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Modulation of amyloid precursor protein processing by synthetic ceramide analogues

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Abstract

Previous studies suggest that membrane lipids may regulate proteolytic processing of the amyloid precursor protein (APP) to generate amyloid-beta peptide (Abeta). In the present study, we have assessed the capacity for a series of structurally related synthetic ceramide analogues to modulate APP processing in vitro. The compounds tested are established glucosylceramide synthase (GS) inhibitors based on the D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) structure. PDMP and related compounds PPMP and EtDO-P4 inhibited Abeta secretion from Chinese hamster ovary cells expressing human APP (CHO-APP) with approximate IC50 values of 15, 5, and 1 mu M, respectively. A trend for reduced secretion of the APP alpha-secretase product, sAPPalpha, was also observed in PDMP-treated cells but not in PPMP- or ETDO-P4-treated cells, whereas levels of the cellular beta-secretase product APP C-terminal fragment, CTFbeta, were increased by both PDMP and PPMP but unaltered with EtDO-P4 treatment. Our data also revealed that EtDO-P4 inhibits endogenous Abeta production by human neurons. In conclusion, this study provides novel information regarding the regulation of APP processing by synthetic ceramide analogues and reveals that the most potent of these compounds is EtDO-P4. (C) 2010 Elsevier B.V. All rights reserved.

Keywords

modulation, amyloid, analogues, protein, ceramide, precursor, synthetic, processing, CMMB

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Modulation of amyloid precursor protein processing by synthetic ceramide analogues

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Abstract

Previous studies suggest that membrane lipids may regulate proteolytic processing of the amyloid precursor protein (APP) to generate amyloid-beta peptide (Abeta). In the present study we have assessed the capacity for a series of structurally related synthetic ceramide analogues to modulate APP processing in vitro. The compounds tested are established glucosylceramide synthase (GS) inhibitors based on the D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) structure. PDMP and related compounds PPMP and EtDO-P4 inhibited Abeta secretion from Chinese hamster ovary cells expressing human APP (CHO-APP) with approximate IC50 values of 15, 5 and 1 microM, respectively. A trend for reduced secretion of the APP alpha-secretase product, sAPPalpha, was also observed in PDMP-treated cells but not in PPMP- or ETDO-P4-treated cells; whereas levels of the cellular beta-secretase product APP C-terminal fragment, CTFbeta, were increased by both PDMP and PPMP but unaltered with EtDO-P4 treatment. Our data also revealed that EtDO-P4 inhibits endogenous Abeta production by human neurons. In conclusion, this study provides novel information regarding the regulation of APP processing by synthetic ceramide analogues and reveals that the most potent of these compounds is EtDO-P4.

Keywords: Glycosphingolipid, sphingolipids, amyloid precursor protein, Alzheimer's disease, neurodegeneration, amyloid-beta peptide

1 Introduction

1.1 Role of amyloid-β peptide (Aβ) in Alzheimer's disease (AD)

A prominent feature of AD is the presence of amyloid plaques in brain regions associated with memory and learning. Amyloid plaques contain Aβ peptides as a major constituent and it is established that Aβ is derived from the amyloid precursor protein (APP) which undergoes two major pathways of enzymatic cleavage in the neuron [1]. The α-secretase pathway, which represents the major pathway for APP processing, does not generate Aβ as the metalloproteases (such as ADAM-10) responsible cleave in the middle of the Aβ sequence. In the second pathway, however, sequential cleavage of APP by β-secretase (BACE-1) and γ-secretase (a complex containing presenilins PS-1 or PS-2 as the catalytic sub-unit) generates Aβ peptide predominantly of 40 or 42 amino acids [2, 3]. Once formed, Aβ peptides may assemble as soluble oligomeric species that lead to protofibrils (Fig 1), which are neurotoxic at sub-micromolar concentrations [4, 5]. It is clear that different macromolecular forms of Aβ regulate inflammation, oxidative stress and lipid metabolism; all processes that are implicated in AD neurodegeneration [6-8]. In addition to factors that regulate the net production of Aβ in the brain, the ratio of Aβ1-40 to Aβ1-42 species generated, their propensity to form macromolecular complexes, and their clearance from the central nervous system (CNS) are all potential therapeutic targets for AD [2, 9]. Despite the recognised role for Aβ in AD neurodegeneration, the factors that modify Aβ production and deposition are not completely understood.

1.2 Glycosphingolipids (GSLs), Aβ and AD

The brain is a rich source of GSLs that represent a large family of complex lipids derived from the sphingolipid biosynthetic pathway (Fig 2). The initial rate limiting enzyme for GSL synthesis is glucosylceramide synthase (GS), an enzyme that catalyses the conversion of ceramide to glucosylceramide (GlcCer). Through the action of glycosyl transferases, GlcCer may be further acted upon to form more complex GSLs that may contain sialic acid residues in which case the GSLs become negatively charged and are referred to as gangliosides (e.g. monosialylated gangliosides GM1, GM2, GM3). Non-sialylated "neutral" GSLs such as lactosyl ceramide (LacCer) and ceramide trihexoside (CTH) are also present in the brain [10].

More than 30 years ago it was reported that reductions in the levels of specific gangliosides were associated with AD; however, it was concluded that this was a "phenomenon accompanying extensive degradation of brain tissue rather than a factor in the aetiology of dementia" [11]. Similar studies performed almost a decade later also reported ganglioside reductions in AD brains and suggested this was due to "reduced density of nerve endings in the demented brains" [12]. These data suggest that the reduction of gangliosides observed in the AD brain may be a consequence of the disease rather than a cause.

Subsequent *in vitro* and *in vivo* studies indicated that ganglioside (particularly GM1) administration could potentiate the trophic effects of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [13-15]. Perhaps prematurely, these observations were set against the background data indicating "reduced" GSL levels in

AD brains and this led to the proposal that intracerebroventricular administration of GM1 could be used to treat AD [16, 17]. Overall this approach appeared to be unsuccessful as a treatment for AD and concerns were raised regarding immunological responses to GM1 administration [18-21].

Separate studies suggested that GM1 and other GSLs may in fact *promote* Aβ production and its assembly into neurotoxic complexes. It is known that GSLs are co-localised with Aβ in amyloid plaques and it has been proposed that GM1 may interact with Aβ to form a seed for amyloid plaque formation [22, 23]. In addition, when GD3 synthase gene knockout mice (phenotype characterised by reductions in the levels of several brain gangliosides) were crossed with APPswe+PSEN1DE9 amyloidogenic mice, both soluble Aβ and plaque load were reduced (85-95%) and this was associated with improved performance in cognitive tests [24]. Other recent studies have shown that GM1 also resolubilises mature Aβ fibrils to regenerate neurotoxic Aβ protofibrils from amyloid plaque [25]. Finally, in vitro studies indicate that GSLs may stimulate both BACE and γ-secretase activity to promote Aβ generation [26, 27].

Together these findings suggest that therapeutic intervention to reduce GSL synthesis may be worth investigating as a novel strategy to reduce Aβ-associated neurodegeneration *in vivo*. Related to this, the synthetic ceramide analogue D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is an established GSL synthesis inhibitor that has been shown to inhibit Aβ secretion from SH-SY5Y neuroblastoma cells [28]. Although PDMP is not suitable for long-term animal studies due to its high hepatic metabolism (plasma $t_{1/2}$ ~1h), PDMP derivatives (Fig 3) including D-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP)

and D-threo-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (EtDO-P4) may provide viable alternatives [29, 30]. EtDO-P4 in particular has been successfully used in long-term studies in mice [31, 32].

The aim of the present study was to investigate the impact that synthetic ceramide analogues PDMP, PPMP and EtDO-P4 have on APP processing and Aβ production using CHO cells that stably express human APP695.

2 Materials and methods

2.1 Materials

Synthetic ceramide analogues D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), D-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (D-PPMP) and L-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (L-PPMP) were purchased from Matreya (Pleasant Gap, PA, USA). D/L-threo-ethylenedioxy-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (D/L-EtDO-P4) was synthesised as described previously [30]. The D-EtDO-P4 enantiomer was purified by preparative normal phase HPLC using a Lux 5 μm Cellulose-2 AXIA packed chiral 250 x 21.2 mm column (Phenomenex, Lane Cove NSW, Australia). An Agilent 1100 HPLC system was used with a mobile phase hexane:isopropanol:diethylamine (85:15:0.1, vol:vol:vol), a flow rate of 10 ml /min and UV detection at 220 nm. D-EtDO-P4 eluted at 25 min and was collected from the column and dried under vacuum before use in experiments. Unless stated otherwise,

all synthetic ceramide analogues used were in the D-threo configuration. The γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was purchased from Sigma (Castle Hill, NSW, Australia). Cell culture media and additives were obtained from Invitrogen (Melbourne, VIC, Australia) unless stated otherwise. Organic solvents were of analytical grade and were purchased from Ajax Finechem (Sydney, NSW, Australia). All other reagents were purchased through standard commercial suppliers.

2.2 Cell culture

The CHO cell line stably expressing the human 695-amino acid APP (CHO-APP) was generated and maintained as described previously [33]. This CHO cell model is an established method for analysis of APP metabolism [34-36]. CHO-APP cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The recombinant plasmid was maintained using puromycin (7.5 μg/ml). Cells were treated with synthetic ceramide analogues or DAPT as indicated for up to 48 h. These compounds were added to cells in complete growth medium containing 10% FCS. Human fetal brain tissues were obtained from 14 to 18 week-old fetuses collected after therapeutic termination following informed consent (ethical approval from the University of New South Wales Human Research Ethics Committee, HREC03187). Neurons were isolated from the brain tissues and cultured as previously described [37].

2.3 Western blotting

CHO-APP cells were routinely cultured in 12 well plates, rinsed with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors). Bicinchoninic acid protein assays were performed on lysates and equal amounts of protein were separated on 12% SDS-PAGE gels and transferred onto 0.2 μm nitrocellulose membranes at 100 volts for 30 min. Membranes were blocked at 22˚C for 2 h in PBS containing 5% non-fat dry milk and probed with the relevant antibodies at 4˚C for 16 h to analyse APP (WO2 monoclonal 1/400), APP plus APPβ C-terminal fragment (CTFβ, rabbit polyclonal 1/10000, Sigma, Cat No. A8717) and β-actin (rabbit polyclonal 1/2000, Sigma, Cat No. A5060). The membranes were washed three times in PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (Dako, 1/2500) or rabbit anti-mouse (Dako, 1/2500) secondary antibody for 2 h. Signals were detected using enhanced chemiluminescence (ECL, Amersham Biosciences) and X-ray films. Membranes were stripped and re-probed with β-actin antibody (rabbit polyclonal 1/5000, Sigma, Cat No. A5060) as a loading control. The signal intensity was quantified using NIH ImageJ software.

Western blotting of secreted Aβ peptides and the secreted products of APP cleavage by α-secretase (sAPPα) was carried out as previously described [35]. Briefly, Aβ in the culture medium was separated on 12% SDS-PAGE gels and transferred onto 0.2 μm nitrocellulose membranes at 65 volts for 17 min. Membranes were boiled in PBS for 5 min, probed with anti-APP WO2 monoclonal antibody followed by rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody and ECL detection applied as described above. Where indicated, the optical density of bands detected in

western blots was measured using NIH ImageJ software and the data regarding expression of APP and its proteolytic products presented as a ratio relative to non-treated cells as previously described [35].

2.4 Cellular GSL analysis

GSL profiles of CHO-APP cells were analyzed as described previously [38]. Briefly, cells grown to confluence in 6-well plates were treated with synthetic ceramide analogues as described above, rinsed three times with PBS and extracted in 2:1 (v/v) chloroform/methanol. The GSL fractions were isolated by silicic acid chromatography, and the glycan moiety was released by ceramide glycanase addition [39]. The GSL glycans were then fluorescently labeled and analyzed by normal phase HPLC as described previously [38]. Total peak area units for the glycan peaks were pooled to calculate the reduction of cellular GSL levels after treatment with ceramide analogues. Values were expressed as a percentage of total GSL levels present in vehicle-treated CHO-APP cells.

2.5 Cell viability assay

CHO-APP cells were seeded in 96 well plates at 80% confluence. Cells were treated with the indicated doses of ceramide analogues for 48 hours. Culture media was removed and 100 μl of media (DMEM, 10% FCS) containing 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Catalog no. M2128, Sigma), was added and the cells incubated for 1 h at 37˚C. Media was discarded and the cells dissolved in DMSO (100 μl /well) and the absorbance of the cell lysates was

measured at 550 nm. Higher absorbance values indicate increased cell viability. Cell number and morphology was also assessed by direct counting of cells in 5 randomly selected fields in each of 3 triplicate wells. The cells remaining attached were captured in digital images recorded under phase contrast (20 X) and the printed images were used for cell counting.

2.6 Statistical Analysis

Unless stated otherwise, experiments were performed in triplicate and repeated at least three times. Data are presented as means with S.E. shown by error bars. Differences were considered significant where $P < 0.05$ as determined by the 2-tailed Student's t-test for unpaired data.

3 Results

3.1 Regulation of Aβ production by ceramide analogues

A previous study reported that PDMP could inhibit the secretion of Aβ from SH-SY5Y neuroblastoma cells [28]. Our initial experiments aimed to examine the impact that two derivatives of PDMP (PPMP and EtDO-P4), that have more potent GS inhibitory activity than PDMP [30], might have on cellular Aβ secretion. Our data indicate that both PPMP and EtDO-P4 inhibit Aβ secretion from CHO-APP cells. Using a 48 h treatment period, we found that the IC_{50} values for the inhibition of Aβ secretion by PDMP, PPMP and EtDO-P4 were 15.8 μM, 5.8 μM and 1.0 μM,

respectively (Fig 4). The reduction in extracellular Aβ levels induced by these compounds (when used at values that are close to their IC_{50} values) is illustrated by the western blots shown in Figure 5. Similar to the previous studies with SH-SY5Y cells [28], we noted a trend for PDMP to also inhibit the secretion of sAPPα from CHO-APP cells (Fig 5); although overall this did not reach statistical significance (P = 0.051). There were no obvious changes in sAPPα secretion when cells were treated with either PPMP or EtDO-P4 at concentrations that reduced Aβ secretion by ~50% (Fig 5). Full-length cellular APP levels were significantly increased by ~ 20% with PPMP treatment whereas a non-significant trend for increased cellular APP was observed with EtDO-P4 treatment (Fig 5). These data suggest that there may be subtle differences in the mechanisms by which these structurally-related ceramide analogues modulate APP processing and Aβ secretion. Intracellular Aβ was not detectable in CHO-APP cells either with or without ceramide analogue treatment (data not shown). The fact that Aβ is very efficiently secreted by the CHO-APP cells is consistent with previous studies [33, 35, 36].

3.2 Toxicity profiles of ceramide analogues

EtDO-P4 is a potent GS inhibitor that has already been used in long-term mouse studies [31, 32]. However, because these ceramide analogues may also exhibit cytotoxicity, especially towards cancer cell lines [40, 41], we also compared their affect on cellular MTT reduction as a marker of cytotoxic potential. Significant decreases in cellular MTT reduction were detected for all three ceramide analogues when tested at concentrations that inhibit Aβ secretion by ~50% (Fig 6). The MTT reduction assay is reliable method for detection of cell death, however, it may also

detect changes in mitochondrial electron transport, cellular metabolic activity and cellular stress well in advance of cell death; particularly under conditions where cells are exposed to Aβ [42, 43]. Therefore cell survival was also checked by anlaysing phase contrast images of CHO-APP cells as described in the Methods section. Gross morphological changes and overt cytotoxicity were only observed when the compounds were used at concentrations ~ 2-fold greater than their IC_{50} values for Aβ secretion. As an example, phase contrast images of CHO-APP cells treated with up to 5 μM EtDO-P4 for 48 reveal significant loss of the cell monolayer and a clearly "rounded-up" cellular morphology (Fig 6). Overall, however, these data indicate that the cytotoxicity of these ceramide analogues is reasonably low when used at concentrations that inhibit Aβ secretion by ~50%.

3.3 Impact of ceramide analogues on APP CTFβ generation

In order to better understand how PDMP, PPMP and EtDO-P4 may be inhibiting cellular Aβ secretion, we also assessed the formation of the APP CTFβ fragment using a specific antibody that recognises the APP C-terminal amino acids 676-695. Increased cellular CTFβ levels in association with reduced Aβ secretion are associated with inhibition of γ-secretase activity. Our data indicate that PDMP and PPMP when used at their ~IC_{50} concentrations both caused a marked 6- to 12-fold increase in cellular CTFβ (Fig 7). In contrast, EtDO-P4 induced only a moderate 20% increase in CTFβ levels (Fig 7). The γ-secretase inhibitor DAPT was used as a positive control in these experiments and was found to increase CTFβ levels by 50-fold and reduce Aβ secretion to undetectable levels (Fig 7). We also assessed the activity of the L enantiomer of PPMP, which does not inhibit GS activity. L-PPMP

had no significant impact on either cellular CTFβ levels or on Aβ secretion (Fig 7). These data indicate that although the inhibition of Aβ secretion that is induced by PDMP and PPMP is associated with increased CTFβ accumulation (and thus presumably γ-secretase inhibition), EtDO-P4 may have a different anti-amyloidogenic mechanism of action. Furthermore, since L-PPMP did not have an impact on either CTFβ or Aβ levels, this suggested that direct inhibition of GS activity may be required for the anti-amyloidogenic activity of these ceramide analogues. One possible caveat with the data related to L-PPMP is that a previous study has indicated that when used at low μM concentrations it may stimulate sphingolipid synthesis (assessed after 48 h treatment of Colo-205 cells) and thereby introduce an non-controlled confounding variable in the present study [44].

3.4 Impact of EtDO-P4 on secretion of endogenous Aβ from primary human neurons

The data above indicate that EtDO-P4 is a potent inhibitor of cellular Aβ secretion that the mechanism for this ceramide analogue is associated with only a moderate CTFβ accumulation. In order to assess whether this action of EtDO-P4 may also be relevant to endogenously synthesised Aβ, we also treated primary human neurons with EtDO-P4. Similar to the results derived from the CHO-APP cell experiments, EtDO-P4 also inhibited Aβ production by human neurons (Fig 8). In this case the IC_{50} for Aβ secretion was found to be ~ 3 μM and, under these conditions, total cellular GSL levels were reduced by ~ 50% (Fig 8).

4 Discussion

Increasing evidence suggests that there may be a link between dysregulation of cerebral lipid homeostasis and AD. For example, there is data indicating that synaptic terminals and isolated lipid raft fractions from AD brains are enriched in cholesterol and specific GSLs, respectively [45, 46]. While causation has not been proved, it has been speculated that such changes in membrane lipid composition could contribute to the acceleration of Aβ deposition in AD [45-47]. If increased neuronal GSL and cholesterol levels do increase Aβ formation *in vivo* then it would be predicted that in genetic diseases resulting in the accumulation of these lipids in the brain, an increase in APP processing to produce Aβ would be observed. While this idea has not been extensively examined, studies in human Niemann-Pick Type C (NPC) brains and NPC mice (in which cholesterol and GSLs accumulate due to defects in endocytic trafficking) do indicate that even though amyloid plaques are not a feature of NPC, Aβ production is significantly increased and it is thought that this may contribute to NPC neurodegeneration [48-50].

There are potentially several mechanisms to explain how GSLs may impact on APP processing, amyloid deposition and Aβ neurotoxicity. It is clear that the amyloidogenic processing of APP by β- and γ-secretases occurs within cell membranes in lipid raft microdomains that are enriched with GSLs and cholesterol (Fig 1) [47, 51-55]. Importantly, even subtle increases in the amount of raft lipids (including GSLs and cholesterol) in the cell membrane enhance APP processing to generate Aβ and there may be multiple mechanistic explanations for this. These include an increase in the proportion of APP and β-secretase (BACE) localised to

lipid rafts, and direct modulation of BACE proteolytic activity [26, 56]. There is also data derived from purified γ-secretase complex reconstituted in phosphatidylcholine (PC) liposomes that indicates strong induction of γ-secretase activity upon inclusion of porcine brain gangliosides to account for from 5% to 25% of the total liposome lipid (whereas increasing the ganglioside content to 50% or above inhibited γ-secretase activity) [27].

In addition there may be direct interaction of cholesterol and GSLs in the membrane that together have an impact on APP processing. Related to the present experiments, our previous research showed that enrichment of cells with GSL promoted membrane cholesterol accumulation and that the PDMP potently stimulated cellular cholesterol efflux via an ABCA1 transporter-dependent pathway [38]. We also showed that PDMP treatment (10μM for 72h) not only reduced cellular GSL expression but also led to a depletion of cholesterol from Triton X-100 insoluble membrane microdomains [38]. A subsequent study showed that 25 μM PDMP inhibited Aβ secretion from SH-SY5Y neuroblastoma cells by ~50% over 48h [28]. These studies led us to examine PDMP-related ceramide analogues that maybe used to test for possible anti-amyloidogenic activity in future studies of amyloidogenic transgenic mice.

The aim of the present study was therefore to examine the potential for synthetic ceramide analogues to inhibit Aβ secretion *in vitro*. We showed that PDMP and related compounds PPMP and EtDO-P4 inhibited Aβ secretion from CHO-APP cells with IC_{50} values of 15.8 μM, 5.8 μM and 1.0 μM, respectively. For reasons that remain to be defined, there were no universal effects of the different compounds on

the levels of full length APP or APP proteolytic products sAPPα and cellular CTFβ. Based on the IC_{50} values and the inactivity of L-PPMP, we speculate that the mechanism by which these ceramide analogues inhibited Aβ may be related to their inhibition of GS activity, however, further experiments are clearly required to identify the precise pathways involved. It has been suggested that changes in cellular ceramide and diglyceride levels may correlate better than GS inhibitory activity with the cytostatic effects of ceramide analogues [40]. Whether differential changes in ceramide and diglyceride homeostasis also contribute to the different effects we have observed using synthetic ceramide analogues and their capacity to modulate different aspects of APP processing remains to be determined.

Regardless, of the remaining details regarding the anti-amyloidogenic mechanism of action, the fact that EtDO-P4 inhibits Aβ secretion from both CHO-APP cells and human neurons at low μM concentrations and is applicable to *in vivo* mouse studies, indicates further investigation of this compound in an experimental animal model of AD is worth pursuing. Due to the rapid hepatic metabolism of PDMP (plasma $t_{1/2}$ ~1h) this compound is not suitable for long-term animal studies. However, EtDO-P4 is ideal for *in vivo* studies with a t½ in mice of ~7h and our own data indicating i.p. injection of $apoE^{-/-}$ mice with EtDO-P4 (10 mg/kg) 3 times per week for 4 months is well tolerated and results in 49% inhibition in plasma GSL levels [32].

EtDO-P4 has a reported IC_{50} for GS (Fig 2) of 100 nM and at levels 100-fold higher than its IC_{50} for GS has no impact on ceramide, sphingomyelin, phospholipid or cholesterol synthesis [30, 31]. EtDO-P4 is a hydrophobic molecule that appears to cross the blood brain barrier (BBB) and has been shown to reduce GlcCer levels in

the brains of Fabry disease (a GSL storage disease) mice by 16% when administered at 10 mg/kg as a complex with phospholipid vesicles (PLV) [31]. The transport of EtDO-P4 across the blood brain barrier is consistent with several earlier short term (few days to 2 weeks) studies that have used the less potent PDMP analogues to modulate cerebral GSL metabolism in rats, gerbils and mice [29, 57-59]. In these "acute" studies, doses of 20 to 40 mg/kg administered i.p. twice per day (using detergent Tween 80 as vehicle) were used. One of these latter studies [29] revealed that a peak brain concentration of 50 μM for D-PDMP was achieved 30 min after a single i.p. injection of 80 mg/kg after which the brain concentration dropped to 6 μM at 3h and plateaued resulting in 4 μM PDMP detected in the brain at the 8h (final) time point. Considering the development of human therapeutics, it is potentially relevant that a GS inhibitor (Genz-112638) is currently in Phase II clinical trials (http://clinicaltrials.gov/show/NCT00358150) to treat Type 1 Gaucher disease, and it may be the case that this compound can also modulate neuronal Aβ secretion; although we are not aware of any study in which this has been assessed.

5 Conclusion

In conclusion, our current study provides novel information regarding the regulation of APP processing by synthetic ceramide analogues and reveals that the most potent of these compounds, EtDO-P4, may regulate APP processing through inhibition of GS. Future studies of the potential impact that EtDO-P4 or related compounds have on Aβ homeostasis in amyloidogenic mouse models appear to be worth pursuing.

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Figure legends

Figure 1. Schematic representation of amyloid precursor protein (APP) processing. Amyloidogenic processing by β-secretase and γ-secretase generates Aβ peptides in the cholesterol- and GSL-enriched lipid raft microdomains within cell membranes. Non-amyloidogenic processing by α-secretase occurs predominantly in non-raft microdomains. Abbreviations are explained in the text.

Figure 2. Simplified scheme of sphingolipid biosynthesis. PDMP, PPMP and EtDO-P4 inhibit glucosylceramide synthase (GS) which catalyses the first step in glycosphingolipid synthesis. (S1P, sphingosine-1-phosphate; C1P, ceramide-1-phosphate; other abbreviations are explained in the text).

Figure 3. Chemical structures of synthetic ceramide analogues used in this study.

Figure 4. Determination of IC_{50} values for inhibition of Aβ production by synthetic ceramide analogues. CHO-APP cells were treated with the concentrations PDMP (A), PPMP (B) or EtDO-P4 (C) indicated for 48 h and Aβ in the culture medium was measured by western blotting. Optical density measurements of the western blots were used to quantify Aβ. Data are derived from 4, 9 and 9 experiments for PDMP, PPMP and EtDO-P4, respectively. Data are mean values with error bars indicating SE.

Figure 5. Impact of synthetic ceramide analogues on sAPPα and Aβ production. CHO-APP cells were treated with 15 μM PDMP (A), 5 μM PPMP (B) or 1 μM

EtDO-P4 (C) for 48 h and cellular APP and secreted sAPPα and Aβ were measured by western blotting. β-actin was used as a loading control. (D) Optical density measurements of the western blots, control (grey bars) and treated (black bars). The bars in the histograms represent (from L to R) levels of APP, sAPPα and Aβ for each analogue tested. Data are mean values with error bars indicating SE, *$p< 0.05$, ***$p<0.001$.

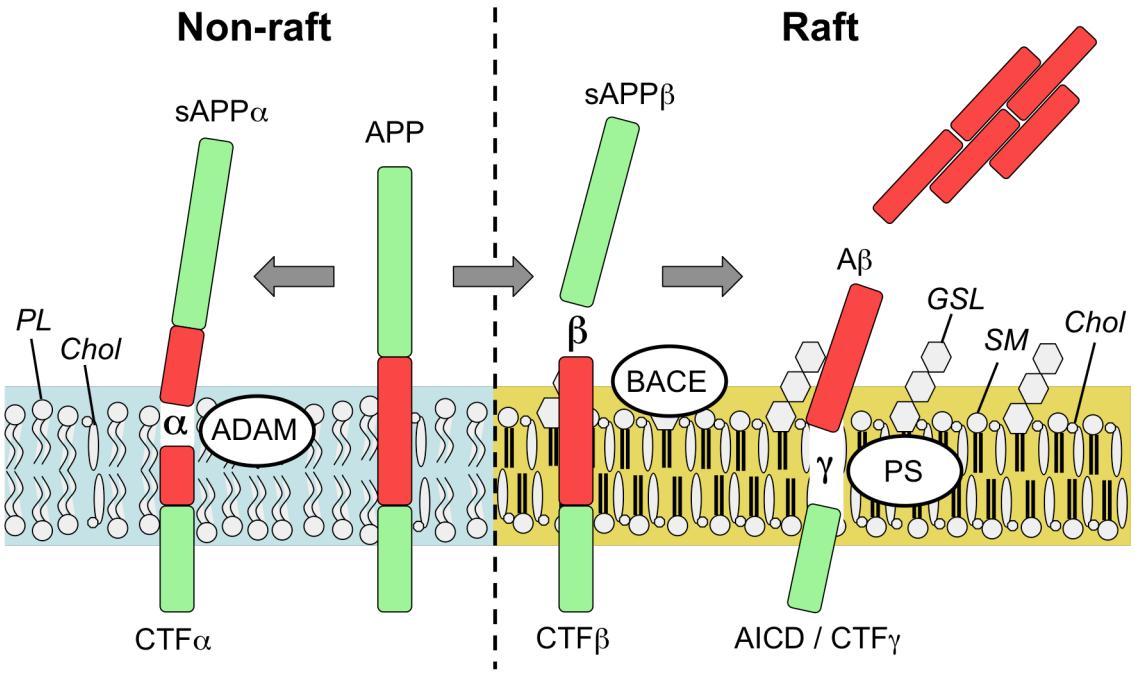
Figure 6. Cytotoxicity assay of synthetic ceramide analogues. CHO-APP cells were treated with PDMP (A), PPMP (B) and EtDO-P4 (C) for 48 h and cytotoxicity was measured using the MTT assay as described in Materials and Methods. Data are mean values (n=3) with error bars indicating SE, *$p< 0.05$, ***$p<0.001$. Phase-contrast microscopy of CHO-APP cells treated with EtDO-P4 at 0 μM (D), 0.1 μM (E), 0.5 μM (F), 1 μM (G) or 5 μM (H) for 48 h.

Figure 7. Effect of synthetic ceramide analogues on the intracellular processing of APP. CHO-APP cells were treated with 15 μM PDMP or DAPT (positive control, 1μM) (A), 5 μM L-PPMP or 5 μM D-PPMP (B) or 1 μM EtDO-P4 (C) for 48 h and cellular APP and CTFβ and secreted Aβ were measured by western blotting. β-actin was used as a loading control. (D–F) Optical density measurements of the western blots, respectively, control (grey bars) and treated (black bars). Data are mean values with error bars indicating SE, *$p< 0.05$, ***$p<0.001$. C, control; PD, PDMP; DA, DAPT; L-P, L-PPMP; D-P, D-PPMP; P4, EtDO-P4.

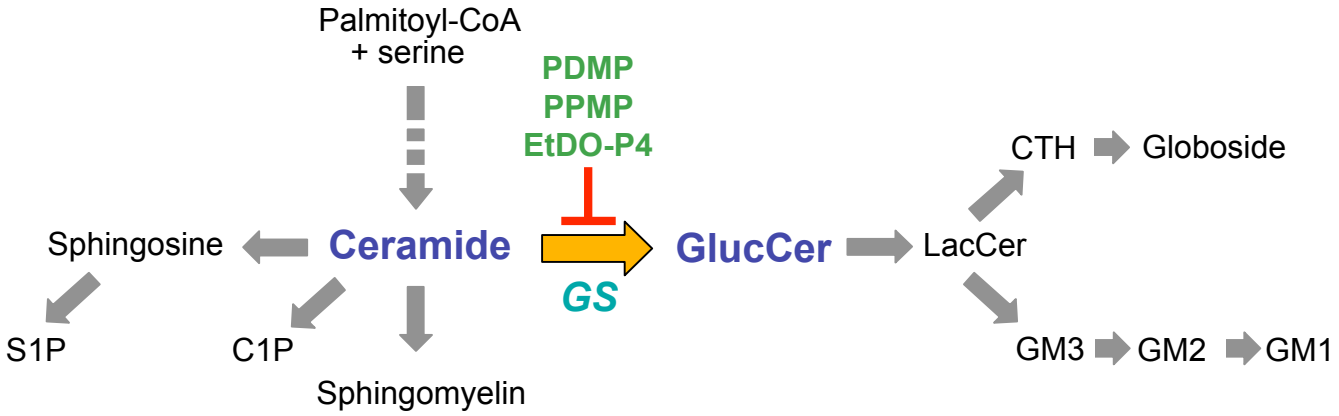
Figure 8. Effect of EtDO-P4 on Aβ and glycosphingolipid levels in primary human neurons. Human neurons were treated with 3 μM EtDO-P4 for 48 h and (A) secreted

Aβ was measured by western blotting and, (B) cellular glycosphingolipid (GSL) by HPLC, control (grey bars) and EtDO-P4 (black bars). Data are mean values (n=3) with error bars indicating SE, $**p<0.01$, $***p<0.001$.

Li et al. Fig 1

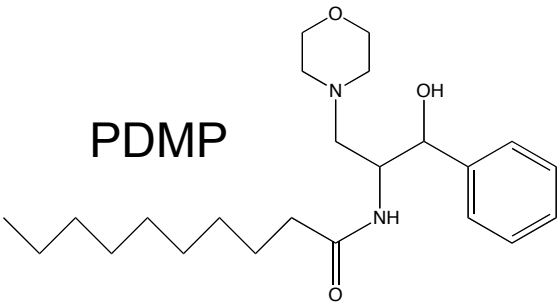

Non-raft
Raft
sAPPα
APP
sAPPβ
Aβ
GSL
SM
Chol
PL
Chol
β
BACE
α
ADAM
γ
PS
CTFα
CTFβ
AICD / CTFγ

Li et al. Fig 2

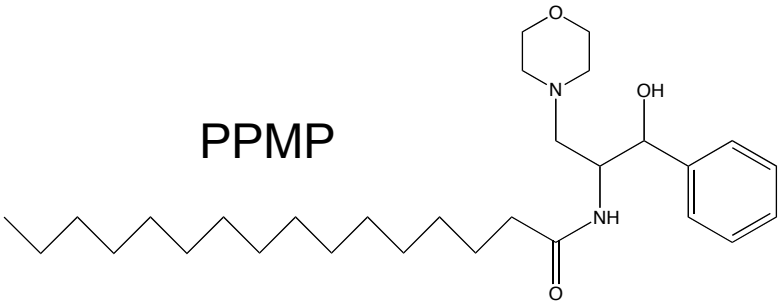

Palmitoyl-CoA
+ serine
PDMP
PPMP
EtDO-P4
CTH
Globoside
Sphingosine
Ceramide
GlucCer
LacCer
GS
S1P
C1P
Sphingomyelin
GM3
GM2
GM1

Li et al. Fig 3

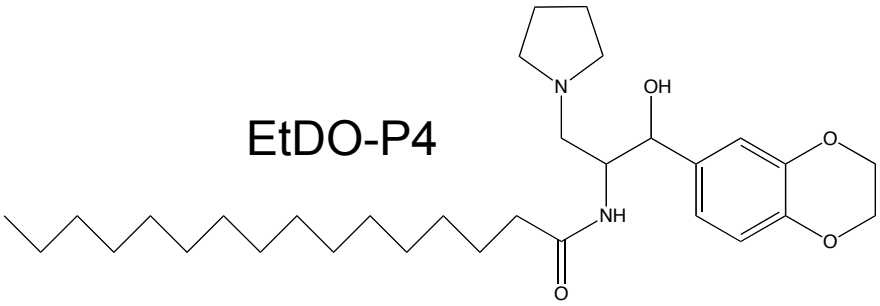
PDMP



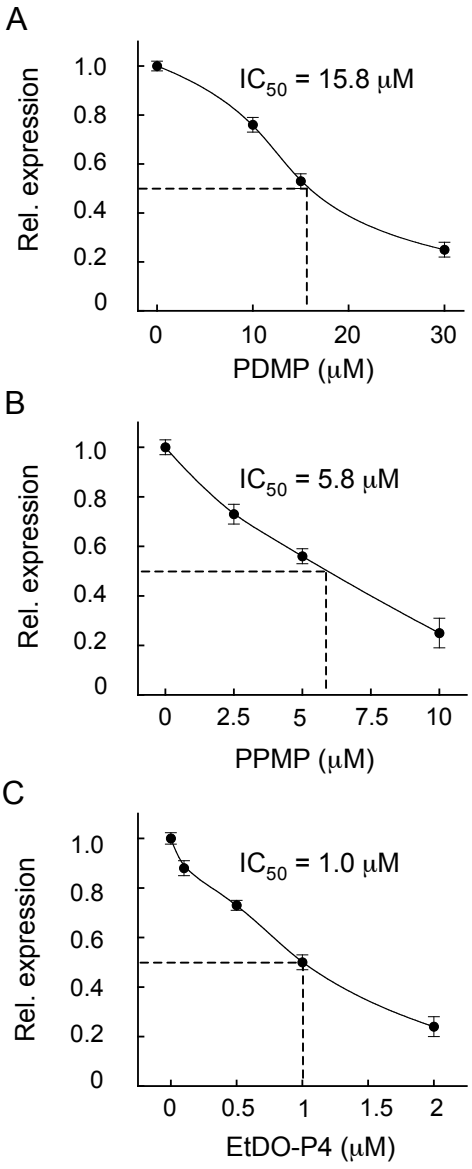
PPMP



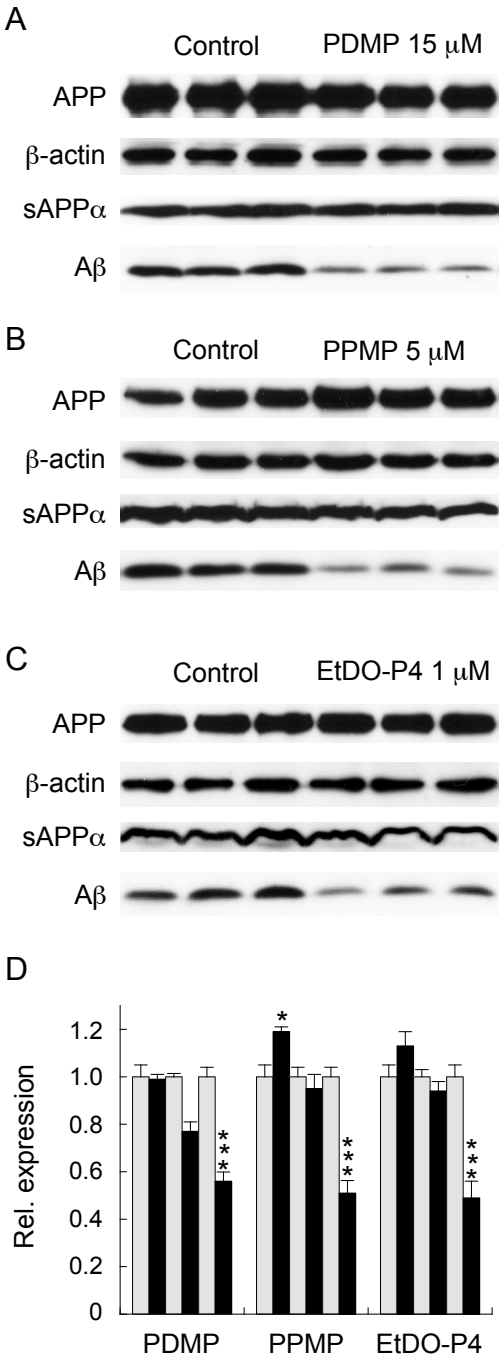
EtDO-P4



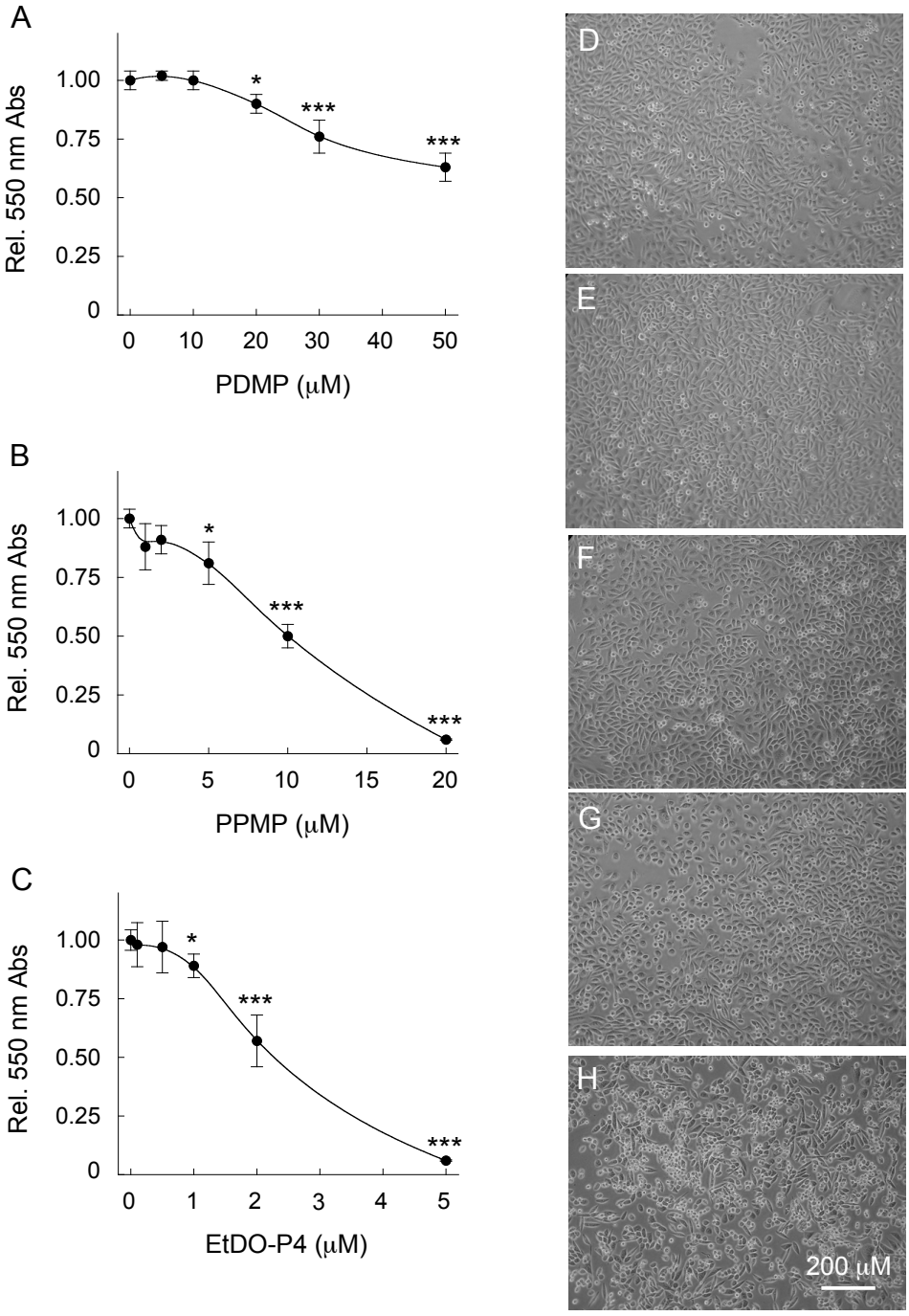
Li et al. Fig 4


A
Rel. expression
1.0
0.8
0.6
0.4
0.2
0
IC50 = 15.8 μM
0
10
20
30
PDMP (μM)
B
Rel. expression
1.0
0.8
0.6
0.4
0.2
0
IC50 = 5.8 μM
0
2.5
5
7.5
10
PPMP (μM)
C
Rel. expression
1.0
0.8
0.6
0.4
0.2
0
IC50 = 1.0 μM
0
0.5
1
1.5
2
EtDO-P4 (μM)

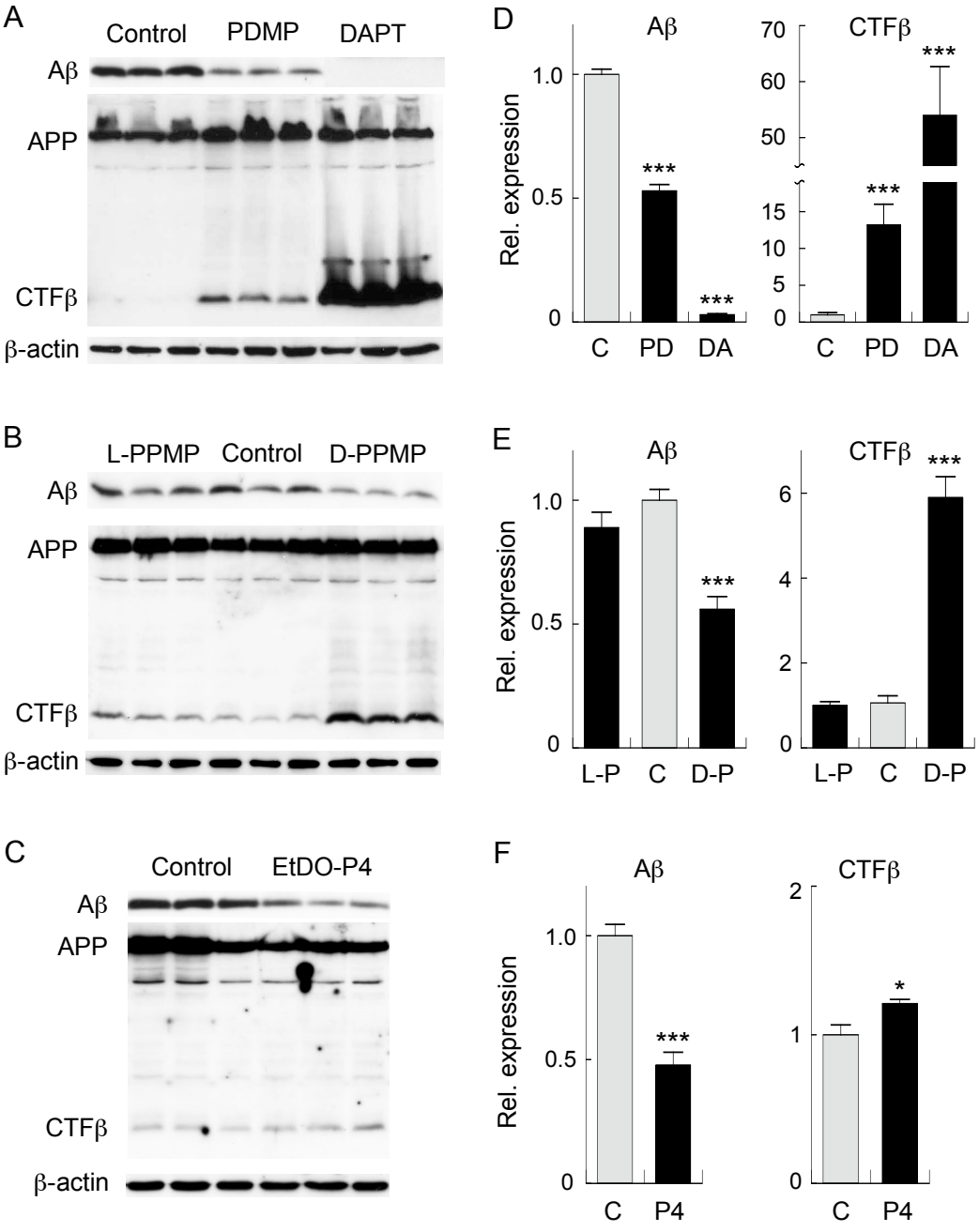
Li et al. Fig 5


A
Control
PDMP 15 μM
APP
β-actin
sAPPα
Aβ
B
Control
PPMP 5 μM
APP
β-actin
sAPPα
Aβ
C
Control
EtDO-P4 1 μM
APP
β-actin
sAPPα
Aβ
D
Rel. expression
1.2
1.0
0.8
0.6
0.4
0.2
0
*
***
***
PDMP
PPMP
EtDO-P4

Li et al. Fig 6


A
Rel. 550 nm Abs
1.00
0.75
0.50
0.25
0
0
10
20
30
40
50
PDMP (μM)
*
***
***
B
Rel. 550 nm Abs
1.00
0.75
0.50
0.25
0
0
5
10
15
20
PPMP (μM)
*
***
***
C
Rel. 550 nm Abs
1.00
0.75
0.50
0.25
0
0
1
2
3
4
5
EtDO-P4 (μM)
*
***
***
D
E
F
G
H
200 μM

Li et al. Fig 7


A
Control
PDMP
DAPT
Aβ
APP
CTFβ
β-actin
B
L-PPMP
Control
D-PPMP
Aβ
APP
CTFβ
β-actin
C
Control
EtDO-P4
Aβ
APP
CTFβ
β-actin
D
Aβ
CTFβ
Rel. expression
1.0
0.5
0
70
60
50
15
10
5
0
***
C
PD
DA
E
Aβ
CTFβ
Rel. expression
L-P
C
D-P
F
Aβ
CTFβ
Rel. expression
P4
*

Li et al. Fig 8