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Plasmalogen Deficit: A New and Testable Hypothesis for the Etiology of Alzheimer's Disease

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1. Introduction

Alzheimer's disease (AD) is a complex cognitive disorder for which the single greatest risk factor is age. The pathophysiological basis for AD is still a matter of debate with no current hypothesis explaining all of the complex pathological changes observed. These include neurofibrillary tangles, amyloid plaques, neuroinflammation, hypomyelination, neuronal shrinkage (eg. N- basalis cholinergic neurons, resulting in a dramatic cholinergic deficit), ocular pathology, microvascular pathology and liver peroxisomal dysfunction. The hypothesis that we are presenting, namely peroxisomal dysfunction resulting in decreased supply of critical ethanolamine plasmalogens to the brain, eye and vascular endothelium, is the first hypothesis that can potentially explain all of these complex pathologies in AD. The value of this hypothesis is that it is imminently testable via resupply of critical ether lipid precursors of plasmalogens. PPI-1011 is such a drug candidate and will be presented after a review of the basis of this hypothesis.

2. Plasmalogens in aging and AD

Advancing age remains the largest risk factor for the development of AD. Pathologically, AD is primarily defined by the accumulation of amyloid plaques (AP) and neurofibrillary tangles (NFT), whereas clinically, AD is primarily defined as the progressive deterioration of mental function the earliest symptoms of which are reduced memory functions. Epidemiologically, these three features exhibit essentially superimposable age-severity plots [compare (Braak and Braak 1997) to (Bachman et al., 1992)], yet the mechanism behind this association remains elusive. In this section we attempt to peel back one more layer of the AD onion and put forward the hypothesis that age-related decline in the peripheral synthesis of ethanolamine plasmalogens (PlsEtn), a critical component of both neuronal membranes and myelin sheaths, is responsible for both the pathological and clinical AD cascade. The underlying cause behind peripheral PlsEtn depletion is currently unknown and therefore represents yet another layer to be uncovered in the future.

The time course of the presence and severity of the pathological features of AD was described in detail (Braak and Braak 1997) using a large population of non-selected post-mortem human

brains (ages 26-95, n= 2661). In regards to amyloid deposition, they reported that initial amyloid patches appear in temporal areas such as the perirhinal and/or ectorhinal fields (Stage A) followed by spreading to adjoining neocortical areas and hippocampus (Stage B), and that the primary areas of the neocortex were the last regions to exhibit plaques (Stage C). Of the three stages, only the prevalence of stage C increases with age. This increase begins in the last half of the 6th decade of life. In regards to NFT, six stages are defined. The earliest signs are observed in the transentorhinal region (temporal lobe) (Stage I), then into the entorhinal region (Stage II), then to the hippocampus and temporal proneocortex (Stage III) then the adjoining proneocortex (Stage IV). The lesions then spread superolaterally (Stage V) and finally into the primary regions of the neocortex (Stage VI). In interpreting their data, Braak and Braak commented that the sequence of pathological progression is the inverse of the sequence of cortical myelination and that poorly myelinated areas were the first affected. This observation is of interest in that white matter, but not gray matter appears to exhibit an appreciable age-related degeneration as well (Meier-Ruge et al., 1992; Bartzokis, 2004). These findings have led some researchers to put forward the hypothesis that age-related myelin degeneration is a causative feature in AD (Bartzokis, 2004; Braak and Del Tredici, 2004). PlsEtn but not phosphatidylethanolamines (PtdEtn) exhibit an age-related decline after age 50-69 (Figure 1).

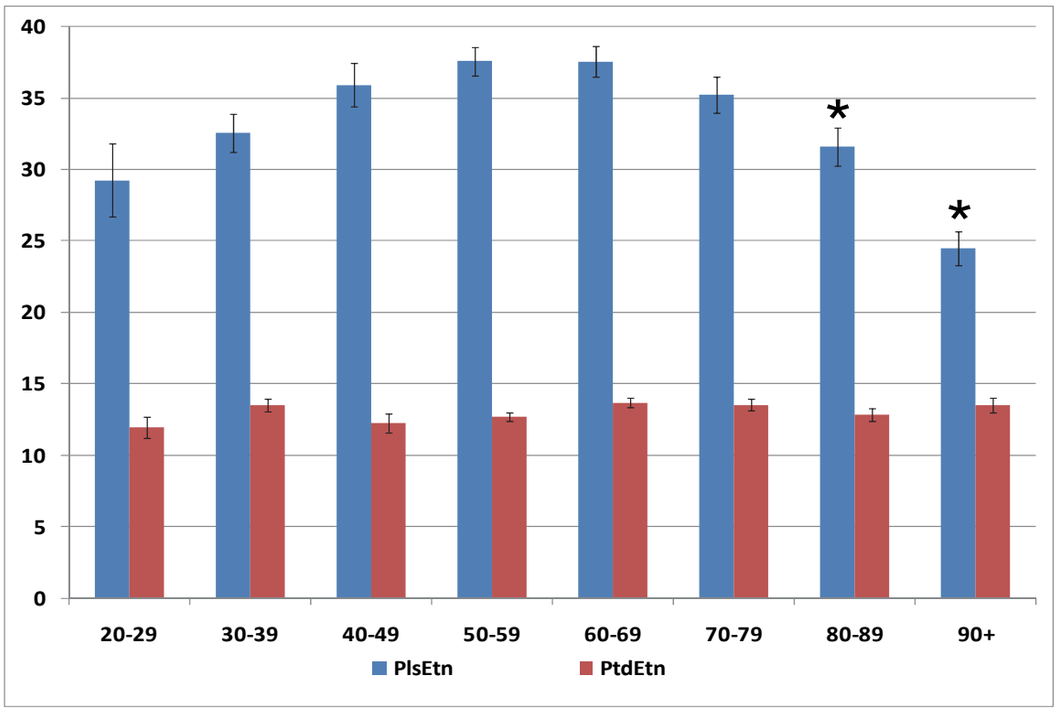


Fig. 1. Effect of age on total serum PlsEtn and PtdEtn. Total PlsEtn and PtdEtn is the sum of 15 sn1/sn2 combinations sn1: 16:0, 18:0, 18:1; sn2: 18:1, 18:2, 18:3, 20:4, 22:6. each normalized to PtdEtn 16:0/18:0. *, $p < 0.05$. Serum samples for the age-association study (Figures 1-2) were provided as anonymized reference samples comprising a random population of 990 control subjects aged 11-99 collected at the provincial clinical testing lab in Regina, Saskatchewan, Canada from the Saskatchewan Disease Control Lab (SDCL) in accordance with SDCL policies and under a protocol approved by the Ethics Department of the University of Saskatchewan. PlsEtn and PlsEtn analyses performed as per Goodenowe et al., 2007.

Interestingly, when the PlsEtn/PtdEtn pairs of the key white matter (18:1) and gray matter (22:6) PlsEtn are compared, the peak age of PlsEtn as a percentage of the ethanolamine phospholipid pool is 50-59. Docosahexaenoic acid (DHA, 22:6) shows the most precipitous decline with age (Figure 2). If PtdEtn and PlsEtn were co-regulated, these ratios should be constant with age. However, this is clearly not the case, indicating that selective plasmalogen decline occurs with age. This age effect is analogous to the above mentioned white matter depletion and AP/NFT accumulation. In addition, DHA-PlsEtn levels decrease in both post-mortem brain and pre-mortem serum samples with increasing Braak scale (Figure 3). These results are consistent with the dementia severity dependent decrease in DHA-PlsEtn in post-mortem brain previously reported (Han et al., 2001). A pharmacologically induced reduction of serum plasmalogens in healthy mice using cuprizone (a molecule that does not cross the blood-brain barrier), preferentially depletes DHA-PlsEtn in the serum and kidney but 18:1-PlsEtn in the CNS, the most abundant PlsEtn of myelin (Figure 4). These data support the hypothesis that a peripheral decline in PlsEtn can cause CNS PlsEtn depletion.

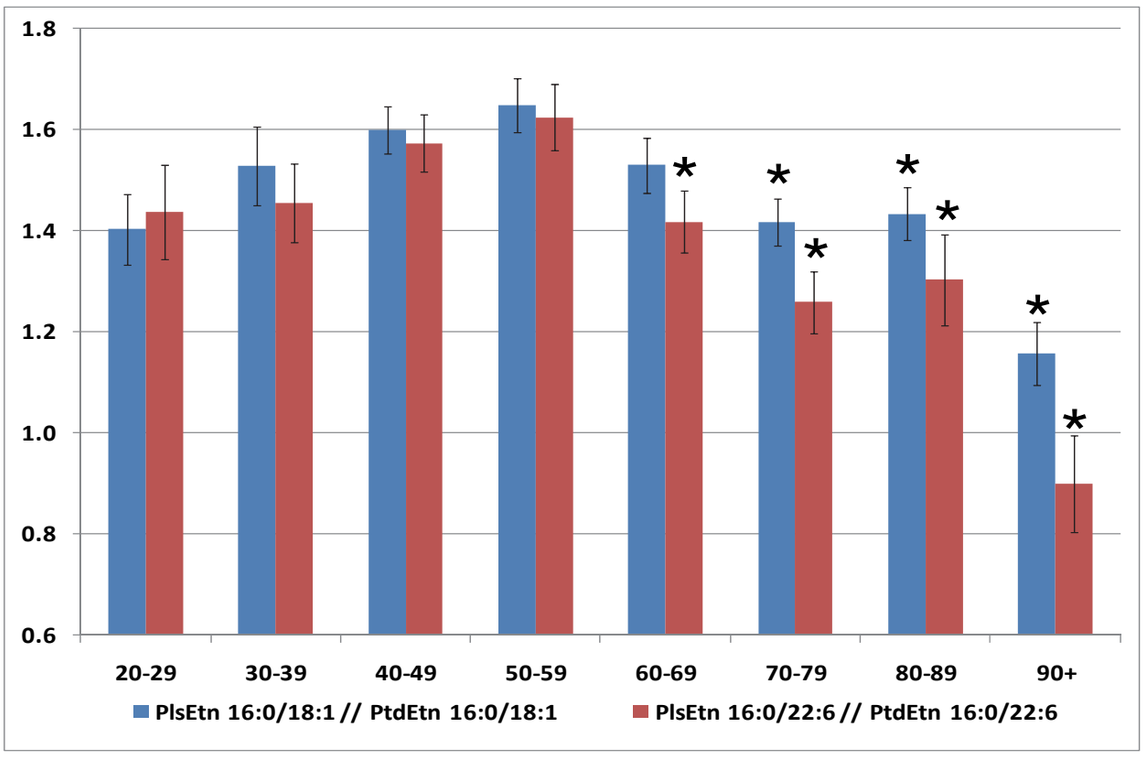


Fig. 2. Effect of age on the relative ratio of specific PlsEtn / PtdEtn species pairs in serum. *, $p < 0.05$.

We have previously reported extensively on the role of plasmalogens in cognition and the association between serum DHA-PlsEtn levels and AD (Goodenowe et al., 2007; Wood et al., 2010). In brief, when compared to cognitively confirmed, age and gender-matched controls (MMSE 29-30, age 77+/-0.8, n=68), subjects with low (ADAS cog 5-19, age 79.3+/-0.8, n=78), moderate (ADAS cog 20-39, age 79.1+/-0.7, n=112), and severe (ADAS cog 40-70, age 82.1+/-1, n=66) dementia had increasingly lower levels of DHA-PlsEtn in their serum (74%,

p=3.0e-4; 66%, p=1.3e-7; 53%, p=3.9e-11, respectively). This finding was confirmed in pre-mortem serum samples collected from subjects later confirmed pathologically to have AD and in Japanese subjects with mild AD (Goodenowe et al., 2007). Subsequently, the effect of serum DHA-PlsEtn levels at enrollment on the rate of cognitive decline over 12 months was investigated (Wood et al., 2010). It was observed that only subjects with serum DHA-PlsEtn of 75% or less of normal levels exhibited cognitive decline over a 12 month period. Using samples collected from Rush University's Memory and Aging Program (Bennett et al., 2006) we investigated the predictability of pre-mortem MMSE scores (within 12 months of death) on post-mortem pathology (Figure 5).

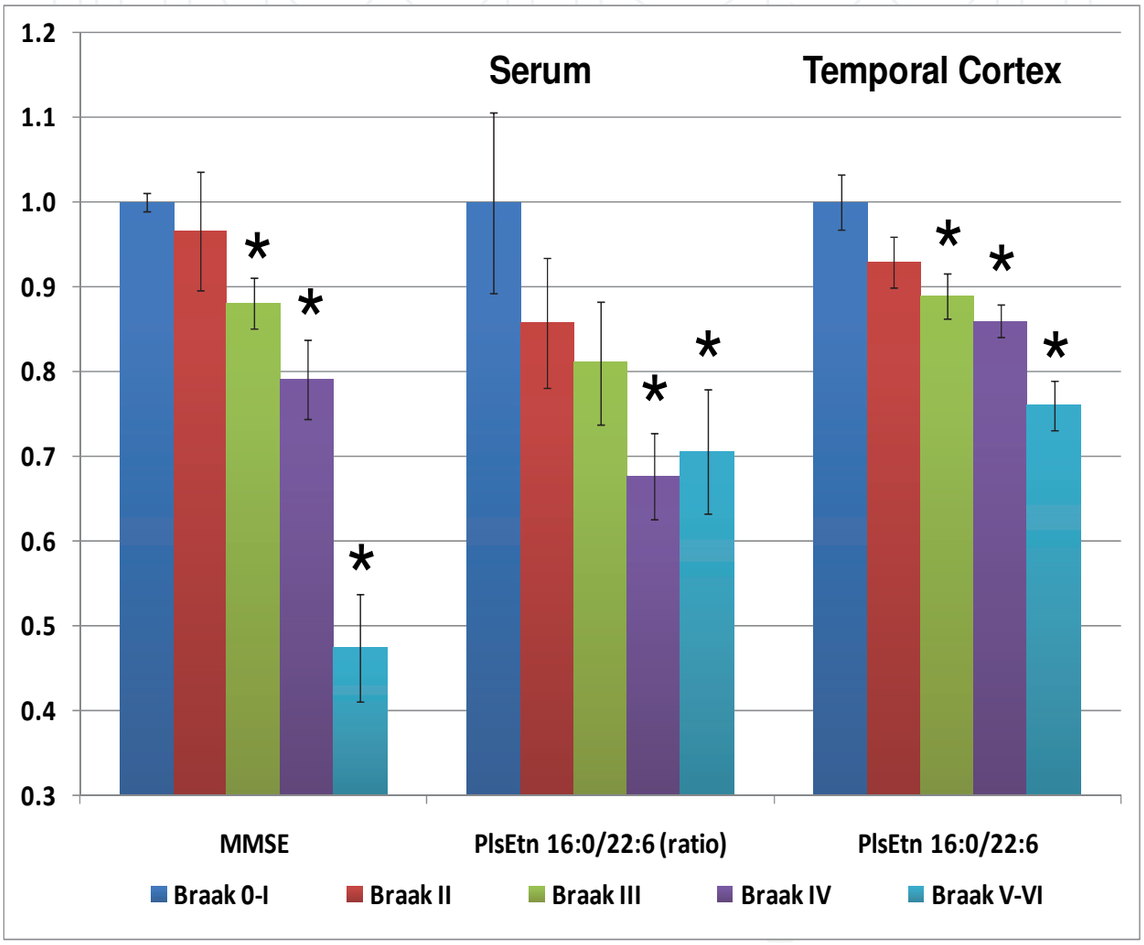


Fig. 3. Effect of Braak Stage on pre-mortem MMSE and Serum DHA-PlsEtn, and post-mortem temporal cortex DHA-PlsEtn levels. Serum levels are normalized to PtdEtn 16:0/18:0, * p<0.05. Braak 0-I (n=11, age 82.9+/-2.6, MMSE=28+/-0.5, 4F); Braak II (n=8, age 86.0+/-1.9, MMSE=25.4+/-3.6, 3F); Braak III (n=23, age 88.2+/-1.0, MMSE 23.9+/-1.2, 14F); Braak IV (n=23, age 91.1+/-0.8, MMSE 22.4+/-1.7, 9F); Braak V-VI (n=21, age 89.1+/-1.0, MMSE 12.5+/-2.1, 14F).

These data indicate that pre-mortem MMSE scores of 25 or less are predictive of increased Braak stage and decreased DHA-PlsEtn levels, whereas MMSE scores of 20 or less are

needed to be predictive of increased Aβ-42 (the key protein in amyloid plaques) levels. Further support for the role of DHA-PlsEtn in cognition is the observation that deficient liver biosynthesis of DHA correlates with cognitive impairment in AD (Astarita et al., 2010) and that phospholipid linked DHA (containing significant levels of plasmalogen) has a positive cognition effect whereas triglyceride linked DHA has a minimal effect (Hiratsuka et al., 2009).

The biosynthesis of DHA and plasmalogens occurs exclusively in peroxisomes (Nagan and Zoeller, 2001), however, it does not appear that the brain is a significant producer of these molecules. The key dietary metabolic precursor of DHA (alpha-linolenic acid) is converted to DHA in the liver, not the brain, and then transported to the brain pre-packaged on phospholipids. Peroxisomal function, as a whole, is known to decline with age (Munn et al., 2003; Mandel et al., 1998). Decreased peroxisomal function leads to decreased synthesis of PlsEtn and DHA (Zoeller and Raetz, 1986; Martinez, 1990). DHA synthesis involves chain elongation and desaturation of 18:3 n-3 fatty acids to 24:6 in the ER. The final step of DHA synthesis, β-oxidation to DHA, occurs in the peroxisome (Voss et al., 1991). Both DHAP synthase (Andre et al., 2006) and β-oxidase (Perichon and Bourre, 1995), exhibit decreased function with age and DHA containing PlsEtn are selectively decreased with age (Favreliere et al., 2000) In addition, the activity of catalase, the principal peroxisomal enzyme responsible for detoxifying H_2O_2 also decreases in activity with age (Perichon and Bourre, 1995; Favreliere et al., 2000; Rao et al., 1990), and is believed to be associated with increased lipid peroxidation with age. Accordingly, there is ample evidence of impaired plasmalogen synthesis and/or increased degradation with increasing age, however, the underlying causes and relative contribution of these processes are currently unknown.

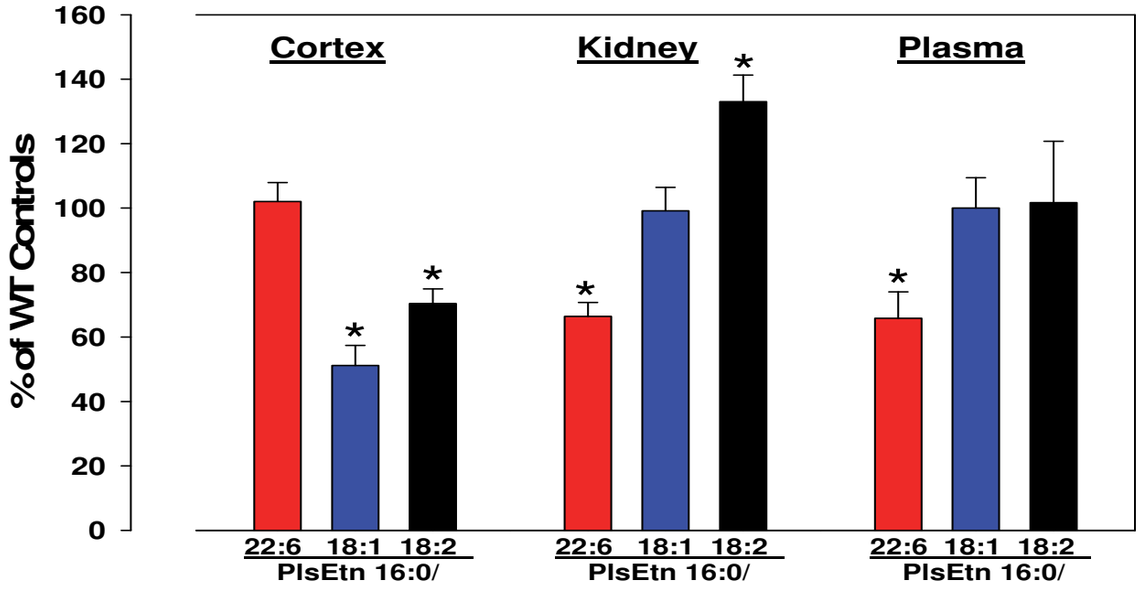


Fig. 4. Effect of cuprizone on plasma, kidney, and cortex 16:0 (sn-1) ethanolamine plasmalogen levels. * $p<0.05$ versus untreated. See PPI-1011 section for methodological description.

3. Plasmalogen biochemistry and its role in AD

The fundamental role of PlsEtn is as a membrane lipid. PlsEtn constitute over 80 mol% of the ethanolamine phospholipid pool in non-neuronal brain membranes and over 60 mol% in neurons and synaptosomes (Han et al., 2001). PlsEtn found in white matter contain predominately 18:1, 20:1 and 22:4 fatty acids at the sn-2 position, whereas in gray matter, 22:6, 20:4, and 22:4 are found in highest concentration (Horrocks and Sharma, 1982). These differences result in dramatically different membrane structures. A high percentage of monounsaturates at *sn*-2 results in very compact and stable membrane conformations (Han and Gross, 1990; 1991) which is consistent with the function of the myelin sheath. A high percentage of polyunsaturates results in more fluid membrane structures that are required for membrane fusion (Lohner et al., 1991; Lohner, 1996; Glaser and Gross, 1994; 1995), which is consistent with the functions performed by neurons. Plasmalogen deficient cells exhibit decreased transmembrane protein function (Perichon et al., 1998) and membrane related intra-cellular (Munn et al., 2003) and extra-cellular (Mandel et al., 1998) cholesterol transport.

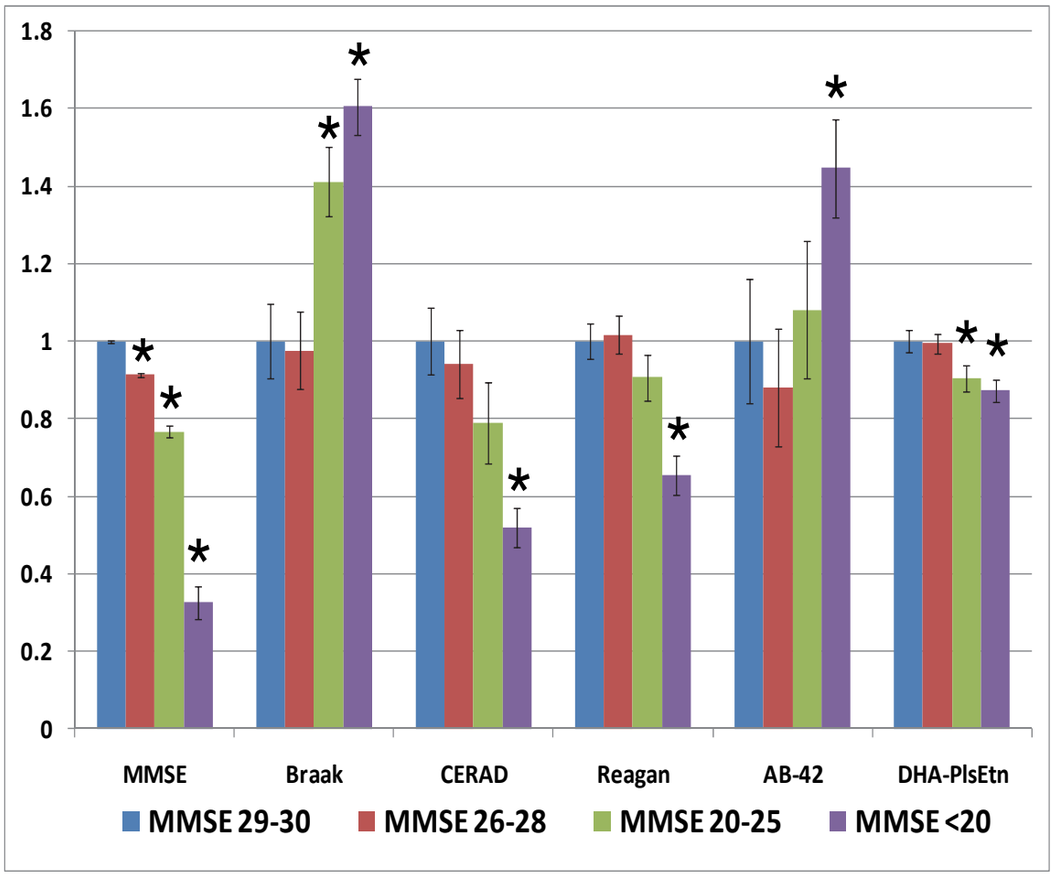


Fig. 5. Effect of pre-mortem MMSE on post-mortem pathology in the temporal cortex. * $p<0.05$. MMSE 29-30 (n=22, age 86.4+/-1.3, 11F); MMSE 26-28 (n=29, age 87.6+/-1.2, 15F); MMSE 20-25 (n=19, age 90.5+/-1.0, 11F); MMSE <20 (n=29, age 89.9 ± 1.0, 14F). MMSE scores obtained <365 days before death. Cognition analysis and pathology analyses as per Bennett et al., 2006. DHA-PlsEtn analyses performed as per Goodenowe et al., 2007; AB-42 by ELISA as per manufacturer (Covance Laboratories, Princeton, NJ)

Abnormal cholesterol regulation has been extensively implicated in AD. One plausible theory that explains the sporadic accumulation of Aβ peptides in AD is a disruption in amyloid precursor protein (APP) processing due to increased membrane cholesterol levels (Puglielli et al., 2003). This theory is supported by evidence that membrane cholesterol increases with age in both rats and humans (Hegner, 1980) and that a high cholesterol diet can increase deposition of Aβ (Refolo et al., 2000) and Aβ accumulation is closely related to the processing of the cholesterol transport protein ApoE (Refolo et al., 2000; Pratico et al., 2001) Furthermore, membrane lipid analyses of post-mortem AD subjects have shown that AD severity is positively correlated with membrane cholesterol (Cutler et al., 2004).
PlsEtn deficient cells have been previously shown to have impaired HDL-mediated cholesterol efflux (Mandel et al., 1998) and impaired intracellular LDL-mediated transport (Munn et al., 2003). In both of these studies, normal functionality was restored by PlsEtn replacement (Munn et al., 2003). Using 1-O-alkyl, 2-acyl glycerols the peroxisomal biosynthesis of plasmalogens can be bypassed and specific classes of plasmalogens selectively enhanced or restored (Figure 6), (Mankidy et al., 2010) and the effect of such manipulations on cholesterol regulation investigated. In brief, we observed that plasmalogen deficient cells (N-Rel) had reduced levels of esterified cholesterol and increased levels of free and total cholesterol in the membrane (Figure 7). We further observed that selective restoration of polyunsaturated rather than saturated PlsEtns was more effective at increasing cholesterol esterification and lowering total and free cholesterol in plasmalogen deficient cells (Table 1, Figure 7).

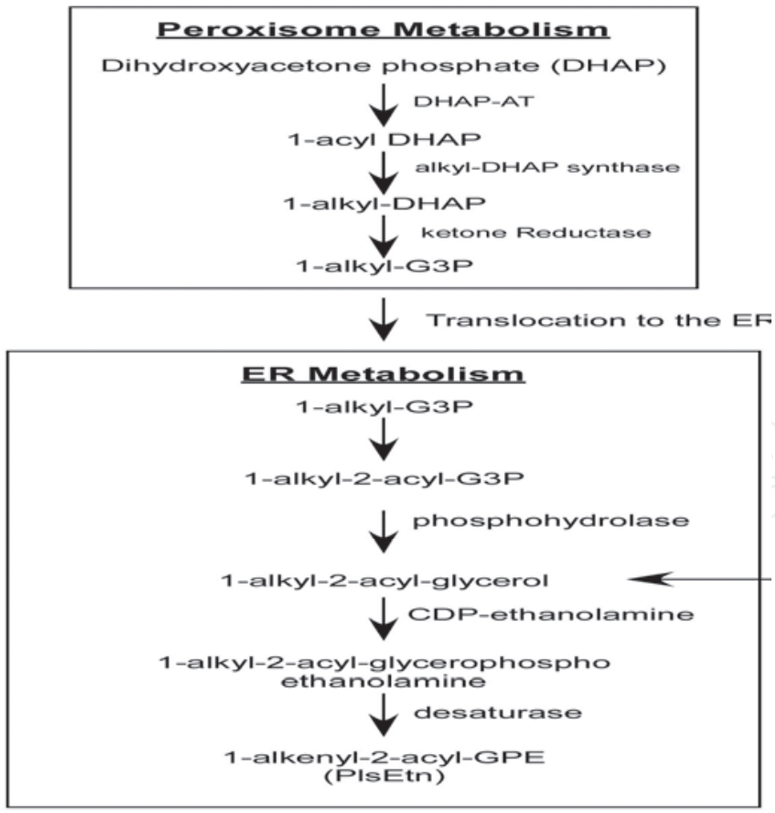


Fig. 6. Plasmalogen biosynthetic pathway. Arrow represents the entry point of sn-2 selective plasmalogen precursors.

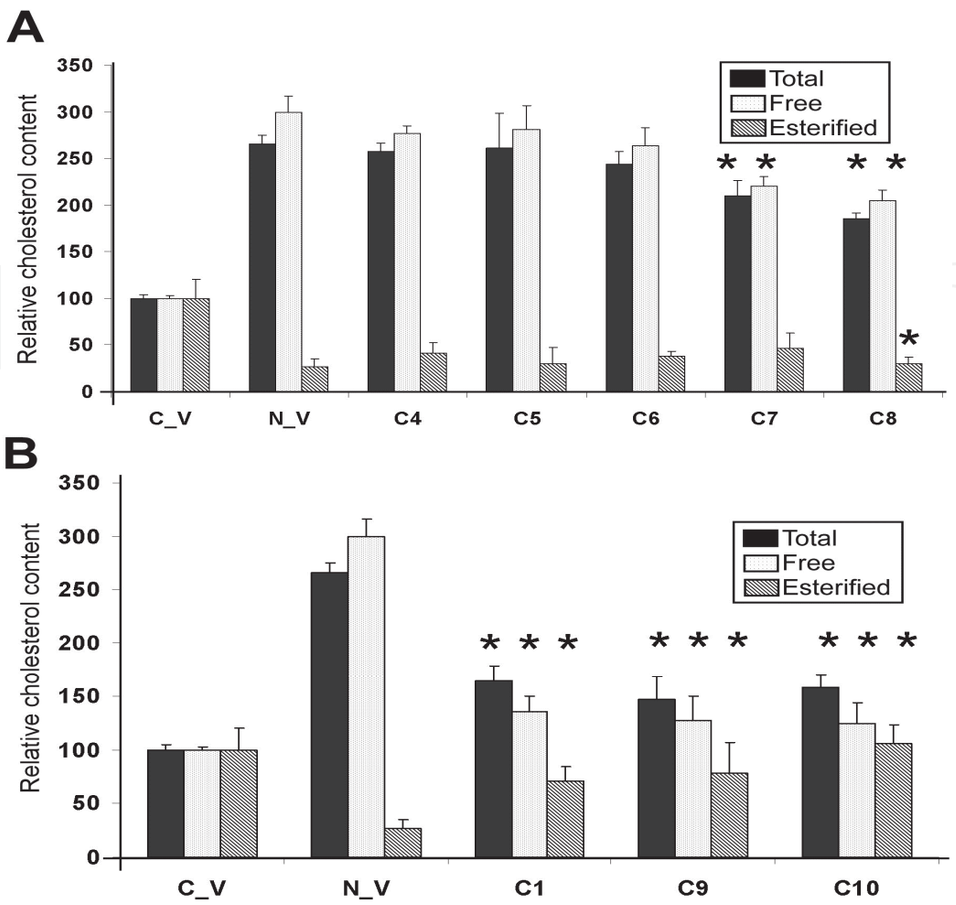


Fig. 7. Effect of plasmalogen deficiency and selective restoration on total, free, and esterified cholesterol levels in N-Rel (N) cells relative to control CHO (C) cells. * $p<0.05$ versus untreated N-Rel cells. Structures of the lipids C1, C9 and C10 are presented in Table 1.

	Compound	sn-1	sn-2	sn-3
1	cis-(±)-2-*O*-docosahexaenoyl-1-*O*-hexadecylglycerol	**16:0 (alkyl)**	**DHA**	**OH**
2	cis-(±)-2-*O*-docosahexaenoyl-1-*O*-octadecylglycerol	**18:0 (alkyl)**	**DHA**	**OH**
3	cis-(±)-2-*O*-docosahexaenoyl-1-*O*-octadec-9-enylglycerol	**18:1 (alkyl)**	**DHA**	**OH**
4	cis-(±)-2-*O*-docosahexaenoyl-1-*O*-palmitoylglycerol	**16:0 (acyl)**	**DHA**	**OH**
5	cis-(±)-2-*O*-docosahexaenoyl-1-*O*-stearoylglycerol	**18:0 (acyl)**	**DHA**	**OH**
6	cis-(±)-2-*O*-stearoyl-1-*O*-hexadecylglycerol	**16:0 (alkyl)**	**18:0**	**OH**
7	cis-(±)-2-*O*-oleoyl-1-*O*-hexadecylglycerol	**16:0 (alkyl)**	**18:1**	**OH**
8	cis-(±)-2-*O*-linoleyl-1-*O*-hexadecylglycerol	**16:0 (alkyl)**	**18:2**	**OH**
9	cis-(±)-2-*O*-α-linolenoyl-1-*O*-hexadecylglycerol	**16:0 (alkyl)**	**18:3**	**OH**
10	cis-(±)-2-*O*-arachidonoyl-1-*O*-hexadecylglycerol	**16:0 (alkyl)**	**20:4**	**OH**

Table 1. Structures of the lipids used in Figures 7-12. See Mankidy et al., 2010 for additional details.

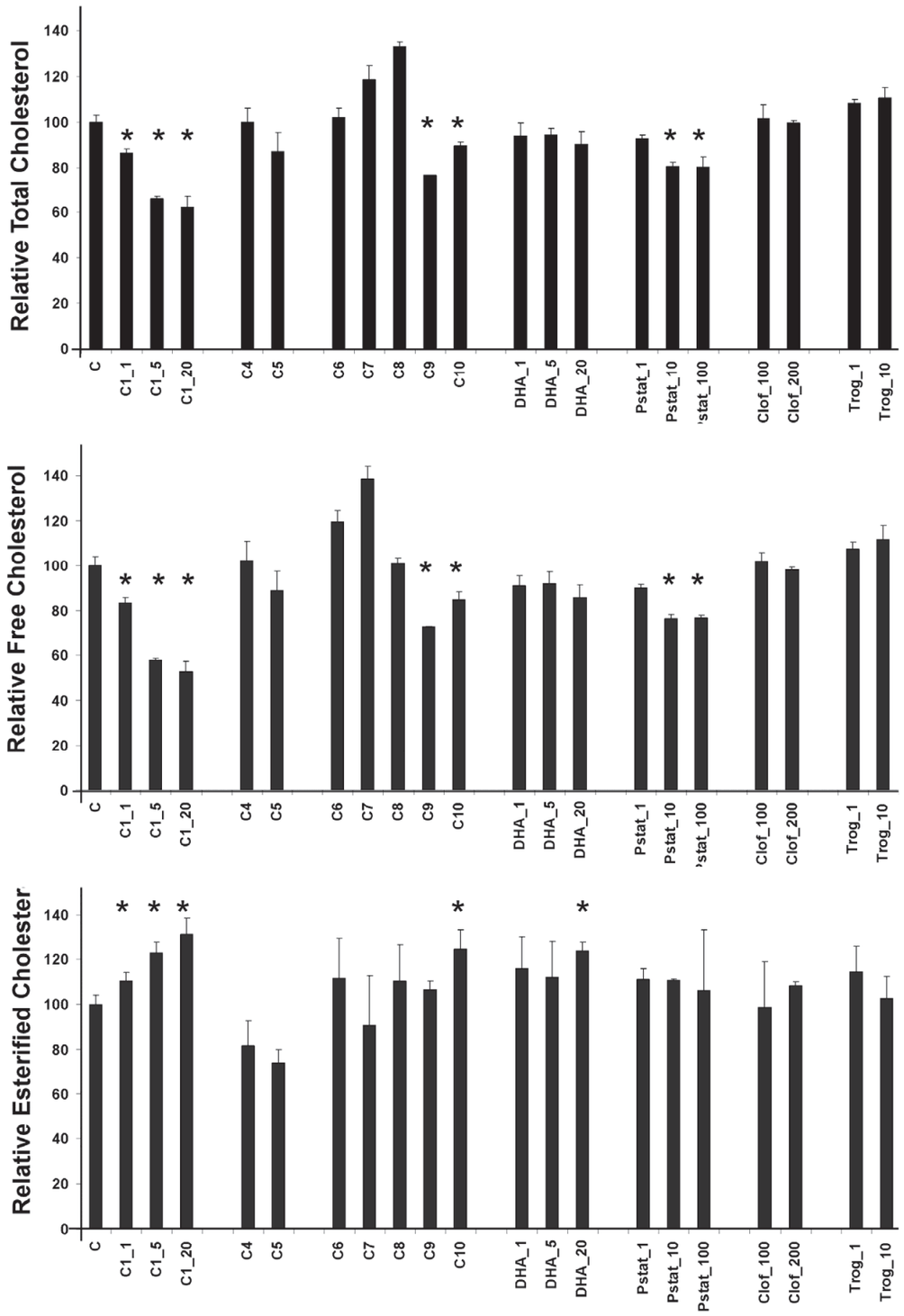


Fig. 8. Effect of plasmalogen precursors on total, free, and esterified cholesterol levels in HEK 293 cells [37]. * $p<0.05$ versus untreated controls. Structures of the lipids C1 to C10, are presented in Table 1. Clof, clofibrate; DHA, docosahexaenoic acid; Pstat, pravastatin; Trog, troglitazone. The numbers for DHA, Clof, Pstat and Trog are μM concentrations.

This effect on cholesterol regulation was also observed in plasmalogen normal, HEK293 cells (Figure 8). The observed increase in cholesterol esterification was further determined to be due to increased levels of sterol-O-acetyltransferase (SOAT) and that this increase was not cholesterol related (no effect of escalating pravastatin dose; Figure 9). These observations explain the increase in esterified cholesterol and an elevated rate of HDL-mediated cholesterol efflux reported by others (Munn et al., 2003; Mandel al., 1998). These effects could not be reproduced by either PPAR agonists or by HMG-CoA reductase antagonists. Clearly membrane PlsEtn composition is a key determiner of cholesterol regulation.

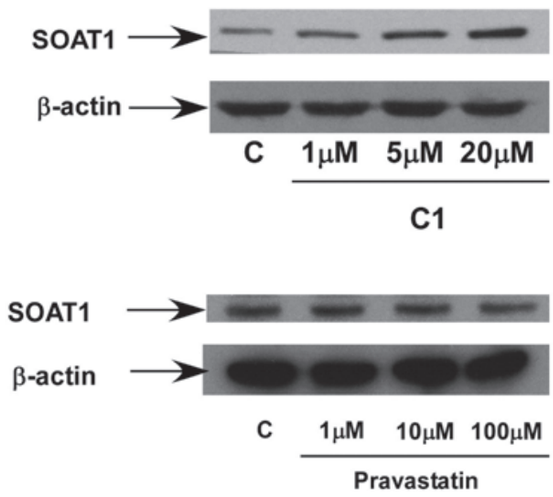


Fig. 9. Effect of the plasmalogen precursor C1 and pravastatin SOAT levels in HEK293 cells (Mankidy et al., 2010). The structures of the lipids C1 is presented in Table 1.

The role of membrane composition on amyloid precursor protein (APP) processing has also been a topic of vigorous experimentation (Puglielli et al., 2003; Chauhan, 2003). The focus of these studies has been on the role of membrane cholesterol. In brief, APP when processed via the β-secretase pathway leads to the pathological accumulation of Aβ peptides. However, under normal conditions, less than 5% of APP is processed via this pathway. The vast majority of APP is processed via the non-pathological α-secretase pathway. Alpha-secretase is located in a phospholipid-rich membrane domain, whereas β-secretase is located in cholesterol-rich lipid rafts. Both of these enzymes are sensitive to changes in membrane cholesterol. When membrane cholesterol is increased, α-secretase activity is decreased (Kojro et al., 2001) and β-secretase activity is increased (Cordy et al., 2003). A similar set of experiments as that described above for cholesterol processing was carried out focusing on APP processing. Consistent with previously published findings, cholesterol loaded HEK293 cells exhibited increased AB42 secretion which was blocked by pravastatin treatment (Figure 10). Interestingly, C1 was observed to dose dependently decrease AB42 secretion. When the proteins involved in APP processing were investigated, it was observed that cholesterol elevated β-secretase levels, but had no effect on α-secretase. C1, on the other hand dose-dependently increased α-secretase levels and decreased β-secretase levels only at the high dose. Levels of sAPPα correlated with α-secretase levels. The effect of C1 versus pravistatin on α-secretase levels was further investigated in HEK293 cells under normal conditions (Figure 11) and it was observed that only C1 was effective at modulating α-secretase levels. Clearly membrane PlsEtn composition is also a key determiner of APP processing.

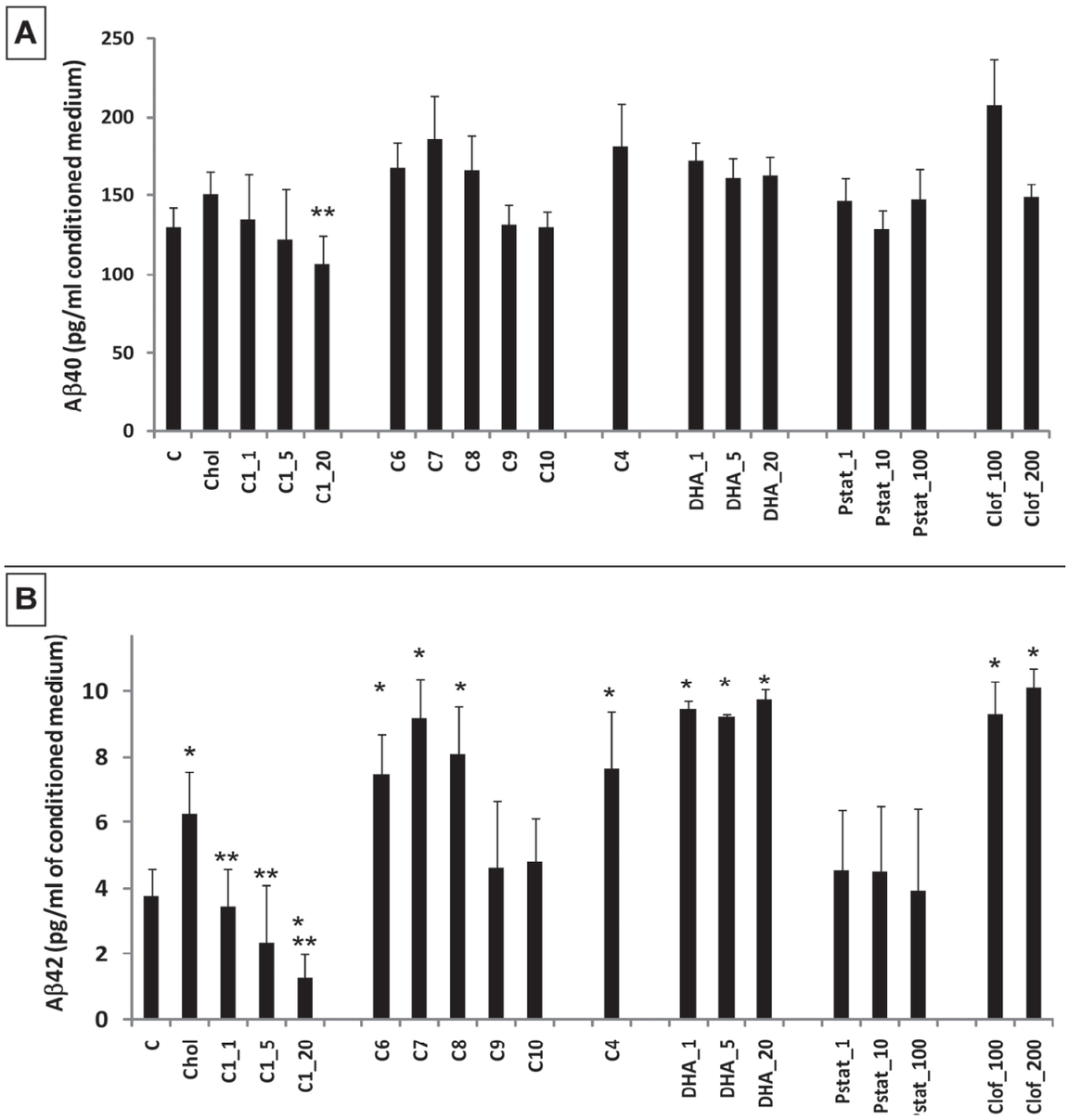


Fig. 10. Effect of various lipid mediators on AB40 and AB42 levels in HEK293 cells. Cell systems prepared as per Mankidy et al., 2010. AB levels determined using ELISA as per manufacturer's instructions (Covance Laboratories, Princeton, NJ). Structures of the lipids C1 to C10, are presented in Table 1. Chol, cholesterol; Clof, clofibrate; DHA, docosahexaenoic acid; Pstat, pravastatin; Trog, troglitazone. The numbers after compound abbreviations are μM concentrations.

In regards to cognition, the most consistent neurochemical observation in AD is decreased choline acetyltransferase (ChAT) activity in the neocortex and hippocampus (Bennett et al., 2006; Perry et al., 1978; Wilcock et al., 1982; DeKosky et al., 1992; Behl et al., 2006). Reductions in cortical ChAT activity, monitored by biopsy or in autopsy samples, correlate with the extent of intellectual impairment in AD patients. In addition, these cortical

cholinergic deficits have been found in patients examined within a year of onset of symptoms and cholinesterase inhibitors which potentiate residual cholinergic transmission, slow the decline in executive memory functions in AD patients (Behl et al., 2006). Furthermore, the inhibition of postsynaptic acetyl-choline (ACh) activity can directly induce cognitive dysfunction in healthy humans (Etienne et al., 1986). Studies of ChAT levels in the N. basalis and cortex in the same autopsy samples have shown that in 50% of AD patients there is a marked loss of cortical ChAT with no reduction in N. basalis ChAT suggesting abnormal axonal transport in AD and that the neurodegeneration originated at the axon terminal, not in the cell body.

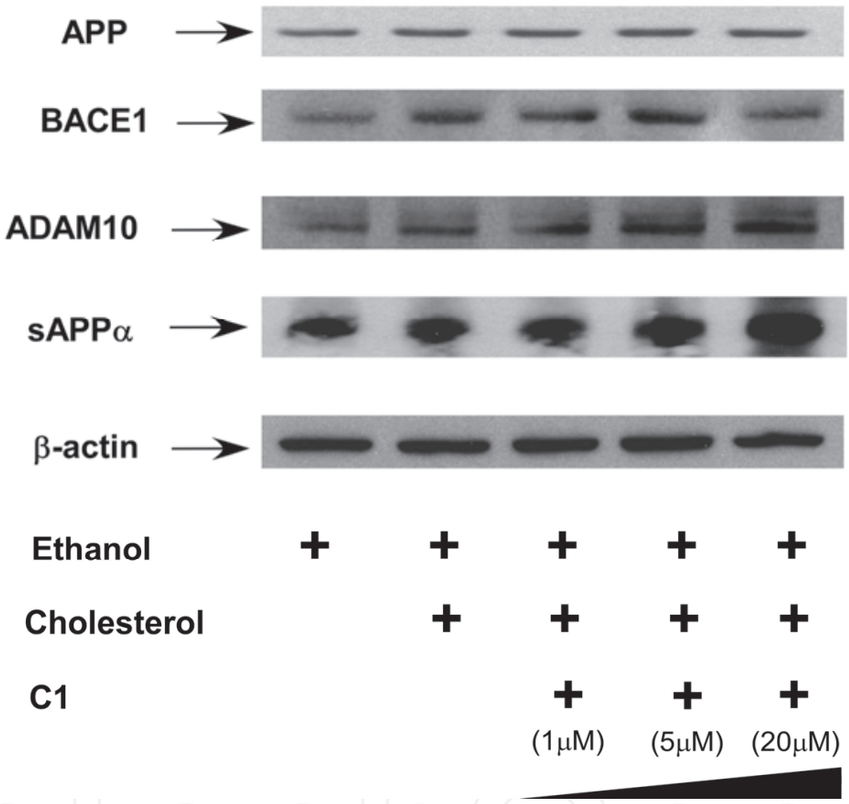


Fig. 11. Effect of escalating dose of C1 on APP, β-secretase (BACE), α-secretase (ADAM10), and sAPPα in cholesterol loaded HEK293 cells. Immunoblotting performed as per Mankidy et al., 2010.

PlsEtn are unique among neuronal lipids in that they have a high propensity to form an inverse hexagonal phase which is the essential transitory phase for successful membrane fusion events (Horrocks and Sharma, 1982). Optimal vesicular fusion is very sensitive to the amount and type of PlsEtn content. Relatively small reductions in either the vinyl ether content and/or the polyunsaturated fatty acid content of vesicles dramatically reduces the number of successful membrane fusion events (Han and Gross, 1991; Lohner et al., 1991). Therefore, this mechanism alone is sufficient to explain the correlation between decreased membrane PlsEtn and the severity of cognitive dysfunction in AD. However, in regards to cholinergic neurons there is yet a second mechanism of importance and that is pre-synaptic ACh synthesis. When a cholinergic nerve terminal releases ACh into the synaptic cleft

during a depolarization event, the released ACh is ultimately degraded to choline and acetate by acetylcholineesterase (AChE). This extracellular choline in the synaptic cleft is then rapidly re-absorbed into the presynaptic terminal by the choline high affinity transporter (CHT). The reabsorbed choline is preferentially utilized by ChAT to re-synthesize ACh, which is then transported into vesicles by an ACh transporter protein and stored for future depolarizing events. Brain slice studies have shown that as long as the CHT is functioning normally, the cholinergic terminal can maintain ACh release for extended periods of time by utilizing membrane stores of PtdCho and PtdEtn (Ulus et al., 1989) and by the extraction of choline from surrounding cells (Farber et al., 1996). This occurs even in the absence of exogenous choline. However, in the presence of the CHT inhibitor, HC-3, the ability to sustain release of ACh is dramatically reduced, even in the presence of exogenous supplied choline (Maire and Wurtman, 1985). These data indicate that the proper functioning of the CHT is essential for the sustained release of ACh from cholinergic neurons. Recently, Ferguson *et al*. made a landmark finding where they showed that the CHT is localized on pre-synaptic vesicles, not constituently expressed on the pre-synaptic membrane (Ferguson et al., 2003; 2004). This finding indicates that the dynamic regulation of choline uptake via the CHT occurs by an increased density of CHTs at the synapse following a nerve impulse and subsequent deactivation by vesicular endocytosis. Impaired vesicular fusion as a result of a PlsEtn deficiency would be expected to have a similar effect on choline uptake as the presence of HC-3, albeit via a different mechanism.

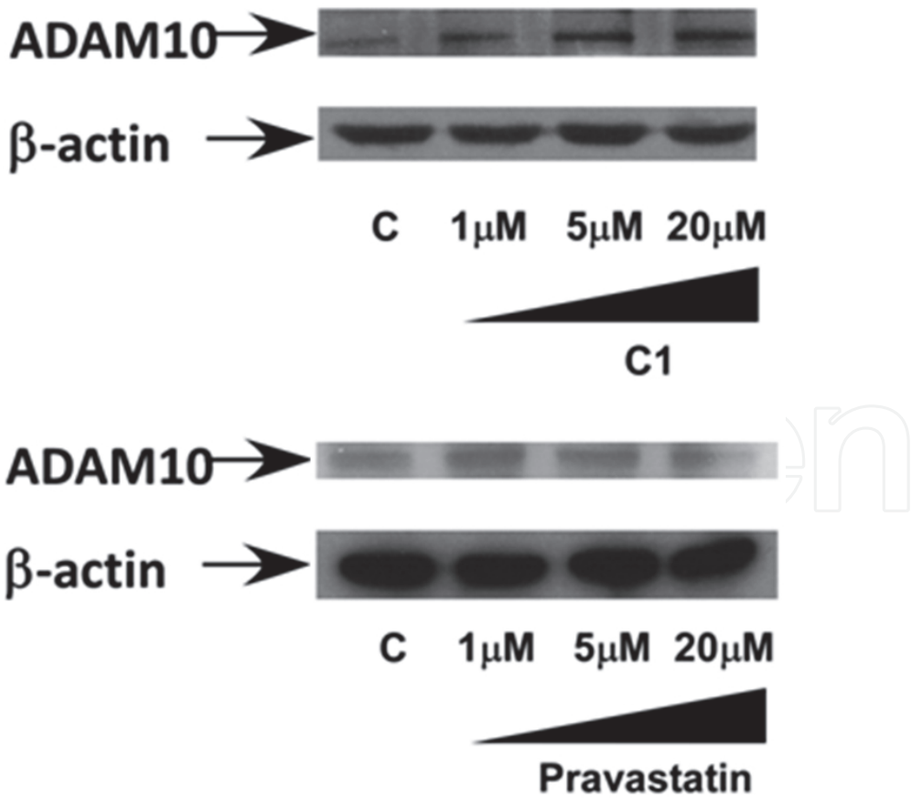


Fig. 12. Effect of escalating dose of C1 (Table 1) and pravastatin on α-secretase (ADAM10) levels in HEK293 cells. Immunoblotting performed as per Mankidy et al., 2010.

In summary, a deficiency in polyunsaturated PlsEtn in the CNS would be expected to reduce non-pathogenic APP processing, impair membrane cholesterol clearance, reduce vesicular release of neurotransmitters, and selectively impair pre-synaptic ACh replenishment following a nerve impulse. Accordingly, a therapeutic strategy aimed at developing an orally bioavailable DHA-PlsEtn precursor warrants clinical investigation in AD.

4. Plasmalogen replacement therapy for Alzheimer's disease

The goal of our research program was to design an orally bioavailable DHA-plasmalogen precursor drug that bypasses age- and disease-sensitive peroxisomal biosynthetic pathways for both plasmalogens and DHA, thereby effectively restoring and/or enhancing DHA-plasmalogen-dependent synaptic and membrane functions that are diminished in AD, and which correlate with both clinical and pathological features of AD.

Since brain plasmalogens are supplied by the liver and intestinal mucosa, we established a research program to design an orally bioavailable ether lipid precursor that would bypass the requirement for peroxisomes to generate the ether lipid linkage at sn-1 of the glycerol backbone. Additionally, the decreased ability of the AD liver to synthesize DHA, along with decreased circulating plasma levels of DHA in AD patients, prompted us to substitute DHA as the fatty acid at sn-2 of the glycerol backbone. A sn-3 substituent was required to stabilize the DHA substitution at sn-2. The requirements for this substitution were: 1) A substituent that did not hinder hydrolysis at sn-3 by gut lipases to provide the alkyl-acyl-glycerol to the gut mucosa for addition of phosphoethanolamine at sn-3; 2) The released sn-3 substituent should be non-toxic and preferably beneficial; 3) Stabilization of the drug product; 4) Ease of synthesis and low cost of goods. A number of candidates were examined and lipoic acid was found to meet all of these criteria, as embodied in the drug development candidate PPI-1011

4.1 PPI-1011: Chemistry

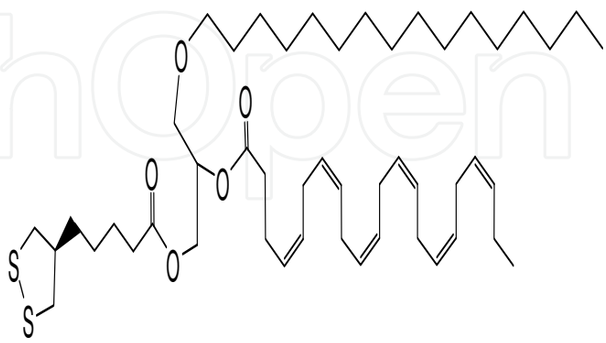
PPI-1011 is an ether lipid plasmalogen precursor possessing palmitic acid at sn-1 of the glycerol backbone; DHA at sn-2 and lipoic acid at sn-3. That lipoic acid at sn-3 was the best moiety for both stabilizing the drug during storage and for synthetic purity.

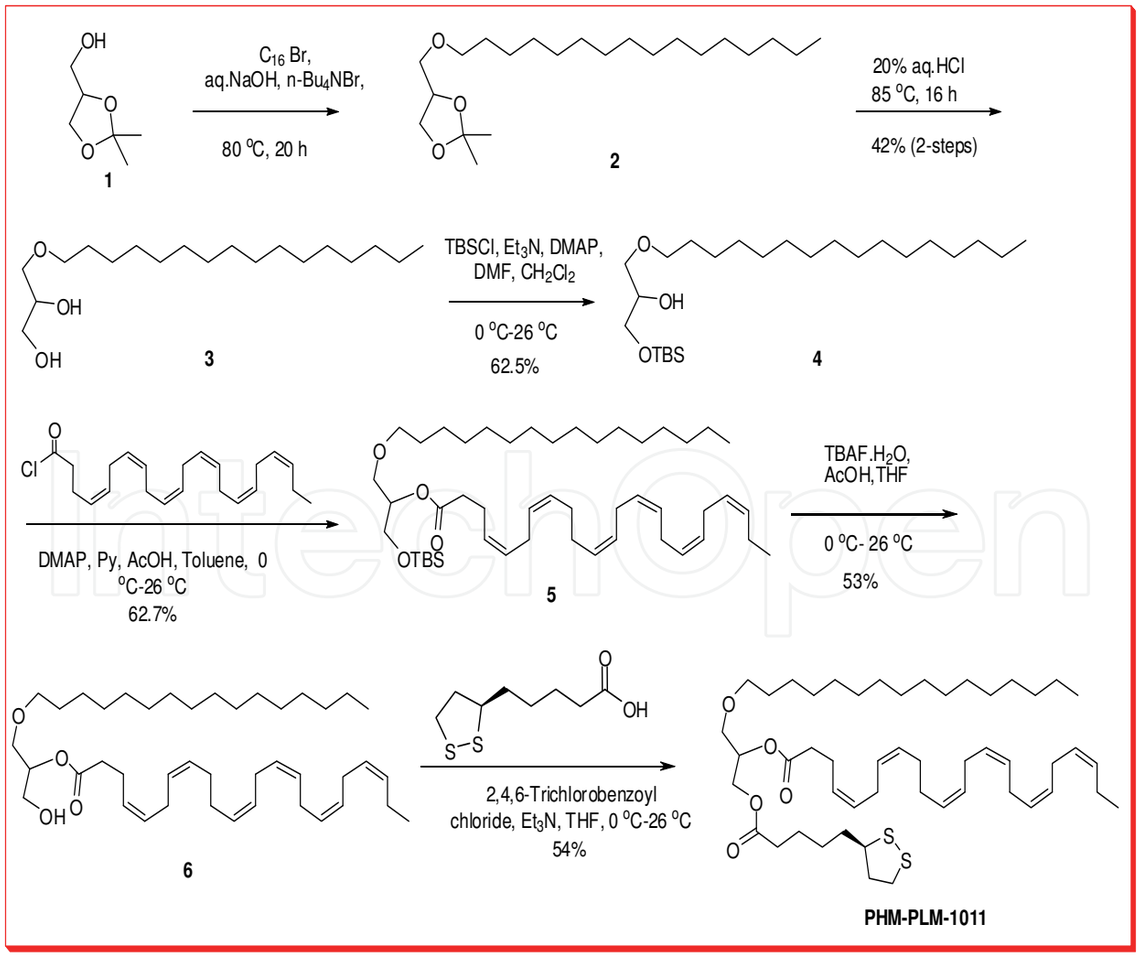
For the synthesis of PPI-1011, solketal **1** was alkylated using cetyl bromide under phase transfer conditions (TBAB / aq. NaOH) to furnish **2**. Acidic hydrolysis of the ketal in **2** gave 1-cetyl glycerol **3** in good yield. The primary hydroxyl at sn-3 position was selectively protected as a TBS ether to give **4.** DHA was activated as its acid chloride using oxalyl chloride and then treated with **4** under basic conditions to furnish **5.** Desilyation of **5** using equimolar mixture of TBAF / acetic acid proceeded smoothly to give **6** as the major product. Initial efforts to carry out the esterification of **6** with R-lipoic acid *via* the acid chloride route did not proceed well, giving low yields of the required product. Esterification under Yamaguchi conditions involving the mixed anhydride of R-lipoic acid afforded the desired lipoic acid conjugate **PPI-1011** in 54% yield.

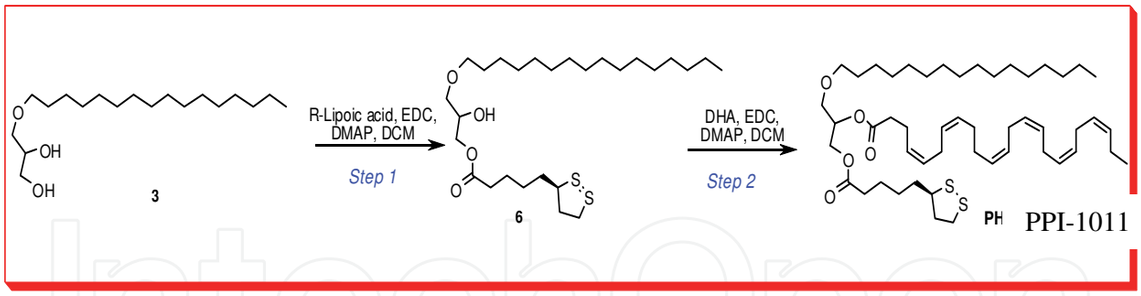
The scheme was further modified to decrease the number of steps and increase the overall yield of the product. The primary alcohol in diol **3** was coupled with R-Lipoic under EDC/DMAP conditions to give **6** as the major product. Bis lipoic acid product formed as the minor product was easily separated by column chromatography. Esterification of the sn-2 position in **6** with DHA was also carried out under EDC/DMAP conditions to give **PPI-**

1011. This route is superior to the previous route in the following ways: i) Protection-deprotection steps are avoided ii) Compound **6** can directly be carried forward for Step 2 (purification can be attempted after step 2) iii) This route is economically viable, easily scalable and can serve as a general protocol for the synthesis of analogues.

Molecular Formula: $C_{49}H_{82}O_5S_2$
Molecular Weight: 815.3
Dosage Form: Gelatin capsule
Glycerol sn-1 : Palmitic acid (alkyl linkage)
Glycerol sn-2 : DHA (acyl linkage)
Glycerol sn-3 : Lipoic acid (acyl linkage)

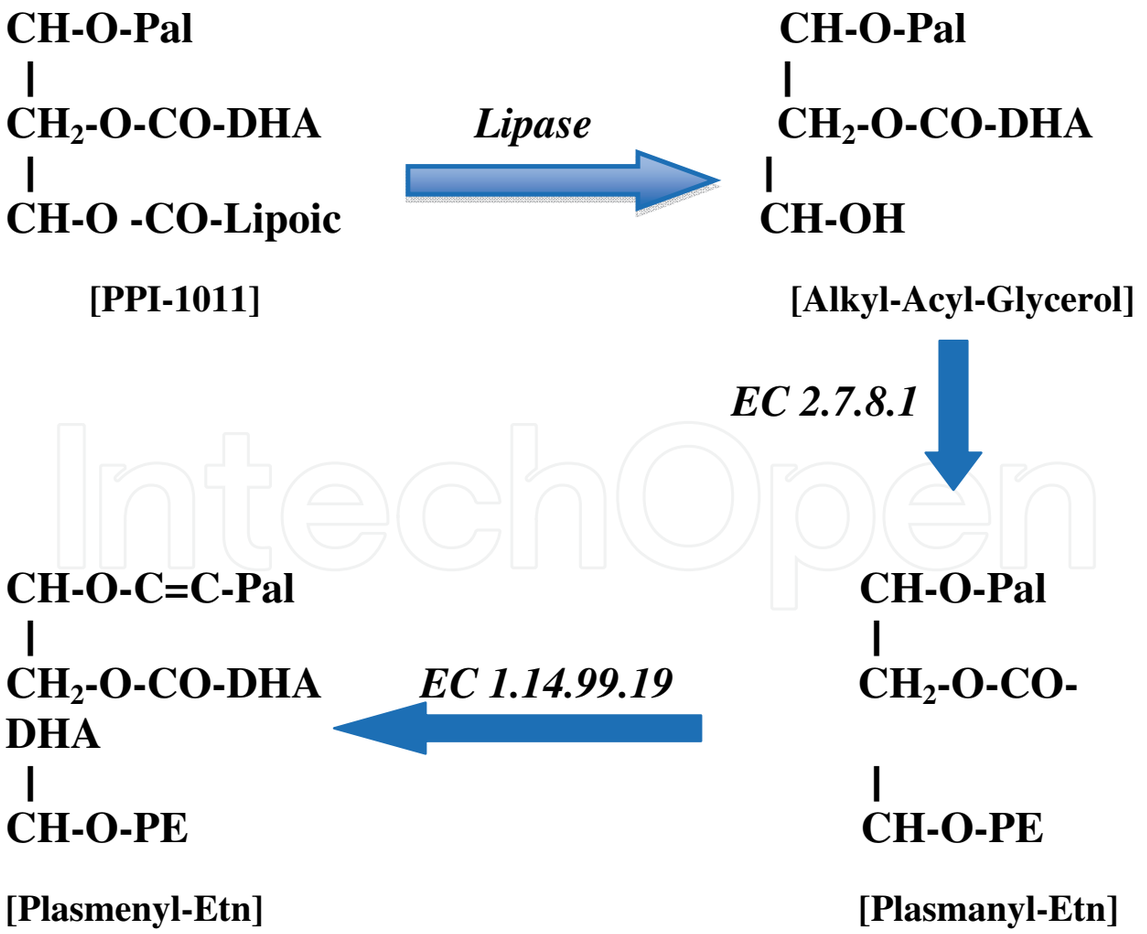






4.2 PPI-1011: In vitro data

In vitro studies with PPI-1011 have demonstrated that PPI-1011 can restore the target PlsEtn 16:0/22:6 in N-Rel cells which lack the peroxisomal enzymes required to generate ether lipids. These mutant Chinese hamster ovary (CHO) cells have plasmalogen levels that are 5 to 10% of control CHO cells, both at the whole cell level and in isolated mitochondria. The target plasmalogen 16:0/22:6 generated by PPI-1011undergoes lipid remodeling with sn-2 deacylation/reacylation thereby restoring other cellular plasmalogens. The released lipoic acid rather than being a prodrug waste product may provide some neuroprotective actions in AD patients (Maczurek et al., 2008).



The *in vitro* effects of PPI-1011 on ethanolamine plasmalogen levels were determined using N-Rel cells which possess a deficiency in DHAPT, a peroxisomal membrane protein, which catalyses the first step in plasmalogen biosynthesis (Nagan et al., 1998). The DHAPT deficiency causes a severe decrease in ethanolamine plasmalogens. However, N-Rel cells do continue to have otherwise functional peroxisomes. N-Rel cells were initially created as a mutagenized cell population of CHO cells. Cell experiments were set up with an n=5 for the control, vehicle and treatment groups. Figure 13 demonstrates the ability of PPI-1011 to bypass peroxisomes and generate the target PlsEtn 16:0/22:6 which undergoes lipid remodeling to also resupply PlsEtns 16:0:20:4, 16:0/18:1 and 16:0/18:2.

The deacylation of DHA (22:6) from the *sn*-2 position of PPI-1011 and reacylation at *sn*-2 with oleic acid (18:1), linoleic acid (18:2), or arachidonic acid (20:4) was observed following PPI-1011 cell treatment. Increases in plasmalogens were both concentration and time-dependent with maximal increases by 72 hr at 100 μM. Utlizing the Alamar blue assay, PPI-1011 was not cytotoxic at 100, 300, 600, 900, 1200, 1500 and 2100 μM with 72 hr incubations. Analyzing whole cell plasmalogen levels, PPI-1011 augmented PlsEtn levels while DHA supplementation was ineffective (Figure 14). These data demonstrate that DHA supplementation is critically dependent upon ether lipids supplied by peroxisomes to effectively augment PlsEtns.

Restoring normal plasmalogen levels in plasmalogen-deficient cells with PPI-1011 also has been demonstrated to have the functional benefit of restoring cholesterol efflux and reducing cellular cholesterol levels in N-Rel cells (Nagan and Zoeller, 2001) as previously reported with other ether lipid precursors (Mankidy et al., 2010).

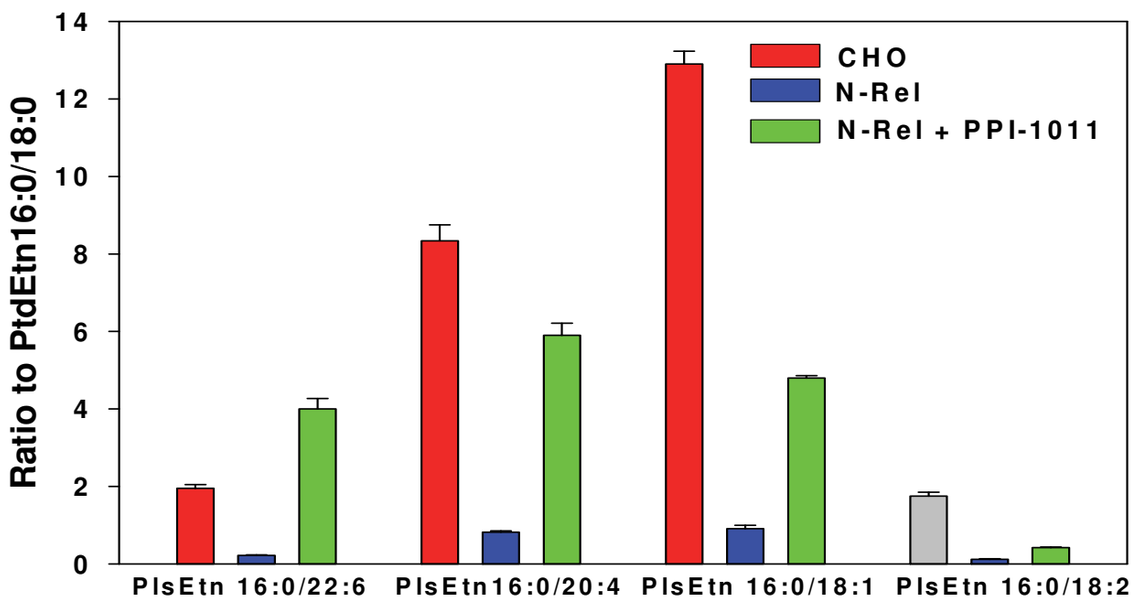


Fig. 13. Augmentation of N-Rel mitochondrial plasmalogens after a 72 hr incubation with 100 μM PPI-1011. Data are expressed as a ratio of the plasmalogen to the housekeeping metabolite phosphatidylethanolamine 16:0/18:0. N= 5; Mean ± SEM. PPI-1011 significantly ($p < 0.01$) restored all 16:0/x plasmalogens.

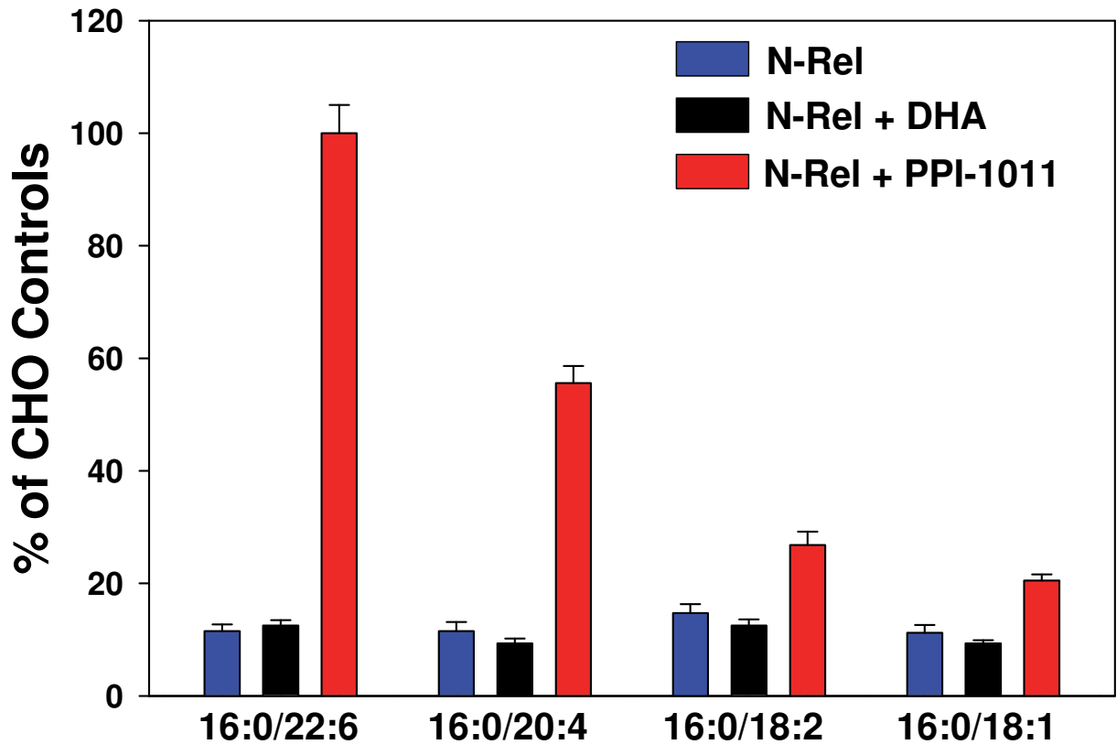


Fig. 14. Augmentation of cellular plasmalogens in N-Rel cells after a 72 hr incubation with PPI-1011 (100 µM) but not with DHA (100 µM; Martek). Data are presented as % of CHO control cells. N= 5; Mean ± SEM. PPI-1011 significantly ($p < 0.01$) restored all 16:0/x plasmalogens. All bars are significantly less than control ($p < 0.01$), except for the 16:0/22:6 in PPI-1011 treated cells.

4.3 PPI-1011: In vivo data

Oral dosing of rabbits (manuscript in preparation) with PPI-1011 in gelatin capsules demonstrated dose (10 to 200 mg/kg)- and time (0 to 48 hr)-dependent augmentation of DHA-containing plasmalogens with further increases in doses (500 and 1000 mg/kg) not further augmenting circulating plasmalogen levels. DHA was also released, via deacylases, with peak plasma DHA levels at 6 hours and maximal plasma levels at a dose of 500 mg/kg rather than 200 mg/kg as observed with plasmalogens. These data support once daily dosing of PPI-1011 and demonstrate that circulating plasmalogens are auto-regulated thereby limiting the potential for toxicity from excess levels.

The critical *in vivo* evaluation of PPI-1011 was to demonstrate restoration of deficient plasmalogens in an animal model. To this end we investigated the murine cuprizone model of extensive cortical demyelination (Skripuletz et al., 2008). C57BL/6J mice were separated into 3 treatment groups: control, cuprizone and cuprizone + PPI-1011 (n=7). Standard rodent chow was ground and 0.3% cuprizone (bis-cyclohexanone oxaldihydrazone) was added to the ground diet of the treated animals. Control or 0.3% cuprizone chow and water were provided to animals *ad libtum*. Diets were fed to the treatment groups for a total of 7 weeks. For the last 2 weeks the cuprizone + PPI-1011 group was given 100 mg/kg/day of PPI-1011 suspended in soybean oil by oral gavage.

Upon completion of the study animals were anesthetized with isoflurane and plasma, kidney and neocortex collected for analyses.

Total RNA was isolated from approximately 2 μg of pulverized cortex tissue using the RNeasy Mini Kit (Qiagen) as per the manufacture's protocol. RNA was quantified by optical density with the NanoVue spectrophotometer (GE Healthcare Life Sciences). Reverse transcription reactions were performed on 800ng RNA using the qScript cDNA SuperMix (Quanta Biosciences). Each cortex samples was analyzed to determine expression of reelin (sense 5'-cccagcccagacagacagtt-3'; antisense 5'-ccaggtgatgccattgttga-3'), as well as the myelin markers myelin basic protein (sense-5' cctcaaagacaggccctcag 3'; antisense-5' cctgtcaccgctaaagaagc 3') and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (EC 3.1.4.37; sense-5' catcctcaggagcaaaggag 3'; antisense-5' tgaatagcgtcttgcactcg 3') and the housekeeping gene β-actin (sense-5' agccatgtacgtagccatcc 3'; antisense-5' ctctcagctgtggtggtgaa 3' (Mack et al., 2007; Heinrich et al., 2006). Specificity of each primer set was determined by analysis of the dissociation curve. Quantitative real-time PCR was carried out in triplicate using the Fast SYBR Green Master Mix (Applied Biosystems) on the StepOne Plus Real-Time PCR System (Applied Biosystems). Thermocycling conditions were: 95°C for 20s followed by 40 cycles of 95°C for 3s and 60°C for 30s. For plasmalogen analyses, 10 mg of tissue powder in 5 mL screw cap tubes were polytroned in 1 mL of PBS + 0.5 mL MeOH. In the case of plasma, 100 μL were vortexed with 1 mL of PBS + 0.5 mL MeOH. Next, 2 mL tert-butylmethylether were added to the plasma samples and tissue homogenates and the samples capped and shaken (1400 rpm) for 15 min at room temperature. The samples were then centrifuged for 8 min in a clinical centrifuge and 1 ml of the upper organic layer isolated for LC-MS/MS analyses of DHA, ethanolamine plasmalogens and phosphatidylethanolamines as reported previously (Wood et al., 2010). For cholesterol and myoinositol analyses, 2 mg of tissue powder in 5 ml screw cap tubes were polytroned in 1.2 ml of acetonitrile:MeOH:formic acid (800:200:2.4) containing [$^2H_7$]cholesterol and [$^2H_6$]myoinositol (Cambridge and CDN Isotopes) as internal standards. The homogenates were transferred to 1.5 ml screw top microtubes and centrifuged at 4^{o}C and 25,000xg for 30 min. Next, 200 μL of the supernatant were dried in a Savant centrifugal evaporator. Trimethylsilylation of the samples was conducted at 80^{o}C for 1 hr with 100 μL acetonitrile and 100 μL of N,O-bis(trimethylsilyl)trifluoro-acetamide and TMCS (10/1). The TMS derivatives were analyzed by GC-MS with the $[M-72]^+$ cations 386.3 and 393.3 monitored under ammonia PCI for cholesterol and [$^2H_7$]cholesterol, respectively and the $[MH]^+$ cations of 613.2 and 619.2 monitored for myoinositol and [$^2H_6$]myoinositol, respectively.

Analysis of plasma phosphatidylethanolamines and ethanolamine plasmalogens revealed selective 20 to 40% decrements in free DHA and DHA containing phospholipids in cuprizone treated mice (Figure 15). Additionally, oral administration of PPI-1011 for 2 weeks fully restored these plasma decrements (Figure 15).

Cuprizone toxicity also resulted in significant decrements in kidney PlsEtns (Figure 16). PPI-1011 treatment restored kidney tissue levels of DHA-containing plasmalogens (PlsEtn 16:0/22:6, 18:0/22:6, 18:1/22:6) and augmented free DHA levels. PlsEtn18:1/18:2 was also restored with PPI-1011 treatment (Figure 16). In the cuprizone model, decrements in circulating and kidney DHA and DHA-containing plasmalogens are most likely the result of cuprizone toxicity in the liver (Heinrich et al., 2006). PPI-1011 clearly demonstrated oral bioavailability in that it was able to fully reverse these effects of cuprizone on circulating and kidney plasmalogens.

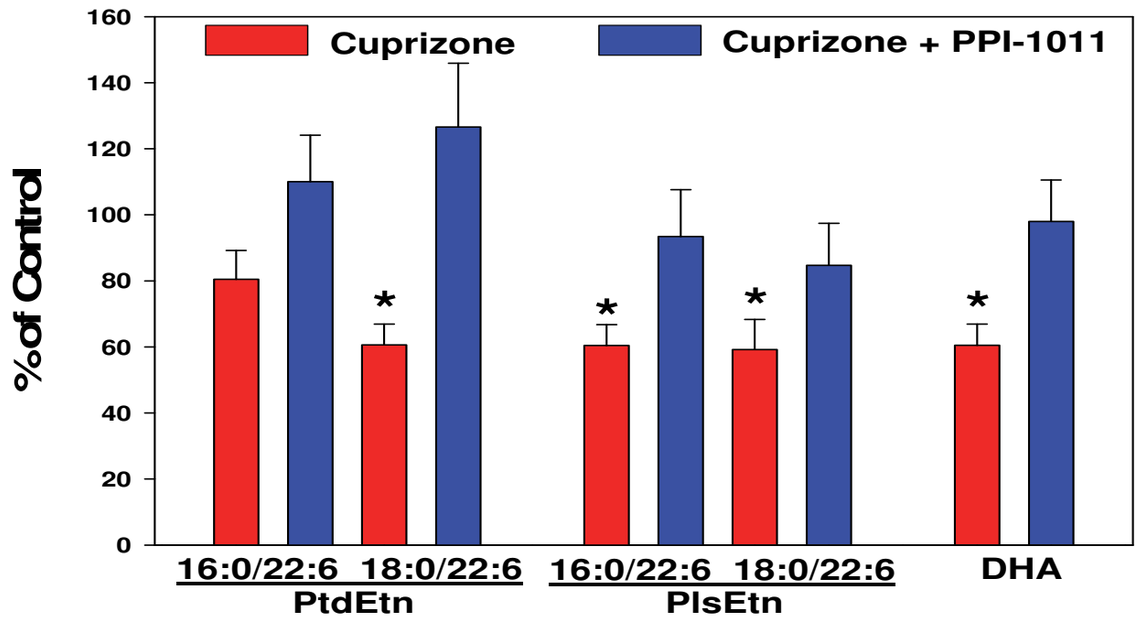


Fig. 15. Plasma DHA and DHA-containing PtdEtns and PlsEtns in mice maintained on a 0.3% cuprizone diet for 7 weeks. One group of cuprizone mice was treated with PPI-1011 (100 mg/kg, po) for weeks 6 and 7. Mean ± SEM; N=6 -7. * $p < 0.05$ vs. control.

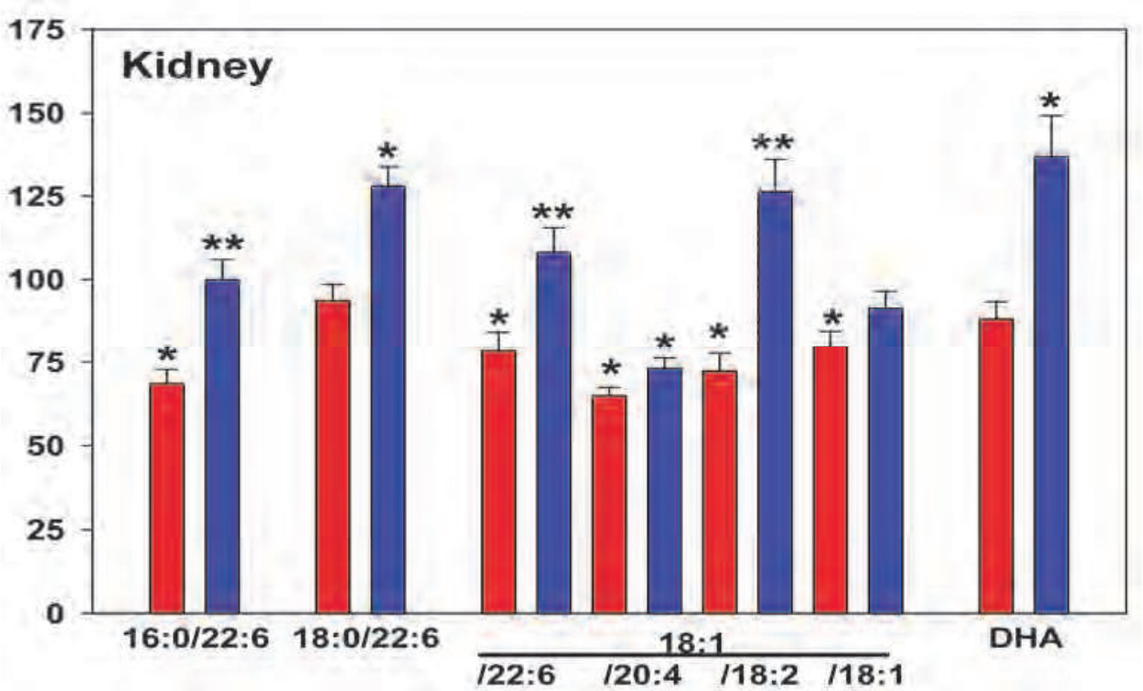


Fig. 16. Kidney PlsEtns and DHA in mice maintained on a 0.3% cuprizone diet for 7 weeks ± PPI-1011 treatment (100 mg/kg, po) for weeks 6 and 7. Mean ± SEM; N=6 -7. * $p < 0.01$ vs. control; **, $p < 0.05$ vs. cuprizone.

Analysis of neocortical ethanolamine phospholipids demonstrated selective and large reductions in white matter plasmalogens (PlsEtn). These included the 16:0/18:1 and 16:0/18:2 plasmalogens (Figure 17- B) as well as the 18:0/18:1, 18:0/18:2 PlsEtns (Figure 17-D). There were also significant reductions in the 18:0/18:1, 18:0/18:2 phosphatidylethanolamines (PtdEtn; Figure 17-C). With PlsEtn precursor treatment (PPI-1011), the decrements in the 16:0/18:2 (Figure 17-B) and 18:0/18:2 (Figure 17-D) PlsEtns and the 18:0/18:2 PtdEtns (Figure 17-C) were significantly reversed. PPI-1011 treatment also significantly augmented PtdEtn 16:0/18:2 in cuprizone treated mice (Figure 17-A). PPI-1011 did not alter PlsEtns with oleic acid (18:1) at sn-2 (Figure 17).

The marked ability of PPI-1011 to restore linoleic (18:2 at sn-2)-containing PlsEtn and phosphatidylethanolamines in both kidney and neocortex, suggests that PPI-1011 stimulates/induces a reacylation mechanism that favors 18:2 addition at sn-2.

To evaluate the potential benefits of plasmalogen replacement on myelin function, we examined the established myelin markers cholesterol, myo-inositol, myelin basic protein mRNA and 2',3'-cyclic-nucleotide 3'-phosphodiesterase mRNA. These markers were all reduced by 20 to 40% with 7 weeks of cuprizone treatment (Figure 18) and were significantly reversed by treatment with PPI-1011 for weeks 6 and 7 (Figure 18). PPI-1011 also induced reelin expression, an extracellular matrix glycoprotein involved in nerve regeneration (Figure 18). These increases in neocortical cholesterol, myo-inositol, myelin basic protein and 2',3'-cyclic-nucleotide 3'-phosphodiesterase and reelin (May et al., 2005) suggest that plasmalogen restoration may stimulate remyelination. Since cholesterol transport is dependent upon plasmalogens (Munn et al., 2003), PPI-1011 replenishment of plasmalogens may also contribute to restoration of cholesterol levels.

These data are relevant to AD in that AD pathology involves extensive hypomyelination as demonstrated by dramatic decrements in a number of myelin markers including plasmalogens (Han et al., 2001) sulfatides (Han et al., 2002); myelin basic protein (Wang et al., 2004) and 2',3'-cyclic nucleotide-3'-phosphodiesterase (Vlkolinsky et al., 2001), in autopsy studies of AD neocortex. Imaging studies also suggest that this process occurs in mild cognitive impairment (MCI) patients, consistent with the observations that myelin plasmalogens decrease early in the disease process (Han et al., 2001). Decrements in myelin and neuronal plasmalogens may well be responsible for the shrinkage in AD neurons that precedes final neuronal cell death (Scott et al., 1992; Stark et al., 2005; Vogels et al., 1990; Artaco-Perula and Insausti, 2007; Vereecken et al., 1994). Our data support previous studies indicating that myelin basic protein mRNA, 2',3'-cyclic-nucleotide 3'-phosphodiesterase mRNA, and plasmalogens with oleic acid and linoleic acid at sn-2 are reliable markers of myelin loss.

Our data for the first time also demonstrate that myelin decrements are reflected by decreases in neocortical phosphatidylethanolamines with oleic acid and linoleic acid at sn-2. With regard to therapeutic implications, these data indicate that plasmalogen precursors have the potential to correct brain plasmalogen and phosphatidylethanolamines deficits in leukodystrophies, multiple sclerosis and Alzheimer's disease.

4.4 PPI-1011: Safety

While PPI-1011 is not an endogenous molecule, it is rapidly cleaved in the gut to yield two endogenous molecules, an alkyl-acyl-glycerol and lipoic acid. Previous human experience indicates that 1-o-octadecyl-sn-glycerol is consumed in the diet up to 100 mg/day and that supplementation with this ether lipid (5 to 10 mg/kg/day for 2 to 4 years) was not associated with any toxicity in children with peroxisomal disorders (Das et al., 1992)

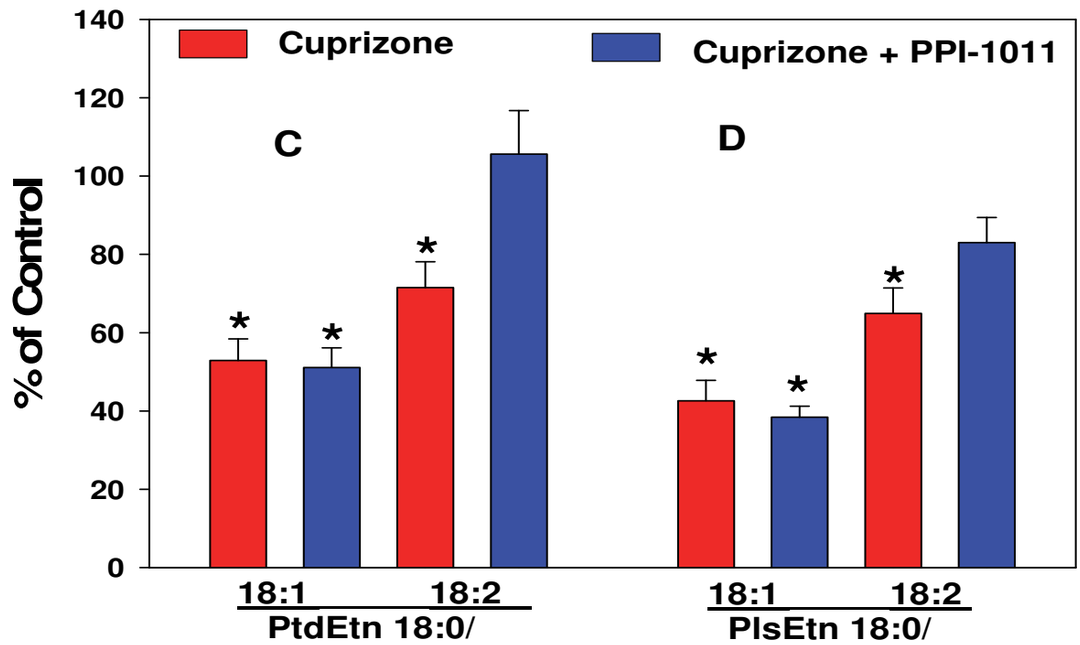


Fig. 17. Neocortical PtdEtns and PlsEtns in mice maintained on a 0.3% cuprizone diet for 7 weeks ± PPI-1011 treatment (100 mg/kg, po) for weeks 6 and 7. Mean ± SEM; N=6 -7. * $p < 0.01$ vs. control.

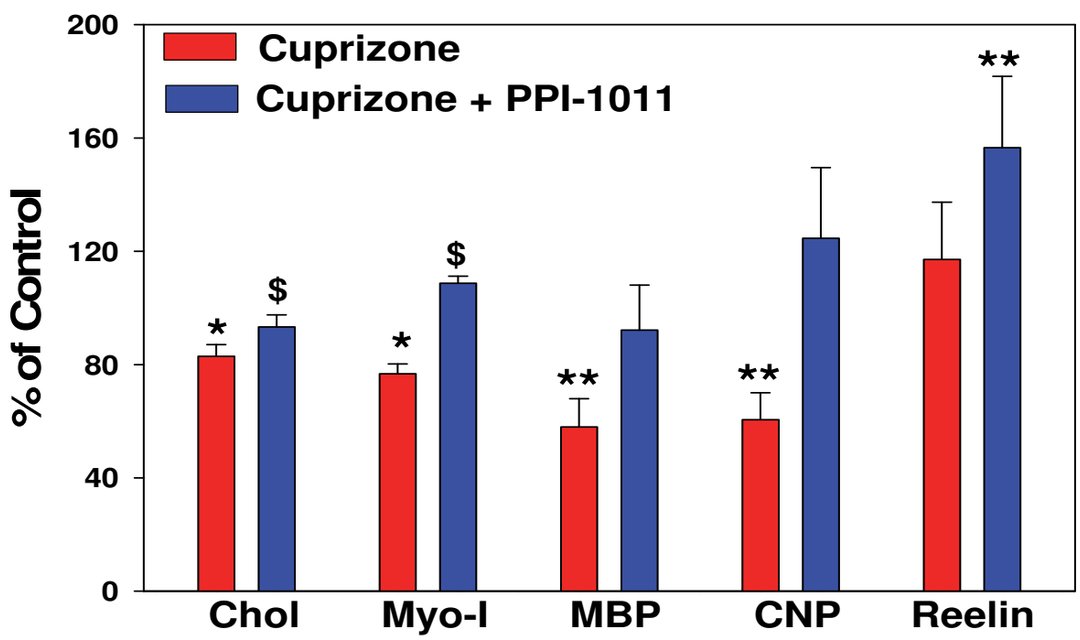


Fig. 18. Neocortical cholesterol (Chol) and myoinositol (Myo-I) levels and mRNA levels for myelin basic protein (MBP), 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) and reelin. Mice were maintained on a 0.3% cuprizone diet for 7 weeks ± PPI-1011 treatment (100 mg/kg, po) for weeks 6 and 7. Mean ± SEM; N=6 -7. * $p < 0.01$ vs. control; ** $p < 0.05$ vs. control; $, $p < 0.01$ vs. cuprizone alone.

AD patients treated with 1.72 g DHA/day for 12 months also have demonstrated no toxicity. Gastrointestinal side effects resulted in several drop-outs (Freund-Levi et al., 2008; Kotani et al., 2006); and 3) AD patients have been dosed with 600 mg of lipoic acid daily for 48 month with no toxicity (Maczurek et al., 2008). Lipoic acid (600 mg) also is an approved therapy for treating diabetic neuropathy, in Germany. These data suggest that PPI-1011 is a safe plasmalogen precursor that should restore neuronal membrane function. This should result in enhanced cholinergic transmission and improve the ADAS-cog scores of AD patients at least as well as a cholinesterase inhibitor. Furthermore, restoring membrane function should halt amyloid deposition and other ongoing pathological processes. PPI-1011 has the potential to be a first-in-class disease-modifying agent for AD.

4.5 PPI-1011: Formulation and stability

PPI-1011 is unstable as an oil but is stable in organic solvents (eg. dichloromethane) and in Neobee. The main impurity is the sulfoxide form of lipoic acid at sn-3. This impurity appears to be minimized by addition of the antioxidant thioglycerol to the formulation. We currently have 14 months of accelerated stability on this formulation.

5. Conclusions

In summary, our data support previous histochemical and biochemical studies demonstrating decrements in markers of neocortical myelin in the cuprizone mouse model (Skripuletz et al., 2008). However, ours is the first data to show that an orally administered plasmalogen precursor is capable of restoring some of these plasmalogens in the brain and that this can also result in remyelination as indicated by increases in cholesterol, myoinositol, myelin basic protein and 2',3'-cyclic nucleotide-3'-phosphodiesterase. Our data suggest that orally bioavailable plasmalogen precursors may offer a new therapeutic approach for neurodegenerative disorders. It has long been known that peripheral sources of DHA lipids are critical for brain and retinal function (Scott and Bazan, 2007). The importance of these observations has been highlighted by recent reports of decreased liver synthesis (Astarita et al., 2010) and circulating levels (Goodenowe et al., 2007) of DHA and DHA phospholipids in Alzheimer's disease, which correlate with cognitive decline and brain decrements in DHA-containing plasmalogens (Han et al., 2001; Goodenowe et al., 2007; Wood et al., 2010). Clearly a next step is to define the site or sites of phospholipid remodeling that allow restoration of myelin plasmalogens and phosphatidylethanolamines. Labeled PPI-1011 is hoped to answer some of these questions. However, there are multiple anatomical sites and multiple enzymes that may participate in phospholipid remodeling processes. The gut, liver, brain and associated endothelial cell populations are all rich in remodeling enzymes. These include: i) the 1-acyl hydrolases, phospholipase A1 (EC 3.1.1.32), lysophospholipase (EC 3.1.1.5), acylglycerol lipase (EC 3.1.1.23); lipoprotein lipase (EC 3.1.1.34) and triacylglycerol lipase (EC 3.1.1.3); ii) the 2-acyl hydolases, phospholipase A2 (EC 3.1.1.4) and acylglycerol lipase (EC 3.1.1.23); iii) the 1-alkenyl hydrolase, alkenylglycerophosphoethanolamine hydrolase (EC 3.3.2.5); iv) the phosphethanolamine hydrolase, phospholipase C (EC 3.1.4.3); and v) the 2-acyltransferases, acylglycerophosphcholine o-acyltransferase (EC 2.3.1.23) and acyl Co-A:lyso-phosphatidylethanolamine acyltransferase (LPEAT2) (Cao et al., 2008). It remains to be defined if these remodeling enzymes can augment plasmalogens with oleic acid (18:1) at sn-2, after longer term dosing with PPI-1011.

6. Acknowledgement

We thank Dr. D.A. Bennett from the Rush Alzheimer's Disease Center, Rush University Medical Center, Chicago, IL, for the human brain samples collected with support from National Institute on Aging grant R01AG17917.

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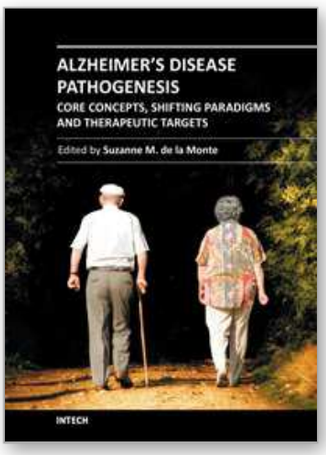

Alzheimer's Disease Pathogenesis-Core Concepts, Shifting Paradigms and Therapeutic Targets

Edited by Dr. Suzanne De La Monte

ISBN 978-953-307-690-4
Hard cover, 686 pages
Publisher InTech
Published online 12, September, 2011
Published in print edition September, 2011

Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

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