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## Effects of fatty acid unsaturation numbers on membrane fluidity and $\alpha$ -secretase-dependent amyloid precursor protein processing

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### Abstract

Fatty acids may integrate into cell membranes to change physical properties of cell membranes, and subsequently alter cell functions in an unsaturation number-dependent manner. To address the roles of fatty acid unsaturation numbers in cellular pathways of Alzheimer's disease (AD), we systematically investigated the effects of fatty acids on cell membrane fluidity and  $\alpha$ -secretase-cleaved soluble amyloid precursor protein (sAPP <sub>$\alpha$</sub> ) secretion in relations to unsaturation numbers using stearic acid (SA, 18:0), oleic acid (OA, 18:1), linoleic acid (LA, 18:2),  $\alpha$ -linolenic acid (ALA, 18:3), arachidonic acid (AA, 20:4), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6). Treatments of differentiated human neuroblastoma (SH-SY5Y cells) with AA, EPA and DHA for 24 h increased sAPP <sub>$\alpha$</sub>  secretion and membrane fluidity, whereas those treatments with SA, OA, LA and ALA did not. Treatments with AA and DHA did not alter the total expressions of amyloid precursor protein (APP) and  $\alpha$ -secretases in SH-SY5Y cells. These results suggested that not all unsaturated fatty acids but only those with 4 or more double bonds, such as AA, EPA and DHA, are able to increase membrane fluidity and lead to increase in sAPP <sub>$\alpha$</sub>  secretion. This study provides insights into dietary strategies for the prevention of AD.

### Keywords

Fatty acids; membrane fluidity; sAPP <sub>$\alpha$</sub> ; Alzheimer's disease

## 1. Introduction

The accumulation of neurotoxic amyloid- $\beta$  peptide (A $\beta$ ) is a pathologically profound characteristic of Alzheimer's disease (AD) (McGeer et al., 1987; Perlmutter et al., 1990; Frautschy et al., 1998; Dickson, 1999; Stalder et al., 1999; Selkoe, 2000). A $\beta$  is derived from amyloid precursor protein (APP) processing through the amyloidogenic pathway, in which APP is cleaved sequentially by  $\beta$ - and  $\gamma$ -secretases (Vassar, 2004). BACE1 is the

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major  $\beta$ -secretase for generation of  $A\beta$  by neurons (Cai et al., 2001). Alternatively, in the non-amyloidogenic pathway, APP is cleaved by  $\alpha$ -secretases between amino acids 16 and 17 within the  $A\beta$  domain to produce  $sAPP_{\alpha}$ .  $\alpha$ -secretases are members of ADAMs (a disintegrin and metalloprotease), including ADAM9, 10, 17 and 19.  $sAPP_{\alpha}$  is neurotrophic and neuroprotective (Thornton et al., 2006) and enhancing APP processing by  $\alpha$ -secretases has been suggested as a potential therapeutic strategy for AD (Cheng et al., 2007). Since APP,  $\alpha$ -,  $\beta$ - and  $\gamma$ -secretases are membrane protein molecules, APP processing should be governed by the local membrane environment. For example, the activity of  $\beta$ -secretase takes place preferentially in highly molecularly-ordered lipid rafts which are cholesterol, saturated phospholipids and sphingolipids-enriched microdomains (Tun et al., 2002; Cordy et al., 2003; Eehalt et al., 2003; Marlow et al., 2003; Kaether and Haass, 2004; Vetrivel et al., 2004), while the activity of  $\alpha$ -secretase is favorable in non-raft domains (Reid et al., 2007). Therefore, APP processing can be manipulated by changing the contents of membrane components, such as cholesterol and sphingolipids (Simons et al., 1998; Kojro et al., 2001; Sawamura et al., 2004; von Arnim et al., 2008).

Since fatty acids are capable of modulating membrane organization and functions (Yehuda et al., 2002; Stillwell et al., 2005; Shaikh and Edidin, 2006; Pepe, 2007; Shaikh and Edidin, 2008), we hypothesized that the effects of fatty acids (e.g. AA) on membrane fluidity and  $sAPP_{\alpha}$  secretion are dependent on their unsaturation numbers (i.e. the number of double bonds in the hydrocarbon chains). In this study, we systematically examined the effects of fatty acids with unsaturation numbers ranging from 0 to 6 double bonds including stearic acid (SA, 18:0), oleic acid (OA, 18:1), linoleic acid (LA, 18:2),  $\alpha$ -linolenic acid (ALA, 18:3), arachidonic acid (AA, 20:4), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) on membrane fluidity and  $sAPP_{\alpha}$  secretion in differentiated SH-SY5Y cells. Since these fatty acids are ingredients in daily food, information derived from this study should provide potential dietary strategy for prevention of AD.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1) and fetal bovine serum (FBS) and 5-hexadecanoylamino fluorescein were from Invitrogen (Carlsbad, CA). Stearic acid (SA, 18:0), oleic acid (OA, 18:1), linoleic acid (LA, 18:2),  $\alpha$ -linolenic acid (ALA, 18:3), arachidonic acid (AA, 20:4), eicosapentaenoic acid (EPA, 20:5), docosahexaenoic acid (DHA, 22:6), *cis*-parinaric acid, Albumin from bovine serum (BSA), phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO) and all-*trans* retinoic acid (RA) were from Sigma-Aldrich (St. Louis, MO). *Cis*-2-eicosenoic acid and *cis*-5, 8, 11-eicosatrienoic acid were from Cayman (Ann Arbor, MI). Farnesyl-(2-carboxy-2-cyanovinyl)-julolidine (FCVJ) was from Dr. Haidekker's Laboratory (University of Georgia) (Nipper et al., 2008).

### 2.2. Cell culture

Human neuroblastoma SH-SY5Y cells ( $1.0 \times 10^6$  cells/dish) were seeded into 60 mm dishes and were cultured in DMEM/F12 medium (1:1) containing 10% FBS. For differentiation, SH-SY5Y cells were exposed to 10  $\mu$ M RA for 6 days. Culture medium was replaced by fresh culture medium every other day. Treatments of cells with different fatty acids including SA, OA, LA, ALA, AA, EPA, DHA, *cis*-2-eicosenoic acid and *cis*-5, 8, 11-eicosatrienoic acid were in DMEM/F12 medium (1:1) containing 1% BSA for 24 h. All cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified incubator.

### 2.3. Fluorescent fatty acids labeled differentiated SH-SY5Y cells

Cells were incubated with 1 $\mu$ M 5-hexadecanoylamino fluorescein or *cis*-parinaric acid for 40 min and excess fluorescent fatty acids were then removed by washing cells with PBS three times. Fluorescent images were obtained at room temperature using a Nikon TE-2000 U fluorescence microscope with an oil immersion 60 $\times$  objective lens. Images were acquired using a CCD camera controlled by a computer running a MetaVue imaging software (Universal Imaging, PA).

### 2.4. Characterization of membrane fluidity by fluorescence microscopy of FCVJ-labeled cells

A fluorescent molecular rotor, farnesyl-(2-carboxy-2-cyanovinyl)-julolidine (FCVJ), was used to measure the relative membrane fluidity in differentiated SH-SY5Y cells. FCVJ was designed to be a more membrane-compatible fluorescent molecular rotor (Haidekker et al., 2001) with the quantum yield strongly dependent on the local free volume. A higher fluorescent intensity of FCVJ reflects the intramolecular-rotational motions being restricted by a smaller local free volume, indicating a more viscous membrane. The hydrocarbon chain of FCVJ has longer to help improve plasma membrane localization and reduce migration into the inner compartments of the cell, cytosolic staining and background fluorescence (Haidekker et al., 2001). Previously, we have verified the application of FCVJ for measuring membrane viscosity by comparing the results obtained using FCVJ with those from the technique of fluorescence recovery after photobleaching (FRAP) (Nipper et al., 2008). In this study, we adapted the protocol from Haidekker et al. (2001) to fluorescently label cells with FCVJ. Briefly, after undergoing different treatments, e.g. SA, OA, LA, ALA, AA, EPA and DHA, SH-SY5Y cells were washed with PBS and incubated in DMEM containing 20% FBS and 1 $\mu$ M FCVJ for 20 minutes. Excess FCVJ was removed by washing cells with PBS three times. Fluorescent intensity measurements were performed at room temperature using a Nikon TE-2000 U fluorescence microscope with an oil immersion 60 $\times$  objective lens. Images were acquired using a CCD camera controlled by a computer running a MetaVue imaging software (Universal Imaging, PA). The fluorescent intensities of FCVJ per cell were measured. Background subtraction was done for all images prior to data analysis.

### 2.5. Western blot analysis of sAPP $\alpha$ released from differentiated SH-SY5Y cells

After different treatments, e.g. SA, OA, LA, ALA, AA, EPA and DHA, culture medium was collected and the same volume of the cell lysate from each sample was used for Western blot analysis using  $\beta$ -actin as internal standard. Medium was centrifuged at 12,000  $\times$  g for 5 min to remove cell debris, and the same volume of medium from each sample (e.g., 40  $\mu$ l) was diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and were incubated overnight at 4 $^{\circ}$ C in 3% (w/v) bovine serum albumin (BSA) with 0.02% (w/v) sodium azide in TBST with a 6E10 monoclonal antibody (1:1000 dilution; Covance, Princeton, NJ) that recognizes residues 1–16 of the A $\beta$  domain of sAPP $\alpha$ . Membranes were washed three times during a 15-min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce (Rockford, IL) to visualize bands. The protein bands detected on x-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad).

## 2.6. Western blot analysis of APP, ADAM9, ADAM10, ADAM17, ADAM19 and BACE1 in differentiated SH-SY5Y cells

After treatments, the protein concentration of the cell lysate was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL) according to manufacture's instruction. Equivalent amounts of protein from each sample (e.g., 30  $\mu$ g) was diluted with Laemmli buffer, boiled for 5 min, subjected to electrophoresis in 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST) and were incubated overnight at 4°C in 3% (w/v) BSA with 0.02% (w/v) sodium azide in TBST with 6E10 monoclonal antibody, anti-ADAM9 antibody or anti-ADAM19 antibody (1:1000 dilution; Abcam, Cambridge, MA), anti-ADAM10 antibody (1:1000 dilution; Millipore, Billerica, MA) or anti-ADAM17 antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-BACE1 antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO). Membranes were washed three times during a 15-min period with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% (w/v) nonfat dry milk in TBST at room temperature for 1 h. After washing with TBST for three times, the membrane was subjected to SuperSignal West Pico Chemiluminescent detection reagents from Pierce (Rockford, IL) to visualize bands. The protein bands detected on x-ray film were quantified using a computer-driven scanner and Quantity One software (Bio-Rad).

## 2.7. Quantification of A $\beta$ <sub>1-42</sub>

After treatments, culture medium and cell lysates were collected, supplemented with protease inhibitor cocktail and centrifuged at 12,000  $\times$  g for 5 min at 4°C to remove cell debris. An aliquot (100  $\mu$ l) of supernatant was used for A $\beta$ <sub>1-42</sub> quantification using an ELISA kit (Invitrogen, Carlsbad, CA) following manufacturer's recommendation. According to the instruction manual, substances including A $\beta$ <sub>1-12</sub>, A $\beta$ <sub>1-20</sub>, A $\beta$ <sub>12-28</sub>, A $\beta$ <sub>22-35</sub>, A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>1-43</sub>, A $\beta$ <sub>42-1</sub> and APP have no cross-reactivity. The minimum detectable dose of A $\beta$ <sub>1-42</sub> is <1.0 pg/ml which is similar to a previous study (Prasanthi et al., 2009). The level of A $\beta$ <sub>1-42</sub> in each sample was measured in duplicates and expressed in pg/ml.

## 2.8. Statistical analysis

Data are presented as mean  $\pm$  SD from at least three independent experiments. There are three trials in each experiment. Comparison between two groups was made with student's *t* test. Comparisons of more than two groups were made with one-way ANOVA, followed by *Bonferroni's* post hoc tests. Values of *p* < 0.05 are considered to be statistically significant.

# 3. Results

## 3.1. Exogenous fatty acids integrated into cellular membranes

In order to study the effects of fatty acids with different saturations on cellular membrane fluidity and sAPP $_{\alpha}$  secretion, we first confirmed if fatty acids were able to integrate into membranes of SH-SY5Y cells. Therefore, we incubated differentiated SH-SY5Y cells for 40 min with 5-hexadecanoylamino fluorescein and *cis*-parinaric acid. Both 5-hexadecanoylamino fluorescein and *cis*-parinaric acid are fluorescent fatty acids representing saturated and unsaturated fatty acids, respectively. Fig. 1A and Fig. 1B showed that 5-hexadecanoylamino fluorescein and *cis*-parinaric acid were incorporated into cellular membranes of SH-SY5Y cells, suggesting that exogenous fatty acids used in this study are capable of integrating into cellular membranes in SH-SY5Y cells.

### 3.2. Fatty acids with 3 or less double bonds did not alter membrane fluidity and sAPP $_{\alpha}$ secretion

To study the effects of fatty acids on membrane fluidity, we applied a fluorescent molecular rotor, FCVJ. As explained in the Materials and Methods section, FCVJ integrated into a highly fluidized membrane exhibits a lower quantum yield, as reflected by a lower fluorescent intensity. The application of this technique for the measurement of membrane fluidity has been validated (Nipper et al., 2008; Yang et al., 2010). FCVJ-labeled differentiated SH-SY5Y cells were exposed to SA, OA, LA and ALA for 24 h. Interestingly, cells did not exhibit a significant change in membrane fluidity as indicated by the unchanged fluorescent intensity of FCVJ as compared to control (Fig. 2A, B, C, D, *right*). These results suggest that SA, OA, LA and ALA do not alter membrane fluidity in SH-SY5Y cells.

To study the effects of SA, OA, LA and ALA on sAPP $_{\alpha}$  secretion, cell culture medium was subjected to western blot analysis of sAPP $_{\alpha}$  after the treatments of cells with SA, OA, LA and ALA. SA, OA, LA and ALA did not alter sAPP $_{\alpha}$  secretion in SH-SY5Y cells (Fig. 2A, B, C, D, *left*). Since it has been reported that PMA, a PKC agonist, increases sAPP $_{\alpha}$  secretion (Caporaso et al., 1992; Slack et al., 1993; Camden et al., 2005), treatment with PMA (10nM) was used as a positive control.

To test whether positions of double bonds plays a role in membrane fluidity and sAPP $_{\alpha}$  secretion, differentiated SH-SY5Y cells were exposed to *cis*-2-eicosenoic acid and *cis*-5, 8, 11-eicosatrienoic acid, which have the same numbers of double bonds as OA and ALA respectively, but the double bonds are closer to the carboxylic groups of fatty acids. Results showed that these two fatty acids also had no effect on membrane fluidity and sAPP $_{\alpha}$  secretion in SH-SY5Y cells, like OA and ALA (Fig. 3), suggesting that the positions of double bonds in fatty acids do not play a role in their effects on membrane fluidity and APP processing.

### 3.3. Fatty acids with 4 or more double bonds increased membrane fluidity and sAPP $_{\alpha}$ secretion

We then tested the effects of fatty acids with 4 or more double bonds including AA, EPA and DHA on membrane fluidity and sAPP $_{\alpha}$  secretion. After treatment with AA, EPA and DHA, cells exhibited more fluidized membranes as indicated by lower fluorescent intensities of FCVJ compared to control (Fig. 4A, B, C, *right*). Western blot analysis showed that AA, EPA and DHA increased sAPP $_{\alpha}$  secretion in differentiated SH-SY5Y cells (Fig. 4A, B, C, *left*).

Together with results from SA, OA, LA and ALA treatments, these data showed that unsaturation numbers of fatty acids, but not the positions of double bonds, determined their effects on membrane fluidity and sAPP $_{\alpha}$  secretion, as summarized in Fig. 5. These results suggested that fatty acids with 4 or more double bonds increased sAPP $_{\alpha}$  secretion through increasing membrane fluidity in differentiated SH-SY5Y cells.

### 3.4. AA and DHA did not alter the expressions of total APP and $\alpha$ -secretases in SH-SY5Y cells

To rule out the changes of sAPP $_{\alpha}$  secretion possibly due to the changes in the expressions of total APP and  $\alpha$ -secretases in cells, western blot analyses of APP and  $\alpha$ -secretases were performed after the treatments of cells with AA and DHA for 24 h. Fig. 6 shows that AA and DHA did not alter the expressions of total APP and different isoforms of  $\alpha$ -secretases including ADAM9, ADAM10, ADAM17 and ADAM19. Mature APP blots were shown in this study. These results suggested that AA, DHA and EPA do not alter  $\alpha$ -secretases and APP expressions to contribute to the increase in sAPP $_{\alpha}$  secretion.

### 3.5. AA, EPA and DHA did not alter BACE1 expression, A $\beta$ <sub>1-42</sub> secretion and expression in SH-SY5Y cells

Since AA, EPA and DHA increase sAPP $_{\alpha}$  secretion, they may alter expression of BACE1, the major  $\beta$ -secretase, and A $\beta$ <sub>1-42</sub> in SH-SY5Y cells. Western blot analysis showed that AA, EPA and DHA did not change BACE1 expression (Fig. 7A). ELISA measurement was used to quantify A $\beta$ <sub>1-42</sub> in culture medium. Fig. 7B showed that AA, EPA and DHA did not change the A $\beta$ <sub>1-42</sub> secretion and expression level in SH-SY5Y cells.

## 4. Discussion

In this study, we have systematically examined how unsaturation numbers of fatty acids affected membrane fluidity and sAPP $_{\alpha}$  secretion in differentiated SH-SY5Y cells. We found that not all unsaturated fatty acids, but only fatty acids with at least 4 or more double bonds increased membrane fluidity and sAPP $_{\alpha}$  secretion (Fig. 5). The effects on membrane fluidity and sAPP $_{\alpha}$  secretion were not dependent on the positions of double bonds in fatty acids. In addition, treatments of cells with fatty acids did not alter the expressions of APP and  $\alpha$ -secretases in differentiated SH-SY5Y cells, which ruled out the effects of fatty acids on sAPP $_{\alpha}$  secretion possibly due to the changes in APP and  $\alpha$ -secretases expressions.

Studies have shown the roles of fatty acids on health and diseases (Bazan, 2007; Das, 2008; Hooijmans and Kiliaan, 2008). Fatty acids are important ingredients in various dietary sources (Connor, 2000; Chow, 2007). For example, ALA, an essential fatty acid, can be found in many vegetable oils (Mozaffarian, 2005), which has been reported to be neuroprotective in *in vivo* model of global ischemia (Lauritzen et al., 2000). SA is common in animal fats, and cocoa butter (Beare-Rogers et al., 2001). OA is a mono-unsaturated  $\omega$ -9 fatty acid, which can be found in peanut oil and poppy seed oil (Untoro et al., 2006). DHA, mainly found in fishes, is also abundant in neuronal cell membranes, especially in synaptic membranes (Bazan and Scott, 1990) and myelin sheaths (Ansari and Shoeman, 1990). DHA is also essential for prenatal brain development and normal brain functions. Its levels in serum and brains are lower in AD patients compared with those in age-matched controls (Tully et al., 2003). Furthermore, greater consumption of DHA significantly reduced the likelihood of developing AD (Schaefer et al., 2006). AA is another abundant fatty acid in the brain. It is a second messenger (Khan et al., 1995) and a precursor for synthesis of eicosanoids (Zhou and Nilsson, 2001). It also helps maintain cell membrane fluidity (Fukaya et al., 2007). The disturbed metabolism of AA is associated with neurological disorder such as AD (Rapoport, 2008).

APP processing depends on the local membrane environment (Tun et al., 2002; Cordy et al., 2003; Eehalt et al., 2003; Marlow et al., 2003; Kaether and Haass, 2004; Vetrivel et al., 2004; Reid et al., 2007) and can be altered by manipulating the membrane lipid composition (Simons et al., 1998; Kojro et al., 2001; Sawamura et al., 2004; von Arnim et al., 2008). Since fatty acids modulate membrane organization and functions (Yehuda et al., 2002; Stillwell et al., 2005; Shaikh and Edidin, 2006; Pepe, 2007; Shaikh and Edidin, 2008), they may affect APP processing. Therefore, we tested the hypothesis that the effects of fatty acids on membrane fluidity and sAPP $_{\alpha}$  secretion depend on the number of double bonds (i.e. the unsaturation number) in the hydrocarbon chains of fatty acids.

To test the hypothesis, we investigated the effects of fatty acids with different unsaturation (from 0 to 6 double bonds) on membrane fluidity and sAPP $_{\alpha}$  secretion in differentiated SH-SY5Y cells. Our data showed that fatty acids with 3 or less double bonds including SA, OA, LA and ALA had no effects on membrane fluidity and sAPP $_{\alpha}$  secretion (Fig. 2). We also exposed SH-SY5Y cells to *cis*-2-eicosenoic acid and *cis*-5, 8, 11-eicosatrienoic acid, which has 1 or 3 double bonds as OA and ALA, respectively, but their double bonds are closer to

the carboxylic group of fatty acids. Results showed that these two acids had no effect on membrane fluidity and sAPP $_{\alpha}$  secretion in differentiated SH-SY5Y cells either, suggesting that the position of the double bonds may not play a role in altering membrane fluidity and APP processing (Fig. 3). On the other hand, fatty acids with 4 or more double bonds including AA, EPA and DHA increased membrane fluidity and sAPP $_{\alpha}$  secretion (Fig. 4). Our data are consistent with other studies that AA increased membrane fluidity in hippocampal neurons *in vivo* (Fukaya et al., 2007) and cultured human umbilical vein, cerebral endothelial cells (Villacara et al., 1989; Beck et al., 1998). In addition, DHA has been shown to increase membrane fluidity and sAPP $_{\alpha}$  secretion in HEK cells and SH-SY5Y cells overexpressing APP (Kogel et al., 2008). Our study demonstrated that not all the unsaturated fatty acids, but only fatty acids of unsaturation number  $\geq 4$ , are able to increase membrane fluidity, and subsequently promote sAPP $_{\alpha}$  secretion.

To explore if AA, EPA, DHA effects on membrane fluidity and secretion of sAPP $_{\alpha}$  have dose dependency, we exposed SH-SY5Y cells to AA, EPA and DHA with lower doses at 10nM and 100nM. At lower doses, AA, EPA and DHA have no effect on membrane fluidity and sAPP $_{\alpha}$  secretion (data not shown). Taken together, our data show that polyunsaturated fatty acids (PUFAs) only begin to have effects on membrane fluidity and APP processing at certain threshold value between 100nM and 1 $\mu$ M.

Membrane fluidity plays important roles in pathogenesis of AD. Hippocampal membranes of AD patients showed a significant lower fluidity compared with membranes from elderly non-demented controls (Eckert et al., 1999). Membrane fluidity in AD patients was correlated with abnormal APP processing and cognitive decline (Zainaghi et al., 2007). The clinical study by Croisile et al. (1993) indicated that long-term and high-dose treatment with piracetam may slow down the progression of AD and increased membrane fluidity (Eckert et al., 1999). DHA protection effect in A $\beta$ -infused rats was associated with increased membrane fluidity (Hashimoto et al., 2006) which also provided oxidative stress resistance in hippocampal cells (Clement et al., 2010). *In vivo*, increased membrane fluidity in rat hippocampus improved memory formation (Muller et al., 1997; Scheuer et al., 1999), whereas reduced fluidity impaired memory (Schaeffer et al., 2005; Schaeffer and Gattaz, 2007). Furthermore, membrane fluidity affected not only APP processing (Kojro et al., 2001), but also A $\beta$  aggregation size and hydrophobicity (Kremer et al., 2000), which is critical for the formation of A $\beta$  plaques and downstream cellular pathways.

PUFAs play a central role in the normal development and functioning of brain (Schuchardt et al., 2010). Diets enriched in  $\omega$ -3 PUFA increased membrane fluidity, affect signal transduction and modulate gene expression for brain function (Horrocks and Farooqui, 2004). Preclinical studies suggested that DHA maintains membrane fluidity, improved synaptic and neurotransmitter functioning, enhanced learning and memory performances and displayed neuroprotective properties (Carrie et al., 2009). Meanwhile, DHA decreased the amount of vascular A $\beta$  deposition (Hooijmans et al., 2007) and reduced A $\beta$  burden (Lim et al., 2005) in aged Alzheimer mouse model. In AD mouse model, DHA modulated APP processing by decreasing both  $\alpha$ - and  $\beta$ -APP C-terminal fragment products and full-length APP (Lim et al., 2005), which is contradictory to the idea that higher membrane fluidity increased sAPP $_{\alpha}$  production. More works are warrant to resolve this discrepancy. Despite favorable effects of DHA, it should be noted that DHA has a potential to increase oxidative stress (Meydani et al., 1991), resulting in lipid peroxidation (Song and Miyazawa, 2001), especially with long-term and high dose treatments (Grundt et al., 2003; Wang et al., 2003). Peroxidation of lipids and lipoproteins, especially low-density lipoprotein (LDL), was thought to lead to the development of atherosclerotic plaques (Wiklund et al., 1991). As such, although the impact of PUFAs on lipid peroxidation is controversial (Nenseter and

Drevon, 1996), considerations should be taken when PUFAs are used for dietary supplementation.

Although we report that AA promoted sAPP $_{\alpha}$  secretion, it is important to note that AA exerts both neurotrophic and neurotoxic effects (Farooqui and Horrocks, 2006). The concentration of AA (<10  $\mu\text{mol/kg}$ ) in the brain tissue is very low under normal conditions. AA performs a variety of functions, such as regulating activity of protein kinase A, protein kinase C, NADPH oxidase, choline acetyltransferase, and caspase-3 (Farooqui and Horrocks, 2006). 1  $\mu\text{M}$  of AA also significantly potentiates the elongation of neurites in hippocampal cultures (Katsuki and Okuda, 1995). Here we report that 1  $\mu\text{M}$  of AA increased membrane fluidity and promoted the secretion of sAPP $_{\alpha}$  which is neurotrophic and neuroprotective. However, under pathological conditions, higher concentrations ( $\sim 0.5$  mmol/kg) of AA are present in the brains (Farooqui and Horrocks, 2006). Higher concentrations of AA result in mitochondrial dysfunction (Schapira, 1996). AA also triggers nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) and increases neuronal death (Toborek et al., 1999). AA can be converted to inflammatory mediators, prostaglandins and leukotrienes, by prostaglandin synthetases and lipoxygenases, respectively (Farooqui and Horrocks, 2006). Therefore, whether AA is beneficial to brains is highly concentration-dependent.

Other compounds capable of altering membrane fluidity can also modulate sAPP $_{\alpha}$  secretion. For example, treatment with methyl- $\beta$ -cyclodextrin (M $\beta$ CD) or lovastatin to reduce cellular cholesterol resulted in increase in membrane fluidity and sAPP $_{\alpha}$  secretion (Kojro et al., 2001). In addition, decreased sAPP $_{\alpha}$  secretion by Pluronic F68 (PF68) is associated with decreased membrane fluidity, while increased sAPP $_{\alpha}$  by benzyl alcohol (C $_6$ H $_5$ OH) is associated with increased membrane fluidity (Peters et al., 2009). These data suggested that sAPP $_{\alpha}$  secretion can be altered by manipulating membrane fluidity (Wolozin, 2001). Taken together, our data and those from Kojro et al (2001) and Peter et al (2009) provide the explanation that fatty acids with 4 or more double bonds promote sAPP $_{\alpha}$  secretion through increasing membrane fluidity in differentiated SH-SY5Y cells.

sAPP $_{\alpha}$  has been shown to be neurotrophic. sAPP $_{\alpha}$  enhanced neuronal survival and neurite extension in a dose-dependent manner with a maximum effect at approximately 100 nM in rat primary cerebral cortical neurons (Araki et al., 1991) and significantly increased neurite length and branching in pheochromocytoma PC12 cells (Milward et al., 1992) and stimulated neurite outgrowth of hippocampal neurons in an isoform-dependent manner (Qiu et al., 1995). ADAM10 over-expression, which led to increased sAPP $_{\alpha}$  production, increased cortical synaptogenesis (Bell et al., 2008). sAPP $_{\alpha}$  is also neuroprotective, although mechanisms are yet to be fully understood. For example, sAPP $_{\alpha}$  enhanced basal glucose and glutamate transport and protected against oxidative impairment of glucose and glutamate transport in synaptosomes by a cyclic GMP-mediated mechanism (Mattson et al., 1999); sAPP $_{\alpha}$  exerted neuroprotective effects through regulating intraneuronal calcium (Mattson et al., 1993); sAPP $_{\alpha}$  antagonized dendritic degeneration and neuron death triggered by proteasomal stress (Copanaki et al., 2010), and this anti-apoptotic effect of sAPP $_{\alpha}$  was associated with inhibition of the stress-triggered c-Jun N-terminal kinase (JNK)-signalling pathway; Statins, widely used cholesterol-lowering drugs, exerted neuroprotective effects through stimulating  $\alpha$ -secretase cleavage of APP (Ma et al., 2009). Obviously, more efforts are needed to further understand the neurotrophic and neuroprotective mechanisms of sAPP $_{\alpha}$ .

Information derived from this study should provide potential dietary strategies for prevention of AD.



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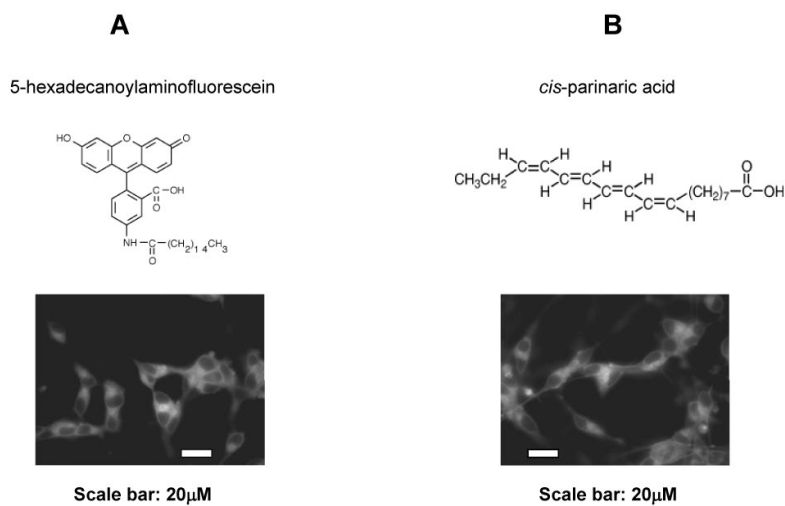
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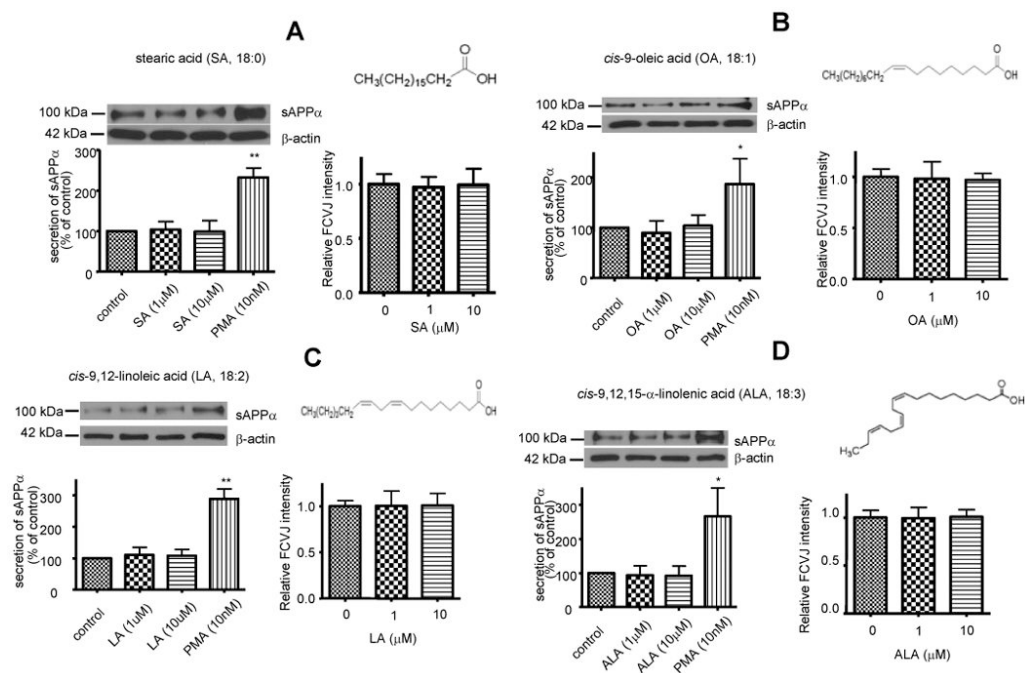
## Abbreviations used

<b>AA</b>	arachidonic acid
<b>AD</b>	Alzheimer's disease
<b>ADAM</b>	a disintegrin and metalloprotease
<b>ALA</b>	$\alpha$ -linolenic acid
<b>APP</b>	amyloid precursor protein
<b>A<math>\beta</math></b>	amyloid- $\beta$ peptide
<b>BCA</b>	bicinchoninic acid
<b>BSA</b>	bovine serum albumin
<b>DHA</b>	docosahexaenoic acid
<b>DMEM</b>	dulbecco's modified Eagle's medium
<b>DMSO</b>	dimethyl sulfoxide
<b>EPA</b>	eicosapentaenoic acid
<b>FBS</b>	fetal bovine serum
<b>FCVJ</b>	farnesyl-(2-carboxy-2-cyanovinyl)-julolidine
<b>FRAP</b>	fluorescence recovery after photobleaching
<b>LA</b>	linoleic acid
<b>M<math>\beta</math>CD</b>	methyl- $\beta$ -cyclodextrin

<b>NF-<math>\kappa</math>B</b>	nuclear factor- $\kappa$ B
<b>OA</b>	oleic acid
<b>PF68</b>	Pluronic F68
<b>PMA</b>	phorbol 12-myristate 13-acetate
<b>PUFA</b>	polyunsaturated fatty acid
<b>RA</b>	all- <i>trans</i> retinoic acid
<b>SA</b>	stearic acid
<b>sAPP<math>_{\alpha}</math></b>	$\alpha$ -secretase-cleaved soluble APP
<b>sPLA<math>_2</math>-III</b>	secretory phospholipase A $_2$ type III



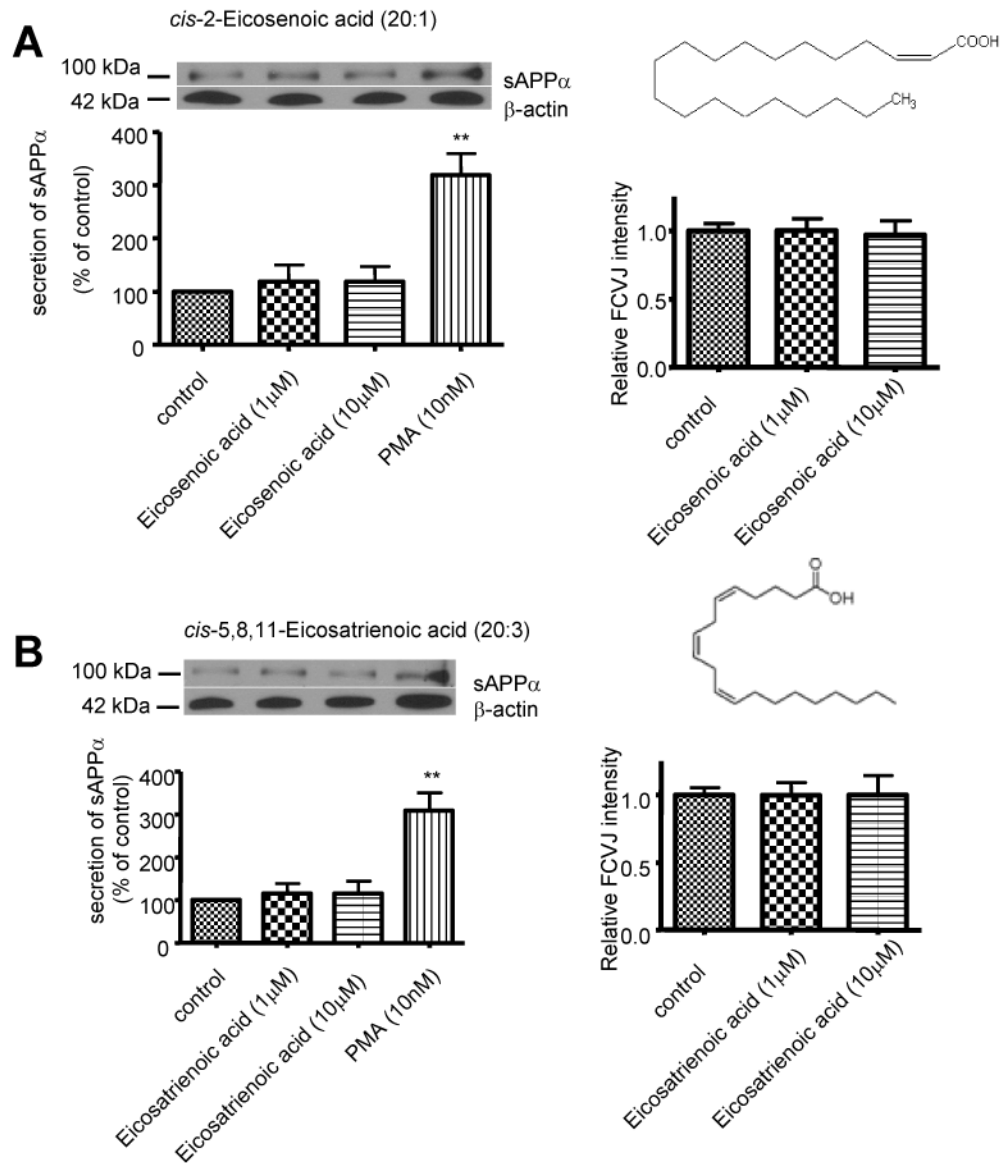
**Figure 1.** Fluorescently labeled fatty acids incorporated into membranes in differentiated SH-SY5Y cells. 5-hexadecanoylamino fluorescein (**A**) and *cis*-parinaric acid (**B**) represent saturated and unsaturated fatty acids, respectively. After incubation for 40 min, fluorescent images were acquired with an oil immersion 60 $\times$  objective lens. Scale bar, 20  $\mu$ m.



**Figure 2. SA, OA, LA and ALA on sAPP $\alpha$  secretion and membrane fluidity in differentiated SH-SY5Y cells**

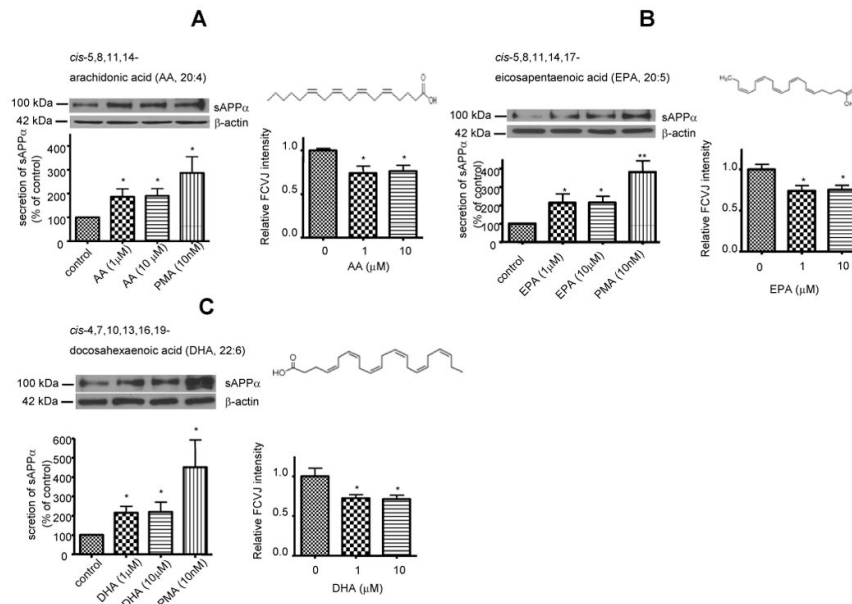
Western blot analysis of sAPP $\alpha$  shows that treatments of cells with SA (A, left), OA (B, left), LA (C, left) and ALA (D, left) did not affect sAPP $\alpha$  secretion to medium from SH-SY5Y cells. PMA treatment known to increase sAPP $\alpha$  secretion in cells was used as a positive control. SA (A, right), OA (B, right), LA (C, right) and ALA (D, right) did not affect membrane fluidity of cells, as the fluorescent intensity of FCVJ-labeled cells remained unchanged after treatments. Data are expressed as mean  $\pm$  SD from at least three independent experiments (\*  $p < 0.05$ , \*\*  $p < 0.01$  compared with control).





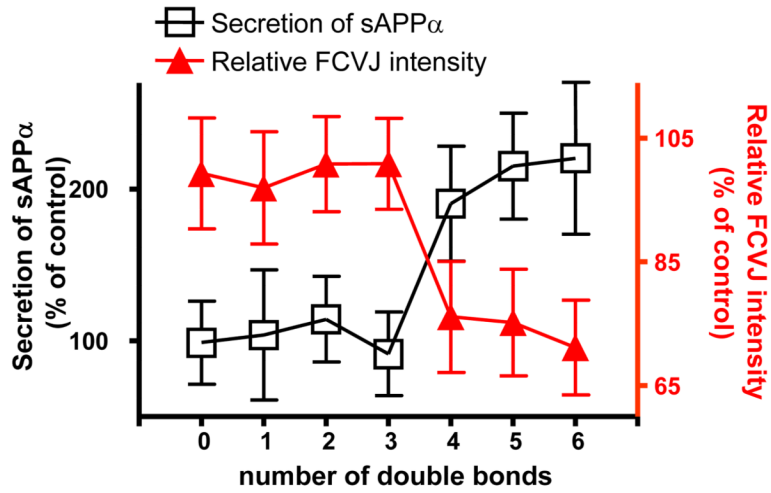
**Figure 3.** *cis*-2-Eicosenoic acid (20:1) and *cis*-5, 8, 11-Eicosatrienoic acid (20:3) on sAPP $\alpha$  secretion and membrane fluidity in differentiated SH-SY5Y cells

Western blot analysis of sAPP $\alpha$  shows that treatments of cells with *cis*-2-Eicosenoic acid (20:1) (A, left) and *cis*-5, 8, 11-Eicosatrienoic acid (20:3) (B, left) did not affect sAPP $\alpha$  secretion from SH-SY5Y cells. PMA treatment was used as a positive control. *cis*-2-Eicosenoic acid (20:1) (A, right) and *cis*-5, 8, 11-Eicosatrienoic acid (20:3) (B, right) did not affect membrane fluidity of cells, as the fluorescent intensity of FCVJ-labeled cells remained unchanged after treatments. Data are expressed as mean  $\pm$  SD from at least three independent experiments (\*\*  $p < 0.01$  compared with control).



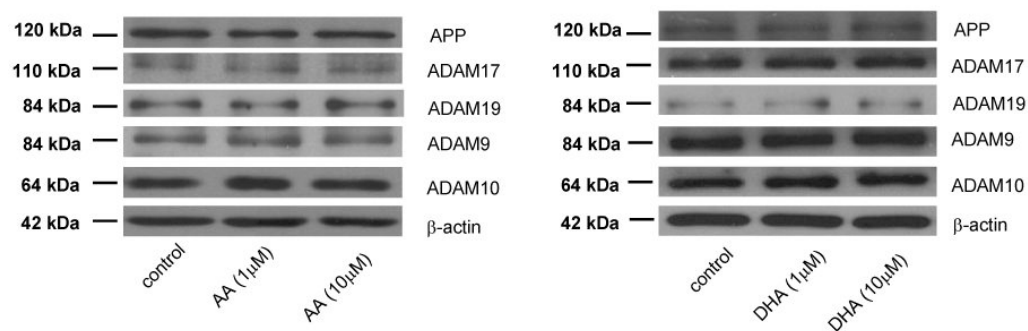
**Figure 4. AA, EPA and DHA increased the sAPP $\alpha$  secretion and membrane fluidity in differentiated SH-SY5Y cells**

Western blot analysis of sAPP $\alpha$  shows that AA (A, left), EPA (B, left) and DHA (C, left) increased sAPP $\alpha$  secretion to medium from SH-SY5Y cells. PMA was used as a positive control. AA (A, right), EPA (B, right) and DHA (C, right) increased membrane fluidity in cells, as indicated by decreased fluorescent intensity of FCVJ-labeled cells. Data are expressed as mean  $\pm$  SD from at least three independent experiments (\* p<0.05, \*\* p<0.01 compared with control).



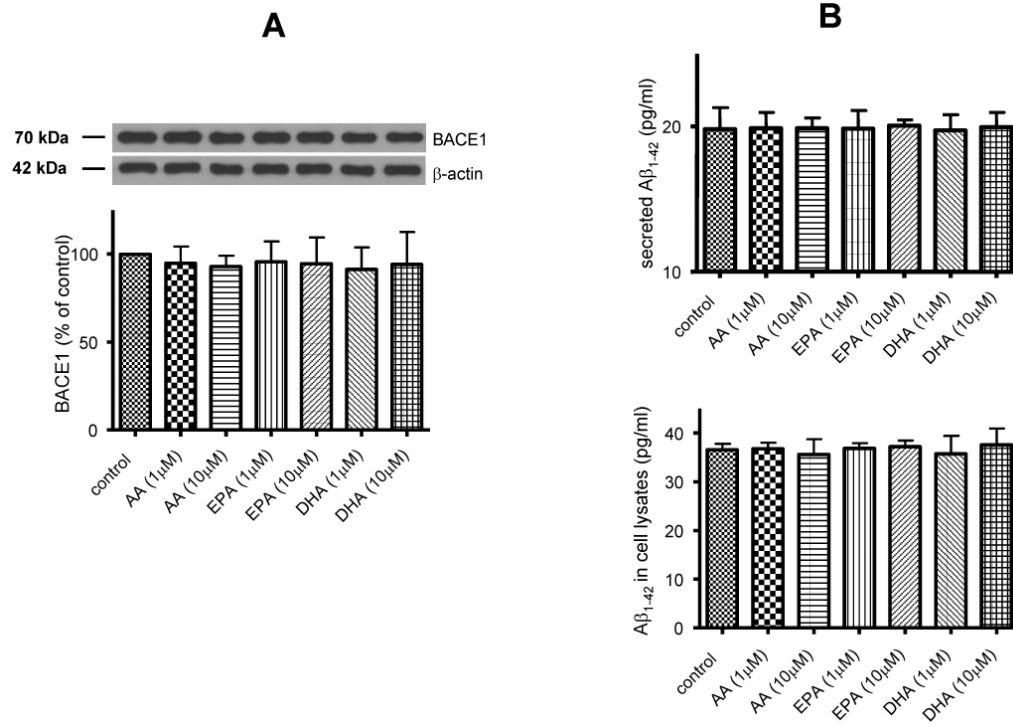
**Figure 5. The effects of the number of double bonds in fatty acids on sAPP $\alpha$  secretion and membrane fluidity in differentiated SH-SY5Y cells**

Our data shows that not all unsaturated fatty acids, but only those with four or more double bonds, including AA, EPA and DHA, promoted sAPP $\alpha$  secretion and increased membrane fluidity. Data are expressed as percentages of control and mean  $\pm$  SD from at least three independent experiments (\*  $p < 0.05$  compared with control).



**Figure 6. Effects of AA and DHA on total APP and  $\alpha$ -secretases expression in differentiated SH-SY5Y cells**

Western blot analysis of total APP and  $\alpha$ -secretases shows that AA and DHA did not alter the expressions of total APP and different isoforms of  $\alpha$ -secretases including ADAM9, ADAM 10, ADAM17 and ADAM 19. Data are representative blots from at least three independent experiments.



**Figure 7. Effects of AA, EPA and DHA on BACE1 expression and Aβ<sub>1-42</sub> from differentiated SH-SY5Y cells**

Treatments of AA, EPA and DHA did not change BACE1 expression in SH-SY5Y cells (A), Aβ<sub>1-42</sub> secretion from SH-SY5Y cells (B, upper) and Aβ<sub>1-42</sub> expression in SH-SY5Y cells (B, lower). Data are expressed as mean ± SD from at least three independent experiments.