *in vivo* [7].

Most A β is composed of 38–43 amino acid residues, all deriving from the transmembrane amyloid precursor protein (APP) after the

sequential proteolytic cleavage by different secretases. There are two concurrent cleavage pathways. In the non-amyloidogenic pathway, APP is first cleaved at the N-terminus by α -secretase within the A β sequence and precludes the formation of A β . α -Secretase is a member of the ADAM (a disintegrin and metalloprotease) family [8]. Via their metalloprotease domain, ADAMs are often implicated in ectodomain shedding, either to release e.g. growth factors or to initiate further intracellular signaling via regulated intramembrane proteolysis. ADAMs 9, 10 and 17, have been proposed to act as α -secretases for APP [9]. Proteolytic cleavage of APP by ADAM-enzymes produces a 105–125 kDa soluble N-terminal APP fragment (sAPP α) and an 83 residue membrane-associated C-terminal fragment (C83). The secreted sAPP α exert anti-apoptotic and neuroprotective effects [10]. Alternatively, A β is produced primarily in the trans-Golgi network and recycling compartments [11–14]. This amyloidogenic pathway involves first APP cleavage at the N-terminus by β -secretase to produce sAPP β and a 99-residue membrane associated C-terminal fragment C99. β -Secretase is a membrane-anchored aspartyl protease with its active site in its ecto-domain and it was shown to be a member of the memapsin family. Subsequent cleavage of C99 by γ -secretase produces Alzheimer's amyloid β -protein precursor (APP) generate intracellular domain fragment (AICD) along with varying lengths of extracellular A β , the most common being A β _(1–40) and A β _(1–42). This model of A β

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formation is now widely accepted. However, the presence and exact contribution of both intracellular and extra-cellular A β is still an issue of great controversy and interest in the field [15].

APP processing by α -, β -, and γ -secretase, which are strictly associated with cellular membranes, strongly depends on membrane fluidity. Modulation of membrane fluidity for example affects the accessibility of secretases acting on APP [16]. Kojro et al. showed that α -secretase activity increased with enhanced membrane fluidity [17]. Decreased membrane fluidity augments γ -secretase activity [18]. Moreover, processing of APP by β -secretase also might be explained by alterations in cell membrane fluidity [19].

Membrane fluidity may contribute to the processing of APP and the cleavage product A β may in turn perturb the membrane environment. Studies have examined the effects of A β peptides on membrane fluidity in model membranes and biological membranes of mice, rats and humans (summarized in [20]). It is well recognized that changes in the physico-chemical state of the membrane can markedly alter activity of various membrane proteins, including α -, β -, and γ -secretase. The cell membrane represents the first site of interaction between A β and neurons, and may be the location where the neurotoxic cascade of A β is initiated. The mechanism by which A β affects membrane fluidity is not well-understood. Yanagisawa et al. discovered that A β binds to monosialoganglioside (GM-1 ganglioside) and induces the assembly of A β in the brain and in vitro [21]. GM-1 ganglioside reduces membrane fluidity of PC12 cells and alters the localization of receptors within the plasma membrane [43]. We examined the hypothesis that A β triggers the amyloidogenic processing of APP by decreasing membrane fluidity, which in turn stimulates its own production. A β perturbation of the membrane may involve complexing with GM-1 ganglioside.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all cell culture reagents were obtained from Gibco/Invitrogen. The inhibitors and substances applied were human A β _(1–40) and rodent A β _(1–42) (Bachem, Heidelberg, Germany), Pluronic F68 (Sigma-Aldrich, Taufkirchen, Germany), Benzyl alcohol (Fluka, Neu-Ulm, Germany), lovastatin (MSD Sharp and Dohme, Haar, Germany) and DAPT (Sigma-Aldrich, Taufkirchen, Germany). The Western blot reagents and Cholera Toxin Subunit B were obtained from Invitrogen (Karlsruhe, Germany); the PVDF membranes, ECL detection kit and hyper films were obtained from GE Healthcare (München, Germany). Trimethylammoniumdiphenylhexatrien (TMA-DPH), 1,1,1,3,3,3- and hexafluoro-2-propanol (HFIP) were obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.1.1. Antibodies

Full length APP and C-terminal fragments (CTF) were detected using mouse IgG C1/6.1 (kindly provided by Dr. Paul M. Mathews, Nathan Kline Institute, Orangeburg, NY, USA) which was raised against the conserved carboxyl-terminal 20 residues of APP (residues 676–695 of APP₆₉₅) [22]. The secreted sAPP α was detected using monoclonal mouse IgG 6E10 (Signet Laboratories, Cat.9320-02), which recognizes the residues 1–17 of A β . Mouse anti-GAPDH was obtained from Chemicon, Hofheim, Germany (Cat.MAB374). Anti-mouse HRP-conjugated secondary antibody was purchased from Calbiochem (Bad-Soden, Germany; (CAT.401253)).

2.2. Cell culture

HEK293-cells and neuroblastoma SH-SY5Y-cells stable transfected with human APP695 were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C and 5% CO₂. The HEK-medium was supplemented with 10% FCS and penicillin/streptomycin. Geneticin

(G418) was added at 3 μ g/ml as a selective antibiotic. For incubation with lovastatin, cells were grown in DMEM medium supplemented with 2% v/v Ultrosor G instead of FCS. Ultrosor G is free of cholesterol, requiring the cells to rely upon their own de-novo synthesis. Cells were treated for 24 h with 1, 2 or 4 μ M lovastatin. Untransfected HEK293-cells were cultured in the same medium without G418. Antibiotic treatments per se had no impact on the effects reported (data not shown). Medium for SH-SY5Y-APP695 cells was supplemented with 1% of glutamine, MEM-vitamins, Hygromycin B (3 μ g/ml), pyruvate and non-essential amino-acids plus 10% FCS. For lovastatin treatment, cells were maintained in serum-free OptiMEM medium supplemented with Hygromycin B. The cells were treated for 24 h with lovastatin (1, 2, or 4 μ M), Pluronic F68 (4.5 or 7.5 μ M) and benzyl alcohol (5 or 10 mM). DAPT [1 μ M] was added to the culture medium for at least 10 passages.

2.3. Preparation of A β _(1–40) peptides

The preparation of monomeric, oligomeric and fibrillar A β _(1–40) was performed as previously reported [23,24]. Briefly, 1 mg of A β _(1–40) was dissolved in 200 μ l 1,1,1,3,3,3-hexafluoro-2-propanol and the peptide solution was evaporated using a speed vacuum for 45 min.

2.3.1. Preparation of monomeric A β _(1–40) peptides

The dried film was re-suspended in 2 μ l DMSO and diluted in 98 μ l DMEM medium to achieve a working solution of 100 μ M. The solution was vortexed for 30 s and immediately used for the experiments.

2.3.2. Preparation of oligomeric A β _(1–40) peptides

The dried film was re-suspended in 2 μ l DMSO and diluted in 98 μ l DMEM medium to achieve a working solution of 100 μ M. The solution was vortexed for 30 s and incubated at 4 °C for 24 h.

2.3.3. Preparation of fibrillar A β _(1–40) peptides

The dried film was re-suspended in 2 μ l DMSO and diluted in 98 μ l HCl 0,01 N to achieve a working solution of 100 μ M. The solution was vortexed for 30 s and incubated at 37 °C for 24 h. A β _(1–40) peptides were characterized using native gel electrophoresis, followed by silver staining and electron microscopy as described below.

2.4. Characterization of A β _(1–40) peptides

Blue native SDS-PAGE gel electrophoresis and silver staining was performed according procedures previously published [25]. Prepared A β _(1–40) samples (see above) were mixed with 6 \times Laemmli buffer in a sample/buffer ratio of 5:1 (Boston bioproduct, Cat # ADP-111R) and placed in wells of 13% polyacrylamide gel (0.75 mm). The gel was run in a 1 \times trisglycerine-SDS buffer from Bio-Rad at a constant voltage (90 mV for 10 min then at 180 mV for another 60 min). Finally, the gel was silver stained (Silver Stain Plus Kit from Bio-Rad (cat # 161-0449) according to the manufacturer's instructions.

Transmission electron microscopy was used for visualization of A β peptides as previously described [26]. Aliquots of A β _(1–40) samples (10 μ l) were pipetted on to the surface of coated copper microscope grids. The grids were air-dried and the samples were then stained with 1% uranyl acetate. Grids were examined in an electron microscope (EM 109, Zeiss, Oberkochen, Germany).

2.5. Western blot analysis

Total protein levels were determined by the Lowry method. The samples were prepared by diluting 20 μ g protein with the reducing agent (10 \times) and loading buffer (4 \times). After denaturation for 10 min at 90 °C, the samples were electrophoresed on a 4–12% NuPage Bis/Tris gel for 40 min at 200V and then transferred on a PVDF membrane for 90 min at 30 V. For the detection of secreted sAPP α , the conditioned medium was collected and the values were normalized to the cell

lysate protein concentration. The membranes were blocked overnight and incubated with the primary antibody for 1 h. After washing, the blot was hybridized with an HRP-conjugated secondary antibody for 30 min. Visualization was done using an ECL detection kit from GE Healthcare. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. Band analysis was performed using BioRad's Quantity One software.

2.6. ELISA detection of $A\beta_{(1-42)}$ and sAPP α

The $A\beta_{(1-42)}$ levels were determined using ELISA-kits from Biosource, Solingen, Germany (Cat.KHB3482 and KHB3544) according to the manufacturer's instructions. Briefly, the conditioned medium was collected and supplemented with PMSF. The cells were collected and washed twice with $1\times$ PBS. After centrifugation ($22,000\times$ rpm, 2 h, 4°C), the cells were resuspended in guanidine hydrochloride (HCL, 5M) and incubated for 4 h. The suspension was diluted and centrifuged at $16,000\times$ g for 20 min. The supernatant was used for the ELISA.

In those experiments where oligo $A\beta_{(1-40)}$ was added to cells, cellular $A\beta_{(1-40)}$ levels could not be determined in parallel, since the antibody used in the ELISA-kit for $A\beta_{(1-40)}$ detects both, cellular endogenous and added exogenous $A\beta_{(1-40)}$ (data not shown).

For the quantitative analysis of sAPP α , wild type APP over-expressing HEK and control cells were plated at equal density in 24 well plates. Conditioned, serum-free media were collected after 24 h and supernatants were centrifuged at $200\times$ g for 4 min to eliminate cell debris. Soluble sAPP α was quantified using the IBL human sAPP α colorimetric sandwich ELISA Kit (IBL Immuno-Biological Laboratories, Hamburg, Germany) according to the supplier's instructions.

2.7. Determination of membrane fluidity in living cells

The membrane fluidity of living cells was determined as earlier described [28]. The fluorescence polarization probe TMA-DPH incorporates very rapidly into plasma membranes of cells, and is specifically localized on the cell surface making its use particularly appropriate for determining plasma membrane fluidity in living cells. Cells were washed twice with warm HBSS and supplemented with $2\mu\text{M}$ TMA-DPH. 700,000 cells per vial were incubated at 37°C for 20 min. The membrane fluorescence polarization was determined using a SLM Aminco Bowman Series 2 luminescence spectrometer. The cuvette temperature was 37°C . The steady-state fluorescence polarization (P_s) was expressed as the anisotropy (r_s) of the probe, using the following equation: $r_s = 2P_s/3 - P_s$. The r_s is inversely correlated to the membrane fluidity, particularly to the acyl-chain flexibility of phospholipids.

2.8. Fluorescence and laser scanning fluorescence microscopy

$A\beta$ was stained using Fluoro- $A\beta_{(1-40)}$ conjugated with Fluorescein (Advanced Bioconcept, Cat. AB2012). GM-1 ganglioside was visualized using fluorescent labeled cholera toxin subunit B. Briefly, 100,000 cells were plated overnight on cover slips coated with polylysine. Next, the medium was supplemented with $2\mu\text{M}$ oligo $A\beta_{(1-40)}$ containing 25 nM of Fluoro- $A\beta_{(1-40)}$ for 24 h. After incubation, $2\mu\text{l}$ of cholera toxin subunit B conjugated with Alexa Fluor 555 were added to the culture medium, which was then incubated for 10 min. The cells were washed three times with PBS and incubated with 1.990 ml medium plus $10\mu\text{l}$ of the anti-cholera toxin antibody (Calbiochem, Darmstadt, Germany; Cat. 227040) for 15 min. Again, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min at 4°C . Cholesterol was stained in fixed cells using filipin ($50\mu\text{g}/\text{ml}$ in PBS) (Sigma-Aldrich, Taufkirchen, Germany; Cat. F9765).

For fluorescence microscopy, Gel Mount was used to fix the object slide. The fluorescent images were acquired on a Nikon ECLIPSE E800 microscope equipped with a Nikon DXM1200C digital camera

connected to a PC. The images were evaluated using the Nikon NIS-Elements Imaging software package.

For confocal laser-scanning fluorescence microscopy, the samples were embedded in Mowiol and analyzed using a confocal laser-scanning microscope (Leica TCS SP5, Wetzlar, Germany), equipped with a 63×1.4 oil-immersion objective. The images were evaluated using the Imaris Imaging and Imaris Coloc Software (Version 6.2.0).

2.9. Determination of unesterified cholesterol

Unesterified cholesterol levels were determined using the CHOD-PAP method (Cholesteroloxidase-Peroxidase-Aminophenazon-Phenol) developed in our lab as reported earlier [29]. Cells were centrifuged

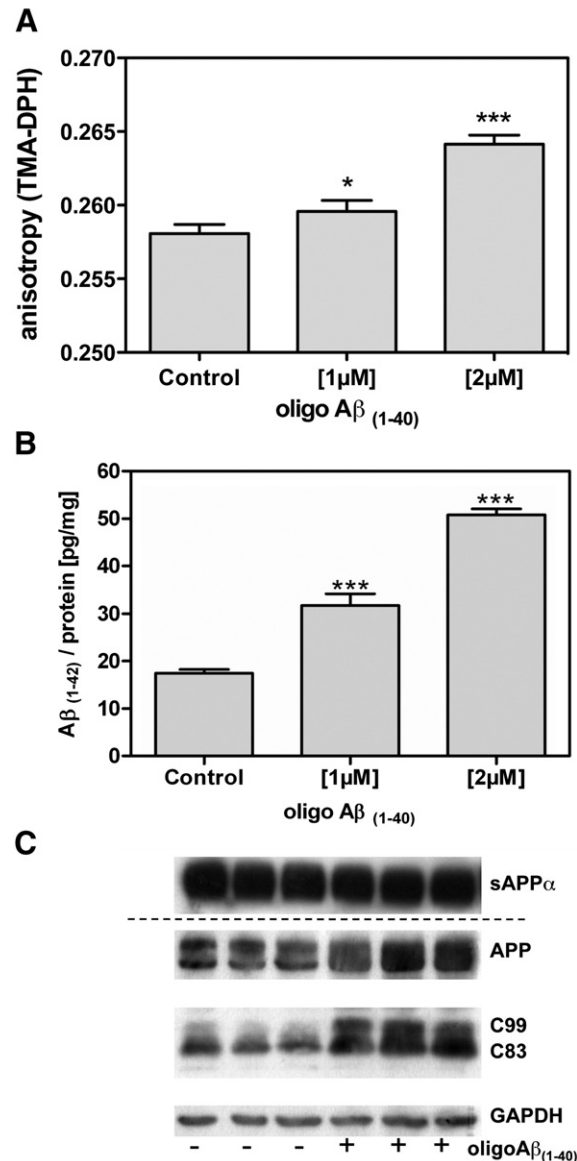


Fig. 1. Effects of oligo $A\beta_{(1-40)}$ on membrane properties and the processing of APP; HEK293-APP695 cells were incubated for 24 h in the presence or absence of $1\mu\text{M}$ oligo $A\beta_{(1-40)}$; (A) Changes in the membrane fluidity, which is inversely correlated to the anisotropy of the fluorescence probe TMA-DPH, were determined using fluorescence polarization spectroscopy in living cells; (B) Levels of endogenous $A\beta_{(1-42)}$ were determined by ELISA [pg/mg protein]; (C) Representative Western blot analysis of full-length APP and its fragments: The supernatants and cells were collected after incubation; proteins were separated by gel electrophoresis and transferred to PVDF membranes; secreted sAPP α was detected in cell supernatants using antibody 6E10; for APP and CTF analyses, cell homogenates were probed with the antibody C1/6.1; GAPDH served as loading control; Data are means \pm SD $n = 6-9$ (* $p < 0.05$; *** $p < 0.001$).

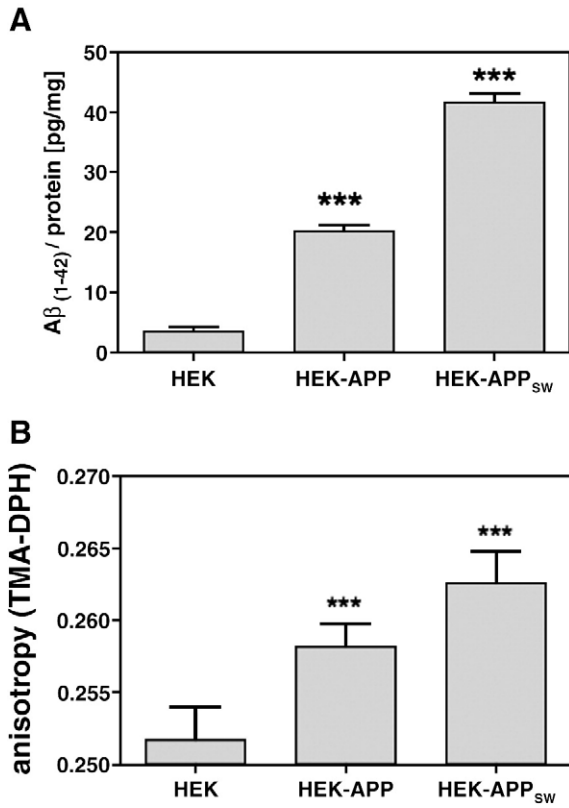


Fig. 2. Effects of endogenous A β levels on membrane fluidity; (A) A β levels were determined by ELISA [pg/mg protein] in HEK293 cells, either transfected with human APP695 (HEK-APP) or human APP695 harboring the Swedish mutation (HEK-APP_{sw}) and in control cells (HEK); (B) Changes in the membrane fluidity, which is inversely correlated to the anisotropy of the fluorescence probe TMA-DPH, were determined using fluorescence polarization spectroscopy in living cells; Data are means \pm SD; $n = 6-9$ (***) $p < 0.001$.

and washed twice with $1 \times$ PBS. The pellets were re-suspended in PBS with a protease-inhibitor cocktail (Roche) and frozen at -80°C . Before use, the cells were homogenized using a Dounce homogenizer fitted with a Teflon pestle and a Brandson sonicator.

2.10. Cytotoxicity

The cytotoxicity of A β , benzyl alcohol, pluronic F68 and lovastatin used in the current study was determined using the MTT assay as previously reported [30]. No cytotoxicity was observed for the concentrations of inhibitors and substances used in the current study (data not shown).

2.11. Statistics

All experiments were done at least in three independent experiments and all assays were done in triplicates. Statistical analyses were performed using one-way ANOVA followed by a Tukey comparison test. All data were expressed as the means \pm SD. The correlations were calculated using the Pearson using GraphPad Prism 4.0 software package (San Diego, USA).

3. Results

3.1. Oligomeric A $\beta_{(1-40)}$ reduces membrane fluidity and enhances β -secretase cleavage of APP

A β interacts with neuronal membranes and decreases fluidity [20]. We tested if A β induced changes in membrane fluidity alters processing

of APP in living cells. Cells stably transfected with human APP695 were incubated with oligomeric A $\beta_{(1-40)}$ (oligoA $\beta_{(1-40)}$). Then fluorescence polarization of TMA-DPH, A $\beta_{(1-42)}$ levels, C-terminal fragments of APP (CTF) and sAPP α levels were determined. Transfecting the APP gene induces an overproduction of the protein, and APP overproduction increases A β peptide secretion [31]. In the following experiments non-neuronal human embryonic kidney cells stably transfected with human APP695 (HEK293-APP695) were used, which provides expression levels that are sufficient for adequate assessment of APP cleavage products, such as sAPP α (Supplementary Fig. 3).

Treatment with oligoA $\beta_{(1-40)}$ significantly decreased plasma membrane fluidity of living cells (Fig. 1A) and increased levels of secreted cellular A $\beta_{(1-42)}$ (Fig. 1B). Western blot analysis revealed that the levels of C99, the β -secretase related C-terminal fragment (CTF), are significantly elevated by oligoA $\beta_{(1-40)}$ (Fig. 1C). α -Secretase

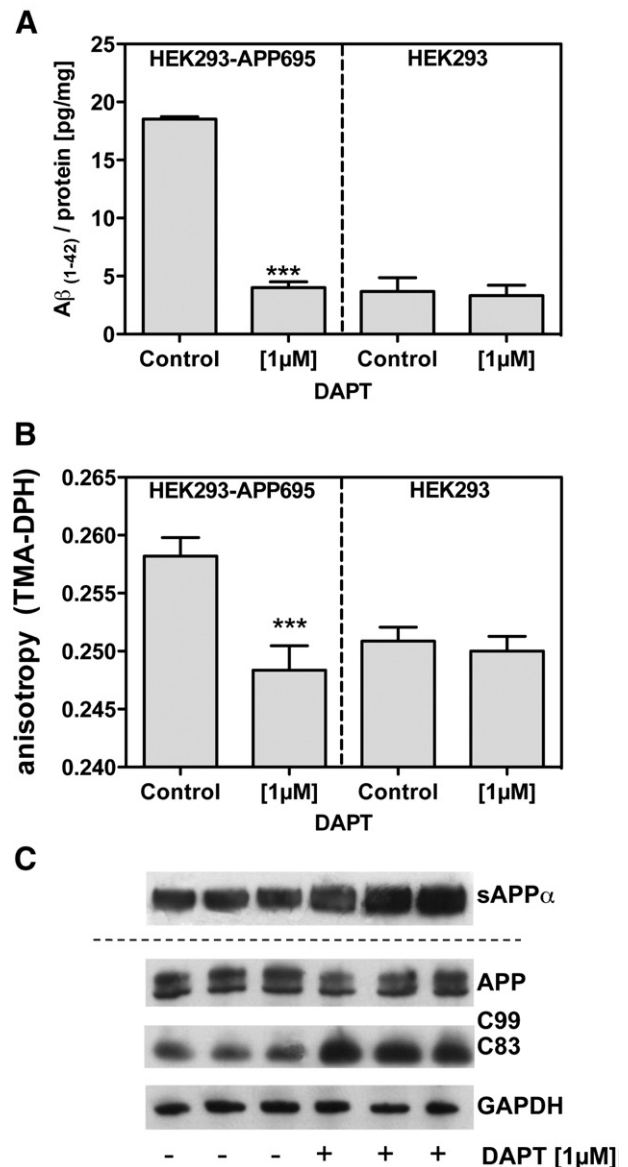


Fig. 3. Effects of the γ -secretase inhibitor DAPT on the A β production and membrane fluidity; HEK293-A β PP695 cells were treated with 1 μM DAPT for at least 10 passages; (A) Changes of cellular A $\beta_{(1-42)}$ -levels were determined using ELISA at the end of the incubation period [pg/mg protein]; (B) Changes of the membrane fluidity in living cells were measured using TMA-DPH anisotropy as described in the Materials and methods; (C) APP processing fragments were analyzed by Western blot analysis (please refer to Fig. 1). Data are means \pm SD; $n = 6-9$ (***) $p < 0.001$.

cleaved CTF C83 and secreted levels of sAPP α (Fig. 1C) remained unchanged.

To assess the influence of peptide aggregation on the membrane fluidity of living cells and the production of A $\beta_{(1-42)}$, we compared the effects of monomeric, oligomeric and fibrillar A $\beta_{(1-40)}$, which were characterized by native gel electrophoresis and electron microscopy (Supplementary Fig. 1). Monomeric A $\beta_{(1-40)}$ has no effect on membrane fluidity and A $\beta_{(1-42)}$ production (Supplementary Fig. 2). Effects of oligoA $\beta_{(1-40)}$ on membrane fluidity and on production of A $\beta_{(1-42)}$ were much more pronounced compared to fibrillar A $\beta_{(1-40)}$ (Supplementary Fig. 2).

3.2. Cellular A β levels determine membrane fluidity of living cells

We next investigated, if endogenous A β produced by cells harboring the human APP695 protein was associated with membrane fluidity of living cells. A majority of the mutations in human APP that have been tested, when transfected in cellular models, induce an increase in A $\beta_{(1-40)}$ and A $\beta_{(1-42)}$ levels [31]. Therefore, we examined fluidity in cells, expressing human APP695 (HEK-APP) and human APP695 harboring the Swedish mutation (HEK-APP_{sw}). Expression of human APP is related to enhanced levels of A β (Fig. 2A). Cells expressing human APP harboring the Swedish mutation released

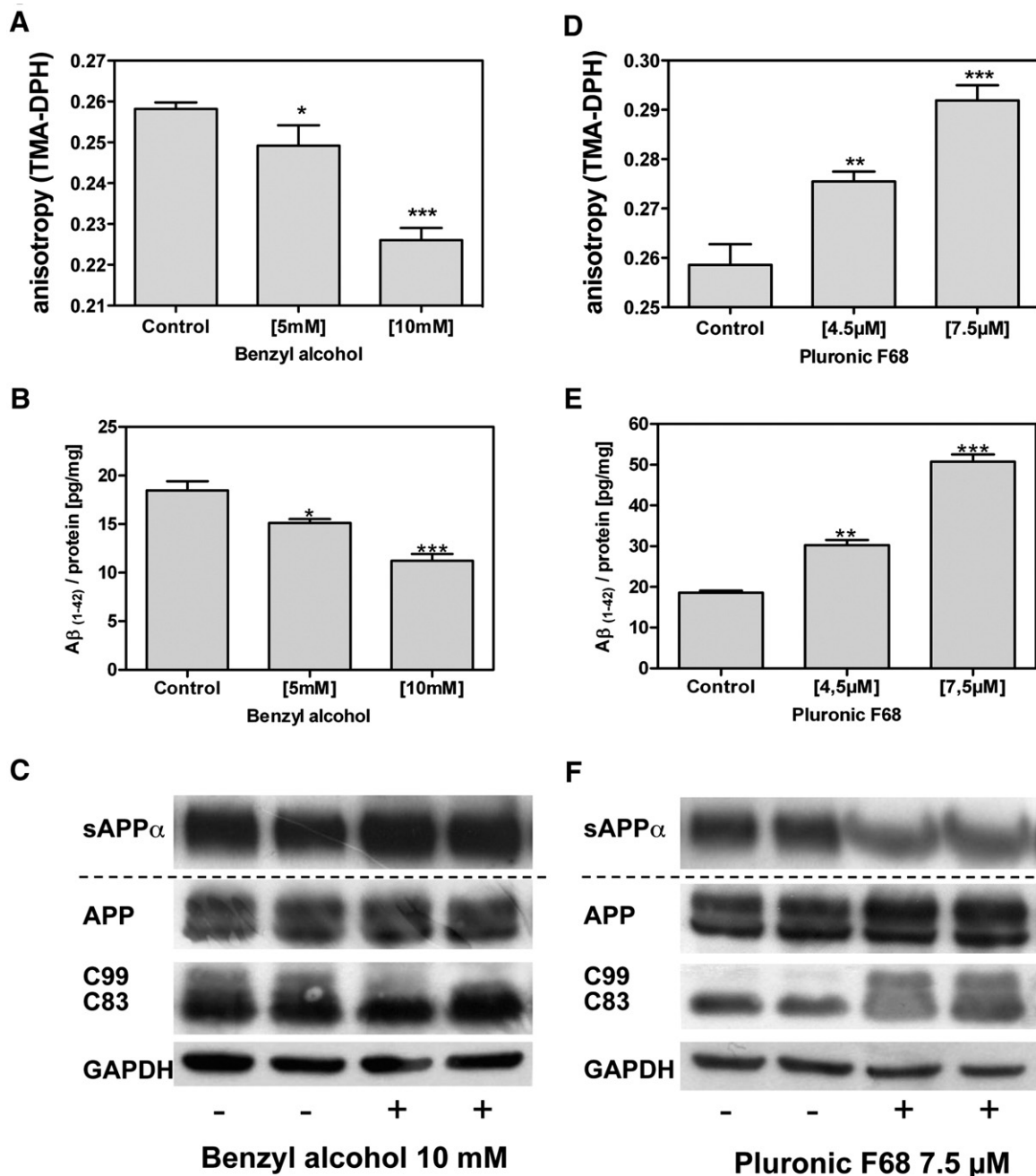


Fig. 4. Impact of membrane fluidity on the cleavage of APP; (A–C) HEK293-APP695 cells were treated with benzyl alcohol or (D–F) Pluronic F68; (B, E) The levels of endogenous A $\beta_{(1-42)}$ were determined using ELISA [pg/mg protein]; (A, D) Changes in the membrane fluidity were measured as described in the Materials and methods (C, F) APP cleavage products were analyzed by Western blot analysis; the secreted sAPP α was detected in the medium using the antibody 6E10; full-length APP and its C-terminal fragments in the cell homogenates were detected with the antibody C1/6.1; GAPDH served as loading control; Band analysis was performed using BioRad's Quantity One; Data are means \pm SD; $n = 6-12$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

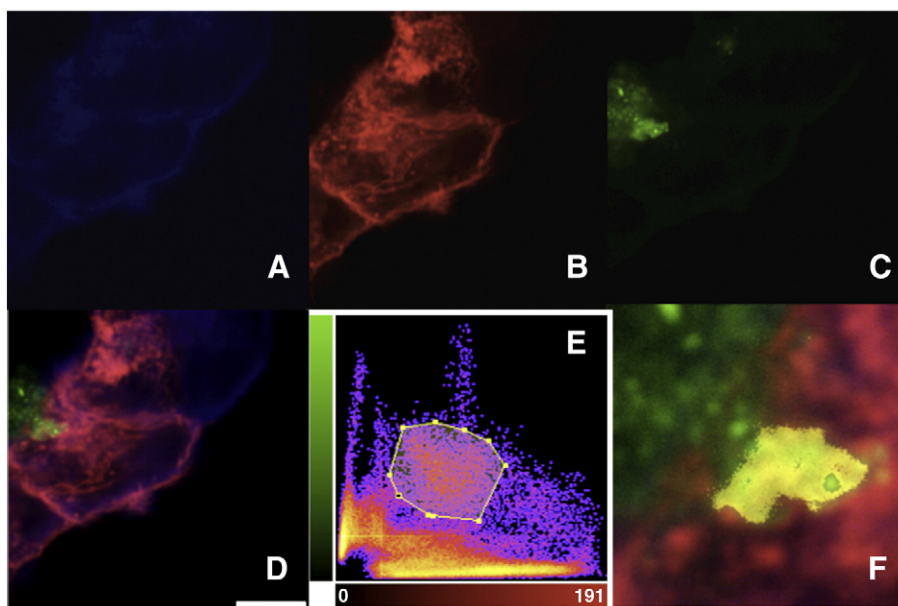


Fig. 5. Binding of A β to GM-1 ganglioside; confocal laser-scanning fluorescence microscopy was used to visualize the cellular localization of A β and GM-1 ganglioside. (A–D) Representative images of the HEK293–APP695 cells; Fluoro-oligoA $\beta_{(1-40)}$ was added to the medium and cells were stained for (A) cholesterol using filipin, (B) GM-1 ganglioside using Alexa 555-conjugated cholera toxin subunit B (CTX-Aexa555) and for (C) A β using Fluoro-oligoA $\beta_{(1-40)}$. (D) Merged pictures reveal that oligoA $\beta_{(1-40)}$ attaches to the cell membrane (Scale bar = 7.5 μ m). Co-localization of oligoA $\beta_{(1-40)}$ and GM-1 was described by the presence of the two fluorochromes at the same physical location. (E) The 2D-histogram displays the degree of overlap of the green (Fluor-oligoA $\beta_{(1-40)}$) and red (CTX-Alexa555) fluorescence and reflects the distribution of pairs of voxel intensities occurring in the two selected channels. The range of intensity pairs considered as co-localized was defined after definition of the region of interest on the 2D histogram, its (F) result is indicated as yellow color in the picture detail.

excessive amounts of A β (Fig. 2A). Enhanced levels of cellular A $\beta_{(1-42)}$ were related to reduced membrane fluidity (Fig. 2B), indicating that endogenous A β perturbed the plasma membrane.

Incubation of HEK–APP cells with the γ -secretase inhibitor DAPT abolished production of endogenous A $\beta_{(1-42)}$ (Fig. 3A) and normalized membrane fluidity in HEK293–APP695 cells (Fig. 3B). These results indicate that the observed changes in membrane fluidity were due to the endogenous production of A β .

3.3. Membrane fluidity determines the processing of APP

To confirm that oligoA $\beta_{(1-40)}$ stimulated β - and γ -secretase cleavage of APP by altering membrane fluidity (Fig. 1), we used compounds that increase (benzyl alcohol) or decrease membrane fluidity (Pluronic F68; PF68) [32,33]. Treatment with benzyl alcohol increased membrane fluidity (Fig. 4A) and reduced cellular A $\beta_{(1-42)}$ levels in HEK293–APP cells (Fig. 4B). Elevated sAPP α levels induced by benzyl alcohol were associated with reduced CTF C99 levels and elevated CTF C83 levels (Fig. 4C), indicating enhanced α -secretase cleavage of APP. Pluronic PF68 reduced membrane fluidity (Fig. 4D) and increased levels of endogenous A $\beta_{(1-42)}$ (Fig. 4E). Secretion of sAPP α in living HEK293–APP695 cells was reduced (Fig. 4F). CTF C99 levels were increased and CTF C83 were decreased after PF68 treatment, indicating enhanced β -secretase cleavage of APP (Fig. 4F).

We also used lovastatin, which increases membrane fluidity by lowering cholesterol levels, and has been shown to stimulate α -secretase-related processing of APP and reducing A β production in cells [17,34,35]. We confirmed that lovastatin reduced both cellular cholesterol and A $\beta_{(1-42)}$ levels and increased secreted levels of sAPP α and membrane fluidity. β -secretase-related CTF C99 was significantly reduced (data not shown). In contrast to lovastatin, benzyl alcohol and Pluronic F68 did not alter membrane cholesterol levels (data not shown), suggesting that the observed effects of membrane fluidity on APP processing were cholesterol independent.

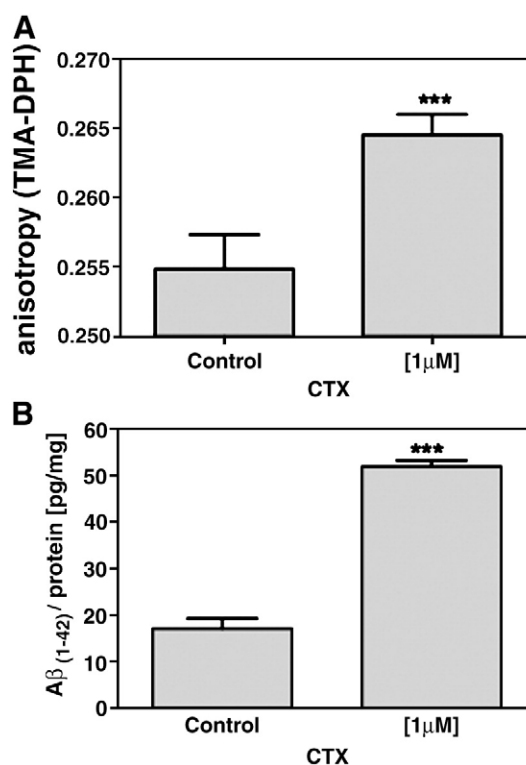


Fig. 6. Impact of GM-1 ganglioside on the production of cellular A β ; HEK293–APP695 cells were treated for 24 h with 1 μ M Cholera toxin subunit B (CTX), which specifically binds to GM-1 ganglioside. (A) Changes in the membrane fluidity after incubation with CTX were measured as described in the Materials and methods (B) The levels of cellular A $\beta_{(1-42)}$ after incubation CTX were determined using ELISA [pg/mg protein]; Data are means \pm SD; $n = 6$ –12 (***) $p < 0.001$.

3.4. OligoA $\beta_{(1-40)}$ binds to GM-1 ganglioside in living cells

A β binds to membrane-bound GM-1 ganglioside and it has been proposed that this binding acts as an endogenous seed for amyloid fibrillization [21]. To test whether the oligoA $\beta_{(1-40)}$ induced reduction of membrane fluidity in HEK293-APP695 cells (Fig. 1B) involves GM-1 ganglioside, we incubated cells with fluorescein-labeled oligoA $\beta_{(1-40)}$ (fluoro-oligoA $\beta_{(1-40)}$). The cells were also stained for GM-1 ganglioside using Alexa555-labelled cholera toxin subunit B and cholesterol using filipin (Fig. 5). Blue fluorescence of filipin indicated plasma membrane bound cholesterol (Fig. 5). Confocal laser scanning fluorescence microscopy revealed co-localization of Alexa555-labelled cholera toxin subunit B and fluoro-oligoA $\beta_{(1-40)}$, which was indicative of oligoA $\beta_{(1-40)}$ binding to GM-1 ganglioside (Fig. 5).

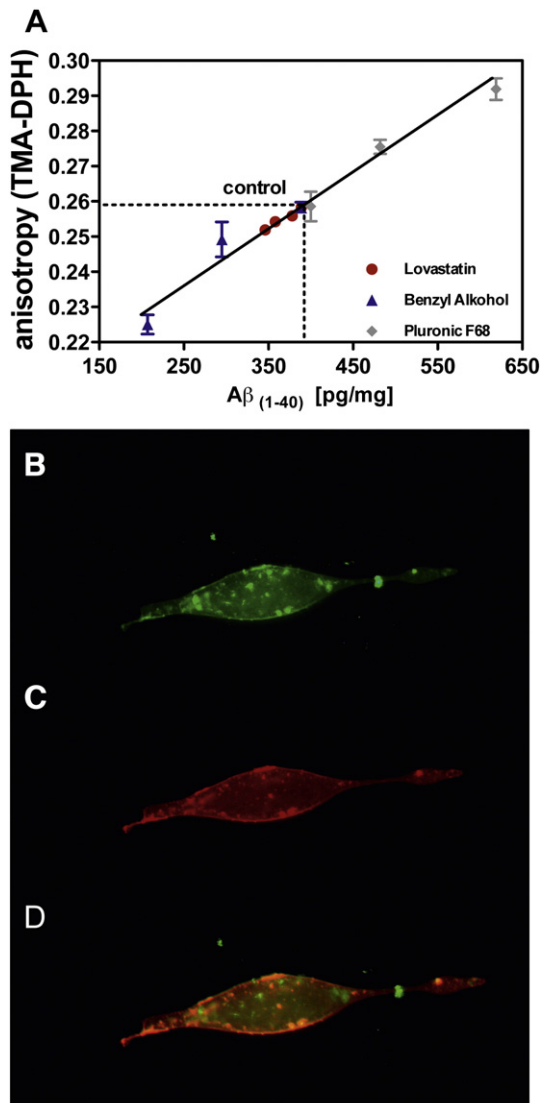


Fig. 7. Membrane fluidity and cleavage of APP in neuronal cells. SH-SY5Y-APP695 cells were treated as described in Materials and methods; (A) Membrane fluidity of living cells was correlated to endogenous A $\beta_{(1-40)}$ levels [pg/mg protein]; cells were treated for 24 h with lovastatin (1, 2 and 4 μ M), Benzyl alcohol (5 and 10 mM) and Pluronic F68 (4.5 and 7.5 μ M); (B–D) Representative fluorescence microscopy images of the SH-SY5Y-A β PP695 cells; (B) Fluoro-oligoA $\beta_{(1-40)}$ was added to the medium and cells were incubated for 24 h; (C) Cells were stained for GM-1 ganglioside using Alexa 555-conjugated cholera toxin subunit B; (D) Co-localization of Fluoro-oligoA $\beta_{(1-40)}$ and Alexa 555-conjugated cholera toxin sub-unit B. Data are means \pm SD; $n = 6-12$.

3.5. Cholera toxin reduces membrane fluidity and enhances cellular A β production

If GM-1 ganglioside contributes to the oligoA $\beta_{(1-40)}$ induced reduction of membrane fluidity and altered processing of APP, then binding of GM-1 ganglioside to other compounds should mimic effects of A β . HEK293-APP695 cells were incubated with cholera toxin subunit B (CTX), which specifically binds to its cell surface receptor GM-1 [36] and it was determined if CTX also reduces membrane fluidity and increases endogenous A β levels in cells. Binding of CTX to GM-1 ganglioside significantly reduced membrane fluidity and increased cellular A $\beta_{(1-42)}$ levels (Fig. 6A and B) findings, which were similar to effects of oligoA $\beta_{(1-40)}$ as described above. These findings suggest that GM-1 gangliosides play a role in A β effects on membrane fluidity and APP cleavage.

In order to investigate possible effects of A β on its own production, we studied the influence of oligoA $\beta_{(1-40)}$ on the release of cellular A $\beta_{(1-42)}$ in HEK293-APP695 cells. A $\beta_{(1-42)}$ could be determined by a specific and very sensitive ELISA. In order to prove that A β also regulates ectodomain shedding of APP in neuronal cells, we used the SH-SY5Y cell line.

Again, a very good correlation was found between membrane fluidity (modulated by Pluronic F68, benzyl alcohol and lovastatin) and cellular A $\beta_{(1-40)}$ production (Fig. 7A). OligoA $\beta_{(1-40)}$ modulation of A $\beta_{(1-42)}$ release could not be investigated, since levels of A $\beta_{(1-42)}$ were below the detection limit.

SH-SY5Y-APP695 cells were also incubated with fluoro-oligoA $\beta_{(1-40)}$ (Fig. 7B) and stained for GM-1 ganglioside using Alexa555-labelled cholera toxin subunit B (Fig. 7C). Co-localization of Alexa555-labeled cholera toxin subunit B and fluoro-oligoA $\beta_{(1-40)}$ is indicative of oligoA $\beta_{(1-40)}$ binding to GM-1 ganglioside (Fig. 7D), also in neuronal cells.

In summary, manipulation of the membrane fluidity using different compounds also modulates cellular A β levels and oligoA $\beta_{(1-40)}$ binds to GM-1 ganglioside in SH-SY5Y-APP695 cells, indicating that these data are confirmatory of the findings we obtained using non-neuronal cells.

4. Discussion

There is evidence that the membrane lipid environment modulates secretase activity and potentially affects the function and conformation of the enzymes, influencing substrate selection and the distribution of APP cleavage sites [37]. In the present study we showed that A β triggers the amyloidogenic processing of APP by decreasing membrane fluidity and stimulated its own production in living cells. The A β perturbation of the membrane was associated with A β complexing with GM-1 ganglioside. Our data showed that inhibition of γ -secretase inhibited A β production, increased membrane fluidity and stimulated α -secretase cleavage of APP in APP695 over-expressing cells underlines this notion. *In vivo* support for the potential importance of membrane fluidity in APP processing are reports that the polyunsaturated fatty acid docosahexaenoic acid enhanced synaptic membrane fluidity in aged mice [38] and decreased A β levels in brains of murine AD models [39, 40].

The A β -induced reduction in fluidity may involve GM-1 ganglioside. This idea is based on several different lines of evidence. The cell surface association of fluoro-oligoA $\beta_{(1-40)}$ with GM-1-ganglioside was observed in plasma membranes of intact cells, which confirms GM-1 ganglioside as a potential target molecule of A β in plasma membranes [21]. These results are in agreement with recent findings showing that GM-1 ganglioside induces A β assembly on the cell surface [41]. Binding of oligoA β to GM-1 ganglioside may induce changes in membrane fluidity, which in turn provides an energy favorable environment for β - and γ -secretase within the plasma membrane. GM-1 ganglioside regulates membrane structure, increases the order

of hydrocarbon chains and decreases fluidity in sphingolipid-enriched membranes [42]. GM-1 ganglioside reduces the membrane fluidity of PC12 cells and alters the localization of receptors within the plasma membrane [43]. Binding of cholera toxin protein to membrane-incorporated GM-1 ganglioside alters the long-range lateral diffusion of fluorescently labeled lipids [44]. Importantly, we demonstrate that binding of cholera toxin to GM-1 reduces membrane fluidity and enhances the production of cellular $A\beta_{(1-42)}$, which are similar to effects of oligo $A\beta_{(1-40)}$. Moreover, previous reports show that GM-1 ganglioside regulates APP cleavage [45–47]. These findings further support the notion that $A\beta$ affects the processing of APP in part by binding to GM-1. While $A\beta$ targeting of GM-1 ganglioside may reduce fluidity, it is worth noting that we have been reported previously that aggregated $A\beta$ has a high binding affinity for cholesterol as compared with phosphatidylcholine and saturated fatty acids [25]. It is well-established that membrane fluidity is reduced when cholesterol levels are increased and that APP processing is influenced by cholesterol abundance [17,34,35]. However, our findings with lovastatin indicate that membrane fluidity determines the processing of APP independent of cholesterol levels.

Sporadic AD is related to advancing age far more than any other risk factor and there is evidence that sporadic AD overlaps with normal aging in many clinical and pathologic features [27,48]. Changes in membrane properties are distinguishing markers of brain aging [49, 50]. In particular, membrane fluidity is less in synaptosomal plasma membranes isolated from brains of aged versus young mice [29]. Hippocampal membranes, isolated from brains of AD patients were significantly less fluid compared to membranes of age-matched controls [51]. Thus, age-related reduction of synaptosomal membrane fluidity may provide the optimal environment for β - and γ -secretase cleavage of APP.

Based on our novel findings, we propose that oligo $A\beta$ interacts with neuronal membranes by decreasing membrane fluidity and binding to GM-1 ganglioside, which accelerates the proteolytic cleavage of APP and starting a vicious circle in which endogenous $A\beta$ stimulates its own production. This process may be further enhanced by reduced membrane fluidity which occurs during brain aging.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmem.2009.01.012.

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