Low-dose γ-secretase inhibition increases secretion of Aβ peptides and intracellular oligomeric Aβ

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Abstract

γ-Secretase inhibitors have been considered promising drug candidates against Alzheimer's disease (AD) due to their ability to reduce amyloid-β (Aβ) production. However, clinical trials have been halted due to lack of clinical efficacy and/or side effects. Recent *in vitro* studies suggest that low doses of γ-secretase inhibitors may instead increase Aβ production. Using a stem cell-derived human model of cortical neurons and low doses of the γ-secretase inhibitor DAPT, the effects on a variety of Aβ peptides were studied using mass spectrometry. One major focus was to develop a novel method for specific detection of oligomeric Aβ (oAβ), and this was used to study the effects of low-dose γ-secretase inhibitor treatment on intracellular oAβ accumulation. Low-dose treatment (2 and 20 nM) with DAPT increased the production of several Aβ peptides. Furthermore, using the novel method for oAβ detection, we found that 2 nM DAPT treatment of cortical neurons resulted in increased oAβ accumulation. Thus, low dose-treatment with DAPT causes both increased production of long, aggregation-prone Aβ peptides, and accumulation of intracellular Aβ oligomers, both believed to contribute to AD pathology.

Keywords: amyloid-β, γ-secretase, DAPT, Duolink, neurons, oligomers

Introduction

Plaques, composed of the aggregation-prone peptide amyloid-β (Aβ), are key pathological hallmarks of Alzheimer's disease (AD). Ever since the amyloid cascade hypothesis was proposed in 1991 (Hardy & Allsop, 1991), much research on AD pathogenesis has focused on the mechanisms behind Aβ generation and degradation. In line with this, several drug candidates have been developed, aiming at reducing Aβ production by inhibiting or modulating the key enzymes involved in the production of Aβ. Aβ is generated by sequential cleavage of the amyloid precursor protein (APP) by β-secretase followed by γ-secretase (Blennow et al, 2006), and therefore both β- and γ-secretase inhibitors have been proposed as possible treatments for AD.

The first γ-secretase inhibitor (GSI) showing *in vivo* reduction of Aβ in the brain was N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Dovey et al, 2001), although failing to reach clinical testing. It has since then been widely used *in vitro* to study different aspects of APP cleavage and Aβ generation. Other GSIs, such as semagacestat (also called LY-450139) and avagacestat, have been developed thereafter, but the phase III trial with semagacestat and the phase II trial with avagacestat were struck by side effects (Coric et al, 2012; Imbimbo et al, 2011), and even leading to deteriorating cognition (Doody et al, 2013). γ-Secretase has many other substrates besides APP, including NOTCH, a protein important during neuronal development and maintenance (Imayoshi et al, 2010). The inhibition of NOTCH cleavage may therefore be one cause for the severe side effects of GSIs (Henley et al, 2014), as could accumulation of toxic APP cleavage products (Mitani et al, 2012). In addition, upon treatment with GSIs, it has been reported that the initial decrease in Aβ levels in blood is followed by an increase in Aβ levels, as the drug concentration in plasma decreases (De Strooper, 2014). Interestingly, both *in*

vivo and *in vitro* studies have shown that low doses of both semagacestat and DAPT increase Aβ levels, instead of decreasing them (Barnwell et al, 2014; Lanz et al, 2006).

Because the Aβ peptide is hydrophobic, accumulation causes it to aggregate into soluble oligomers and further on into insoluble fibrils, the constituent of Aβ plaques (Blennow et al, 2006). It has been reported that deposits of extracellular Aβ do not correlate with cognitive symptoms and that Aβ plaques may be present several years before symptom onset in AD (Perrin et al, 2009). Additionally, compact plaques consisting of Aβ fibrils are found in non-demented individuals (Guillozet et al, 2003), and it is now believed that the soluble oligomeric Aβ (oAβ) is the most toxic species (Benilova et al, 2012). Soluble forms of synthetic as well as naturally produced oAβ are toxic to cells *in vitro* and *in vivo* (Hoshi et al, 2003; Lambert et al, 1998; Takuma et al, 2004), and have the ability to disrupt cognitive function (Cleary et al, 2005). Due to the importance of oAβ in AD pathogenesis, there is a need for reliable methods to detect oAβ. Previously, oligomer-specific antibodies such as the polyclonal anti-oligomer antibody A11, have been developed. However, this antibody also detects oligomers from other proteins such as insulin, prion protein and lysozyme (Kayed et al, 2003). Different dyes, such as congo red and thioflavin S/T stain Aβ fibrils and protofibrils, but do not recognize smaller oligomers of Aβ (Walsh et al, 1999). In addition, small probes (luminescent conjugated oligothiophenes; LCOs) have been shown to bind to aggregated Aβ in diffuse plaques (Nilsson et al, 2006), and seem to detect smaller Aβ aggregates than traditional dyes, but are not specific for Aβ aggregates. It is therefore of importance to develop new sensitive and specific methods for detection of oAβ. Recently, a method based on the proximity ligation

assay (PLA) for detection of α-synuclein oligomers was described (Roberts et al, 2015). Here, we used the same approach to develop an assay for detection and quantification of intracellular oAβ. This oAβ method, along with immunochemical quantification of Aβx-38, Aβx-40, and Aβx-42 and immunoprecipitation-mass spectrometry (IP-MS) analysis of secreted Aβ peptides, was used to examine the effects of low-dose γ-secretase inhibition on production of a variety of Aβ peptides and the potential accumulation of intracellular oAβ in a previously established human neuronal model (Shi et al, 2012) that produces all relevant Aβ peptides (Bergström et al, 2016).

Results

Low-dose γ-secretase inhibition increases secretion of Aβ42 without affecting intracellular C-terminal fragment of APP

To test the hypothesis that low-dose γ-secretase inhibition increases production of long Aβ peptides, human induced pluripotent stem cell (iPSC)-derived cortical neurons were treated with 2, 20 or 200 nM DAPT for 48 hours. Thereafter, cell culture media were collected and analysed for secreted Aβx-38, Aβx-40, and Aβx-42. Treatment with 2 or 20 nM DAPT tended to increase the secreted levels of Aβx-38 (Figure 1A) and Aβx-40 (Figure 1B) compared with control, although the difference did not reach statistical significance, while secretion of Aβx-42 (Figure 1C) was significantly increased upon treatment with 2 and 20 nM DAPT. Treatment with 200 nM DAPT significantly decreased the secretion of all three peptides compared with control. This shows that low-dose treatment with the γ-secretase inhibitor DAPT increases the secretion of all Aβ peptides quantified, although only

reaching statistical significance for Aβx-42. A higher concentration (200 nM) decreased the production of all three Aβ peptides.

In addition, the effect of DAPT treatment on the Aβx-42/38 and Aβx-42/40 ratios was also investigated. Treatment with 20 nM DAPT increased the Aβ42/38 ratio, which was further increased upon treatment with 200 nM DAPT (Figure 1D). Treatment with 200 nM DAPT also significantly increased the Aβx-42/40 ratio (Figure 1E). To investigate if the increased secretion of Aβ was accompanied by a change in the APP C-terminal fragment (the substrate for γ-secretase), western blot analysis was performed on cell lysates. While treatment with 2 or 20 nM DAPT did not change the levels of intracellular C-terminal fragments compared with control, they were significantly increased upon 200 nM DAPT treatment (Figure 1F).

Low-dose γ-secretase inhibition affects different Aβ peptides differently and dose-dependently

A more detailed investigation of the effects of low-dose GSI treatment on Aβ peptide secretion, was performed by analysing conditioned cell culture media using immunoprecipitation (IP) followed by mass spectrometry (MS). The relative levels of all secreted peptides were calculated by dividing the peak area of each peptide by the total peak areas for all peptides detected. Relative secretion levels of the short Aβ1-16 (Figure 2A) was not affected by 2 or 20 nM DAPT but was, as expected (Portelius et al, 2009), increased when cells were exposed to 200 nM DAPT. This suggests that γ-secretase is not involved in the production of Aβ1-16, but rather that the decrease in γ-secretase activity allows for an increase of other secretases. In contrast, Aβ1-17 was significantly decreased upon treatment with 200 nM DAPT, with a trend towards

decrease upon 20 nM DAPT treatment (Figure 2B). A similar tendency was seen for Aβ1-19 (Figure 2C), although not reaching statistical significance, indicating that production of these two peptides can be γ-secretase-dependent. Interestingly, Aβ1-20 had a similar secretion pattern as Aβ1-16, with a tendency to increase dose-dependently upon DAPT treatment (Figure 2D). Secretion of Aβ1-33 was not significantly decreased by low doses of DAPT, but completely abolished upon treatment with 200 nM (Figure 2E). A direct dose-dependent decrease in secretion of Aβ1-34 (Figure 2F) was seen upon treatment with increasing doses of DAPT. The longer peptides Aβ5-40 (Figure 2G), Aβ1-37 (Figure 2H), Aβ1-38 (Figure 2I), Aβ1-39 (Figure 2J), Aβ1-40 (Figure 2K) and Aβ1-42 (Figure 2L) all follow the same trend, however statistically non-significant, towards increased secretion upon treatment with 2 and 20 nM DAPT, which was strongly reduced upon treatment with 200 nM DAPT. This suggests that one set of short Aβ peptides, especially Aβ1-16, increases upon DAPT treatment. Other shorter peptides tend to decrease already at low doses of DAPT, being significant for Aβ1-34, whereas longer Aβ peptides, starting with Aβ1-37, only decrease with high doses. It has earlier been shown that Aβ1-34 is the Aβ peptide that is most sensitive to γ-secretase inhibition (Portelius et al, 2010). Indeed, we found a dose-dependent decrease in relative Aβ1-34 secretion, starting already at 2 nM DAPT. In addition, γ-secreatse dependent cleavage of Notch results in expression of a number of Notch response genes, including the *HES*-genes (Kageyama et al, 2008). We measured the expression of the neuronal-relevant *HES* gene *HES1* upon treatment with 2, 20 and 200 nM DAPT and found that all three doses decreased *HES1* expression to a similar level, compared to control

(Supplementary figure 1). Taken together, this shows that as low concentration as 2 nM DAPT is enough to reduce γ-secretase activity.

A novel method to detect oligomeric Aβ

As low doses of the γ-secretase inhibitor DAPT increase the production/secretion of longer, more aggregation-prone Aβ peptides and decrease, or do not affect, the production of shorter, we were interested in investigating the effects of low-dose γ-secretase inhibition on intracellular oAβ accumulation. A novel method for oAβ detection was developed using proximity ligation assay (PLA; Duolink), by conjugating PLUS and MINUS Duolink probes to the N-terminal specific Aβ antibody 82E1 (Horikoshi et al, 2004). The method was optimized on oAβ1-42 (Supplementary figure 1A) fed to SH-SY5Y cells, as previously described (Domert et al, 2014). Using immunocytochemistry, it was evident that the 82E1 antibody was able to detect intracellular Aβ1-42 (Supplementary figure 1B, first panel), but as predicted, the control peptide Aβ42-1 was not detected (Supplementary figure 1B, second panel). SH-SY5Y cells were thereafter fed with increasing concentrations of oAβ1-42 (125, 250 and 500 nM) and re-seeded to contain only intracellular oAβ. Using PLUS and MINUS probe-conjugated 82E1 antibody to visualize the oAβ, we found that intracellular Aβ oligomers could be detected in cells fed with 125 nM oAβ (Figure 3A, first panel). Increasing concentrations of oAβ increased the number of positive Duolink spots dose-dependently (Figure 3A, second and third panels). Staining of the reverse peptide Aβ42-1 only resulted in very few positive Duolink spots (Figure 3A, last panel), regarded as background staining.

Quantification of the number of Duolink spots (one spot representing one oligomeric Aβ assembly) was performed using the Volocity software, and calculated as number of Duolink spots per cell. Already at 125 nM oAβ, the number of spots per cell increased significantly compared with the control peptide Aβ42-1 (Figure 3B), and there were significant differences between all concentrations tested. This shows that the Duolink method successfully detects intracellular oAβ at as low concentrations as 125 nM.

To test that the Duolink method does not detect monomeric Aβ, SH-SY5Y cells were also fed with the non-oligomerizing peptide Aβ1-15. However, this peptide was not efficiently taken up by the SH-SY5Y cells, and could not be detected by immunocytochemistry using the 82E1 antibody (Supplementary figure 1B, third panel). Instead, pure protein solutions of oAβ1-42 or Aβ1-15 were added to glass slides and let to dry. Samples were incubated with Duolink probes and reagents, followed by immunocytochemistry to detect both oligomeric and monomeric Aβ. While monomeric Aβ1-15 was detected with immunocytochemistry, using the 82E1 antibody, the Duolink method did not give a positive signal (Supplementary figure 1C, upper panels). On the contrary, oAβ1-42 was successfully detected with both immunocytochemistry and Duolink (Supplementary figure 1C, lower panels). In conclusion, this shows that oligomeric Aβ can be specifically and sensitively detected using the Duolink method.

Low-dose γ-secretase inhibition causes intracellular accumulation of oligomeric Aβ in cortical neurons

iPSC-derived neurons were treated with low concentrations of DAPT and thereafter analysed for oAβ using the Duolink method described above. A few Duolink spots were seen in control cells (Figure 4A, first panel), whereas an increased number of Duolink spots were seen in cells treated with 2 nM of DAPT (Figure 4A, second panel). Treatment with 20 or 200 nM DAPT did not seem to increase the number of Duolink spots compared with control (Figure 4A, third and fourth panel). Analysis of the number of Duolink spots per neuron showed that treatment with 2 nM DAPT almost doubled the number of Duolink spots per cell compared with control. There was no difference between control cells and cells treated with 20 or 200 nM DAPT (Figure 4B). In addition, we measured the levels of secreted oAβ, and found that iPSC-derived cortical neurons secreted measurable levels of oAβ (around 60-100 fM), but that DAPT treatment did not affect secretion (Supplementary figure 3). This suggests that treatment of neurons with low doses of the γ-secretase inhibitor DAPT not only results in increased secretion of long, aggregation prone Aβ peptides, but also causes an intracellular accumulation of oligomeric Aβ.

Discussion

The failed clinical trials based on γ-secretase inhibition have led to an abandonment of these drugs in AD drug development. It is, however, still important to fully investigate the effect of partial γ-secretase inhibition, as is seen when blood and brain concentrations of γ-secretase inhibitors fluctuate due to once-a-day administration (De Strooper, 2014). In this study, we performed a detailed investigation of the effects of partial γ-secretase inhibition on secreted Aβ peptides, as well as on intracellular oAβ accumulation, using a human iPSC-derived neuronal cell model.

We found that low-dose γ-secretase inhibition in human iPSC-derived cortical neurons tended to increase the relative levels of longer Aβ peptides (including all peptides ending at amino acid 37 through 42), and decreased the relative levels of shorter non-aggregation prone peptides such as Aβ1-34. Furthermore, treatment with 2 nM DAPT increased the number of intracellular oAβ aggregates, which are suggested to function as seeds for further Aβ aggregation and plaque formation (Gouras et al, 2010).

Quantitative analysis of Aβx-38/40/42 indicated an increase in secretion of Aβx-42 when cells were treated with 2 and 20 nM DAPT, whereas Aβx-38 and Aβx-40 did not increase significantly. The ratio of Aβ42 over Aβ38 was significantly increased upon treatment with 20 nM DAPT, whereas the Aβ42/40 ratio was unchanged. Interestingly, although the highest dose of DAPT tested decreased the secretion of Aβ38/40/42, the ratio of both Aβ42/38 and Aβ42/40 increased, suggesting that the effect of γ-secretase inhibition is greater on shorter Aβ peptides also at higher concentrations. In support of this, it was shown that semagacestat more effectively inhibited production of Aβ1-40 than Aβ1-42 in a human trial (Lanz et al, 2006). Taken together, these results suggest that GSIs may tilt the ratio towards longer, more aggregation-prone Aβ peptides.

To further investigate the effects of low-dose DAPT treatment on neurons, we also performed IP-MS, enabling simultaneous detection of a variety of secreted Aβ peptides (Portelius et al, 2007). It showed three different types of Aβ peptide secretion patterns upon DAPT treatment. The shortest peptide, Aβ1-16, was not affected by 2 or 20 nM DAPT, but increased dramatically upon 200 nM DAPT treatment, further supporting that generation of this peptide is γ-secretase-independent

(Portelius et al, 2010). A similar trend was seen for the Aβ1-20 peptide, suggesting that generation of neither peptide involves γ-secretase cleavage. We have earlier shown that Aβ1-16 is produced by concerted α/β-secretase cleavage (Portelius et al, 2011), and a similar mechanism could be true for Aβ1-20. However, both a second β-secretase (BACE2) and insulin-degrading enzyme have been suggested to cleave Aβ at position 20 (Andreasson et al, 2007). Reduced γ-secretase activity could leave more APP C-terminal fragment available for these alternative, normally less common, cleavage pathways.

A dose-dependent decrease in secretion was seen for Aβ1-34 for all doses tested, and a similar trend was observed for Aβ1-17 and 1-19 upon treatment with 20 and 200 nM DAPT. This suggests that cleavage of these peptides is very sensitive to γ-secretase inhibition (Portelius et al, 2010). All is not known regarding the generation of these peptides, but it has been suggested that Aβ1-34 is generated by a second BACE1 cleavage, requiring a preceding γ-secretase cleavage upstream (Portelius et al, 2014), which our data supports.

A third group of Aβ peptides, starting at Aβ5-40 and including all longer peptides up until Aβ1-42 all had a general tendency to increase upon treatment with 2 and 20 nM DAPT. The relative levels of all these peptides were significantly decreased upon treatment with 200 nM DAPT, supporting the results obtained by the quantification of Aβx-38/40/42.

We hypothesised that partial inhibition of γ-secretase would result in increased levels of longer Aβ peptides, as the step-wise removal of amino acids from the C-terminal end of Aβ would be hampered, based on the finding that cleavage of APP by γ-secretase starts at the ζ- and ε-sites (resulting in 46 or 49 amino acid long peptides)

(Zhang et al, 2012). Thereafter amino acids are removed step by step by γ-secretase, resulting in Aβ peptides varying from 38-43 amino acids. γ-Secretase inhibition of CHO cells overexpressing the C-terminal fragment of APP resulted in increased levels of Aβ43 and Aβ46, whereas Aβ40 decreased (Yagishita et al, 2006). Although we found a relative increase in all Aβ peptides from 37 amino acids of length, the increased Aβ42/38 ratio at 20 nM, and both Aβ42/38 and Aβ42/40 at 200 nM DAPT treatment could further support this idea.

As oAβ is thought to play an important role in AD pathogenesis (Benilova et al, 2012), there is a need for specific and sensitive methods for oAβ detection in cells and tissue. Several methods have been developed for detection of oligomeric and fibrillar Aβ, although most being conformational specific and lacking specificity for Aβ (Kayed et al, 2003), or only recognizing larger Aβ aggregates (Walsh et al, 1999). We therefore developed a novel method, based on proximity ligation, utilizing the N-terminal specificity of the 82E1 antibody, together with the epitope blocking upon binding (Horikoshi et al, 2004). Therefore, signals detected were specific for binding of two 82E1 antibodies, conjugated to one PLUS and one MINUS probe respectively. However, due to the even ratio of PLUS and MINUS probe labelled antibody, there is also a chance of two PLUS or two MINUS probes binding in close proximity, resulting in detection of only 50% of the possible oligomers. In spite of this, we could detect intracellular Aβ from cells fed with a starting concentration of 125 nM Aβ, hence being both a specific and sensitive assay for oAβ detection.

Intracellular accumulation of Aβ has for long been suggested to play a role in AD pathogenesis (Ferreira et al, 2007), with endosomes being a suggested site for Aβ production (Small & Gandy, 2006). The acidic environment within the endosomes

could favour oligomerization, as could an increased production of longer, aggregation-prone Aβ peptides. We therefore went further to investigate the effects of low-dose γ-secretase treatment on intracellular accumulation of oAβ. Treatment with 2 nM DAPT resulted in an increased number of Duolink-positive oAβ spots per cell. This effect was abolished already at 20 nM DAPT and no difference was seen between 200 nM compared to control. There may be several explanations for this specific effect of 2 nM DAPT. It was recently reported that presenilin (PSEN)1 and PSEN2, the two variants of the catalytically active subunit of γ-secretase, cleave Aβ in different manners. PSEN1 cleavage also takes place at the plasma membrane, resulting in secretion of Aβ, whereas the majority of PSEN2 cleavage takes place intracellularly in late endosomes and lysosomes, resulting in intracellular accumulation of Aβ (Sannerud et al, 2016). DAPT might, at low doses, predominantly inhibit PSEN1 and therefore increase the amounts of APP undergoing intracellular Aβ production by PSEN2, hence causing oligomerization. One could also speculate that γ-secretase inhibition generally affects intracellular and membrane-bound γ-secretase differently, resulting in this discrepancy. It would of course be interesting to repeat these experiments using other GSIs as well. However, as the main focus of the study was to develop a method for oAβ detection and to verify this in a neuronal model, this will be the scope for a future study.

In conclusion, this study shows that exposure of human iPSC-derived neurons to low nanomolar concentrations of DAPT induces biochemical changes thought to contribute to AD pathology. This includes increased production of long, aggregation-prone Aβ peptides, together with decreased relative levels of shorter Aβ peptides, as well as accumulation of intracellular oAβ. These effects could contribute to

explaining why there were no positive effects upon treatment with γ-secretase inhibitors in clinical trials.

Material and methods

Induced pluripotent stem cells and neuronal differentiation

The human iPS cells (iPSCs) were reprogrammed from fibroblasts obtained from a healthy donor as described previously (Sposito et al, 2015). The iPSCs were differentiated towards cortical neurons according to the protocol by Shi *et al*. (2012), as previously described (Bergstrom et al., 2016). One iPSC line was used in the experiments, and the experiments were performed using three separate differentiations.

DAPT treatment and sample collection

Neurons differentiated for 75-90 days were treated with 2, 20 or 200 nM N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sigma-Aldrich) in 2 ml of neural maintenance media (NMM) for 48 hours. DMSO (Sigma-Aldrich) was used as vehicle control. After 48 hours of DAPT treatment, conditioned NMM was collected and centrifuged at 360g for five min before the supernatant was transferred to new tubes and stored at -80 °C. For protein analysis, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), removed by scraping, centrifuged at 400g, and DPBS was removed before storage of cell pellet at -80 °C until further analysis. For mRNA analysis, cells were washed once in DPBS and thereafter lysed directly in the well by adding 600 µl buffer RLT supplemented with 4mM dithiothreitol (DTT). Extraction of total RNA and cDNA synthesis was performed as described previously (Bergström et al, 2016).

SH-SY5Y cell culturing

SH-SY5Y neuroblastoma cells (ETCC, Sigma-Aldrich) were cultured in DMEM/F12 medium (Life Technologies) supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich) and 1 % Penicillin/Streptomycin (Hyclone, GE Health Care) in a humified chamber at 37 °C with 5 % CO_2. The cells were differentiated using 10 μM retinoic acid (RA; Sigma-Aldrich) 5-7 days prior to experiments.

Immunochemical quantification of Aβ peptides

Media concentrations of Aβx-38/40/42 were measured using the MSD Human (6E10) Abeta Triplex Assay as described by the manufacturer (Meso Scale Discovery). This assay employs C-terminally specific antibodies to capture Aβx-38/40/42, respectively, and the 6E10 antibody in combination with a SULFO-TAG-labelled anti-6E10 to quantify the peptides. The limit of detection was set to the value of the lowest standard point.

Immunoprecipitation and mass spectrometry

Cell-conditioned media collected throughout differentiation was investigated for Aβ peptides using IP-MS as described previously (Portelius et al, 2007) Briefly, 4 μg of the anti-Aβ antibodies 6E10 and 4G8 (Biolegend) was separately added to 50 μl each of magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen) and used for IP-MS measurements were performed using a Bruker Daltonics UltraFleXtreme matrix-assisted-laser-desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) instrument or a Bruker Daltonics AutoFlex MALDI TOF (Bruker Daltonics). All samples were analysed in duplicate. The area of each peak was normalized against the

sum of all areas for all Aβ peaks in the spectrum (providing a relative MALDI signal).

Quantitative PCR

Quantitative PCR was performed using inventoried TaqMan Gene Expression Assays with FAM reporter dye in TaqMan Universal PCR Master Mix II with UNG (Applied Biosystems) according to protocol, but in a total reaction volume of 25 µl. qPCR reactions were carried out on Micro-Amp 96-well optical microtitre plates on a 7900HT Fast QPCR System (Applied Biosystems), using standard settings for Standard Curve qPCR. TaqMan Gene Expression Assays for the following genes were used: Hairy And Enhancer Of Split 1, (Drosophila) (Hes1: Hs00172878_m1); ribosomal protein L27 (RPL27: Hs03044961_g1); hypoxanthine phosphoribosyltransferase 1 (HPRT1: Hs02800695_m1). 2.5 ng cDNA was used in the PCR and all samples were run in duplicates. PCR results were analysed with the SDS 2.3 software (Applied Biosystems) and the relative quantity was determined using the ΔΔCT method (Livak & Schmittgen, 2001), with the sample with highest expression as calibrator and average CT:s of RPL27 and HPRT1 as endogenous reference.

Western blot

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl pH 7.5, 150 nM NaCl, 1 nM EDTA, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS) supplemented with protease inhibitor cocktail (Complete Mini; Roche), and protein concentration was determined using the Pierce bicinchoninic acid (BCA) protein assay (ThermoFisher Scientific). Equal amounts of protein were mixed with 4x NuPAGE® LDS sample buffer (Life Technologies)

and 50 mM DTT, boiled and then loaded onto a NuPAGE 4-12 % Bis-tris gel. For oAβ, 1 μM samples were diluted 1:20, mixed with 4x sample buffer, and loaded onto a NuPAGE 4-12 % Bis-tris gel. Proteins were blotted onto a 0.2 μm nitrocellulose membrane using semi-dry technique. Membranes were blocked using 5 % milk before incubation with primary antibody over night at 4 °C. Primary antibodies used were anti-C-terminal APP (1:5000; Millipore 171610) and anti-β-Amyloid 1-16 (1:1000, clone 6E10; Biolegend). After washing, membranes were incubated with a horseradish peroxidase (HRP)-linked Anti-Rabbit IgG or HRP-linked Anti-Mouse IgG (both 1:2000; Cell Signalling Technology). Proteins were visualized using the SuperSignal® West Dura Extended Duration Substrate (ThermoFisher Scientific) by the ChemiDoc XRS+ and Image Lab 3.0 software (both from Bio-Rad Laboratories). Membranes were stripped using Restore stripping buffer (ThermoFisher Scientific) and re-probed using HRP-conjugated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:20000; Novus biological). Densitometry quantification was performed using ImageJ (NIH).

Aβ oligomerization

Aβ1-15, 1-42 and 42-1 (all from Bachem) were dissolved to 443 μM in 10 mM NaOH, aliquoted and stored at -80 °C until further use. Aβ peptide in 10 mM NaOH was mixed with 10 mM HCl and DPBS in a 1:1:2 ratio in protein LoBind tubes (Eppendorf) and incubated for 48 hours at 37 °C.

Treatment of cells with Aβ

Oligomerized Aβ1-42 and Aβ42-1 were delivered to RA differentiated SH-SY5Y cells as previously described (Domert *et. al.,* 2014). Briefly, oAβ was dissolved in serum free DMEM/F12 (125, 250 or 500 nM for Aβ1-42, 500 nM for Aβ42-1) and cells were incubated with oAβ containing medium for 3 hours. Thereafter, cells were washed with PBS, incubated with fresh PBS for 10 min at 37 °C, trypsinized, resuspended in DMEM/F12 media and centrifuged at 400g for 5 min. Thereafter, the cells were resuspended in fresh DMEM/F12 media and re-seeded onto glass coverslips.

Immunocytochemistry

Aβ-fed cells were fixed with 4 % PFA 24 hours after re-seeding. Thereafter, the samples were blocked with 5 % donkey serum (Sigma-Aldrich) in 0.3 % Triton-X100 in TBS for 1 hour at room temperature (RT). The antibody (Anti human Aβ, clone 82E1; IBL International) was diluted 1:100 in block buffer and incubated over night at 4 °C. Secondary goat anti mouse Alexa 562-antibody (Life Technologies) was diluted 1:400 in block buffer and incubated for 1h at RT. Samples were incubated with cell mask (Life Technologies; 1:20000 in TBS) for 10 minutes (for visualization of cytosol) and after washing, the samples were mounted onto microscope slides using prolong gold antifade mounting media (Life Technologies).

Duolink assay

Lyophilized anti human Aβ antibody (clone 82E1, IBL international) in PBS without BSA or preservatives was reconstituted in H_2O to a concentration of 1 mg/ml. Thereafter one aliquot of the antibody was conjugated to PLUS probe, and

one to MINUS probe (all reagents from Sigma-Aldrich if not stated otherwise) respectively, according to the manufacturer's instructions. Samples were fixed in 4 % PFA in PBS and blocked using 5 % donkey serum in TBS with 0.3 % triton-X100. Antibody/probe was diluted 1:1000 (for PLUS and MINUS respectively) in block buffer containing 1x Duolink assay reagent. Samples were incubated with antibody/probe solution over night at 4 °C. The remaining steps were performed according to the manufacturer's instructions using Duolink wash buffers and detection reagent red. After polymerase reaction, samples were incubated with cell mask (1:20000), and mounted using Duolink mounting media with DAPI and sealed with nail polish. Samples were stored at -20 °C until further analysis.

Confocal microscopy and image analysis

The samples were analysed using a Zeiss LSM700 inverted confocal microscope with 40-63x objectives and the ZEN2000 software (Zeiss). From each sample, 10 images were captured in a randomized manner. Image analysis was performed using the Volocity 6.3 Software (PerkinElmer). Duolink spots were detected based on fluorescence intensity and size. DAPI-stained nuclei were detected based on fluorescence and size. The number of Duolink spots and number of nuclei per image was analysed and the number of Duolink spots per nuclei (cell) was calculated.

Statistical analysis

All results where statistical analysis is performed are presented at means ± S.E.M. from three independent repeats. One-way ANOVA was used to compare differences between groups, followed by Dunnet's correction for multiple comparisons. All

statistical analyses were performed using Graph Pad Prism 6.0 (GraphPad Software Inc).

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Author contributions

LA designed the study, performed Duolink and DAPT experiments, analysed secretion and Duolink data and wrote the manuscript. MC performed Duolink experiments, analysed Duolink data and revised the manuscript. EG performed IP-MS experiments, and analysed IP-MS data. PK developed the Aβ oligomerization protocol, helped designing the study, and revised the manuscript. GB analysed IP-MS data and revised the manuscript. KB took part in revision of manuscript for important intellectual content. PB took part in study design, interpretation of data and revised the manuscript. EP planned mass spectrometry experiments, analysed mass spectrometry data, and wrote the manuscript. HZ initiated and designed the study, interpreted data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

KB has served on advisory boards or as a consultant for Alzheon, Eli Lilly, Fujirebio Europe, IBL International and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based company at the University of Gothenburg. HZ is co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. All other authors have no conflicts of interest.

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Figure legends

Figure 1. Low-dose γ-secretase inhibition increases secretion of Aβ x-42. iPSC-derived human cortical neurons were treated with various doses (2, 20 and 200 nM) of the γ-secretase inhibitor DAPT for 48 hours and thereafter conditioned media and cells were collected. A-C. Immunochemical analysis of secreted Aβx-38 (A), Aβx-40 (B) and Aβx-42 (C). Treatment with 2 and 20 nM DAPT increases the secretion of Aβx-42 significantly compared to control, whereas no difference in Aβx-38 or Aβx-40 secretion is observed. On the contrary, treatment with 200 nM DAPT decreases the secreted levels of Aβx-38, Aβx-40 and Aβx-42. D. The ratio of Aβx-42 to Aβx-38 is significantly increased after treatment with 20nM of DAPT, and further increased upon treatment with 200 nM DAPT. E. The ratio of Aβx-42 to Aβx-40 is significantly increased after treatment with 200nM of DAPT. F. Western blot analysis of the C-terminal fragment of APP (APP C-term) shows that treatment with 2 or 20 nM DAPT does not result in increased levels of APP C-terminal fragments, whereas treatment with 200 nM of DAPT increases the levels significantly. One representative blot out of three is shown. GAPDH was used as loading control, and densitometric analysis was used to calculate the ratio of APP C-term to GAPDH. Bars represent mean ± SEM, n = 3. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 2. DAPT treatment affects Aβ peptides differentially and dose-dependently. iPSC-derived human cortical neurons were treated with various doses (2, 20 and 200 nM) of the γ-secretase inhibitor DAPT for 48 hours and thereafter conditioned media and cells were collected and analysed using immunoprecipitation followed by mass spectrometry (IP-MS). A. Treatment with 2 or 20 nM DAPT does not change the relative levels of Aβ1-16, but 200 nM DAPT treatment increases

secretion of Aβ1-16 compared with control. B. DAPT treatment tends to decrease the relative levels of Aβ1-17 upon 20 nM DAPT treatment, being significantly reduced upon 200 nM DAPT compared with control. C. DAPT treatment similarly tends to decrease the relative levels of Aβ1-19 upon treatment with 20 or 200 nM DAPT. D. Treatment with 20 or 200 nM DAPT tends to increase relative levels of Aβ1-20, although not reaching statistical significance. E. Treatment with 200 nM DAPT abolishes the levels of Aβ1-33, compared with control. F. DAPT treatment decreases the levels of Aβ1-34 dose-dependently. G. Relative levels of Aβ5-40 are not affected by 2 or 20 nM DAPT, but decrease upon treatment with 200 nM. H-I. Relative levels of Aβ1-37 and 1-38 tend to be slightly increased upon treatment with 2 or 20 nM DAPT, but decrease upon treatment with 200 nM compared with control. J. Relative levels of Aβ1-39 are not affected by 2 or 20 nM DAPT, and decrease upon treatment with 200 nM. K-L. Relative levels of Aβ1-40 and 1-42 tend to slightly increase dose-dependently upon treatment with 2 or 20 nM DAPT, and decrease upon treatment with 200 nM compared with control. Bars represent mean ± SEM, n = 3, * = $p < 0.05$, ** = $p < 0.01$.

Figure 3. Detection of oligomeric Aβ using a proximity ligation based assay. SH-SY5Y cells were fed with 125, 250 or 500 nM oligomeric Aβ (oAβ), or with control peptide (Aβ42-1) for three hours, and were thereafter washed and reseeded to remove extracellular Aβ peptides. 24 hours after re-seeding, cells were fixed and the intracellular oligomeric Aβ was investigated using the proximity ligation assay developed by Duolink. A. Representative images of Duolink staining of SY-SY5Y cells that have been fed with control peptide (Aβ42-1) or increasing concentrations of

oAβ1-42. Increasing concentration of oAβ1-42 results in increasing numbers of Duolink spots (arrowheads). Duolink spots in red, DAPI-stained nuclei in blue and cell mask in green. Scale bar = 25 μm. B. Quantification of Duolink spots per cell (DAPI-stained nuclei). The Duolink signal is significantly increased different from control in cells fed with 125 nM oAβ1-42. A significant difference was also seen between 125 nM and 250 and 250 nM and 500 nM respectively. Bars represent mean ± SEM, n = 3. ** = $p < 0.01$, *** = $p < 0.001$.

Figure 4. Treatment with low-dose γ-secretase inhibitor DAPT increases the intracellular accumulation of oligomeric Aβ. iPSC-derived neurons were treated with various doses (2, 20 and 200 nM) DAPT for 48 hours and thereafter analysed for oligomeric Aβ using a Duolink-based method. A. Control cells contain a few positive Duolink spots (red), and treatment with 2 nM DAPT increases the number of Duolink spots (see arrowheads). Treatment with 20 or 200 nM DAPT does not increase the number of Duolink spots compared with control. DAPI-stained nuclei in blue and cell mask in green. Scale bar = 15 μm. B. Quantification of number of oAβ Duolink spots per cell. Treatment with 2 nM DAPT increases the number of oAβ spots significantly compared to control, and is also significantly higher than cells treated with 20 nM DAPT. Treatment with 20 and 200 nM DAPT does not increase the number of oAβ spots compared with control. Bars represent mean ± S.E.M., n=3. * = $p < 0.05$, ** = $p < 0.01$.

Supplementary figure 1. mRNA expression of Notch response gene *HES1* is reduced upon low-dose DAPT treatment. Quantitative PCR analysis of Notch response gene *HES1* in iPSC-derived cortical neurons upon treatment with 2, 20 and

200 nM DAPT for 48. A similar reduction in *HES1* mRNA is observed for all doses of DAPT tested, compared to control. All samples are related to control, n=1.

Supplementary figure 2. A. Western blot of oligomerized Aβ1-42 (oAβ). Short exposure (lane 1) shows the presence of dimers and trimers as well as monomeric Aβ. Longer exposure (lane 2) also reveals a trail of positive Aβ staining indicating presence of larger Aβ species. B. Immunocytochemistry shows that the n-terminal specific Aβ antibody 82E1 detects oAβ1-42 taken up by SH-SY5Y cells (arrowheads). Aβ42-1 is used as negative control. Aβ1-15 is not detected intracellularly. Scale bar = 15 μm. C. Oligomerized Aβ1-15 and 1-42 was added to glass slides and investigated using Duolink (red) followed by immunocytochemistry (green). The antibody 82E1 successfully detects Aβ1-15, but this Aβ peptide is not detected with the Duolink method. Aβ1-42 is detected both using immunocytochemistry (both monomeric and oAβ) and the Duolink method (oAβ only).

Supplementary figure 3. Secretion of oligomeric Aβ upon low-dose DAPT treatment. Detectable levels of oligomeric Aβ were secreted from iPSC-derived neurons. No difference in secretion was seen upon treatment with 2, 20 or 200 nM DAPT. Bars represent mean +/- SEM, n=2.