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Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increase Aβ-degradation by affecting insulin-degrading enzyme (IDE)

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Abstract:

Omega-3 polyunsaturated fatty acids (PUFAs) have been proposed to be highly beneficial in Alzheimer's disease (AD). AD pathology is closely linked to an overproduction and accumulation of β-amyloid (Aβ) peptides as extracellular senile plaques in the brain. Total Aβ levels are not only dependent on its production by proteolytic processing of the amyloid precursor protein (APP), but also on Aβ-clearance mechanisms, including Aβ-degrading enzymes. Here we show that the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increase Aβ-degradation by affecting insulin-degrading enzyme (IDE), the major Aβ-degrading enzyme secreted into the extracellular space of neuronal and microglial cells. The identification of the molecular mechanisms revealed that EPA directly increases IDE enzyme activity and elevates gene expression of IDE. DHA also directly stimulates IDE enzyme activity and affects IDE sorting by increasing exosome release of IDE, resulting in enhanced Aβ-degradation in the extracellular milieu. Beside the known positive effect of DHA by reducing Aβ production, EPA and DHA might ameliorate AD pathology by increasing Aβ turnover.

Key words: Polyunsaturated omega-3 fatty acids, eicosapentaenoic acid, docosahexaenoic acid, Aβ-degradation, insulin-degrading enzyme

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Introduction

Alzheimer´s disease (AD) is a devastating neurodegenerative disorder and the most common cause of dementia in the elderly. The severe accumulation of amyloid-β (Aβ) peptides in brain tissue resulting in the formation of extracellular senile plaques is proposed to be the initial pathological event of the disease (Hardy and Selkoe 2002; Hardy and Higgins 1992). Cerebral Aβ level are not only dependent on Aβ-production by sequential proteolytic processing of the amyloid precursor protein (APP), but also on Aβ-clearance mechanisms including the transport across the blood-brain barrier, microglial phagocytosis and the enzymatic degradation within brain parenchyma (Miners et al. 2011; Saido and Leissring 2012).

Several *in vitro* and cell-based studies have shown an involvement of insulin-degrading enzyme (IDE) in Aβ-degradation (Chesneau et al. 2000; Fernandez-Gamba et al. 2009; Kurochkin and Goto 1994; Perez et al. 2000; Qiu et al. 1998; Sudoh et al. 2002). Utilizing IDE deficient mice the enzyme has been identified as one of the major Aβ-degrading enzymes in brain tissue (Farris et al. 2003; Miller et al. 2003). IDE is a zinc metallopeptidase which is most abundant in the cytosol, but also localized in several other subcellular compartments (Saido and Leissring 2012). Additionally, IDE is the major Aβ-degrading enzyme secreted into the medium of neuronal and microglial cell cultures (Farris et al. 2003; Qiu et al. 1998; Qiu et al. 1997; Vekrellis et al. 2000). The protein lacks a typical secretory signal sequence and is not exported via the classical secretory pathway (Zhao et al. 2009), but it has been discussed to be secreted by exosome release (Bulloj et al. 2010).

Several epidemiological trials indicate that the dietary intake of polyunsaturated fatty acids (PUFAs) from fish and marine oils, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is associated with a reduced risk for the development of AD (Barberger-Gateau et al. 2007; Boudrault et al. 2009; Grimm et al. 2013b; Hartmann et al. 2007; Jicha and Markesbery 2010; Mett et al. 2016; Morris et al. 2003). Reduced levels of

these fatty acids have been found in the serum/plasma and in *post mortem* brain tissue of AD patients (Conquer et al. 2000; Grimm et al. 2011a; Soderberg et al. 1991; Tully et al. 2003). Furthermore, dietary supplementation of PUFAs seems to improve the cognitive functions of AD animal models (Calon et al. 2004; Hashimoto et al. 2002; Hashimoto et al. 2005; Hooijmans et al. 2012; Hooijmans et al. 2009; Hosono et al. 2015) and of patients with early stages of AD (Chiu et al. 2008; Freund-Levi et al. 2006; Kotani et al. 2006). We and others analysed the underlying mechanisms showing that DHA reduces Aβ-generation via pleiotropic mechanisms in cell cultures and *in vivo* (Green et al. 2007; Grimm et al. 2011b; Grimm et al. 2013b; Lim et al. 2005; Torres et al. 2014). Additionally, PUFAs have been shown to negatively affect Aβ-aggregation and Aβ-induced toxicity in neuroblastoma cells (Hashimoto et al. 2011; Hashimoto et al. 2008; Hossain et al. 2009) and further to improve microglial phagocytosis of Aβ-peptides (Hjorth et al. 2013). However, the influence of PUFAs on enzymatic Aβ-degradation is still unclear. The aim of this study was to clarify whether EPA- and DHA-containing phosphatidylcholines (PC) have beneficial effects on Aβ-degradation compared to PCs containing saturated fatty acids with the same chain length. Our results revealed that EPA and DHA have, in addition to their positive effects described above, a stimulating influence on IDE-dependent Aβ-degradation further indicating a potential of these PUFAs in preventing AD or slowing down its progression.

Material and methods

Materials

Cell culture materials and chemicals were purchased from Sigma (Taufkirchen, Germany) if not stated otherwise.

The phospholipids 1,2-Di-arachidoyl-sn-glycero-3-phosphocholine (PC20:0/20:0), 1,2-Di-behenoyl-sn-glycero-3-phosphocholine (PC22:0/22:0) and 1,2-Di-docosahexaenoyl-sn-glycero-3-phosphocholine (PC22:6/22:6) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Di-eicosapentaenoyl-sn-glycero-3-phosphocholine (PC20:5/20:5) was acquired from Sigma (Taufkirchen, Germany). Due to the limited solubility of PC20:0/20:0 and PC22:0/22:0 all used phospholipids were uniformly dissolved in pre-warmed (37 °C) ethanol to a final concentration of 2 mM by vortexing.

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Cell culture

Cultivation: Mouse neuroblastoma Neuro-2a (N2a) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany), 0.1 mM non-essential amino acid solution (MEM), penicillin/streptomycin (100 U/ml and 0.1 mg/ml), 2 mM L-glutamine and 1 mM sodium pyruvate. For stable N2a IDE knock-down and the corresponding mock-transfected control cells (Grimm et al. 2015), culture medium was additionally supplemented with 400 µg/ml hygromycin B (PAN Biotech, Aidenbach, Germany).

Incubations: In order to reduce the lipid content of the cell culture medium during incubation with phospholipids the concentration of FCS was reduced to 0.1 % as described earlier (Grimm et al. 2005). Cells were cultured until reaching confluence and cultivated in FCS-reduced culture medium (DMEM/ 0.1% FCS) for 6 hours (h) prior to incubation. Then pre-warmed (37 °C) culture medium (DMEM/ 0.1% FCS) was supplemented with phospholipids

to a final concentration of 10 µM in glass vials under continuous vortexing and incubated on cells over a period of 18 h. As an example for the uptake we measured the shortest saturated (PC20:0/20:0) and the longest unsaturated (PC22:6/22:6) phospholipids in incubated cells utilizing mass spectrometry analysis. As illustrated in Supplemental figure 1 and already published earlier (Grimm et al. 2013a), the PC-profile of the treated cells is altered compared to cells incubated with solvent indicating the phospholipids to be efficiently taken up.

For inhibition of exosome release GW4869 (Merck Millipore, Darmstadt, Germany), an inhibitor of neutral sphingomyelinase known to affect exosome secretion (Li et al. 2013; Trajkovic et al. 2008), was added to the incubation medium in a final concentration of 20 µM.

Transfections: Transient transfection was performed by using Lipofectamine 2000 and Opti-MEM (Invitrogen, Karlsruhe, Germany) according to manufacturer's protocol. Incubations of transfected cells were started 48 h after transfection. For the generation of stably transfected IDE knock-down and the corresponding control cells (Grimm et al. 2015), N2a cells were transfected with SureSilencingTM–Insulin degrading enzyme shRNA plasmid or control vector (SABioscience, Frederick, MD, USA) as described above followed by selection for stable clones with 400 µg/ml hygromycin B (PAN Biotech, Aidenbach, Germany).

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Determination of protein concentration

Determination of protein concentration was performed using bicinchoninic acid (Smith et al. 1985) as described in detail earlier (Rothhaar et al. 2012). Samples were adjusted to equal protein amount prior to further usage in experiments.

Mass spectrometry analysis

PC content in samples was measured by using a 4000 quadrupole linear-ion trap (QTrap) equipped with a Turbo Spray ion source (AB Sciex, Darmstadt, Germany) connected to a

1200 Agilent HPLC (Agilent, Böblingen, Germany). For measuring phospholipid uptake N2a cells were washed three times with ice cold PBS, scraped off, sonificated in water and adjusted to equal protein amount prior to mass spectrometry analysis. The PC-profile of the samples was determined as described in detail earlier (Grimm et al. 2011a) with minor modifications. To evaluate the binding properties of phospholipids to IDE, samples were transferred to a 96-deep well plate (Nunc, Langenselbold, Germany), which was covered with a silicone cap and shaken for 2 minutes (min) at 450 rpm at room temperature. The plate was placed into the autosampler. Each sample's PC content was measured three times by injection of 20 µl sample volume by precursor ion scan of m/z 184.1 (representing the PC head group) in positive mode with the following parameters: curtain gas (CUR) 20 psi, collision gas (CAD) medium, ion spray voltage (IS) 5500 V, temperature (TEM) 200 °C, ion source gas 1 (GS1) 40 psi, ion source gas 2 (GS2) 50 psi, interface heater (ihe) on, declustering potential (DP) 83 V, entrance potential (EP) 10 V, collision energy (CE) 48 V and collision cell exit potential (CXP) 15 V. For detection and evaluation, Analyst 1.5 software (AB Sciex, Darmstadt, Germany) was used.

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Assessment of cell viability

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity in cell culture supernatant of incubated cells utilizing the Cytotoxicity Detection Kit PLUS (Roche Diagnostics, Mannheim, Germany) in accordance to the manufacturer's protocol. Under the used conditions cell viability was unaltered (data not shown).

Preparation of cell lysates and collection of conditioned culture medium

Conditioned culture medium was collected and centrifuged for 5 min at 13000 x g and 4 °C. Supernatant was used for further analysis. Cells were washed with ice cold phosphate

buffered saline (PBS) and scraped off on ice. After lysis on ice for 1 h in cell lysis buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.4, 2 mM EDTA, 0.1 % NP-40, 0.1 % Triton-X 100) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), samples were centrifuged for 5 min at 13000 x g and 4 °C. Supernatants were used for further experiments.

Measurement of total Aβ-degradation

Determination of total Aβ-degradation was performed as described earlier (Grimm et al. 2014). Accordingly, N2a cells were incubated with 10 µM phospholipids for 18 h as described above. Incubation medium was replaced by DMEM/0.1 % FCS containing 10 µM phospholipids and 0.5 µg/ml synthetic human Aβ40-peptides (B. Penke, Szeged, Hungary), which was incubated on cells for 6 h. Remaining, not degraded human Aβ40 peptides in the cell culture medium were quantified by western blot analysis using W02-antibody detecting only the supplemented human, but not endogenous murine Aβ40 peptides.

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Western Blot analysis

Samples were adjusted to equal protein content and loaded on 10-20 % tricine gels (Anamed Elektrophorese, Groß-Bieberau, Germany). For detection of extracellular IDE proteins, conditioned cell culture medium was enriched by the use of Amicon Ultra Filters with a cutoff of 30 kDa (Merck Millipore, Darmstadt, Germany) prior to gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). Immobilized IDE was detected by using the antibody ST1120 (Merck Millipore, Darmstadt, Germany), detection of human Aβ-Peptides was performed with W02-antibody (Merck Millipore, Darmstadt, Germany). Anti-rabbit HRP-coupled antibody W401 (Promega, Mannheim, Germany) and anti-mouse HRP-coupled antibody P0260 (Dako, Hamburg, Germany) were

utilized as secondary antibodies. Proteins were detected with ECL solutions (Perkin Elmer, Rodgau-Jügesheim, Germany). Band intensity was quantified densitometrically by using Image Gauge V3.45 software after the background signal was subtracted.

Quantitative real-time PCR

Extraction of total RNA was performed by the use of Trizol Reagent (Invitrogen, Karlsruhe, Germany). Reverse transcription of RNA was carried out with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany) according to manufacturer´s instructions. Quantitative real-time PCR was performed by utilizing the Fast SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany), PikoReal Real-Time PCR System (Thermo Scientific, Waltham, USA) and the following primers (Eurofins MWG Operon, Eberberg, Germany): β-actin 5′-CCTAGGCACCAGGGTGTGAT-3′ and 5′-TCTCCATGTCGTCCCAGTTG-3′; insulin-degrading enzyme 5′-GCTACGTGC AGAAGGACCTC-3′ and 5′-TGGACGTATAGCCTCGTGGT-3′. Changes in gene expression were calculated with the 2(-ΔΔCt)-method according to Livak and Schmittgen (Livak and Schmittgen 2001) after normalization to β-actin.

Assessment of IDE-promoter activity

For measurement of IDE promoter activity cells were transiently transfected with the GLuc-ON Promoter Reporter plasmid HPRM22553-PG04 (GeneCopoeia, Rockville, USA) comprising the *Gaussia* luciferase gene regulated by the IDE promoter region as reporter gene and the constitutively expressed tracking gene encoding for secreted alkaline phosphatase (SEAP). 48 h after transfection, cells were incubated with phospholipids as described above. The activities of *Gaussia* luciferase and SEAP in conditioned culture medium of the incubated cells were measured by using the Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia,

Rockville, USA) according to manufacturer´s instructions. Luminescence was detected in an Infinite M1000Pro-Fluorometer/ Luminometer (Tecan, Crailsheim, Germany). For each sample the signal of *Gaussia* luciferase activity was normalized to the signal of SEAP activity eliminating variations in transfection efficiency or cell confluence.

***In vitro* incubation of recombinant IDE with phospholipids**

50 ng recombinant human IDE (R&D Systems, Minneapolis, USA) was incubated for 15 min with 10 µM phospholipids in IDE assay buffer (100 mM Tris-HCl pH 7.0, 50 mM NaCl, 10 µM $ZnCl_2$) in glass vials under continuous shaking at 37° C.

Determination of the direct effect of phospholipids on IDE activity

After *in vitro* incubation, 50 µl of IDE/phospholipid mixture (corresponding to 12.5 ng recombinant IDE) were pipetted onto a black 96-well plate. After adding of 5 µM substrate Mca-RPPGFSAFK(Dnp)-OH (R&D Systems, Minneapolis, USA), resulting fluorescence was detected continuously at an excitation wavelength of 320±10 nm and an emission wavelength of 405±10 nm in a Safire^2 Fluorometer (Tecan, Crailsheim, Germany).

Determination of binding properties of phospholipids to IDE

50 ng recombinant human IDE was *in vitro*-incubated with equimolar amounts of PC20:5/20:5 and PC20:0/20:0 or PC22:0/22:0 and PC22:6/22:6 (10 µM). Samples containing phospholipids in absence of IDE were used as negative control. To remove excessive phospholipids, samples were transferred to Amicon Ultra Filters with a cutoff of 30 kDa (Merck Millipore, Darmstadt, Germany) and centrifuged at 13000 x g. Supernatant containing IDE was washed several times with 10 mM Tris/HCl pH 7.5. For co-immunoprecipitation IDE/phospholipid-complexes were incubated with 20 µl protein G-Sepharose (Sigma,

Taufkirchen, Germany) and anti-IDE antibody ST1120 (1.5 µg/ml) (Merck Millipore, Darmstadt, Germany) in an end-over-end shaker for 3 h at room temperature and centrifuged at 13000 x g for 1 min. After removal of supernatant protein G-Sepharose beads were washed three times with 10 mM Tris/HCl pH 7.5 and boiled in ethanol. Phospholipid content of the precipitates was determined by mass spectrometry.

Measurement of the activity of Aβ-degrading enzymes in living cells after pre-incubation with phospholipids

N2a cells were seeded on a 96-well plate, incubated for 18 h with 10 µM phospholipids as described above and washed twice with warm (37 °C) cell imaging solution (140 mM NaCl, 5 mM KCl, 8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 0.1 % FCS, pH 7.4). Cell imaging solution containing the substrate Mca-RPPGFSAFK(Dnp)-OH (10 µM) (R&D Systems, Minneapolis, USA), EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 25 µM γ-secretase inhibitor IV (Merck Millipore, Darmstadt, Germany) and 1 µM β-secretase inhibitor (Merck Millipore, Darmstadt, Germany) was allocated to the cells. These inhibitors were supplemented to the assay to enhance the specificity of substrate cleavage by Aβ-degrading enzymes. Resulting fluorescence was detected at an excitation wavelength of 320±10 nm and an emission wavelength of 405±10 nm in a Safire^2 Fluorometer (Tecan, Crailsheim, Germany) as described above.

Statistical analysis

All quantified data presented here are based on an average of at least three independent experiments. The effects of PC20:5/20:5 and PC22:6/22:6 were calculated in comparison to PC20:0/20:0 or PC22:0/22:0 (set at 100%), respectively, if not stated otherwise. Error bars

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represent standard error of the mean. Statistical significance was determined by ANOVA or two-tailed Student's t-test; significance was set at * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

Results

The omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) increase Aβ-degradation via insulin-degrading enzyme (IDE)

To analyse whether EPA and DHA influence Aβ-degradation, we treated mouse N2a cells with PC containing EPA or DHA in the sn-1 and sn-2 position (PC20:5/20:5 = 1,2-Di-eicosapentaenoyl-sn-glycero-3-phosphocholine; PC22:6/22:6 = 1,2-Di-docosahexaenoyl-sn-glycero-3-phosphocholine). As control we used phospholipids containing the corresponding saturated fatty acids in the sn-1 and sn-2 position (PC20:0/20:0 = 1,2-Di-arachidoyl-sn-glycero-3-phosphocholine; PC 22:0/22:0 = 1,2-Di-behenoyl-sn-glycero-3-phosphocholine) to determine the effect of the double-bonds within the fatty acid carbon chain esterified to PC. After an 18 h incubation period with 10 µM phospholipids cells were incubated for 6 h with 10 µM phospholipids and 0.5 µg/ ml synthetic human Aβ40 peptide. Remaining, not degraded human Aβ40 peptides were quantified by western blot analysis using W02-antibody detecting only the supplemented human, but not endogenous murine Aβ40 peptides.

In the presence of PC20:5/20:5 remaining Aβ peptides were reduced compared to PC20:0/20:0 treated cells, indicating that EPA increases Aβ-degradation (Figure 1). The stimulating effect on Aβ-degradation was even more pronounced for DHA. PC22:6/22:6 treated N2a cells showed an even higher reduction in remaining Aβ compared to PC22:0/22:0 incubated cells (Figure 1). The DHA- and EPA-induced positive effect on Aβ-degradation

was not observed in N2a cells with reduced expression of IDE, one of the main Aβ-degrading enzymes (Farris et al. 2003; Qiu et al. 1998). In the used N2a IDE knock-down cell line IDE both protein level and Aβ-degrading activity were reduced as illustrated in Supplemental figure 2 and published earlier (Grimm et al. 2015). IDE knock-down cells incubated with PC20:5/20:5 showed a slight but not significant increase in remaining, not-degraded Aβ peptides in comparison to IDE knock-down cells treated with PC20:0/20:0. Also for DHA, the positive effect on Aβ-degradation was not observed in IDE knock-down cells (Figure 1). These results indicate that EPA and DHA increase Aβ-degradation via IDE.

Influence of EPA and DHA on IDE gene expression

As outlined above, EPA and DHA increase Aβ-degradation via IDE. To further clarify the molecular mechanisms involved in elevating Aβ-degradation, we first analysed IDE expression in the presence of PC20:5/20:5 and PC22:6/22:6, again compared to the corresponding saturated phospholipids PC20:0/20:0 and PC22:0/22:0. Real-time (RT)-PCR analysis revealed that EPA-containing phospholipids significantly increased IDE expression whereas DHA-containing phospholipids showed no significant effect on IDE expression (Figure 2a). The elevation of IDE expression in the presence of EPA-containing phospholipids could be further verified by the determination of IDE promoter activity. Therefore N2a cells were transiently transfected with a promoter reporter plasmid comprising a luciferase gene regulated by the IDE promoter region. 48 h after transfection cells were incubated for 18 h with PC20:5/20:5 or the control saturated phospholipid PC20:0/20:0. IDE promoter activity was significantly increased in the presence of EPA-containing phospholipids (Figure 2b), comparable to the effect of EPA-containing phospholipids on IDE

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expression determined by RT-PCR (Figure 2a). These results clearly indicate that EPA increases Aβ-degradation by elevated gene expression of IDE.

DHA affects cellular sorting of IDE

IDE is mainly a cytosolic protein lacking any secretory signal sequence (Zhao et al. 2009). However, secreted IDE plays a major role in the extracellular clearance of secreted Aβ (Jiang et al. 2008; Vekrellis et al. 2000). Therefore we analysed whether EPA- or DHA-containing phospholipids influence cellular sorting of IDE. In the presence of PC20:5/20:5 compared to PC20:0/20:0 the level of secreted IDE tends to be increased while the intracellular IDE level was significantly elevated (Figure 2c). Extracellular IDE level were significantly elevated in PC22:6/22:6 incubated cells compared to PC22:0/22:0 treated cells whereas intracellular IDE levels tend to decrease, resulting in a significantly elevated ratio of extracellular/intracellular IDE for DHA-containing phospholipids (Figure 2c). This result indicates that DHA affects IDE sorting by increasing the secretion of IDE. In contrast, the ratio of extracellular/intracellular IDE was not significantly changed for PC20:5/20:5 (Figure 2c). Thus EPA does not seem to affect IDE sorting but increases Aβ-degradation by elevating IDE gene expression in general (Figure 2a).

As the IDE protein lacks a typical secretory signal sequence, IDE is not released through the classic exocytic pathway (Zhao et al. 2009), but most probably by exosome release (Bulloj et al. 2010). As we found increased secretion of IDE for DHA-containing phospholipids, resulting in elevated degradation of extracellular added human Aβ peptides (Figure 1), we addressed whether exosome release of IDE is affected in the presence of PC22:6/22:6. Therefore we analysed Aβ-degradation in cells treated with PC22:6/22:6 or PC22:0/22:0 in the presence of GW4869, known to inhibit exosome release (Li et al. 2013; Trajkovic et al.

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2008), or the solvent DMSO. Total Aβ-degradation is strongly reduced in N2a cells treated with GW4869 and PC22:0/22:0 compared to cells incubated with DMSO and the same lipid as illustrated in Supplemental figure 3, which is in line with the described effect of GW4869 to inhibit exosomal release and the resulting reduced IDE secretion.

As expected, remaining not-degraded human Aβ peptides were significantly decreased in the presence of PC22:6/22:6 and DMSO compared to PC22:0/22:0 and DMSO (Figure 2d). In the presence of PC22:6/22:6 and GW4869 remaining Aβ peptides were decreased to a lesser extent in comparison to PC22:0/22:0 and GW4869 (Figure 2d), indicating that DHA-induced increased release of IDE and thus elevated Aβ-degradation is at least partially mediated via exosome release of IDE.

EPA and DHA directly affect IDE activity

In order to analyse whether EPA and DHA directly affect IDE activity we performed an *in vitro* assay in the presence of recombinant IDE, phospholipids and a suitable substrate. Both, EPA and DHA, dramatically increased IDE activity *in vitr*o (Figure 3a), indicating that EPA and DHA also directly affect IDE activity. Binding properties of the applied phospholipids were determined by Co-immunoprecipitation studies with recombinant IDE and equimolar amounts of unsaturated and saturated phospholipids followed by mass spectrometry analysis. In the presence of recombinant IDE and equimolar amounts of PC20:0/20:0 and PC20:5/20:5 we found that the unsaturated phospholipid PC20:5/20:5 co-immunoprecipitated stronger with IDE than the saturated PC20:0/20:0 phospholipid, resulting in a significant increase in the ratio of PC20:5/20:5 to PC20:0/20:0. Similarly, unsaturated PC22:6/22:6 was also found to bind more efficiently to IDE than the corresponding saturated phospholipid PC22:0/22:0. The

ratio of PC22:6/22:6 to PC22:0/22:0 was also significantly elevated (Figure 3b). Both results indicate that the unsaturated phospholipids have a higher affinity to IDE than the saturated phospholipids resulting in increased IDE activity in the presence of PC20:5/20:5 and PC22:6/22:6.

To investigate whether these direct effects of EPA- and DHA- containing phospholipids on IDE are mainly responsible for the increased Aβ-degradation in cells incubated with PC20:5/20:5 or PC22:6/22:6 (Figure 1), we measured the activity of Aβ-degrading proteases in N2a cells after pre-incubation with these lipids in comparison to PC20:0/20:0 or PC22:0/22:0. After incubation the medium was removed and cells were washed before the substrate Mca-RPPGFSAFK(Dnp)-OH, which is cleaved by Aβ-degrading enzymes, was added. In the absence of supplemented phospholipids the cleavage of the substrate is slightly, but significantly increased in cells pre-incubated with PC20:5/20:5 and not significantly altered in cells pre-incubated with PC22:6/22:6 (Supplemental Figure 4). These results indicate that several mechanisms contribute to the observed effect of these lipids on IDE-mediated Aβ-degradation. However, the direct effect of the lipids under these conditions seems to be more pronounced than the other observed mechanisms.

Discussion

An increased dietary intake of omega-3 PUFAs, such as EPA and DHA, are discussed to ameliorate cognitive deficits or neuropathology associated with AD (Boudrault et al. 2009). One of the main pathological hallmarks of AD is the overproduction and accumulation of Aβ peptides (Masters et al. 1985). The level of pathogenic Aβ peptides is determined by the

balance between its generation and turnover (Miners et al. 2011; Saido and Leissring 2012). Whereas several studies addressed the influence of omega-3 PUFAs, especially DHA, on Aβ production (Green et al. 2007; Grimm et al. 2011b; Lim et al. 2005; Torres et al. 2014), very little is known about the effect of PUFAs on Aβ-degradation. In this study we aimed to examine the effect of EPA and DHA on Aβ-degradation including the identification of the underlying molecular mechanisms.

PC represent 58 % of membrane phospholipids being the most abundant phospholipids of mammalian membranes (Kadowaki and Grant 1995) and the brain is highly enriched in fatty acids including PUFAs, especially DHA, which accounts for about 8 % of the dry weight of the brain (Muskiet et al. 2006). Correspondingly, in the present study EPA and DHA were applied as phospholipids PC20:5/20:5 and PC22:6/22:6 and a neuroblastoma cell line was selected as cell culture model. It has to be mentioned that synthetic phospholipids were used containing PUFAs both in the sn1 and sn2 position. Under physiological conditions PUFAs are mainly bound in the sn2 position. However, this approach used here allowed us to address the question whether PUFAs are more beneficial than saturated fatty acids in respect to Aβ-degradation more directly than using phospholipids containing a mixture of saturated and unsaturated fatty acids. Both, PC20:5/20:5 and PC22:6/22:6, elevated the degradation of extracellular added synthetic human Aβ peptides via IDE, one of the main Aβ-degrading enzymes (Farris et al. 2003; Qiu et al. 1998). For EPA-containing phospholipids, elevated Aβ-degradation is caused by an increased gene expression of IDE and a direct effect of PC20:5/20:5 on IDE enzyme activity. In contrast, DHA-containing phospholipids revealed no effect on IDE gene expression in mouse N2a cells consistent with the study by Hosono et al. reporting that a DHA-enriched diet did not alter the expression of Aβ-degrading enzymes in APPswedish transgenic mice (Hosono et al. 2015). However, another study found up-regulated IDE protein levels in primary hippocampal neurons incubated with DHA as free

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fatty acid (Du et al. 2010). In our study increased Aβ-degradation in the presence of DHA-containing phospholipids is mediated by changes in IDE sorting, resulting in an elevated secretion of IDE into the extracellular space. By inhibition of exosome release we could further show that DHA at least partially affects the exosome release of IDE into the extracellular space. However, we cannot rule out that the used exosome release-inhibitor GW4869, which inhibits neutral sphingomyelinase itself, influences Aβ-degradation by other mechanisms than exosome secretion. To identify the exact mechanism how DHA influences the exosome release of IDE further studies are needed. In contrast, the sorting of IDE is not affected by EPA. More likely, extracellular and intracellular IDE levels trend to be elevated as a consequence of increased IDE gene expression in the presence of EPA-containing phospholipids. Similar to PC20:5/20:5, DHA-containing phospholipids also directly stimulated IDE enzyme activity. In contrast, palmitic (C16:0) and linoleic acid (C18:2) have been reported to inhibit IDE activity (Hamel et al. 2003). The PUFAs in PC20:5/20:5 and PC22:6/22:6 bound more efficiently to IDE compared to the corresponding saturated fatty acids in PC20:0/20:0 and PC22:0/22:0 *in-vitro*. Binding of fatty acids to IDE is further supported by a proposed cytosolic fatty acid binding protein signature within the IDE protein (Hamel et al. 2003). The co-immunoprecipitation experiments presented here are a proof of the concept that PUFAs bind more efficiently to IDE than saturated fatty acids. Under physiological conditions the phospholipids do not contain PUFAs in both the sn1- and the sn2-position and are mostly bound to lipoproteins, which might affect the interaction of the fatty acids to IDE.

In summary, EPA and DHA increase Aβ turnover by affecting the Aβ-degrading enzyme IDE (Figure 4). Both omega-3 PUFAs directly stimulate IDE activity. In addition EPA increases IDE gene expression whereas DHA affects IDE sorting resulting in elevated extracellular IDE

levels. Beside the positive effect of EPA and DHA on Aβ-degradation via IDE, DHA and EPA were found to increase Aβ clearance by stimulating microglial phagocytosis of Aβ42 (Hjorth et al. 2013), substantiating the finding that treatment with PUFAs, especially EPA and DHA, might protect against AD pathology by increasing Aβ turnover.

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Conflict of interest

The authors declare no conflict of interest.

References

Barberger-Gateau, P., Raffaitin, C., Letenneur, L., Berr, C., Tzourio, C., Dartigues, J.F., and Alperovitch, A. 2007. Dietary patterns and risk of dementia: the Three-City cohort study. Neurology **69**(20): 1921-1930. doi: 10.1212/01.wnl.0000278116.37320.52.

Boudrault, C., Bazinet, R.P., and Ma, D.W. 2009. Experimental models and mechanisms underlying the protective effects of n-3 polyunsaturated fatty acids in Alzheimer's disease. The Journal of nutritional biochemistry **20**(1): 1-10. doi: 10.1016/j.jnutbio.2008.05.016.

Bulloj, A., Leal, M.C., Xu, H., Castano, E.M., and Morelli, L. 2010. Insulin-degrading enzyme sorting in exosomes: a secretory pathway for a key brain amyloid-beta degrading protease. Journal of Alzheimer's disease : JAD **19**(1): 79-95. doi: 10.3233/JAD-2010-1206.

Calon, F., Lim, G.P., Yang, F., Morihara, T., Teter, B., Ubeda, O., Rostaing, P., Triller, A., Salem, N., Jr., Ashe, K.H., Frautschy, S.A., and Cole, G.M. 2004. Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. Neuron **43**(5): 633-645. doi: 10.1016/j.neuron.2004.08.013.

Chesneau, V., Vekrellis, K., Rosner, M.R., and Selkoe, D.J. 2000. Purified recombinant insulin-degrading enzyme degrades amyloid beta-protein but does not promote its oligomerization. The Biochemical journal **351 Pt 2**: 509-516.

Chiu, C.C., Su, K.P., Cheng, T.C., Liu, H.C., Chang, C.J., Dewey, M.E., Stewart, R., and Huang, S.Y. 2008. The effects of omega-3 fatty acids monotherapy in Alzheimer's disease and mild cognitive impairment: a preliminary randomized double-blind placebo-controlled study. Progress in neuro-psychopharmacology & biological psychiatry **32**(6): 1538-1544. doi: 10.1016/j.pnpbp.2008.05.015.

Conquer, J.A., Tierney, M.C., Zecevic, J., Bettger, W.J., and Fisher, R.H. 2000. Fatty acid analysis of blood plasma of patients with Alzheimer's disease, other types of dementia, and cognitive impairment. Lipids **35**(12): 1305-1312.

Draft

Du, J., Zhang, L., Liu, S., and Wang, Z. 2010. Palmitic acid and docosahexaenoic acid opposingly regulate the expression of insulin-degrading enzyme in neurons. Die Pharmazie **65**(3): 231-232.

Farris, W., Mansourian, S., Chang, Y., Lindsley, L., Eckman, E.A., Frosch, M.P., Eckman, C.B., Tanzi, R.E., Selkoe, D.J., and Guenette, S. 2003. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proceedings of the National Academy of Sciences of the United States of America **100**(7): 4162-4167. doi: 10.1073/pnas.0230450100.

Fernandez-Gamba, A., Leal, M.C., Morelli, L., and Castano, E.M. 2009. Insulin-degrading enzyme: structure-function relationship and its possible roles in health and disease. Current pharmaceutical design **15**(31): 3644-3655.

Freund-Levi, Y., Eriksdotter-Jonhagen, M., Cederholm, T., Basun, H., Faxen-Irving, G., Garlind, A., Vedin, I., Vessby, B., Wahlund, L.O., and Palmblad, J. 2006. Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial. Archives of neurology **63**(10): 1402-1408. doi: 10.1001/archneur.63.10.1402.

Green, K.N., Martinez-Coria, H., Khashwji, H., Hall, E.B., Yurko-Mauro, K.A., Ellis, L., and LaFerla, F.M. 2007. Dietary docosahexaenoic acid and docosapentaenoic acid ameliorate amyloid-beta and tau pathology via a mechanism involving presenilin 1 levels. The Journal of neuroscience : the official journal of the Society for Neuroscience **27**(16): 4385-4395. doi: 10.1523/JNEUROSCI.0055-07.2007.

Grimm, M.O., Grimm, H.S., Patzold, A.J., Zinser, E.G., Halonen, R., Duering, M., Tschape, J.A., De Strooper, B., Muller, U., Shen, J., and Hartmann, T. 2005. Regulation of cholesterol and sphingomyelin metabolism by amyloid-beta and presenilin. Nature cell biology **7**(11): 1118-1123. doi: 10.1038/ncb1313.

Draft

Grimm, M.O., Grosgen, S., Riemenschneider, M., Tanila, H., Grimm, H.S., and Hartmann, T. 2011a. From brain to food: analysis of phosphatidylcholins, lyso-phosphatidylcholins and phosphatidylcholin-plasmalogens derivates in Alzheimer's disease human post mortem brains and mice model via mass spectrometry. Journal of chromatography. A **1218**(42): 7713-7722. doi: 10.1016/j.chroma.2011.07.073.

Grimm, M.O., Haupenthal, V.J., Rothhaar, T.L., Zimmer, V.C., Grosgen, S., Hundsdorfer, B., Lehmann, J., Grimm, H.S., and Hartmann, T. 2013a. Effect of Different Phospholipids on alpha-Secretase Activity in the Non-Amyloidogenic Pathway of Alzheimer's Disease. International journal of molecular sciences **14**(3): 5879-5898. doi: 10.3390/ijms14035879.

Grimm, M.O., Kuchenbecker, J., Grosgen, S., Burg, V.K., Hundsdorfer, B., Rothhaar, T.L., Friess, P., de Wilde, M.C., Broersen, L.M., Penke, B., Peter, M., Vigh, L., Grimm, H.S., and Hartmann, T. 2011b. Docosahexaenoic acid reduces amyloid beta production via multiple pleiotropic mechanisms. The Journal of biological chemistry **286**(16): 14028-14039. doi: 10.1074/jbc.M110.182329.

Grimm, M.O., Lehmann, J., Mett, J., Zimmer, V.C., Grosgen, S., Stahlmann, C.P., Hundsdorfer, B., Haupenthal, V.J., Rothhaar, T.L., Herr, C., Bals, R., Grimm, H.S., and Hartmann, T. 2014. Impact of Vitamin D on amyloid precursor protein processing and amyloid-beta peptide degradation in Alzheimer's disease. Neuro-degenerative diseases **13**(2-3): 75-81. doi: 10.1159/000355462.

Grimm, M.O., Stahlmann, C.P., Mett, J., Haupenthal, V.J., Zimmer, V.C., Lehmann, J., Hundsdorfer, B., Endres, K., Grimm, H.S., and Hartmann, T. 2015. Vitamin E: Curse or Benefit in Alzheimer's Disease? A Systematic Investigation of the Impact of alpha-, gamma- and delta-Tocopherol on Ass Generation and Degradation in Neuroblastoma Cells. The journal of nutrition, health & aging **19**(6): 646-656. doi: 10.1007/s12603-015-0506-z.

Draft

Grimm, M.O., Zimmer, V.C., Lehmann, J., Grimm, H.S., and Hartmann, T. 2013b. The impact of cholesterol, DHA, and sphingolipids on Alzheimer's disease. BioMed research international **2013**: 814390. doi: 10.1155/2013/814390.

Hamel, F.G., Upward, J.L., and Bennett, R.G. 2003. In vitro inhibition of insulin-degrading enzyme by long-chain fatty acids and their coenzyme A thioesters. Endocrinology **144**(6): 2404-2408. doi: 10.1210/en.2002-0007.

Hardy, J., and Selkoe, D.J. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science **297**(5580): 353-356. doi: 10.1126/science.1072994.

Hardy, J.A., and Higgins, G.A. 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science **256**(5054): 184-185.

Hartmann, T., Kuchenbecker, J., and Grimm, M.O. 2007. Alzheimer's disease: the lipid connection. Journal of neurochemistry **103 Suppl 1**: 159-170. doi: 10.1111/j.1471-4159.2007.04715.x.

Hashimoto, M., Hossain, S., Shimada, T., Sugioka, K., Yamasaki, H., Fujii, Y., Ishibashi, Y., Oka, J., and Shido, O. 2002. Docosahexaenoic acid provides protection from impairment of learning ability in Alzheimer's disease model rats. Journal of neurochemistry **81**(5): 1084-1091.

Hashimoto, M., Katakura, M., Hossain, S., Rahman, A., Shimada, T., and Shido, O. 2011. Docosahexaenoic acid withstands the Abeta(25-35)-induced neurotoxicity in SH-SY5Y cells. The Journal of nutritional biochemistry **22**(1): 22-29. doi: 10.1016/j.jnutbio.2009.11.005.

Hashimoto, M., Shahdat, H.M., Yamashita, S., Katakura, M., Tanabe, Y., Fujiwara, H., Gamoh, S., Miyazawa, T., Arai, H., Shimada, T., and Shido, O. 2008. Docosahexaenoic acid disrupts in vitro amyloid beta(1-40) fibrillation and concomitantly inhibits amyloid levels in

Draft

cerebral cortex of Alzheimer's disease model rats. Journal of neurochemistry **107**(6): 1634-1646. doi: 10.1111/j.1471-4159.2008.05731.x.

Hashimoto, M., Tanabe, Y., Fujii, Y., Kikuta, T., Shibata, H., and Shido, O. 2005. Chronic administration of docosahexaenoic acid ameliorates the impairment of spatial cognition learning ability in amyloid beta-infused rats. The Journal of nutrition **135**(3): 549-555.

Hjorth, E., Zhu, M., Toro, V.C., Vedin, I., Palmblad, J., Cederholm, T., Freund-Levi, Y., Faxen-Irving, G., Wahlund, L.O., Basun, H., Eriksdotter, M., and Schultzberg, M. 2013. Omega-3 fatty acids enhance phagocytosis of Alzheimer's disease-related amyloid-beta42 by human microglia and decrease inflammatory markers. Journal of Alzheimer's disease : JAD **35**(4): 697-713. doi: 10.3233/JAD-130131.

Hooijmans, C.R., Pasker-de Jong, P.C., de Vries, R.B., and Ritskes-Hoitinga, M. 2012. The effects of long-term omega-3 fatty acid supplementation on cognition and Alzheimer's pathology in animal models of Alzheimer's disease: a systematic review and meta-analysis. Journal of Alzheimer's disease : JAD **28**(1): 191-209. doi: 10.3233/JAD-2011-111217.

Hooijmans, C.R., Van der Zee, C.E., Dederen, P.J., Brouwer, K.M., Reijmer, Y.D., van Groen, T., Broersen, L.M., Lutjohann, D., Heerschap, A., and Kiliaan, A.J. 2009. DHA and cholesterol containing diets influence Alzheimer-like pathology, cognition and cerebral vasculature in APPswe/PS1dE9 mice. Neurobiology of disease **33**(3): 482-498. doi: 10.1016/j.nbd.2008.12.002.

Hosono, T., Mouri, A., Nishitsuji, K., Jung, C.G., Kontani, M., Tokuda, H., Kawashima, H., Shibata, H., Suzuki, T., Nabehsima, T., and Michikawa, M. 2015. Arachidonic or Docosahexaenoic Acid Diet Prevents Memory Impairment in Tg2576 Mice. Journal of Alzheimer's disease : JAD **48**(1): 149-162. doi: 10.3233/JAD-150341.

Hossain, S., Hashimoto, M., Katakura, M., Miwa, K., Shimada, T., and Shido, O. 2009. Mechanism of docosahexaenoic acid-induced inhibition of in vitro Abeta1-42 fibrillation and

Draft

Abeta1-42-induced toxicity in SH-S5Y5 cells. Journal of neurochemistry **111**(2): 568-579. doi: 10.1111/j.1471-4159.2009.06336.x.

Jiang, Q., Lee, C.Y., Mandrekar, S., Wilkinson, B., Cramer, P., Zelcer, N., Mann, K., Lamb, B., Willson, T.M., Collins, J.L., Richardson, J.C., Smith, J.D., Comery, T.A., Riddell, D., Holtzman, D.M., Tontonoz, P., and Landreth, G.E. 2008. ApoE promotes the proteolytic degradation of Abeta. Neuron **58**(5): 681-693. doi: 10.1016/j.neuron.2008.04.010.

Jicha, G.A., and Markesbery, W.R. 2010. Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. Clinical interventions in aging **5**: 45-61.

Kadowaki, H., and Grant, M.A. 1995. Relationship of membrane phospholipid composition, lactosylceramide molecular species, and the specificity of CMP-N-acetylneuraminate:lactosylceramide alpha 2,3-sialyltransferase to the molecular species composition of GM3 ganglioside. Journal of lipid research **36**(6): 1274-1282.

Kotani, S., Sakaguchi, E., Warashina, S., Matsukawa, N., Ishikura, Y., Kiso, Y., Sakakibara, M., Yoshimoto, T., Guo, J., and Yamashima, T. 2006. Dietary supplementation of arachidonic and docosahexaenoic acids improves cognitive dysfunction. Neuroscience research **56**(2): 159-164. doi: 10.1016/j.neures.2006.06.010.

Kurochkin, I.V., and Goto, S. 1994. Alzheimer's beta-amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme. FEBS letters **345**(1): 33-37.

Li, J., Liu, K., Liu, Y., Xu, Y., Zhang, F., Yang, H., Liu, J., Pan, T., Chen, J., Wu, M., Zhou, X., and Yuan, Z. 2013. Exosomes mediate the cell-to-cell transmission of IFN-alpha-induced antiviral activity. Nature immunology **14**(8): 793-803. doi: 10.1038/ni.2647.

Lim, G.P., Calon, F., Morihara, T., Yang, F., Teter, B., Ubeda, O., Salem, N., Jr., Frautschy, S.A., and Cole, G.M. 2005. A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. The Journal of neuroscience :

Draft

the official journal of the Society for Neuroscience **25**(12): 3032-3040. doi: 10.1523/JNEUROSCI.4225-04.2005.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**(4): 402-408. doi: 10.1006/meth.2001.1262.

Masters, C.L., Simms, G., Weinman, N.A., Multhaup, G., McDonald, B.L., and Beyreuther, K. 1985. Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proceedings of the National Academy of Sciences of the United States of America **82**(12): 4245-4249.

Mett, J., Hartmann, T., and Grimm, M.O. 2016. The Effects of Glycerophospholipids and Fatty Acids on APP Processing: Implications for Alzheimer's Disease. Handbook of Lipids in Human Function: Fatty acids(Edited by Ronald Ross Watson and Fabien De Meester): 377-421. doi: 10.1016/B978-1-63067-036-8.00013-5.

Miller, B.C., Eckman, E.A., Sambamurti, K., Dobbs, N., Chow, K.M., Eckman, C.B., Hersh, L.B., and Thiele, D.L. 2003. Amyloid-beta peptide levels in brain are inversely correlated with insulysin activity levels in vivo. Proceedings of the National Academy of Sciences of the United States of America **100**(10): 6221-6226. doi: 10.1073/pnas.1031520100.

Miners, J.S., Barua, N., Kehoe, P.G., Gill, S., and Love, S. 2011. Abeta-degrading enzymes: potential for treatment of Alzheimer disease. Journal of neuropathology and experimental neurology **70**(11): 944-959. doi: 10.1097/NEN.0b013e3182345e46.

Morris, M.C., Evans, D.A., Bienias, J.L., Tangney, C.C., Bennett, D.A., Wilson, R.S., Aggarwal, N., and Schneider, J. 2003. Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease. Archives of neurology **60**(7): 940-946. doi: 10.1001/archneur.60.7.940.

Draft

Muskiet, F.A., van Goor, S.A., Kuipers, R.S., Velzing-Aarts, F.V., Smit, E.N., Bouwstra, H., Dijck-Brouwer, D.A., Boersma, E.R., and Hadders-Algra, M. 2006. Long-chain polyunsaturated fatty acids in maternal and infant nutrition. Prostaglandins, leukotrienes, and essential fatty acids **75**(3): 135-144. doi: 10.1016/j.plefa.2006.05.010.

Perez, A., Morelli, L., Cresto, J.C., and Castano, E.M. 2000. Degradation of soluble amyloid beta-peptides 1-40, 1-42, and the Dutch variant 1-40Q by insulin degrading enzyme from Alzheimer disease and control brains. Neurochemical research **25**(2): 247-255.

Qiu, W.Q., Walsh, D.M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M.B., Rosner, M.R., Safavi, A., Hersh, L.B., and Selkoe, D.J. 1998. Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. The Journal of biological chemistry **273**(49): 32730-32738.

Qiu, W.Q., Ye, Z., Kholodenko, D., Seubert, P., and Selkoe, D.J. 1997. Degradation of amyloid beta-protein by a metalloprotease secreted by microglia and other neural and non-neural cells. The Journal of biological chemistry **272**(10): 6641-6646.

Rothhaar, T.L., Grosgen, S., Haupenthal, V.J., Burg, V.K., Hundsdorfer, B., Mett, J., Riemenschneider, M., Grimm, H.S., Hartmann, T., and Grimm, M.O. 2012. Plasmalogens inhibit APP processing by directly affecting gamma-secretase activity in Alzheimer's disease. TheScientificWorldJournal **2012**: 141240. doi: 10.1100/2012/141240.

Saido, T., and Leissring, M.A. 2012. Proteolytic degradation of amyloid beta-protein. Cold Spring Harbor perspectives in medicine **2**(6): a006379. doi: 10.1101/cshperspect.a006379.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Analytical biochemistry **150**(1): 76-85.

Soderberg, M., Edlund, C., Kristensson, K., and Dallner, G. 1991. Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. Lipids **26**(6): 421-425.

Draft

Sudoh, S., Frosch, M.P., and Wolf, B.A. 2002. Differential effects of proteases involved in intracellular degradation of amyloid beta-protein between detergent-soluble and -insoluble pools in CHO-695 cells. Biochemistry **41**(4): 1091-1099.

Torres, M., Price, S.L., Fiol-Deroque, M.A., Marcilla-Etxenike, A., Ahyayauch, H., Barcelo-Coblijn, G., Teres, S., Katsouri, L., Ordinas, M., Lopez, D.J., Ibarguren, M., Goni, F.M., Busquets, X., Vitorica, J., Sastre, M., and Escriba, P.V. 2014. Membrane lipid modifications and therapeutic effects mediated by hydroxydocosahexaenoic acid on Alzheimer's disease. Biochimica et biophysica acta **1838**(6): 1680-1692. doi: 10.1016/j.bbamem.2013.12.016.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B., and Simons, M. 2008. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science **319**(5867): 1244-1247. doi: 10.1126/science.1153124.

Tully, A.M., Roche, H.M., Doyle, R., Fallon, C., Bruce, I., Lawlor, B., Coakley, D., and Gibney, M.J. 2003. Low serum cholesteryl ester-docosahexaenoic acid levels in Alzheimer's disease: a case-control study. The British journal of nutrition **89**(4): 483-489. doi: 10.1079/BJN2002804.

Vekrellis, K., Ye, Z., Qiu, W.Q., Walsh, D., Hartley, D., Chesneau, V., Rosner, M.R., and Selkoe, D.J. 2000. Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. The Journal of neuroscience : the official journal of the Society for Neuroscience **20**(5): 1657-1665.

Zhao, J., Li, L., and Leissring, M.A. 2009. Insulin-degrading enzyme is exported via an unconventional protein secretion pathway. Molecular neurodegeneration **4**: 4. doi: 10.1186/1750-1326-4-4.

Draft

Figure Legends

Figure 1: Influence of EPA and DHA on Aβ-degradation. Mouse neuroblastoma N2a cells (-) and N2a IDE knock-down cells (+) were incubated with PC20:5/20:5 or PC22:6/22:6. As control, cells were treated with PC20:0/20:0 or PC22:0/22:0. Aβ-degradation was determined by addition of human synthetic Aβ peptides to the cell culture medium and western blot analysis of remaining not-degraded Aβ peptides with the antibody W02 (N2a cells, PC20:5/20:5: remaining Aβ = 66.4 % ± 7.1 %, $p \leq 0.001$; N2a cells, PC22:6/22:6: remaining Aβ = 39.5 % ± 3.2 %, $p \leq 0.001$; N2a IDE knock-down cells, PC20:5/20:5: remaining Aβ = 113.1 % ± 4.4 %, $p = 0.669$; N2a IDE knock-down cells, PC22:6/22:6: remaining Aβ = 105.4 % ± 3.0 %, $p = 0.918$).

All quantified data represent an average of at least three independent experiments. Error bars represent standard error of the mean. Asterisks show the statistical significance compared to control (* $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$).

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Figure 2: Effect of EPA and DHA on IDE gene expression and cellular sorting of IDE. **(a)** IDE gene expression determined via RT-PCR in the presence of PC20:5/20:5 and PC22:6/22:6 in comparison to PC20:0/20:0 or PC22:0/22:0 (PC20:5/20:5: IDE gene expression = 117.6 % ± 3.6 %, $p = 0.003$; PC22:6/22:6: IDE gene expression = 106.7 % ± 11.4 %, $p = 0.567$). **(b)** Influence of PC20:5/20:5 compared to PC20:0/20:0 on IDE promoter activity using luciferase as reporter gene regulated by the IDE promoter region (PC20:5/20:5: IDE promotor activity = 116.7 % ± 1.4 %, $p = 0.003$). **(c)** Influence of PC20:5/20:5 and PC22:6/22:6 on extracellular and intracellular IDE level in comparison to PC20:0/20:0 or PC22:0/22:0. Extracellular and intracellular IDE proteins were determined by western blot analysis using the antibody ST1120 (PC20:5/20:5: extracellular IDE level = 134.6 % ± 22.1

%, p = 0.26; intracellular IDE level = 116.5 % ± 3.6 %, p = 0.05; ratio extracellular/intracellular IDE level = 109.5 % ± 18.9 %, p = 0.77; PC22:6/22:6: extracellular IDE level = 128.3 % ± 5.6 %, p = 0.01; intracellular IDE level = 79.3 % ± 7.7 %, p = 0.09; ratio extracellular/intracellular IDE level = 161.5 % ± 12.2 %, p = 0.03). **(d)** Influence of PC22:6/22:6 compared to PC22:0/22:0 on Aβ-degradation, determined as described for Figure1, in the presence of the exosome release inhibitor GW4869 or the solvent DMSO (PC22:6/22:6/DMSO: remaining Aβ = 54.5 % ± 2.2 %, p ≤ 0.001; PC22:6/22:6/GW4869: remaining Aβ = 72.7 % ± 4.3 %, p = 0.004). Analysis and statistical significance as described for Figure 1.

Figure 3: (a) Direct effect of EPA and DHA on IDE enzyme activity. *In vitro* assay measuring IDE enzyme activity by the use of purified IDE enzyme in the presence of PC20:5/20:5 and PC22:6/22:6 or of PC containing the corresponding saturated fatty acids (PC20:5/20:5: IDE activity = 268.2 % ± 16.3 %, p ≤ 0.001; PC22:6/22:6: IDE activity = 520.8 % ± 63.7 %, p ≤ 0.001). **(b) Binding properties of polyunsaturated fatty acids (PUFA) and saturated fatty acids to IDE**. *In vitro*, equimolar amounts of PC20:0/20:0 and PC20:5/20:5 or PC22:0/22:0 and PC22:6/22:6 were incubated with recombinant IDE protein. Phospholipids co-immunoprecipitated (IP) with IDE were determined by mass spectrometry analysis (ratio PC20:5/20:5 to PC20:0/20:0 = 285.7 % ± 22.1 %, p ≤ 0.001; ratio PC22:6/22:6 to PC22:0/22:0 = 242.9 % ± 22.5 %, p = 0.004). Analysis and statistical significance as described for Figure 1.

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Figure 4: Summary of the mechanisms involved in enhanced Aβ-degradation in the presence of EPA and DHA.

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figure 1

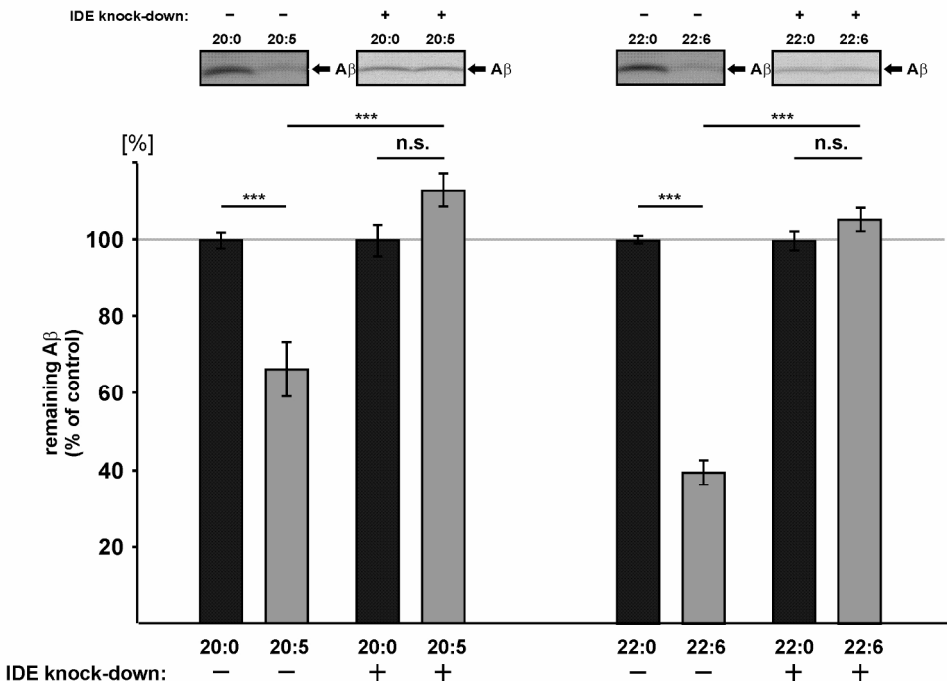

Figure 1

figure 2

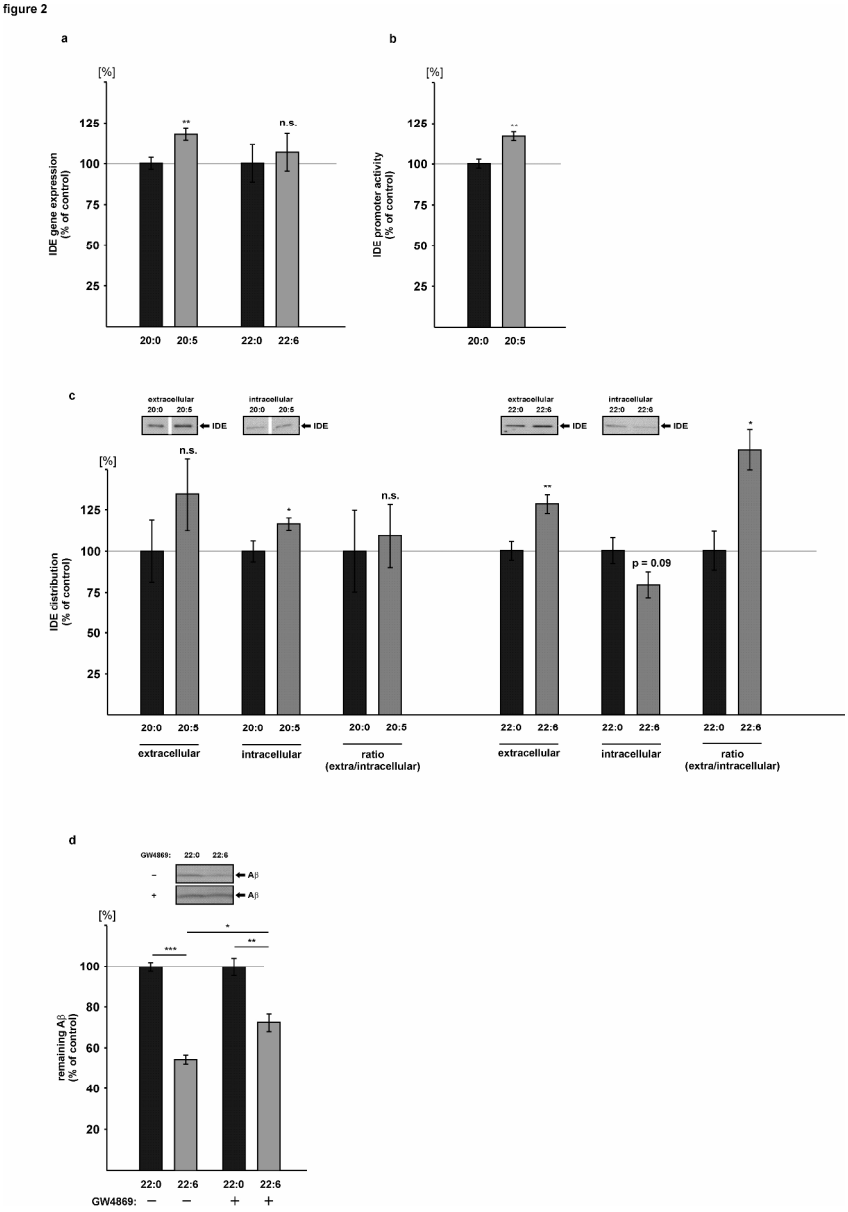

Figure 2

figure 3

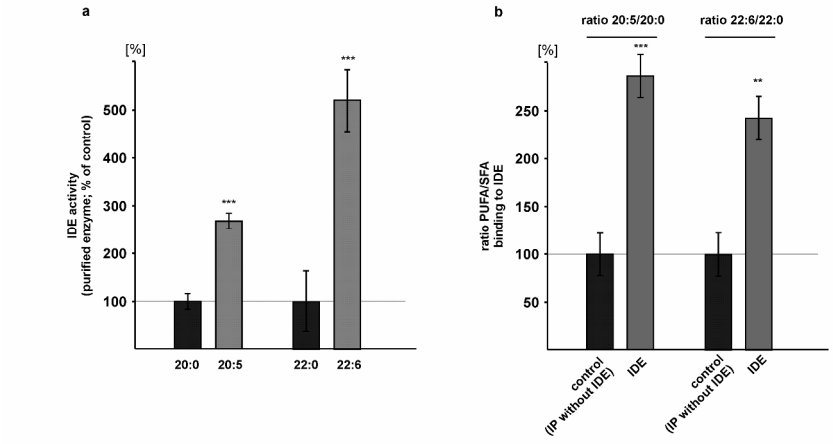

Figure 3

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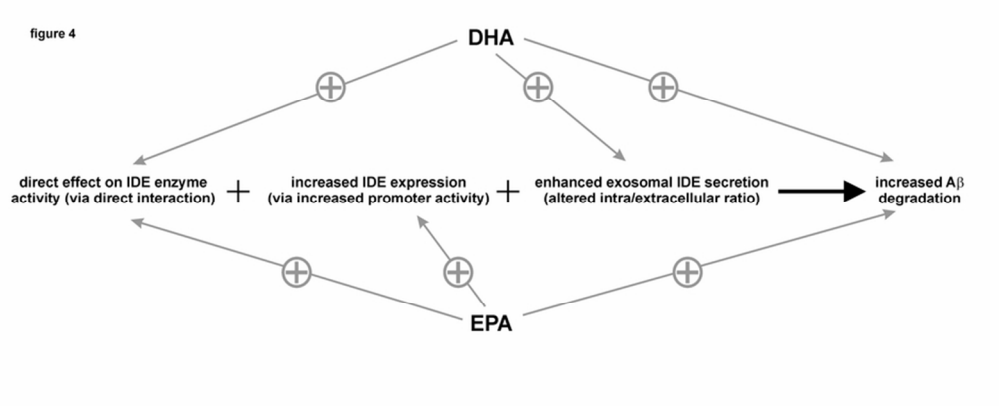

Figure 4
79x32mm (300 x 300 DPI)

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