Universidade do Minho
Escola de Ciências da Saúde

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The role of phospholipase D2 in Alzheimer's disease.

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The role of phospholipase D2 in Alzheimer's disease.

Tese de Doutoramento
Medicina - Medicina

Trabalho efectuado sob a orientação de
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Columbia University, New York, NY – USA
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Escola de Ciências da Saúde,
Universidade do Minho, Braga – Portugal

Outubro de 2010

Aceito o desafio.
Que poeta se nega
A um aceno do acaso?
Tenho o prazo
Acabado,
O que vier é ganho.
Na lonjura
Da última aventura
É que a alma revela o seu tamanho.

Miguel Torga

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Abstract

Growing evidence implicates aberrant lipid signaling in Alzheimer's disease (AD). While phospholipases A2 and C have been recently shown to mediate key actions of amyloid β-peptide (Aβ) through a dysregulation of arachidonic acid and phosphatidylinositol-4,5-bisphosphate metabolism, respectively, the role of phospholipase D (PLD) has so far remained elusive. PLD produces phosphatidic acid (PA), a bioactive lipid involved in multiple aspects of cell physiology, including signaling and membrane trafficking processes. Here we show that oligomeric Aβ enhances PLD activity in cultured neurons and that this stimulatory effect does not occur upon ablation of PLD2 via gene targeting. Aβ fails to suppress long-term potentiation in PLD2-deficient hippocampal slices, suggesting that PLD2 is required for the synaptotoxic action of this peptide. *In vivo* PLD activity, as assessed by detection of phosphatidylethanol levels using mass spectrometry (MS) following ethanol injection, is also increased in the brain of a transgenic mouse model of AD (SwAPP). Furthermore, *Pld2* ablation rescues memory deficits and confers synaptic protection in SwAPP mice despite a significant Aβ load. MS-based lipid analysis of *Pld2* mutant brains in the presence or absence of the SwAPP transgene unmasks striking crosstalks between different PA species. This lipid analysis shows an exquisite acyl chain specificity and plasticity in the perturbation of PA metabolism, with the notable elevation in SwAPP brains of a pool of PA previously linked to degeneration. Collectively, our results point to specific molecular species of PA as key modulators of AD pathogenesis and identify PLD2 as a novel potential target for therapeutics. Moreover we expanded our MS analysis of the *Pld2*/SwAPP mice to other lipid groups, other than PA. We found that overexpression of the SwAPP transgene leads to significant increase in the ganglioside, GM3. Remarkably, *Pld2* ablation leads to a decrease in GM3 in the non-transgenic background and to a rescue to normals in the SwAPP background. This lipidomic analysis uncovered interesting lipid signaling crosstalks that are modulated by PLD2 in the context of AD models.

Resumo

Trabalhos anteriores indicam que distúrbios no metabolismo dos lípidos estão relacionados com a doença de Alzheimer (AD). Enquanto que as fosfolipases A2 e C foram demonstradas como mediadoras das acções do péptido, amilóide beta (Aβ), através da desregulação do metabolismo do ácido araquidónico e fosfoinositol-4,5-bifosfato, respectivamente, o papel da fosfolipase D (PLD) permanence por esclarecer. A PLD produz ácido fosfatídico (PA), um lípido envolvido em múltiplos aspectos da fisiologia cellular, como vias de sinalização e tráfico membranar. Neste trabalho, mostramos que oligómeros de Aβ levam a um aumento da actividade da PLD em culturas primárias de neurónios e que esse efeito estimulatório não ocorre após a delecção genética da PLD2.

A Aβ perde o seu efeito suppressor de potenciação de longo termo em fatias de hipocampo de ratinhos *Pld2*$^{-/-}$, sugerindo que a PLD2 é necessária para o efeito sinaptotóxico deste péptido. A actividade da PLD *in vivo*, medida através da detecção dos níveis de fosfatidiletanol, por espectrometria de massa (MS) após injecção de etanol, também está aumentada no cérebro de um modelo transgénico de AD (SwAPP). Para além disso a ablação da *Pld2* recupera os défices de memória e leva a uma protecção sináptica em ratinhos SwAPP, apesar dos altos níveis de Aβ. Análise de lípidos por MS de cérebros de ratinhos mutantes para a *Pld2* na presença ou ausência do transgene SwAPP revela haver uma intensa intraregulação nas espécies de PA. Esta análise lipídica mostra uma especificidade e plasticidade no modo como o metabolismo do PA está alterado, com uma marcada elevação nos cérebros SwAPP de uma espécie de PA previamente ligado a processos neurodegenerativos. Em suma, os nossos resultados apontam para espécies específicas de PA como moduladores da patogénese da AD e identificam a PLD2 como um novo alvo potencial terapêutico. Além disso, expandimos a nossa análise por MS para outros lípidos, para além de PA. Observámos que a sobreexpressão do transgene SwAPP leva a um aumento do gangliosídeo, GM3. A ablação genética de *Pld2* leva a um decréscimo de GM3 nos animais não-transgénicos e a uma renormalização

dos valores nos animais SwAPP. Em conclusão, esta análise lipidómica revelou ligações mecanísticas interessantes reguladas pela PLD2, no contexto da AD.

CONTENTS

Abbreviations

α7nAchR - alpha7 nicotinic acetylcholine receptor

Aβ - amyloid β-peptide

AD - Alzheimer's disease

AICD - APP intracellular COOH-terminal domain

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APLP2 - APP-like-protein 2

APP - β-amyloid precursor protein

BACE1 - Beta-site APP cleavage enzyme 1

BST - Basal synaptic transmission

CDK5 - cyclin-dependent kinase 5

Cer - ceramides

cPLA2 - cytosolic phospholipase A2

CTF - COOH-terminal fragment

DAG - diacylglycerol

ERK - extracellular signal-regulated kinase

fEPSPs - field-excitatory post-synaptic potentials

FPRL1 - formyl-peptide-receptor-like 1

Glu-Cer - glucosyl-ceramide

GSK3 - glygogen synthase kinase 3

KO - knock-out

LPA - lysophosphatidic acid

LTP - long-term potentiation

LC - Liquid chromatography

MAPT - microtubule-associated protein tau

MARK - microtubule-affinity-regulating kinase

MEFs - mouse embryonic fibroblasts

MS - mass spectrometry

mTOR – mammalian target of rapamycin

NFTs - neurofibrillary tangles

NMDAR - *N*-methyl *D*-aspartate receptor

PA - phosphatidic acid

PC - phosphatidylcholine

PE - phosphatidylethanolamine

PG - phosphatidylglycerol

PH - pleckstrin homology

PI - phosphatidylinositol

$PI(4,5)P_2$ - phosphoinositol-4,5-bisphosphate

PLA2 - phospholipase A2

PLC - phospholipase C

PLD - phospholipase D

PrP - prion protein

PS - phosphatidylserine

PS1 - presenilin 1

PS2 - presenilin 2

PSD95 – post synaptic density 95

PtdEtOH - phosphatidylethanol

PtdBut - phosphatidylbutanol

PX - phox

SM - sphingomyelin

SwAPP – Swedish APP

WT - wild type

Chapter 1

Introduction

Introduction

1.1. Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease characterized clinically by progressive memory deficits, impaired cognitive function, altered behavior, and a decline in language function (Tanzi and Bertram 2005; Haass and Selkoe 2007; Querfurth and LaFerla 2010). The brain of AD patients displays cortical atrophy, loss of neurons and synapses, and typically presents at the anatomopathological analysis with plaques and neurofibrillary tangles (NFTs). While senile plaques are largely composed of aggregated amyloid β-peptide (Aβ) (Tanzi and Bertram 2005; Haass and Selkoe 2007), tangles consist of pairs of ~10 nm filaments wound into helices, also called paired helical filaments, and contain hyperphosphorylated forms of the microtubule-associated protein tau (MAPT). Growing evidence indicates that there is crosstalk between Aβ and tau pathogenesis (Small and Duff 2008). Genetic studies of families with AD have identified several genes, β-amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2), that are important in the pathogenesis of AD. APP is the precursor protein that after cleavage gives rise to Aβ, and the presenilins have been identified as components of the γ-secretase complex, which, alongside the β-secretase, are responsible for the generation of Aβ (Tanzi and Bertram 2005; Haass and Selkoe 2007) (figure 1).

Aβ is derived from the sequential cleavage of type I transmembrane protein APP by membrane-bound proteases, β- and γ-secretase (Selkoe, Podlisny et al. 1988; Landman and Kim 2004; Wilquet and De Strooper 2004). Beta-site APP cleavage enzyme 1 (BACE1) has been identified as the major β-secretase activity that mediates the first cleavage of APP in the β-amyloidogenic pathway (Sinha and Lieberburg 1999; Haass 2004). BACE1-mediated cleavage leads to the release of the APP ectodomain sAPPβ into the extracellular space. The remaining COOH-terminal fragment (CTF) undergoes subsequent cleavage by γ-secretase to release Aβ and the APP intracellular COOH-terminal domain (AICD). The presenilins have been proposed to be the major catalytic component of the γ-secretase complex, whose sequential intramembrane cleavage of APP produces a spectrum of Aβ peptides varying in length by a few amino acids at the COOH-terminus (Haass 2004; Landman and Kim 2004). The majority of Aβ normally ends at amino acid 40 (Aβ40), but the 42-amino acid variant (Aβ42) is more amyloidogenic, and has been hypothesized to nucleate senile plaque formation (Haass 2004; Landman and Kim 2004; Haass and Selkoe 2007). In the past few years it has been shown that soluble oligomeric Aβ40/42 in various assembly states better correlate with synaptic malfunction and

cognitive impairment than neuritic plaques *in vivo*, consistent with mounting evidence that oligomeric Aβ is significantly synaptotoxic (Haass and Selkoe 2007) (figure 1).

As mentioned above, a number of links exist between Aβ and tau pathologies. Importantly, in an AD mouse model associated with a significant Aβ burden, the genetic ablation of tau was neuroprotective in the context of Aβ-induced deficits (Roberson, Scearce-Levie et al. 2007) (Ittner, Ke et al. 2010), supporting an intimate crosstalk between Aβ and tau. Even though, it is not clear whether the precise reasons for the protective role(s) of tau ablation are related directly to tau's pathogenic potential, it has been shown that the ablation of tau decreases the excitotoxic potential induced by Aβ, by decreasing the NMDAR/PSD95 interaction in a Fyn-dependent way (Ittner, Ke et al. 2010). Overall, this relationship between the two major pathogenic AD hallmarks remains under intense study (Small and Duff 2008). Nevertheless, development of an improved understanding of tau-related pathology may inform not only our knowledge of Aβ-related dysfunction in AD but the illness as a whole. While no familial genetic studies have shown a direct cause effect for tau mutations in AD, mutations in the *MAPT* gene were shown to cause a distinct neurodegenerative disorder, frontal temporal dementia, which is not associated with neuritic plaques. There is a single MAPT gene, which, through alternative splicing, can lead to the expression of six different isoforms in the human brain. MAP tau has a well-established known function of stabilizing microtubules. The microtubule-binding capacity of tau is known to be regulated by post-translational modifications. The most well established process is phosphorylation, mediated by a number of kinases including glygogen synthase kinase 3 (GSK3), cyclin-dependent kinase 5 (CDK5) and microtubule-affinity-regulating kinase (MARK). The formation of NFTs is thought to be a sequential process that starts with impaired tau phosphorylation, detachment of tau from microtubules, accumulation of misfolded tau, tau aggregation in pretangles, formation of paired helical filaments and finally NFTs (Ballatore, Lee et al. 2007).

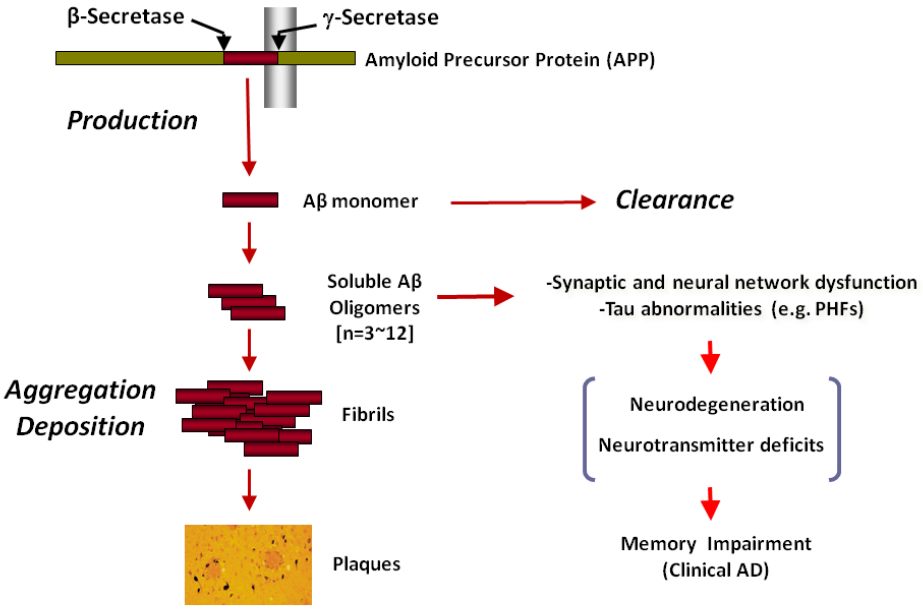

Figure 1. After Aβ is produced from the sequential cleavage of APP, if not cleared, it can be aggregated into oligomers, fibrils and plaques. It has been proposed that the Aβ oligomers are toxic species that can lead to neuronal dysfunction. PHF – paired helical filaments, AD – Alzheimer Disease.

1.2. Phospholipids and Alzheimer's Disease

Lipid-mediated signaling regulates a plethora of physiological processes, including multiple aspects of brain function. Dysregulation of lipid pathways has been involved in a growing number of neurodegenerative disorders. While much attention has been given to the sterol link to AD (Puglielli, Tanzi et al. 2003), growing evidence, suggests that other classes of lipids, such as phospholipids, either mediate or modulate key pathological processes associated with AD (Landman, Jeong et al. 2006; Berman, Dall'Armi et al. 2008; Sanchez-Mejia, Newman et al. 2008).

The interaction of both tau and Aβ with cellular and artificial membranes has been extensively investigated. Given direct binding of tau to membrane phospholipids, it was hypothesized that this interaction may be relevant for the physiological function of tau as well as AD pathogenesis (Baudier and Cole 1987). Similarly, Aβ can directly alter artificial lipid bilayers by forming pores that are permeable to various ions (Arispe, Rojas et al. 1993). More recently, after the discovery of the synaptotoxic properties of soluble Aβ oligomers, studies showed that these oligomers destabilize membranes and trigger Ca^{2+} influxes through unknown mechanisms (Demuro, Mina et al. 2005). In

part reflecting the pathophysiological relevance of oligomer-induced Ca^{2+} dyshomeostasis, acute and chronic treatments of neurons with soluble oligomers of Aβ were shown to disrupt the metabolism of phosphoinositol-4,5-bisphosphate [$PI(4,5)P_2$] in a phenomenon requiring both extracellular $Ca^{2+}$and PLC activity (Berman, Dall'Armi et al. 2008). Similarly, a recent study showed that dysregulation of the group IV A phospholipase A2 pathway mediates some aspects of synaptic and neurobehavioral dysfunction in a mouse model of AD (Sanchez-Mejia, Newman et al. 2008). These two studies provide further support to a prior hypothesis maintaining that phospholipases are highly dysregulated in AD (Farooqui, Rapoport et al. 1997).

Given the body of evidence presented above demonstrating the importance of phospholipids and AD, development of an improved understanding of phospholipid signaling is essential. Increasing evidence (see below) has pointed to one enzyme family especially in regulating biosynthesis and metabolism of phospholipids: phospholipase D (PLD). We conducted this work addressing the hypothesis that in AD there is an overall dysfunction of phospholipases.

1.3. Phospholipase D – Structure, Function and Localization

In the last two decades the purification (Wang, Dyer et al. 1993) and cloning (Wang, Xu et al. 1994) of PLD in plants boosted the follow-up studies in mammals with the cloning of two PLD isozymes, PLD1 (Hammond, Altshuller et al. 1995) and PLD2 (Colley, Sung et al. 1997; Kodaki and Yamashita 1997). In mammals there are three isozymes of PLD: PLD1, PLD2 and the recently-identified mitochondrion-associated mitoPLD (Jenkins and Frohman 2005; Choi, Huang et al. 2006; Donaldson 2009). The majority of studies have so far focused on PLD1 and PLD2 (figure 2A), which share: (i) two HxKxxxxD (HKD) motifs that are essential for catalysis; (ii) a phox (PX) consensus sequence and (iii) a pleckstrin homology (PH) domain, which are phosphoinositide-binding modules that are required for proper targeting of PLD; and (iv) a PIP2-binding site, which is fundamental for the enzymatic activity. However, PLD1 differs from PLD2 by the presence of a loop region, which has been proposed to function as a negative regulatory element for catalysis (figure 2A) (Jenkins and Frohman 2005). In the presence of water, both PLD1 and -2 hydrolyze phosphatidylcholine (PC) to generate phosphatidic acid (PA) and free choline. However in the presence of primary alcohols, such as ethanol and 1-butanol, PLD preferentially (~1000-fold) uses these nucleophiles over water for the transphosphatidylation reaction, thus leading to the formation of non-naturally occurring phospholipids, phosphatidylethanol (PtdEtOH) or phosphatidylbutanol (PtdBut), respectively (Gustavsson 1995) (figure 2B). This atypical property of

the enzyme has been exploited in a myriad of studies either to block the production of bioactive lipid PA or to measure PLD activity in intact cells or tissues.

PA has unique bioactive properties and can modify both the physical and signalling properties of lipid bilayers. Structurally, it is composed of a three-carbon glycerol backbone, two fatty acid chains and a small phosphate headgroup, thus referred to as a 'cone shape' lipid (i.e., a lipid with a small head groups relative to a large hydrophobic domain) (Jenkins and Frohman 2005; Cazzolli, Shemon et al. 2006). This property not only confers PA a higher affinity for negative curvature within lipid bilayers, but it also reduces the energy barrier for bending membranes, thus acting as a fusogenic lipid. Somewhat reminiscent of phosphoinositides, PA also plays an important role at the membrane-cytosol interface through a direct interaction with effector proteins, such as PIP kinases, mTOR, SNARE proteins and sphingosine kinase (Stace and Ktistakis 2006). However, unlike a variety of phosphoinositide-binding modules (e.g., pleckstrin homology or FYVE domains), PA-binding (poly)peptides generally do not consist of well defined three dimensional folds, but instead involve basic residues in unstructured parts of effector proteins. Additionally, PA can be metabolized to other lipids with potent bioactivity. For instance, PA can be converted to diacylglycerol (DAG) by PA phosphatases (Sciorra and Morris 2002; Reue 2009) and to lysophosphatidic acid (LPA), which has an inverted cone shape and thus prefers positive curvature. Finally, PA can serve as a precursor for other lipids in the biosynthetic pathway, where it is consumed for the generation of lipids such as PI via the CDP-DAG pathway. It should be noted that PLD is not the only source of PA, as it can be produced by DAG kinases, LPA acid acyltransferase, mitoPLD and other enzymes in the biosynthetic pathway (Choi, Huang et al. 2006; Haucke and Di Paolo 2007) (figure 2C). However, the fatty acyl composition of PA pools varies, depending on the specific pathways mediating its production. For example, PLD-derived PA species harbor mainly saturated or monounsaturated fatty acids, rapidly giving rise to a pool of DAG with the same properties (i.e., a cone shape), although the negative charge of PA may confer distinct properties and allow for the binding to a different set of effector proteins compared to DAG. In contrast, the pool of DAG resulting from $PI(4,5)P_2$ cleavage by PLC predominantly harbors polyunsaturated fatty acids, likely achieving different physiological functions (Pettitt, Martin et al. 1997; Pettitt, McDermott et al. 2001).

While PLD1 localizes to the Golgi complex, secretory granules and endosomes, PLD2 is concentrated at the plasma membrane, with smaller pools present in the Golgi apparatus, caveolae and in endosomes (Freyberg, Sweeney et al. 2001; Freyberg, Bourgoin et al. 2002; Freyberg, Siddhanta et al. 2003; Bader and Vitale 2009). In part consistent with their subcellular localization,

PLD1 has been implicated in the budding and fusion of *trans*-Golgi–derived secretory vesicles, whereas PLD2 mediates the internalization and recycling of a variety of receptors. Importantly, PLD1 translocates to the plasma membrane upon various stimuli and follows the endosomal internalization route (Jenkins and Frohman 2005; Roth 2008; Donaldson 2009).

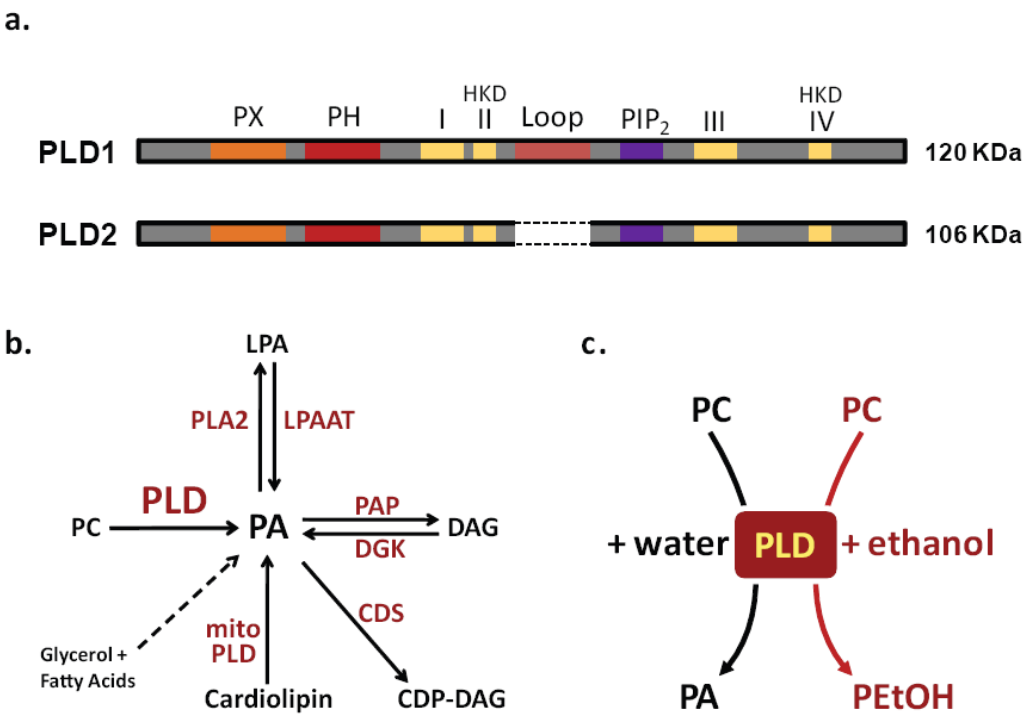

Figure 3. PLD structure, PA metabolism and reactions catalyzed by PLD. (a) Structure of PLD isozymes. Structurally, the two isozymes differ by the presence of a loop domain in the PLD1 isozyme. (b) PA metabolism. Besides the PLD source, PA can be generated from other sources and further metabolized as represented in the figure. The dashed arrow represents the biosynthetic pathway. The enzymes catalyzing the respective reactions are shown in red. (c) PLD activity. In the presence of water, PLD produces PA. In the presence of primary alcohols, such as ethanol, PLD has a 1000-fold higher affinity for primary alcohols as nucleophiles, leading to a preferential generation of phosphatidylethanol (PEtOH). PLD, Phospholipase D; PC, phosphatydilcholine; PA, phosphatidic acid; LPA, lyso-PA; DAG, diacylglycerol; CDP-DAG, cytidine diphosphate-DAG; PLA2, phospholipase A2; LPAAT, LPA acyltransferase; PAP, PA phosphatase; DGK, DAG kinase; CDS, CDP-DAG synthase; mitoPLD, mito-phospholipase D. (adapted from (Oliveira and Di Paolo 2010))

1.4. Lipids and Lipidomics

Although genomic and proteomic studies are nowadays commonly used in research, the field of lipidomics is only now having increased attention, which was in part allowed by technological advances in liquid chromatography and mass spectrometry (Wenk 2005). Lipidomics studies allow the characterization of a vast array of lipid species from a given sample. For instance, as for genomics and proteomics, lipidomics studies allow the identification of potential pathways, in this case involving lipids, which are dysregulated in a certain disease, such as neurodegenerative disorders (Wenk 2005). In the case of AD, this approach is starting to be employed to identify new possible targets, using human and mouse model brain samples (Sanchez-Mejia, Newman et al. 2008) (Han 2010). Lipidomics can also be used to understand mechanistically the lipid alterations of a given alteration.

1.5. Phospholipase D and Alzheimer's disease

Previously, some studies have addressed the link between PLD and AD. Kanfer et al using PEtOH production as a read-out for PLD activity in ethanol-incubated brain homogenates, found an increase in PLD activity in AD brain extracts relative to those from control subjects (Kanfer, Singh et al. 1996). Also, using cell culture models the impact of APP overexpression and extracellular Aβ applications on PLD activity was assessed. Overexpression of the neuronal isoform of human wild type APP in P19 mouse embryonic cells caused an increase in PLD activity (Lee, Oh et al. 2001). Aβ application experiments were used as well with various peptide preparation types. In LA-N-2 cells, a neuroblastoma cell line, an increase in PLD activity was observed after incubating cells with Aβ25-35, a peptide sequence whose pathophysiological significance has been questioned (Singh, Mccartney et al. 1995). Additionally, the same group showed that indomethacin (a non-steroid anti-inflammatory drug), nordihydroguaiaretic acid (an anti-oxidant drug) and nicotine inhibited the increase in PLD activity produced by Aβ25-35 applications (Singh, Sorrentino et al. 1997; Singh, Sorrentino et al. 1998). Furthermore, alanine substitution for the amino acids on the position 29-34 of Aβ25-35 prevented the peptide from having an effect on PLD activity (Singh, Sato et al. 1997). Finally, pre-treatment with Aβ25-35 desensitized the cells, which did not exhibit a PLD activity increase in response to a new Aβ25-35 treatment, thus prompting the authors to suggest that Aβ25-35 might mediate its effects on PLD upon receptor binding (Singh, Sorrentino et al. 1998). Aβ1-40 also produced an increase in PLD activity in rat hippocampal primary cultures, but this effect was seen with high concentrations of Aβ aged at 37°C (likely reflecting a requirement for some aggregated state of Aβ for this phenomenon to

occur) (Cox and Cohen 1997). The increase in PLD activity induced by Aβ correlated with increased release of cytosolic protein lactate dehydrogenase, suggesting it is associated with Aβ-induced toxicity (Cox and Cohen 1997). Besides neurons, Aβ1-42 used in the low micromolar range caused an increase in PLD activity in astrocytes and microglia in a process dependent upon formyl-peptide-receptor-like 1 (FPRL1). Specifically, Aβ1-42 was internalized alongside the FPRL1 receptor and stimulated a downstream signaling pathway involving the phosphorylation of extracellular signal-regulated kinase (ERK). Because both Aβ1-42 internalization and FPRL1-mediated signaling were abrogated by a primary alcohol, a role for PLD in this process was proposed (Brandenburg, Konrad et al. 2008).

While the above studies addressed the effects of Aβ on PLD, there were other groups that addressed the role of PLD on the trafficking of APP and its cleavage machinery. Because Aβ originates from the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases, tremendous effort has been put into attempting to understand the molecular mechanisms regulating the subcellular localization and intracellular sorting of APP and its cleaving enzymes, all of which are transmembrane proteins or protein complexes. Generally, an increasingly popular hypothesis in the field is that perturbation of the trafficking of APP, BACE1 and γ-secretase complex [including its catalytic components, presenilins] may profoundly affect amyloidogenesis and thus impact AD pathogenesis (Small and Gandy 2006). While the bulk of the studies addressing the relationship between the γ-secretase complex and APP focus on the APP processing, some evidence indicates that presenilin can also regulate the trafficking of APP independently of its catalytic activity. Specifically, TGN-derived secretory vesicles produced from PS1-deficient cells contain higher levels of APP, thus resulting in increased cell surface delivery of APP. The converse phenomenon was observed in cells expressing an FAD mutant version of PS1 (i.e., ΔE9), suggesting that these findings may be relevant for the pathogenesis of AD (Cai, Leem et al. 2003). Collectively, these results converged onto a transport pathway involving APP, PS and PLD1, although evidence for a bona fide crosstalk between these three molecules in the context of TGN-to-plasmalemma traffic emerged in subsequent studies.

Accordingly, PLD1 overexpression was shown to promote the formation of APP-containing secretory vesicles from TGN, thus mimicking the effect of PSEN1 nullizygosity (Cai, Zhong et al. 2006). However, primary alcohols failed to rescue this phenotype in PS1 knockout cells, suggesting that the increased biogenesis of APP-containing vesicles observed in PS1-deficient cells is PLD-independent. While these data suggested that WT PS1 and PLD1 may regulate the traffic of APP through independent pathways, there is a clear functional link between these two proteins in the FAD mutant

background (at least, for the PS1ΔE9 mutant). For instance, a subcellular fractionation experiment showed that expression of FAD PS1ΔE9 mutant in cells leads to an enrichment of PLD1 in the Golgi/TGN fraction at the expense of lighter fractions (which may in part represent endosomal structures) and that this redistribution was concomitant with a decrease in total PLD activity (Cai, Zhong et al. 2006). Importantly, overexpression of catalytically-active PLD1 (but not the lipase-dead mutant) was found to rescue the defect in the budding of APP-containing vesicles from the TGN as well in the cell surface delivery of APP induced by the PS1 ΔE9 (Cai, Zhong et al. 2006). Whether PLD1 interacts with APP or not is a matter of debate as conflicting data were obtained in independent studies (Jin, Kim et al. 2006; Liu, Zhang et al. 2009).

While the aforementioned study highlights the effects of PLD1 on APP trafficking, Cai et al. also addressed the role of PLD1 in amyloidogenesis. First, it was shown that PLD1 (but not PLD2) physically interacts with the cytoplasmic loop region of PS1 and that through this binding PS1 mediates the recruitment of PLD1 to the Golgi complex. Furthermore, in N2a cells expressing the PS1 mutant ΔE9, overexpression of PLD1 decreased the levels of Aβ, whereas silencing PLD1 and expression of a lipase-dead PLD1 mutant produced the converse effect. Interestingly, co-precipitation experiments showed that PLD1 regulates the assembly of the γ-secretase complex through a direct effect on PS1 (but not the other components of the complex: Pen-2, nicastrin and APH1), although this phenomenon appears to be independent of the lipase activity of PLD1 (Cai, Netzer et al. 2006).

Since PLD1 regulates the traffic of APP and that APP, in turn, may affect the transport of PS1, it was hypothesized that PLD1 could regulate the trafficking of PS1 indirectly, through APP. However, recently published evidence has suggested that PLD1 positively regulates the delivery of PS1 to the cell surface in an APP-independent fashion (Liu, Zhang et al. 2009). Indeed, analysis of PS1 localization in mouse embryonic fibroblasts (MEFs) lacking both APP and its related family member APP-like-protein 2 (APLP2) (APP dKO) showed increased cell surface delivery of PS1. This phenomenon was mimicked by the overexpression of catalytically-active PLD1, but it also occurred in APPdKO cells, thus suggesting that it is independent of an effect of PLD1 on APP transport (Liu, Zhang et al. 2009).

In summary, there appears to be a significant crosstalk between PLD1, APP and PS1 with important implications for amyloidogenesis. However, it is unclear whether this crosstalk also occurs in vivo, which should be best addressed with genetic models. Importantly, PLD1 and PLD2 are likely to play distinct roles in AD pathogenesis, likely reflecting their differential subcellular localization, expression levels/profile, and regulation as well as their ability to control different aspects in the biology of the key proteins involved in AD.

1.6. Aims

The main goal of this proposal is to understand the role of PLD2 in the pathogenesis of AD. The specific aims of this project are:

1. **To characterize the effects of Aβ on the PLD pathway.**
2. **To test the effects of PLD2 ablation in an AD mouse model.**
3. **To investigate the role of PLD2 in APP processing and Aβ generation.**

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Chapter 2

Experimental work

Chapter 2.1.

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Phospholipase D2 ablation ameliorates Alzheimer's disease-linked synaptic dysfunction and cognitive deficits.

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Abbreviated title: Role of Phospholipase D2 in Alzheimer's disease

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Abstract

Growing evidence implicates aberrant lipid signaling in Alzheimer's disease (AD). While phospholipases A2 and C have been recently shown to mediate key actions of amyloid β-peptide (Aβ) through a dysregulation of arachidonic acid and phosphatidylinositol-4,5-bisphosphate metabolism, respectively, the role of phospholipase D (PLD) has so far remained elusive. PLD produces phosphatidic acid (PA), a bioactive lipid involved in multiple aspects of cell physiology, including signaling and membrane trafficking processes. Here we show that oligomeric Aβ enhances PLD activity in cultured neurons and that this stimulatory effect does not occur upon ablation of PLD2 via gene targeting. Aβ fails to suppress long-term potentiation in PLD2-deficient hippocampal slices, suggesting that PLD2 is required for the synaptotoxic action of this peptide. *In vivo* PLD activity, as assessed by detection of phosphatidylethanol levels using mass spectrometry (MS) following ethanol injection, is also increased in the brain of a transgenic mouse model of AD (SwAPP). Furthermore, *Pld2* ablation rescues memory deficits and confers synaptic protection in SwAPP mice despite a significant Aβ load. MS-based lipid analysis of *Pld2* mutant brains in the presence or absence of the SwAPP transgene unmasks striking crosstalks between different PA species. This lipid analysis shows an exquisite acyl chain specificity and plasticity in the perturbation of PA metabolism, with the notable elevation in SwAPP brains of a pool of PA previously linked to degeneration. Collectively, our results point to specific molecular species of PA as key modulators of AD pathogenesis and identify PLD2 as a novel potential target for therapeutics.

Introduction

Cerebral accumulation of amyloid beta (Aβ) is believed to mediate many aspects of Alzheimer's disease (AD)-associated pathogenesis. Aβ is produced by the sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. While Aβ40 is the predominant cleavage product, the longer peptide Aβ42 is more cytotoxic and aggregate-prone (Small and Gandy, 2006; Haass and Selkoe, 2007; Vassar et al., 2009; De Strooper et al., 2010). Although AD brains typically harbor senile plaques that consist of insoluble aggregates of Aβ, different assemblies of Aβ, including fibrils as well as soluble dimers, trimers and dodecamers, may differentially contribute to AD pathogenesis at various stages of this disorder (Lambert et al., 1998; Walsh et al., 2002; Haass and Selkoe, 2007). Importantly, elevation of soluble Aβ oligomers strongly correlates with cognitive decline, consistent with the synaptotoxic properties exhibited by these peptides in various systems (Haass and Selkoe, 2007). For instance, Aβ oligomers disrupt synaptic plasticity, the trafficking of glutamate receptors, dendritic spine dynamics and Ca^{2+} homeostasis (Demuro et al., 2005; Snyder et al., 2005; Hsieh et al., 2006; Haass and Selkoe, 2007; Shankar et al., 2007; Green and LaFerla, 2008). Importantly, recent work has suggested that Aβ oligomers may exert their effects upon binding to the cellular prion protein on the neuronal membranes (Lauren et al., 2009).

Mounting evidence indicates that Aβ perturbs the metabolism of intracellular signaling lipids and that this phenomenon contributes to the pathogenic actions of this peptide (Hartmann et al., 2007). In particular, work from our laboratory has shown that Aβ42 oligomers promote the hydrolysis of a major regulatory lipid phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] through a stimulation of the phospholipase C (PLC) pathway and that preventing PI(4,5)P_2 deficiency protects against the synapse-impairing actions of Aβ (Berman et al., 2008). Similarly, Aβ stimulates cytosolic phospholipase A2 (cPLA2), thereby enhancing the levels of arachidonic acid, and reduction of the relevant PLA2 isoform by genetic means confers protection against the peptide (Sanchez-Mejia et al., 2008). Collectively, these studies suggest that phospholipases may be primary mediators of Aβ's action.

Phospholipase D (PLD), another family of phospholipases, has also been implicated in AD pathogenesis (Singh et al., 1995; Cai et al., 2006b; Brandenburg et al., 2008; Liu et al., 2009). PLD1 and PLD2, which are differentially localized in cells, hydrolyze phosphatidylcholine (PC) into choline and a bioactive lipid, phosphatidic acid (PA) (Freyberg et al., 2003; Jenkins and Frohman, 2005; Roth, 2008; Donaldson, 2009). This lipid regulates membrane dynamics and signaling processes through its

intrinsic physical properties (i.e., 'cone shape') and interaction with effector proteins (Jenkins and Frohman, 2005; Stace and Ktistakis, 2006; Haucke and Di Paolo, 2007; Roth, 2008; Raghu et al., 2009a). In the present study, we tested the involvement of PLD2 in AD pathogenesis and more specifically, in the synaptotoxic action of Aβ oligomers. We reasoned that PLD2 was likely to mediate the previously-reported increase in total PLD activity induced by Aβ, based on the predominant localization of this isozyme at the cell surface (Du et al., 2004), a major site of action for Aβ. We show that ablating PLD2 through genetic means blocks the synaptotoxic action of Aβ42 oligomers and rescues memory deficits in a transgenic model of AD independently of brain Aβ levels.

Materials and Methods

Cell culture. PC12 cells were maintained in Dulbecco's modified Eagle's medium with sodium pyruvate (Invitrogen) supplemented with 5% fetal bovine serum, 10% horse serum, glutamine (4 mM), penicillin (200 units/mL), streptomycin (200 μg/mL); cells were maintained at 37°C in 5% CO_2. Twenty-four hours before transfection, PC12 cells were plated (at 50% confluence) on coverslips pre-coated with polylysine (20μg/mL) for 1 hr at 37°C. Transfections of GFP-mouse PLD2 construct (kind gift of Michael Frohman, SUNY Stony Brook, New York), which was previously described (Du et al., 2004), were obtained using Lipofectamine 2000 (Invitrogen). Primary cultures from cortical neurons were generated from newborn wild-type mice. Briefly, cortices were dissected out, trypsinized for 30min, and then cortical cells dissociated with a Pasteur pipette and plated on poly-ornithine-coated 10mm dishes at a density of 25,000 cells/cm^2 in Neurobasal-A medium containing 1mM kynurenic acid to reduce enhanced synaptic transmission due to the high density of the cultures. Treatments with oA□42 were typically performed after 15 DIV, with 200nM concentration and with time duration of 4hours for primary neuronal cultures and 5, 30 and 60 minutes in PC12 cells. Ionomycin was used with 2μM concentration for 30 minutes.

Peptide preparation. Aβ□oligomers were prepared as described previously (Dahlgren et al., 2002). Synthetic Aβ (1-42) was purchased from American Peptide (Sunnyvale, CA) and stored at -20°C. The vial containing the peptide was allowed to equilibrate to room temperature for at least 30 min before resuspension. In a fume hood the peptide was diluted to 1mM in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) by pipette mixing and immediately aliquoted in polypropylene microcentrifuge tubes. The

solution was vortexed briefly and allowed to evaporate in the fume hood for 2 hours. The resulting peptide films were dried in a Speed Vac for 10 minutes at 800xg and stored at -20°C. Prior to use, the peptide film was resuspended to 1mM in dimethyl sulfoxide (DMSO) by pipette mixing followed by bath sonication for 10 minutes. The solution was aliquoted in polypropylene microcentrifuge tubes and stored at -20°C. The peptide was used within two weeks of dilution in DMSO. Oligomeric forming conditions: the 1mM DMSO solution was diluted to 100 M in cold PBS, vortexed for 30 seconds, and incubated overnight at 4°C (minimum incubation of 12 hours). Immediately before use, the Aβ-PBS solution was further diluted in culture media to the required final concentration and vortexed briefly. This preparation contains a mixture of monomers, trimers and tetramers as well as traces of dimers and high molecular weight oligomers, as shown previously (Berman et al., 2008).

Mouse strains and breeding strategy. The genetic background of the *Pld2* mice is mixed (C57BL/6-129svj). *Pld2*$^{-/-}$ females were crossed with Tg2576 males (Taconic- mixed background C57BL/6-SJL/N), which express human APP carrying the double mutation K670N and M671L found in a Swedish family with early onset of Alzheimer's disease, under the regulation of the hamster prion protein promoter (Hsiao et al., 1996). From the F_1 hybrid generation we used as breeders *Pld2*$^{-/-}$/no tg females and *Pld2*$^{-/-}$/SwAPP males. For all our animal studies we used littermate mice (or in some cases, mice sharing at least one parent) derived from the F_2 generation. The survival rate of adult mice was >90% for all genotypes within the first 12 months of age (and thus the impact of *Pld2* deletion on the survival of SwAPP mice was not investigated further).

ELISA analysis. Brains were homogenized in 10 volumes of 50 mM Tris–HCl buffer, pH 7.6, containing 250 mM sucrose and protease inhibitor cocktail (Sigma, St. Louis, MO). Soluble and total Aβ were extracted in 0.4% diethylamine (DEA) and 70% formic acid, respectively, as previously described (Schmidt et al., 2005). Levels of full-length Aβ 1–40 and 1–42 were quantified using antibodies donated by Centocor according to previously-published ELISA procedures (Schmidt et al., 2005). Murine Aβ was measured according to a previously-published procedure (Burns et al., 2003).

Western Blot analysis. The DEA fraction of brain extracts from *Pld2*/SwAPP mutant mice was immunoblotted using a rabbit polyclonal antibody to the COOH terminus of PLD (kind gift of Dr. Sung

Ho Ryu, Pohang University of Science and Technology), rabbit polyclonal antibody to PLD1 (Cell Signaling), mouse monoclonal antibody to APP (6E10, Covance), mouse monoclonal antibody to PSD95 (6G6-1C9, Abcam), rabbit polyclonal Synaptophysin (G95, kind gift of Dr. Pietro De Camilli, Yale University) and a mouse monoclonal antibody to α-tubulin (B-5-1-2, Sigma). Quantification was performed using ImageJ software.

PLD activity. Ten day-old neurons were incubated with [^{3}H] palmitic acid (2 μCi/ml) for approximately 5 days to label cellular phospholipids. Following treatments with vehicle or oAβ42, neurons were incubated in the presence of 0.3% butanol for 30 min, which leads to the production and accumulation of [^{3}H]phosphatidylbutanol via a PLD-specific transphosphatidylation reaction. Radiolabeled lipids, including phosphatidylbutanol, were isolated by solvent extraction and separated by thin layer chromatography. The total amount of [^{3}H]phosphatidylbutanol is expressed as a percentage of total [^{3}H]-labeled lipids (Morris et al., 1997).

Confocal microscopy. Analysis of GFP-PLD2 internalization. Twenty-four hours after transfection with a plasmid encoding GFP-PLD2, PC12 cells were incubated with vehicle or 200 nM oAβ42 for 5, 30 and 60 minutes. Other treatments included 2 μM ionomycin (Sigma-Aldrich), 2 mM EGTA (company), 250 nM U-73122 (Calbiochem) and 20 μM AACOCF3 (Calbiochem) for 30 min, as indicated in the figure legend. Cells were then washed in phosphate buffer and fixed with 4% paraformaldehyde. Confocal z-stack images (0.5μm) of PC12 were obtained using Nikon EZ-C1.2.30 confocal microscope and an oil immersion objective (100x). Quantification of GFP intensity was calculated using the ImageJ software: for each cell in a given image, a line intensity profile across the cell was obtained. The relative decrease in plasma membrane localization was calculated as the ratio between the plasma membrane fluorescence intensity and the average cytosolic fluorescence intensity, as previously described (Berman et al., 2008).

Electrophysiology. Transverse hippocampal slices (400 μm) were cut with a tissue chopper (EMS, PA) and maintained in an interface chamber at 29 °C for 90 min prior to recording, as previously reported (Puzzo et al., 2005). CA1 field-excitatory post-synaptic potentials (fEPSPs) were recorded by placing both the stimulating and the recording electrodes in CA1 stratum radiatum. Basal synaptic

transmission (BST) was evaluated either by plotting the stimulus voltages (V) against slopes of fEPSP, or by plotting the peak amplitude of the fiber volley against the slope of the fEPSP, to generate input-output relations. During baseline recordings, responses were evoked at an intensity of approximately 35% of the maximum evoked response LTP was induced using a θ-burst stimulation (4 pulses at 100 Hz, with bursts repeated at 5 Hz and each tetanus including 3 ten-burst trains separated by 15 sec). oAβ42 was applied for 20 min prior to the θ-burst.

Fear Conditioning. The mice's capacity for contextual and cued memory was tested as before (Paylor et al., 1994; LeDoux, 2000; Gong et al., 2006), with slight modifications. Briefly, mice were first exposed for 2 min to the context before the onset of a tone (a 30 s, 85 dB sound at 2800 Hz) serving as a conditioning stimulus (CS). In the last 2 s of the CS, mice received a 2 s, 0.50 mA foot shock (unconditioned stimulus, US) through the bars of the floor. Freezing, which is defined as a species-specific defensive reaction characterized by lack of movement, associated with crouching posture was measured right after the end of the CS/US for 30 s using the Freezeview software (MED Associates Inc.). The contextual memory test was performed 24h later, by re-exposure of the mice to the same context and by measuring the proportion of freezing time during 5 min. To evaluate cued fear learning, 24h after contextual testing, mice were placed into a novel context for 2 min (pre-CS test), followed by an exposure to the CS for 3 min (CS test), during which freezing was measured. No differences were found between the six genotypes in this control test (data not shown). For all the fear conditioning experiments, the conditioning chamber is located inside a sound-attenuating box (72 cm × 51 cm × 48 cm). A clear Plexiglas window (2 cm × 12 cm × 20 cm) allows the researcher to digitally record the mouse performance with a camera placed on a tripod and connected to Freezeframe software (MED Associates Inc.).

Radial Arm Water Maze. The test was performed as before (Trinchese et al., 2008) in a white tank filled with milky water, and containing stainless steel walls positioned to produce six arms, radiating from a central area. Spatial cues were presented on the walls of the testing room. At the end of one of the arms there was a clear 10 cm plexiglass submerged (1.5 cm) platform, which remained in the same location for every trial in one day, but was moved randomly each day. On each trial, the mouse started the task from a different randomly chosen arm. The mouse could not use long-term memory of the location of the platform on previous days, but had to rely on the short-term memory of its location in

the same day based on spatial cues. Each trial lasted 1 min and errors are counted each time the mouse entered the wrong arm with four paws, or needed more than 20 s to reach the platform. After each error, the mouse was gently pulled back to the start arm for that trial. After four consecutive trials, it was placed in its home cage for 30 min, and then administered a retention trial. Testing was considered complete when wild-type mice reached asymptomatic performance (below one error on trials four and five; 10 training days). Scores for each mouse on the last 3 days of testing were averaged and used for statistical analysis. All behavioral experiments were done blind to the genotype. Visible-platform tests to detect visual, motor or motivational impairments were performed in the same pool, but without arms and with the platform marked with a black flag, once the radial arm water-maze study was completed. Platform location was varied randomly to eliminate any contribution of external spatial cues. Four trials per day were given over 2 days. Each animal was allowed to swim for 1 min from a random location. Once the mouse reached the platform (or with help if it did not reach it on its own), it was allowed to rest there for 30 s. Failures to reach the platform are scored as 60 s. Data was recorded using a ceiling-mounted camera and analyzed with an HVS- 2020 video tracking system.

In vivo PLD activity. Non-trangenic and SwAPP mice were injected intraperitoneally with 3g/kg ethanol. Mice were killed 1 hour post-injection. Forebrains were then removed from the mice and levels of phosphatidylethanol (PEtOH) produced via a PLD-specific transphosphatidylation reaction were measured in lipid extracts via mass spectrometry, as described below.

Mass Spectrometry. Lipid extracts were prepared from mice forebrain using a modified Bligh and Dyer method and analyzed by LC-MS. Polar glycerophospholipids and sphingolipids were separated via a Luna silica column (3µm, 2mmx150mm; Phenomenex) with a solvent gradient of 100% chloroform/methanol/water/ammonia solution (90:9.5:0.5:0.32, by vol.) changing to 100% chloroform/methanol/water/ammonia solution (50:48:2:0.32, by vol.) over 40min (Pettitt et al., 2001). Lipid species were measured using a triple quadrupole instrument ABI 4000 Q-Trap (Applied Biosystems, Foster City, CA) operated in multiple reactions monitoring mode (MRM). Both PA and PEtOH species were measured using MRM transition pair of parent ion m/z to fatty acyl chain m/z using instrument settings as described previously (Chan et al., 2008; Fei et al., 2008). PA levels were quantified by referencing to known amounts of spiked internal standard diC17-PA (Avanti Polar Lipids,

Alabaster, AL). PEtOH levels were referenced to spiked internal standard diC16-PEtOH (Avanti Polar Lipids), which was added in excess of endogenous levels of this species.

Statistics. Statistical analysis was performed using two-tailed equal variance and Student's *t* test, unless indicated otherwise. All the experiments were performed in blind to the genotypes.

Results

Reduction in PLD2 levels blocks Aβ-induced PLD activation

To begin to address the role of PLD in Aβ pathogenesis and to determine which of the two isoforms may be stimulated by Aβ, we tested whether oligomeric Aβ signaling alters the subcellular localization of either PLD1 or PLD2. Indeed, previous work from others has indicated that stimulation of these enzymes enhances their transport to and from the cell surface (Du et al., 2003; Laulagnier et al., 2004). Pheochromocytoma cell line PC12 was transfected with constructs encoding either GFP-PLD1 or GFP-PLD2, whose localization was analyzed after an acute treatment with 200nM Aβ42 oligomers (oAβ42). As expected (Du et al., 2004), in the absence of treatment or in the presence of vehicle, the fluorescence of GFP-PLD2 was concentrated at the plasma membrane (Fig.1*A, B*) while the fluorescence of GFP-PLD1 was more intracellular (data not shown), likely reflecting the predominant localization of this isoform in the Golgi complex as well as in secretory granules and endosomes (Jenkins and Frohman, 2005; Roth, 2008; Bader and Vitale, 2009). Incubation of PC12 cells with 200nM oAβ42 for 5, 30, and 60 minutes did not produce an obvious effect in localization pattern of GFP-PLD1 (data not shown). On the other hand, while incubation of PC12 cells with oAβ42 did not significantly affect the localization of GFP-PLD2 after 5 min, it triggered a partial internalization of this probe after 30 and 60 min (Fig.1*A, B*). This effect was mimicked by a treatment with 2 μM ionomycin, a Ca^{2+} ionophore and a known PLD2 activator (Kim et al., 1999), and it was blocked by pre-incubation with 2mM EGTA, an extracellular Ca^{2+} chelator, indicating that the oAβ42 effect on PLD2 is Ca^{2+}-dependent (Fig. 1*C*). Based on previous work showing an activation of both the PLC and cPLA2 pathways downstream of Aβ (Kanfer et al., 1998; Kriem et al., 2005; Berman et al., 2008; Sanchez-Mejia et al., 2008; Oliveira and Di Paolo, 2010), we tested whether Aβ-induced GFP-PLD2 relocalization was dependent upon PLC or cPLA2 using pharmacological inhibitors (*i.e.*, U-73122 and AACOCF3, resp.). While the PLC inhibitor had no effect on the localization of GFP-PLD2, the cPLA2 inhibitor blocked the relocalization of GFP-PLD2 in response to oAβ42, suggesting that this phenomenon is independent or upstream of PLC but likely downstream of cPLA2 (Fig. 1*D*).

To test whether PLD2 mediates cytotoxic actions of Aβ oligomers, we developed a mouse genetic model lacking PLD2 (Suppl. Fig. 1*A,B*). Mutant mice do not exhibit any overt phenotype. Western blot analysis using specific antibodies showed that the PLD2 immunoreactivity is absent and approximately decreased by 50% in adult brain tissue derived from *Pld2*$^{-/-}$ and *Pld2*$^{+/-}$ mice, respectively (Fig. 6*A*). In order to confirm that PLD2 ablation produces a significant decrease in total PLD activity in brain tissue, we developed an *in vivo* PLD activity assay relying on the i.p. injection of adult mice with ethanol and measurement of phosphatidylethanol (PEtOH) (*i.e.*, the product of PLD) one hour post-injection in the adult brain of *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice using liquid chromatography-mass spectrometry (LC-MS) (Pettitt et al., 2001; Chan et al., 2008). We found a 40% decrease of PEtOH levels in the brain of *Pld2*$^{-/-}$ mice (Supp. Fig. 1*C*), thus indicating that PLD2 contributes a significant fraction of total PLD activity in the adult brain (with the remainder likely accounted for by PLD1).

To begin to determine the role of PLD2 in the Aβ signaling pathway, we tested whether the oligomeric peptide preparation used in this study (oAβ42) enhances total PLD activity in primary cortical neurons. Indeed, previous work from others had shown that treatment of neuronal cell lines with micromolar concentrations of Aβ25-35 increases PLD activity (Singh et al., 1995; Kanfer et al., 1998; Singh et al., 1998; Oliveira and Di Paolo, 2010). Here, total PLD activity was analyzed in cultured neurons following metabolic labeling with [^{3}H]-myristic acid in the presence of low concentrations of *1*-butanol and quantification of PLD's transphosphatidylation product, phosphatidylbutanol (Fig. 2). In the absence of Aβ oligomer treatment, basal PLD activity was significantly decreased by 30±11% and 74±13% in *Pld2*$^{+/-}$ and *Pld2*$^{-/-}$ neurons, respectively. Remarkably, oAβ42 application led to a ~2-fold increase in total PLD activity in *Pld2*$^{+/+}$ neurons (Fig. 2), an effect comparable to that obtained with 2μM ionomycin (*i.e.,* a 92±11% increase, n=6, $p < 0.001$). However, oAβ42 failed to increase this activity in *Pld2*$^{+/-}$ as well as in *Pld2*$^{-/-}$ neurons (Fig. *2*), indicating that the PLD2 isoform is involved in the Aβ signaling cascade.

Ablation of PLD2 blocks the suppressive effect of Aβ oligomers on LTP.

If the activation of PLD is necessary for oAβ42-induced synaptic dysfunction, the ablation of *Pld2* may confer a resistance against the peptide with respect to neurophysiology. To test this, the effect of oAβ42 on synaptic transmission was assessed in adult hippocampal slices from *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$

mice, where recordings were performed in the CA1 hippocampal region after stimulation of the Schaffer collateral pathway. First, basal neurotransmission (input-output) was investigated and found to be normal in *Pld2*$^{-/-}$ slices in the absence of the peptide (Suppl. Fig. 2). Next, long-term potentiation (LTP) was induced by a tetanic stimulation after exposing slices from the two genotypes to 200nM oAβ42 or vehicle for 20 minutes. LTP in *Pld2*$^{+/+}$ slices was comparable to that obtained in *Pld2*$^{-/-}$ slices in the presence of vehicle (Fig. 3). However, while oAβ42 partially impaired LTP in *Pld2*$^{+/+}$ control slices, as reported before (Lambert et al., 1998; Vitolo et al., 2002; Haass and Selkoe, 2007), the effect of this crude oligomer preparation on LTP was strongly suppressed in *Pld2*$^{-/-}$ slices (Fig. 3), suggesting that the ablation of PLD2 prevents Aβ42 oligomers from exerting their synaptotoxicity.

PLD activity is increased in the brain of a transgenic model of AD.

To understand the *in vivo* relevance of the PLD pathway in an AD model, we subjected the transgenic line Tg2576, which expresses the Swedish APP (SwAPP) mutant (Hsiao et al., 1996) to acute ethanol injections, so as to conduct *in vivo* measurements of PLD activity as described above. We used aged mice (*i.e.*, 14 month-old) for these studies, because Aβ levels are known to increase in an age-dependent fashion in the forebrain of these animals, thus leading to well-established cognitive deficits (Hsiao et al., 1996). We found that total PEtOH levels are increased by 25±11% (n=6, $p < 0.05$) in the forebrain of SwAPP mice relative to controls, indicating an overall enhancement of PLD activity in mutant animals. More specifically, there was a significant increase in a subset of molecular species of PEtOH (32:1, 34:2, 34:1 and 34:1) and an overall trend for an increase in the other PEtOH species analyzed, with the exception of 38:2 (Fig. 4; see also Suppl. Table 1 for absolute levels of PEtOH species). This is to our knowledge the first *in vivo* evidence in support of a dysfunction of the PLD pathway in an AD mouse model.

Reducing PLD2 levels ameliorates the memory deficits of a transgenic model of AD.

Next, the impact of *Pld2* ablation on learning behavior was assessed in the SwAPP mice. To this goal, mice of the three *Pld2* genotypes (+/+, +/–, –/–) in the transgenic (SwAPP) or non-transgenic (no SwAPP) background were subjected to the contextual fear conditioning paradigm, which assesses a form of emotional learning that requires normal amygdala and hippocampus function. In this task, an innocuous conditioned stimulus (tone) elicits fear response after being associatively paired with an aversive unconditioned stimulus (footshock). The fear response is measured by the frequency of

freezing behavior, which is defined as a stereotyped motionless crouching posture. For these experiments, 5-6 month-old mice were utilized, because our preliminary studies had shown that SwAPP mice, in this age category, exhibit learning deficits, which are not associated with a significant neuritic plaque burden and thus likely reflect the effects of soluble Aβ assemblies. Mice from all six genotypes did not show any major differences in the baseline levels (pre-testing). As expected, the SwAPP mice, unlike non-transgenic mice, exhibited little contextual fear response 24h after the pre-test, thus denoting an impairment in contextual learning in the presence of a normal copy number for *Pld2*. However, transgenic mice lacking one (SwAPP/*Pld2*$^{+/-}$) or two (SwAPP/*Pld2*$^{-/-}$) copies of *Pld2* performed better than the SwAPP/*Pld2*$^{+/+}$ mice (Fig. 5*A*), suggesting that genetic ablation of *Pld2* is protective.

Next, mice were subjected to a second learning task, the radial arm water maze (RAWM) paradigm, which assesses spatial working memory. In this test, mice have to find a hidden platform, at the end of one of six arms, remaining in the same location for every trial in one day, but moved to another location from day to day. For each trial, mice start the task from a different randomly chosen arm. After four consecutive trials mice are returned to the cage and submitted to a fifth trial (retention test) 30 min later. While the SwAPP/*Pld2*$^{+/+}$ mice showed impaired memory after 10 days of training (see also ref. (Trinchese et al., 2008)), mice lacking one (SwAPP/*Pld2*$^{+/-}$) or two (SwAPP/*Pld2*$^{-/-}$) copies of *Pld2* perform similarly as animals that do not express SwAPP (Fig. 5*B*), indicating that, as in the fear conditioning test, reduction of PLD2 was protective for the deficits induced by overexpression of the transgene and increased Aβ load. The six groups of mice analyzed showed no overall major differences in the escape latency in the visible platform task, as well as in the swimming speed (Suppl. Fig. 3) indicating that vision, locomotor activity or motivation did not influence the outcome of the RAWM testing.

Reducing PLD2 levels maintains synaptic protein levels in a transgenic model of AD.

Since a reduction or ablation of PLD2 confers protection against the deleterious effects of oAβ42 on LTP (Fig. 3) and of SwAPP overexpression on learning and memory (Fig. 5), a likely possibility is that lack of PLD2 exerts a synapse-protecting effect. To test this, levels of pre- and post-synaptic proteins which are known to be affected by Aβ elevation and SwAPP overexpression [see for instance (Almeida et al., 2005)] were investigated. We found that ablation of either one or two copies of PLD2 does not affect the forebrain levels of synaptophysin (presynaptic marker) and PSD95 (postsynaptic marker) in

the absence of SwAPP transgene. However, while expression of the SwAPP transgene leads to a significant decrease in the levels of PSD95 and, to a lesser extent, synaptophysin, PLD2 ablation restored the levels of these two synaptic proteins to normal levels, with partial phenotypes observed for SwAPP/*Pld2*$^{-/-}$ mice (Fig. 6). These biochemical data are in agreement with the notion that PLD2 ablation may confer synaptic protection in the context of the SwAPP background. Because synaptic protein levels are not altered by the *Pld2* genotype (Fig. 6) and that the electrophysiology analysis shows a normal input-output relationship in mutant hippocampi in the absence of SwAPP transgene (Suppl. Fig. 2), we speculate that the protective mechanism likely does not involve a gross increase in the number of functional synapses in the mutant.

Effect of the APP transgene expression and PLD2 ablation on brain PA metabolism.

To gain insight into the molecular basis underlying AD pathogenesis as well as the protection conferred by PLD2 ablation, we focused our analysis on PA, the product of PLD. A mass spectrometry analysis of mutant animals was performed covering different species of PA. Total forebrain lipids were extracted from 11-12 month-old *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice in the presence or absence of the SwAPP transgene and analyzed using LC-MS. Three classes of potential lipid alterations were investigated: (i) changes produced by the ablation of PLD2; (ii) changes occurring in response to the overexpression of SwAPP; and (iii) changes reflecting interactions between the SwAPP transgene and the *Pld2* genotypes. Results are expressed either in the form of molar percentages in a table (Suppl. Table 2) or in the form of bar graph relative to control brains (Pld2$^{+/+}$, no SwAPP) (Fig. 7).

The data shows that ablation of PLD2 significantly decreases the levels of two molecular species of PA based on the different fatty acyl composition (see legend), namely, PA 32:1 (~50 %) and 38:4 (~40%), while a trend for a decrease (~20%) was observed for two other species, PA 34:1 and 34:0 (Fig. 4*B*; Suppl. Table 2). Surprisingly, the PA 32:0 and 38:2 species were upregulated (by 25% and ~30%, resp.), suggesting the occurrence of compensatory mechanisms in *Pld2* knockout brains. Indeed, the total amount of PA (i.e., sum of all species measured) was overall unaffected by the *Pld2* genotype, thus denoting a tremendous plasticity in the metabolism of PA. In agreement with this report's findings showing increased PLD activity in response to Aβ application in neuronal cultures (Fig. 2), SwAPP overexpression leads to a selective 35% increase in a low abundance, yet, functionally critical (Raghu et al., 2009b) (see below) species of PA, PA 34:2, out of the eleven molecular species of PA analyzed. Although PA 34:2 is not affected by the *Pld2* genotype in the absence of the transgene, it

no longer accumulates in the SwAPP forebrain upon ablation of PLD2, suggesting it may be a *bona fide* product of PLD2 upon SwAPP overexpression.

Reduction in PLD2 levels does not alter APP or Aβ levels in SwAPP mice

An important question is whether the rescue of the learning performance reflects a reduction in amyloidogenesis. Thus a biochemical analysis of the brains from 12 month-old SwAPP/*Pld2* mice was conducted after the RAWM test. Western blot analysis showed that full-length APP levels are not affected by ablation of one or two copies of *Pld2* (Fig. 8*A,B*). Importantly, no significant differences were found in the levels of soluble and insoluble human $A\beta_{1-40}$ and $A\beta_{1-42}$ (Fig. 8*C,D*) and total murine $A\beta_{1-40}$ and $A\beta_{1-42}$ (Suppl. Fig. 4) upon disruption of *Pld2* gene, as measured by ELISA. Overall, ablation of PLD2 protected from the memory deficits in the SwAPP mice, and this protection was concomitant with a high Aβ burden further suggesting that PLD2 acts downstream of Aβ signalling in this AD model.

Discussion

Previous studies have implicated PLD in AD pathogenesis either as a mediator of APP trafficking, presenilin regulation or downstream target of Aβ (Kanfer et al., 1986; Kanfer et al., 1996; Singh et al., 1997a; Singh et al., 1997b; Kanfer et al., 1998; Singh et al., 1998; Cai et al., 2006a; Cai et al., 2006b; Jin et al., 2007; Brandenburg et al., 2008; Liu et al., 2009; Oliveira and Di Paolo, 2010). Importantly, an increase in total PLD activity was reported in AD brain homogenates, using an *in vitro* enzymatic assay (Kanfer et al., 1996). However, these studies have established neither the pathophysiological relevance of the PLD pathway in AD *in vivo* models nor the precise role of PLD2 in AD pathogenesis. For instance, cell culture studies have established a role for PLD1, but not PLD2, in the trafficking of both APP and PS1 with important implications for APP metabolism and Aβ secretion (Cai et al., 2006a; Cai et al., 2006b; Liu et al., 2009; Oliveira and Di Paolo, 2010). Our study suggests that PLD2, unlike PLD1, may be a downstream target of Aβ oligomers.

Although the synaptotoxic actions of Aβ oligomers are beginning to be understood, the underlying molecular mechanisms are still unclear. This study provides experimental and genetic evidence demonstrating that PLD, and PLD2 in particular, is pathophysiologically relevant in the context of mouse models of AD and in the synaptotoxic actions of Aβ42 oligomers. Specifically, PLD2 ablation

confers protection against the synapse-impairing actions of Aβ42 in an *ex vivo* model of synaptic plasticity (Fig. 3) and against the cognitive deficits and synapse impairment induced by overexpression of SwAPP (Fig. 5 and 6). This protective effect occurs despite high Aβ levels (Fig. 8), as observed previously in various other AD mouse models (Roberson et al., 2007; Sanchez-Mejia et al., 2008; Dziewczapolski et al., 2009; Gimbel et al., 2010). Thus, our data is consistent with scenarios whereby PLD2 and its enzymatic product (i) mediate key signaling cascades downstream of Aβ; (ii) regulate the (cell surface) availability of putative Aβ receptors at synapses; or (iii) alter the capacity of Aβ to bind to its putative receptors. Because a pool of PLD isozymes is associated with lipid rafts and that Aβ has a high affinity for these lipid microdomains (Yanagisawa, 2007; Ariga et al., 2008; Hebbar et al., 2008), a tantalizing hypothesis is that PLD2 ablation may disrupt the raft-dependent signalling of Aβ. Importantly, ablation of one or two copies of PLD2 not only reduces basal PLD activity, but it also blocks oAβ42-induced increase in this activity. Therefore, the protective effect may reflect either changes in PLD activity or a combination of both. Finally, although our data suggest that neurons expressing reduced amounts of PLD2 may play a central role in this protective effect, a contribution of other cell types, such as glial cells, cannot be ruled out.

Importantly, our results expand on previous studies showing that impairment of signaling lipids plays a key role in AD pathogenesis in animal models. Specifically, Aβ oligomers have been shown to dysregulate the PLC and PLA2 pathways with major implications for the signaling downstream of $PI(4,5)P_2$ and arachidonic acid in the brain (Singh et al., 1995; Berman et al., 2008; Sanchez-Mejia et al., 2008). This study provides further support to the hypothesis that phospholipase signaling may be globally altered in AD and that Aβ-induced Ca^{2+}-dyshomeostasis through glutamate receptors or other mechanisms may be underlying these changes. Indeed, we find that oligomeric Aβ affects the localization of PLD2 in a manner dependent upon extracellular Ca^{2+}, thus suggesting a role for Ca^{2+} entry in the activation of three phospholipase families, PLC, PLA2 and PLD2. Here, we show that pharmacological inhibition of PLC does not prevent Aβ-induced PLD2 relocalization, suggesting that PLD2 either lies upstream or acts independently of PLC in the Aβ signalling pathway (Fig. 1*D*). Moreover, we show that PLD2 relocalization induced by oAβ42 is blocked by inhibition of cPLA2 (Fig. 1*D*), in agreement with other studies showing that PLD2 activation occurs downstream of Ca^{2+} entry and cPLA2 stimulation in a lymphocytic leukemia cell line (Kim et al., 1999). More careful molecular

genetic/genetic approaches should provide a definitive answer concerning the crosstalk between these enzymes and their precise role in this cytotoxic signaling pathway.

To understand the impact on PLD activity and PA metabolism of *Pld2* ablation, SwAPP overexpression as well as the interaction between these two genetic manipulations, we have employed LC-MS. The *in vivo* PLD activity assay relies on the measurement of PEtOH, which is the product of PLD catalysis in the presence of ethanol. In contrast to PA, PEtOH is a specific product of PLD and is very stable, due to its poor consumption by lipases (Gustavsson, 1995). Importantly, changes in specific molecular species of PEtOH can be correlated with those in the corresponding species of PA, thus providing clues on the contribution of PLD to the synthesis of specific pools of PA. Results from our analysis suggest that a dysregulation of PLD function and PA metabolism may be associated with AD pathogenesis and that specific pools of PA may be affected. Indeed, while brains from SwAPP mice showed an overall increase in PEtOH levels and thus total PLD activity (Fig. 4*A*), a more detailed analysis of PEtOH species showed that only a subset of species, such as PEtOH 32:1, 34:2, 34:1 and 34:1, were increased in mutant brain (Fig. 4*B*; Suppl. Table 1). A direct comparison of these PEtOH species with the corresponding molecular species of PA (Fig. 7) revealed a remarkable and specific increase in both PEtOH 34:2 and PA 34:2 in the brain of SwAPP mice, and since the latter increase was rescued by PLD2 ablation, we speculate that this PA species may be a *bona fide* product of a hyperactivated PLD2 in the SwAPP brain. Interestingly, PA 34:2 is the only molecular species of PA that was found to accumulate in *Drosophila melanogaster* photoreceptors upon overexpression of PLD out of seventeen species of PA measured (Raghu et al., 2009b). Consistent with a pathogenic role of this lipid, overexpression of the fly ortholog of PLD in the photoreceptors results in the degeneration of photoreceptors. Collectively, these data suggest that increased PLD levels/activity and the resulting accumulation of PA 34:2 may be detrimental to cell physiology, at least in some cell types. In contrast to PEtOH 34:2, other molecular species of PEtOH (*i.e.*, 36:2, 36:1, 36:0, 38:3, 38:2 and 38:1) were not affected by SwAPP overexpression (Fig. 4*B*) and the corresponding PA species were equally insensitive to the presence of the transgene (Fig. 7). Altogether, this combined *in vivo* analysis of PEtOH and PA species has allowed for the implication of specific PLD products in AD. However, future studies will be required to determine whether these lipid products are simple AD biomarkers or key contributors to AD pathogenesis.

In addition, our LC-MS analysis uncovered interesting changes as well as some plasticity in the metabolism of PA as a result of PLD2 manipulation. For instance, of the two molecular species that were significantly decreased in the *Pld2*$^{-/-}$ forebrains (in the absence of SwAPP transgene), PA 32:1 and 38:4, only the former is believed to be a direct product of PLD (Hodgkin et al., 1998; Pettitt et al., 2001). Indeed, the primary source of PA 38:4 is thought to originate from DAG kinase in the PLC pathway (Hodgkin et al., 1998). However, ethanol injections led to the production of PEtOH 38:4 in mouse brain (Fig. 4B; Suppl. Table 1), suggesting that the corresponding molecular species of PC can be a *bona fide* substrate for PLD *in vivo*. On the other hand, hints that compensatory changes in the metabolism of PA can occur as a result of PLD2 ablation are suggested by the fact that knocking out PLD2 causes a modest, but significant increase in brain PA 32:0, a phenomenon exacerbated by SwAPP overexpression (Fig. 7; Suppl. Table 2). Considering the complexity of PA metabolism, adaptive changes can indeed be expected, perhaps allowing PLD2-deficient cells, tissues and organisms to cope with the deficiency of specific pools of PA that are normally produced by PLD2. Such compensatory mechanisms in the metabolism of PA may not be as efficient when PLD is either overexpressed (Raghu et al., 2009b; Raghu et al., 2009a) or hyperactivated upon stress conditions, such as during Aβ elevation (this study). We speculate that PLD2 ablation, which results in a 40% decrease in brain-associated total PLD activity (Suppl. Fig. 1*C*), may confer protection during Aβ elevation by correcting some of the aberrant features of PA metabolism occurring in this condition.

Finally, our study expands on previous work showing that blocking the action of Aβ may be beneficial for the treatment of AD [see *e.g.* ref.(Gong et al., 2006; Trinchese et al., 2008)]. Collectively, results presented in this study identify PLD2 as a new player in AD pathogenesis and, together with the lack of obvious detrimental phenotypes in *Pld2*$^{-/-}$ mice, suggest that PLD2 inhibitors may be valuable therapeutic agents.

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LEGENDS TO FIGURES

Figure 1. PLD2 lies in the Aβ signaling pathway. *A*, PC12 cells were transfected with a plasmid expressing GFP-PLD2. Representative examples show internalization of GFP-PLD2 after treatments with 200 nM oAβ42. Pictures show the fluorescence of GFP-PLD2. *B*, oAβ42 (200nM) was applied to PC12 cells in cultures for 5, 30 and 60 minutes and relocalization of GFP-PLD2 was quantified as PM/cytosol ratio (0min: n=31; 5min: n=29; 30min: n=32; 60min: n=30). *C*, Effect of 30 min treatments with ionomycin (2μM) and EGTA (2 mM) on the localization of GFP-PLD2 in the presence or absence 200 nM oAβ42. The number of cells analyzed was as follows: vehicle (n = 37), oAβ42 (n = 33), ionomycin (n = 26), EGTA (n = 33), EGTA + oAβ42 (n = 38). *D*, Effect of 30 min treatments with pharmacological inhibitors of PLC (U73122, 250 nM) and PLA2 (AACOCF3, 20 μM) on the localization of GFP-PLD2 in the presence or absence of 200 nM oAβ42. The number of cells analyzed was as follows: vehicle (n = 97), oAβ42 (n = 81), U73122 (n = 41), U73122 + oAβ42 (n = 42), AACOCF3 (n = 50), AACOCF3 + oAβ42 (n = 45). For *B-D*, values denote means ± SEM. *, $p < 0.05$; ***, $p < 0.001$).

Figure 2. Ablation of PLD2 reduces basal PLD activity and abolishes the stimulatory effect of Aβ oligomers on PLD activity in cultured neurons. Primary cortical cultures were labeled with [^{3}H]myristic acid at day 12, treatments were performed at day 15, lipids were subsequently extracted and the ratio [^{3}H]PhosphatidylButanol counts/total counts was used as a measure of PLD activity. Four-hour treatments with vehicle or oAβ42 200nM were performed prior to PLD activity measurement in *Pld2*$^{+/+}$ (n=19 and 12 for vehicle and oAβ42 treatment, resp.), *Pld2*$^{+/-}$ (n=7), and *Pld2*$^{-/-}$ (n=5 and 7, resp.). Values denote means ± SEM. ns - non significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. LTP is unaffected by oAβ42 in *Pld2*$^{-/-}$ hippocampal slices. There was no difference in LTP between *Pld2*$^{+/+}$ slices and *Pld2*$^{-/-}$ slices in the presence of vehicle ($F_{1,17} = 0.01$, p=0.9473). Although *Pld2*$^{+/+}$ slices showed a reduction of LTP following bath application of 200 nM oAβ42 ($F_{1,16} = 5.19$, p=0.0367, relative to vehicle), LTP was not reduced by the peptide in *Pld2*$^{-/-}$ slices ($F_{1,14} = 0.01$, p=0.9185, relative to vehicle). fEPSP, CA1 field-excitatory postsynaptic potential. The bar represents the time of bath application of oAβ42. The three arrows represent the θ-burst stimulation used to induce potentiation. Animals were approximately 3 month-old. Values denote means ± SEM (n=8-9).

Note: the vehicle traces for both *Pld2* genotypes (*i.e.*, black filled triangles and circles) are largely overlapping.

Figure 4. PLD activity is enhanced in the forebrain of aged SwAPP mice. Mice, with and without SwAPP transgene, were injected with 3g/kg ethanol and their forebrain lipids were extracted and subjected to LC-MS analysis. The production and accumulation of PEtOH was used as a reporter of *in vivo* PLD activity. Relative individual PEtOH species measured in mutant mice compared to control mice. The nomenclature for phospholipids fatty acid composition are denoted as total chain length:number of unsaturated bonds. Values denote mean ± SEM (n=6). ** $p < 0.01$; *** $p < 0.001$. Absolute amounts of the various molecular species of PEtOH are presented in Supplementary Table 1.

Figure 5. PLD2 ablation improves learning and memory in SwAPP mice. *A*, SwAPP mice (Tg2576) were crossed with *Pld2* knockout mice and the resulting offspring [*Pld2*$^{+/+}$/no tg (n=14); *Pld2*$^{+/-}$/no tg (n=14); *Pld2*$^{-/-}$/no tg (n=11); *Pld2*$^{+/+}$/SwAPP (n=10); *Pld2*$^{+/-}$/SwAPP (n=12); *Pld2*$^{-/-}$/SwAPP (n=11)] were subjected to training for contextual fear memory which was assessed 24h after the foot shock, using 5-6 month old animals. *, $p < 0.05$ in Student's one-tail t-test. *B*, Twelve month-old mice were subjected to Radial Arm Water Maze (RAWM) testing. Errors were scored in the last 3 days of testing. The n value was 8 for all the genotypes, except for *Pld2*$^{-/-}$/SwAPP (n=7) and *Pld2*$^{+/+}$/SwAPP (n=6). **, $p < 0.01$. Values denote means ± SEM.

Figure 6. PLD2 ablation confers synaptic protection in the forebrain of SwAPP mice. After RAWM testing, forebrains from 12 month-old *Pld2*$^{-/-}$SwAPP mice were processed for biochemical analysis. *A*, Protein levels were evaluated by Western blot analysis of PLD2, PLD1, PSD95, synaptophysin and tubulin (representative blots are shown). *B*, Quantification of PSD95 levels by densitometric analysis. *C*, Quantification of synaptophysin levels by densitometric analysis. Values denote means ± SEM. n=4. * $p < 0.05$.

Figure 7. Effect of SwAPP overexpression and *Pld2* genotypes on PA levels. After RAWM testing, forebrains from 12 month-old *Pld2*$^{-/-}$SwAPP mice were processed for lipid biochemical analysis. Forebrain lipids were extracted from *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice with and without SwAPP transgene and

subjected to LC-MS analysis. Relative amounts of PA species measured in mutant mice compared to control mice (*Pld2*$^{+/+}$, no SwAPP). Values denote mean ± SEM (n=6-8). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The nomenclature for phospholipids fatty acid composition are denoted as total chain length:number of unsaturated bonds. Absolute amounts of the various molecular species of PA are presented in Supplementary Table 2.

Figure 8. Effect of SwAPP overexpression and *Pld2* genotypes on APP processing. *A-D*, After RAWM testing, forebrains from 12 month-old *Pld2*/SwAPP mice were processed for biochemical analysis. *A*, Protein levels were evaluated by Western blot analysis of APP and tubulin (representative blots are shown). *B*, Quantification of full-length human APP levels by densitometric analysis. Values denote means ± SEM (n = 6). *C,D*, ELISA analysis of the levels of soluble Aβ40 and Aβ42 (*C*); insoluble Aβ40 and Aβ42 (*D*). Values denote means ± SEM. *Pld2*$^{+/+}$/SwAPP (n=6); *Pld2*$^{+/-}$/SwAPP (n=8); *Pld2*$^{-/-}$/SwAPP (n=7).

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Table 1. PEtOH levels in forebrain extracts from non-transgenic and SwAPP mice (14 month old). The relative abundance of individual lipid species is expressed as mean value ± SEM (n = 6). PEtOH 32:0 was not quantified as it was used as a lipid standard for the MS analysis. The statistical differences in relevant sample groups were analyzed using Student's t-test and the *p* values of these analyses are given.

Supplementary Table 2. PA levels in forebrain extracts from *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice with and without SwAPP transgene. The relative abundance of individual lipid species are expressed as mean value ± SEM (n = 6-8). The statistical differences in relevant sample groups were analyzed using Student's t-test and the *p* values of these analyses are given.

Supplementary Figure 1. Generation of *Pld2* knockout mice. *A*, Gene targeting strategy - an FRT-NEO-FRT-lox*P* cassette was inserted downstream of exon 15 from the *Pld2* gene at the SspI site and the second lox*P* sequence was subcloned upstream of exon 13 at the MfeI site. Exon 14 contains the sequence encoding the first "HKD" motif of PLD2, which is essential for the catalytic activity of PLD2.

Pld2$^{Flox\ Neo/+}$ mice were bred with a "deleter" strain of mice expressing Cre recombinase (Rosa26) to eliminate exons 13-15 and produce *Pld2*$^{+/-}$ mice. *Pld2*$^{+/-}$ mice were then intercrossed to create *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice. The genetic background of the animals is mixed (C57Bl6, 129SVJ). *B*, Southern blot analysis of genomic DNA that was extracted from the tail of mice derived from *Pld2*$^{+/-}$ mouse intercrosses (right panel). DNA was digested with PstI and hybridized with a [^{32}P] labeled-probe located to the 5' region (outside the targeting vector) of the targeted genomic region. The autoradiogram shows the wild type (+/+), knockout (–/–) and heterozygous (+/–) genotypes. The sizes of the wild-type and mutant alleles are indicated (4.2 kb and 11.2 kb, respectively). *C*, Total PLD activity was assessed through the measurement of total PEtOH by LC-MS analysis in the brain of adult mice one hour post-injection with 3g/Kg of EtOH. Values denote means ± SEM *(*n = 8-9*). ***, $p < 0.001$.

Supplementary Figure 2. Basal synaptic transmission in *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ hippocampal slices. CA1 field-excitatory post-synaptic potentials (fEPSPs) were recorded by placing both the stimulating and the recording electrodes in CA1 stratum radiatum. Summary graph of field input/output relationships for *Pld2*$^{+/+}$ (white squares) and *Pld2*$^{-/-}$ slices (black squares). Values denote means ± SEM (n = 8-9).

Supplementary Figure 3. Visible platform task in *Pld2*/SwAPP mice following RAWM testing in the same pool with no arms. *A*, Summary graph of the time needed to find the visible platform. *B*, Summary graph of the swimming speed during the task. Values denote means ± SEM (n = 6-8).

Supplementary Figure 4. Murine Aβ levels in the forebrain of 9 month-old *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice. ELISA analysis of the levels of murine Aβ40 and Aβ42. Values denote means ± SEM (n=7).

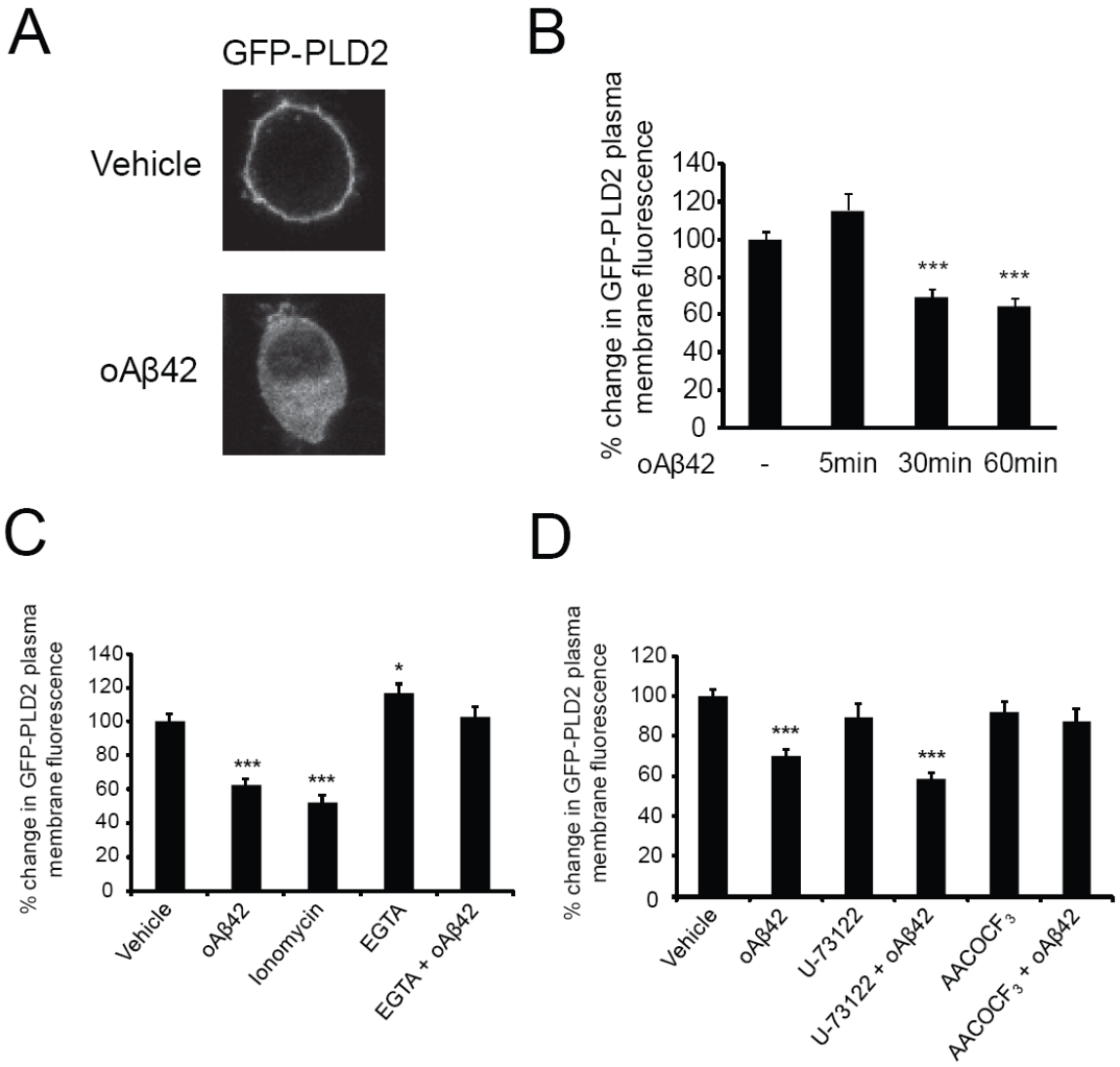
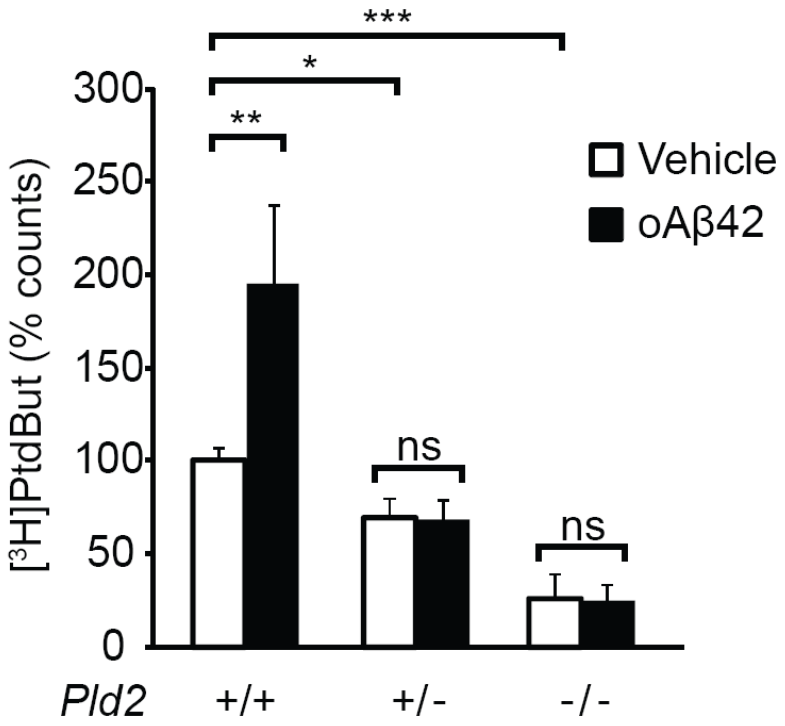


Figure 1 (Oliveira et al)



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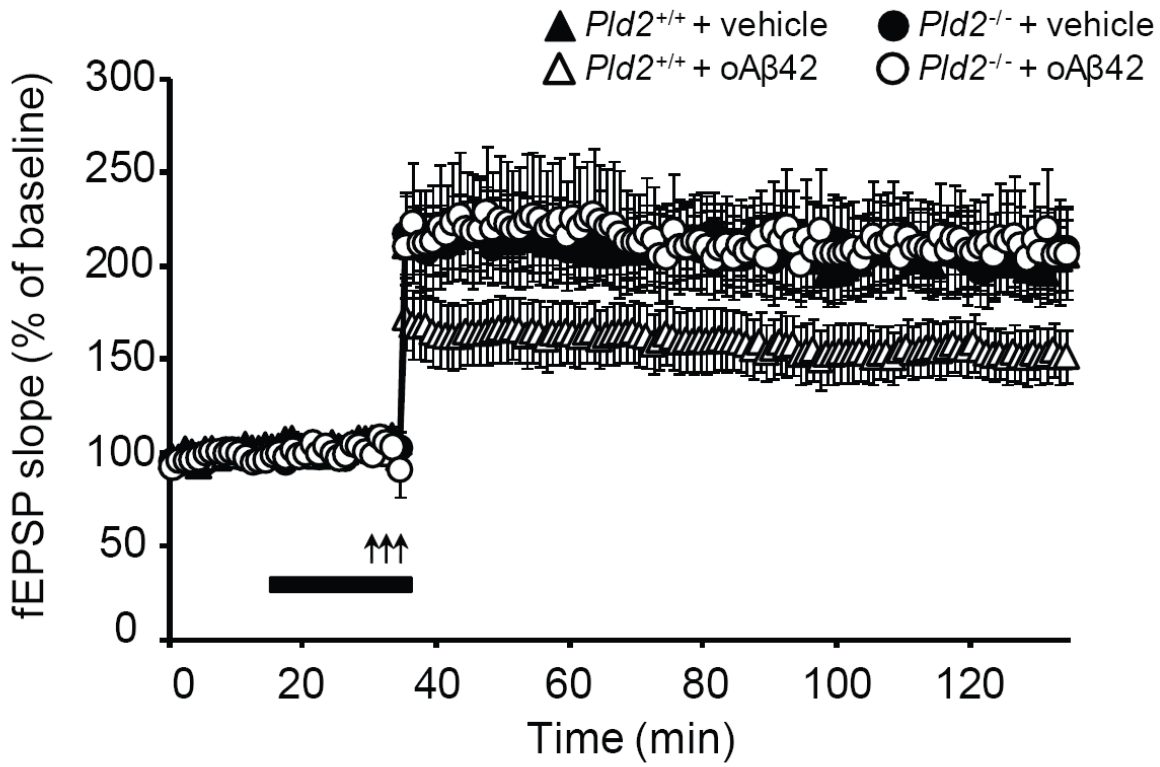

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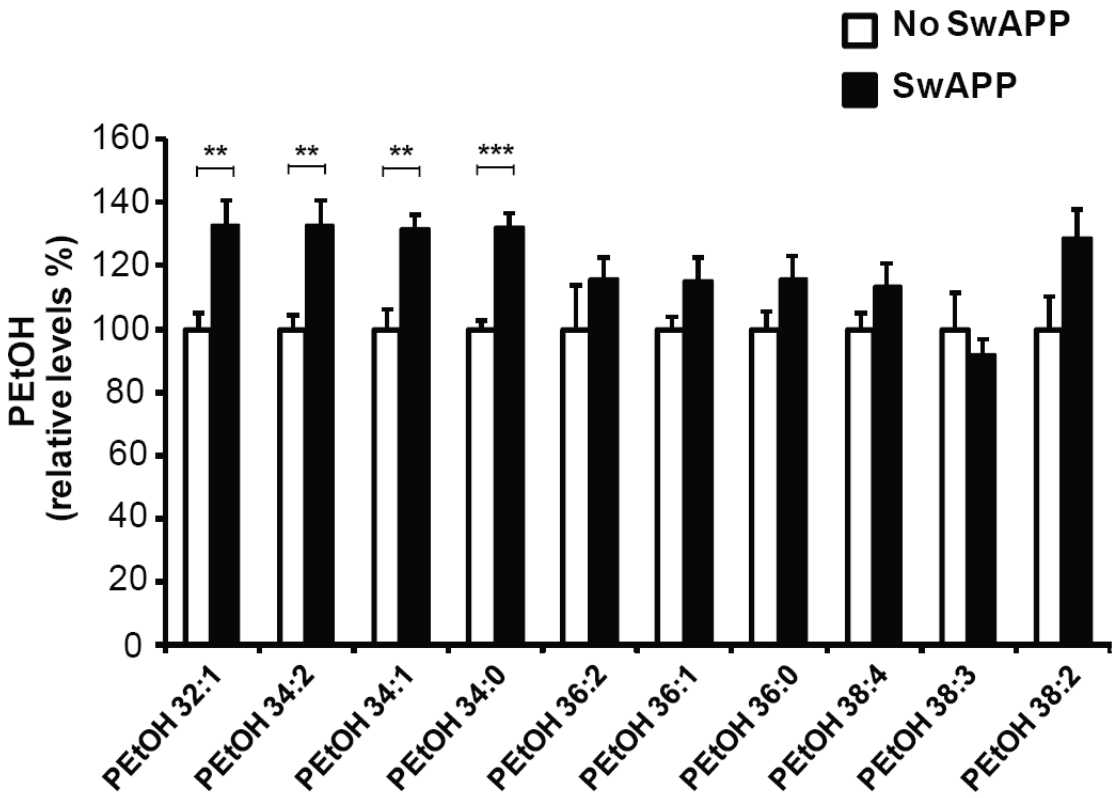

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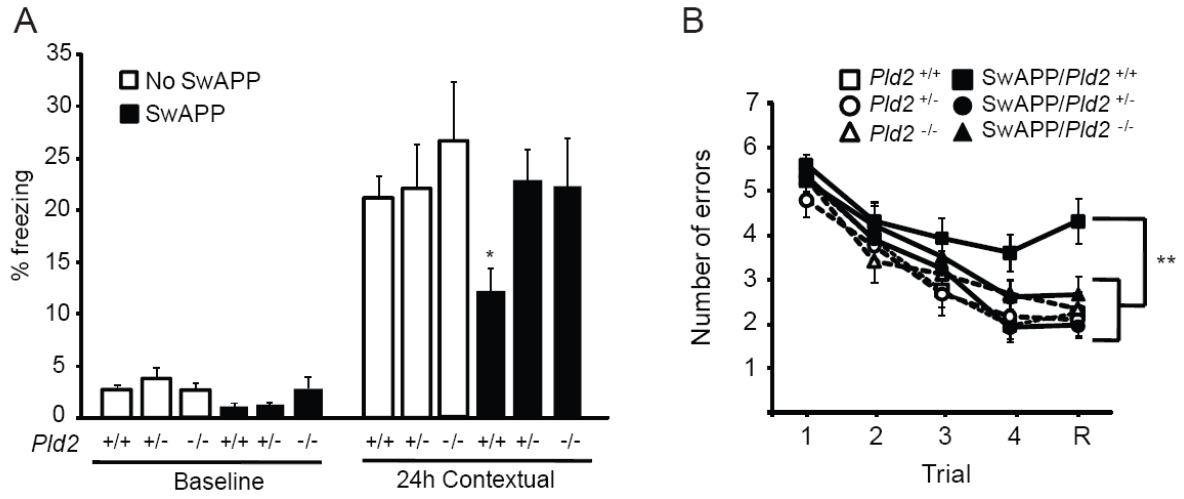

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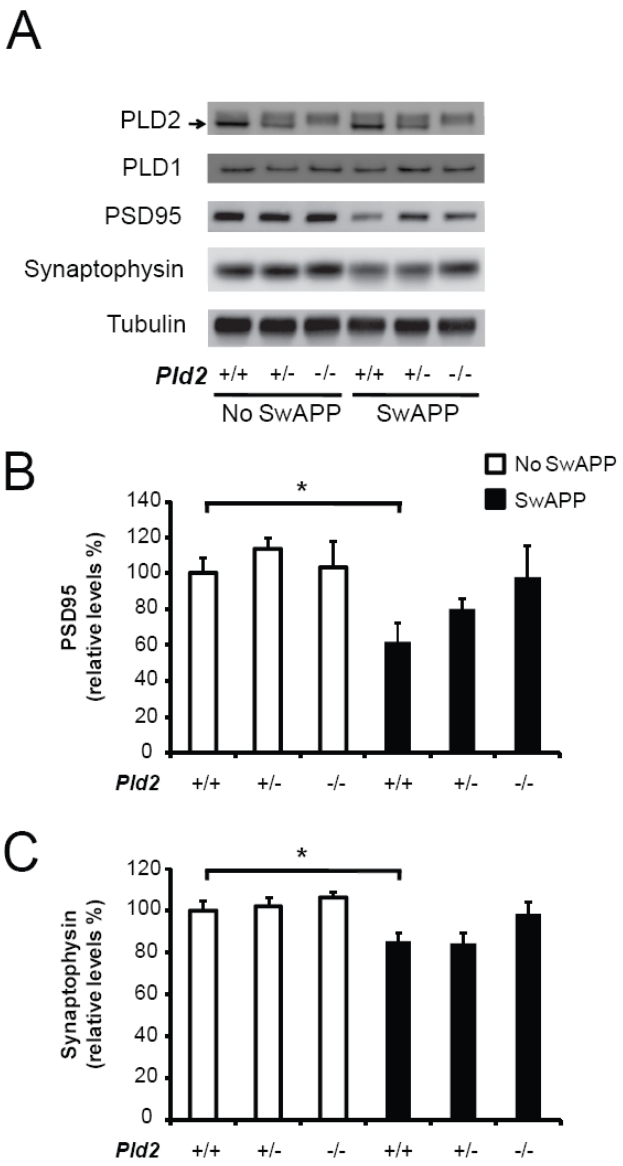

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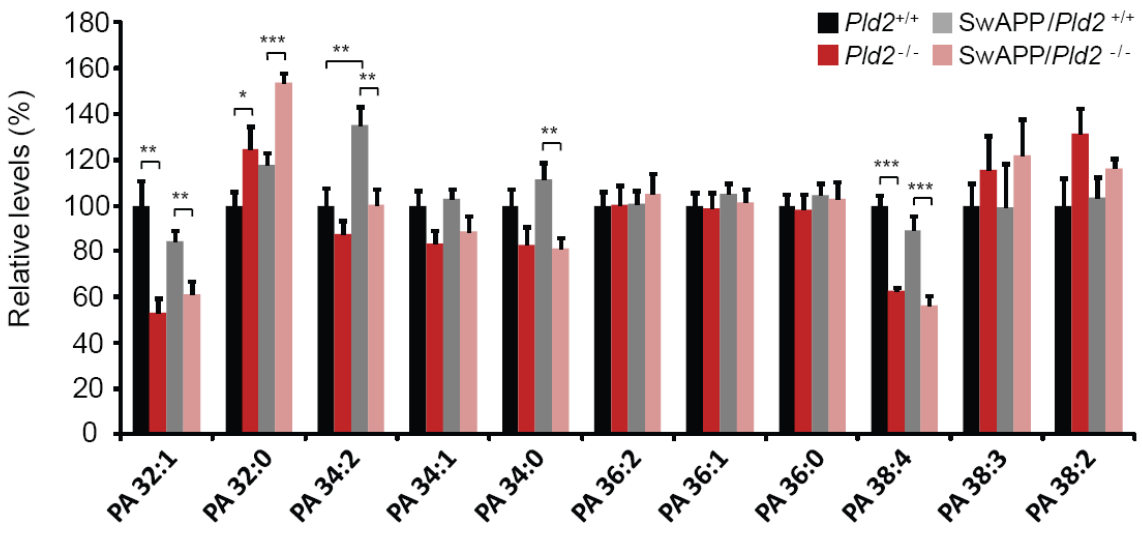


Figure 7 (Oliveira et al)

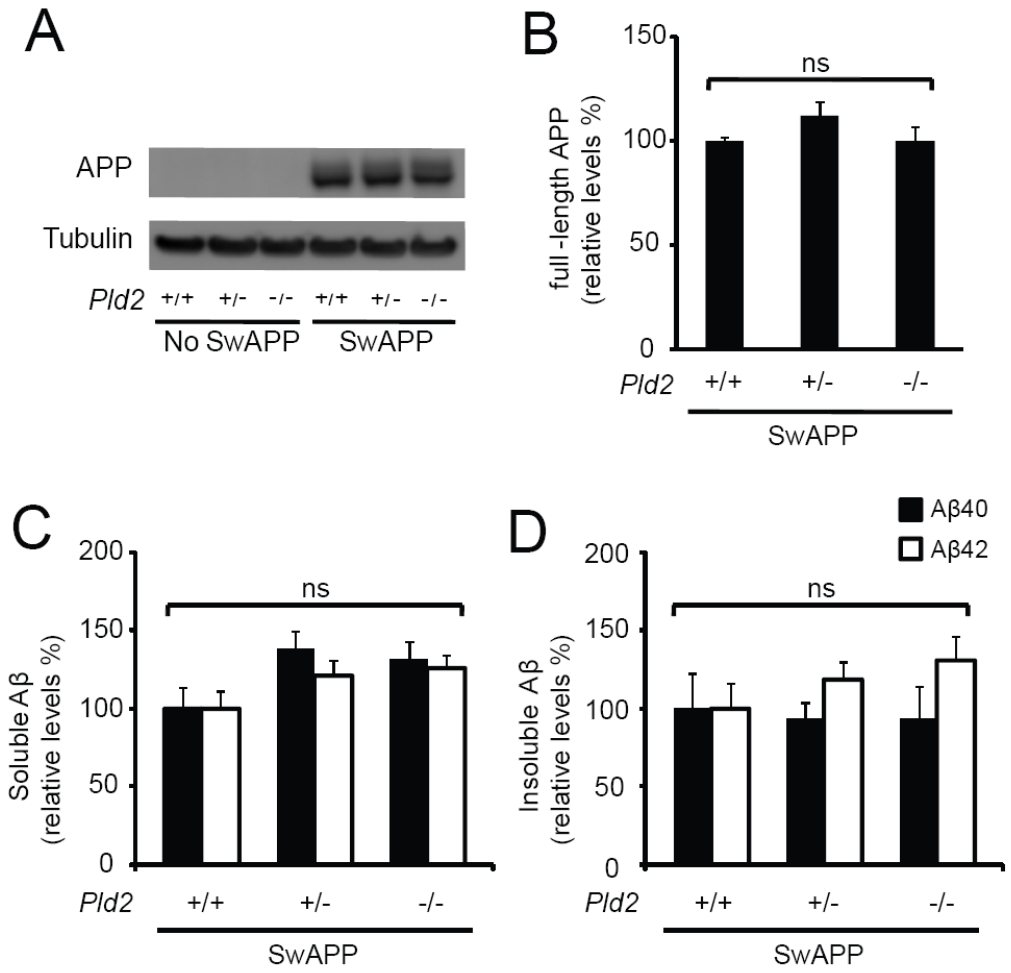
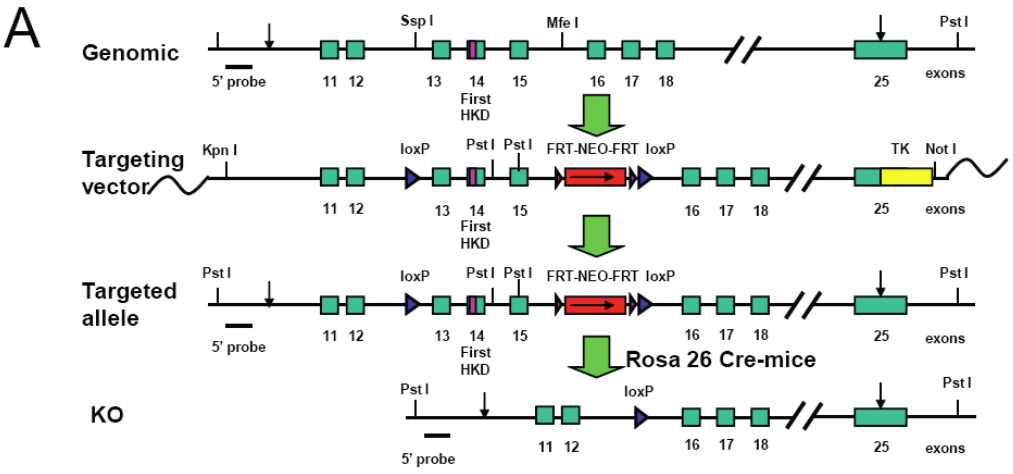
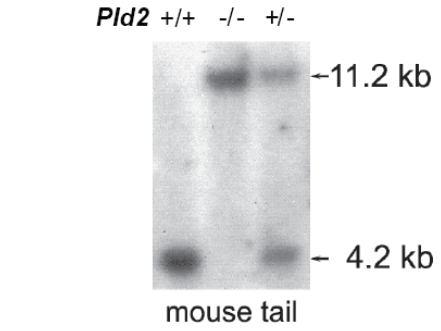
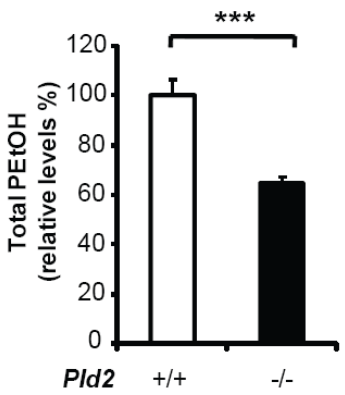


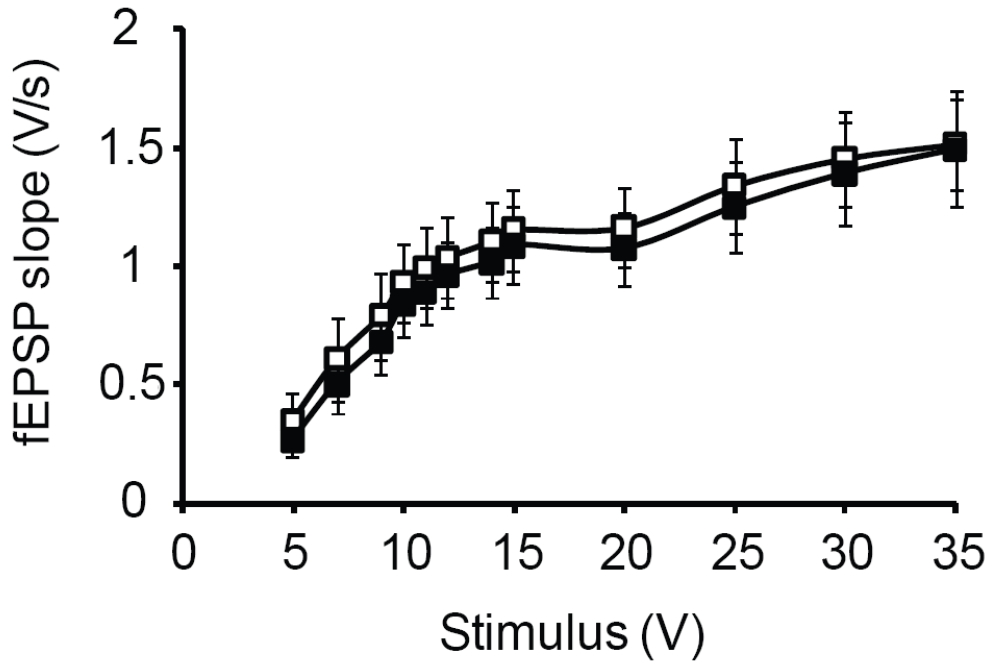
Figure 8 (Oliveira et al)



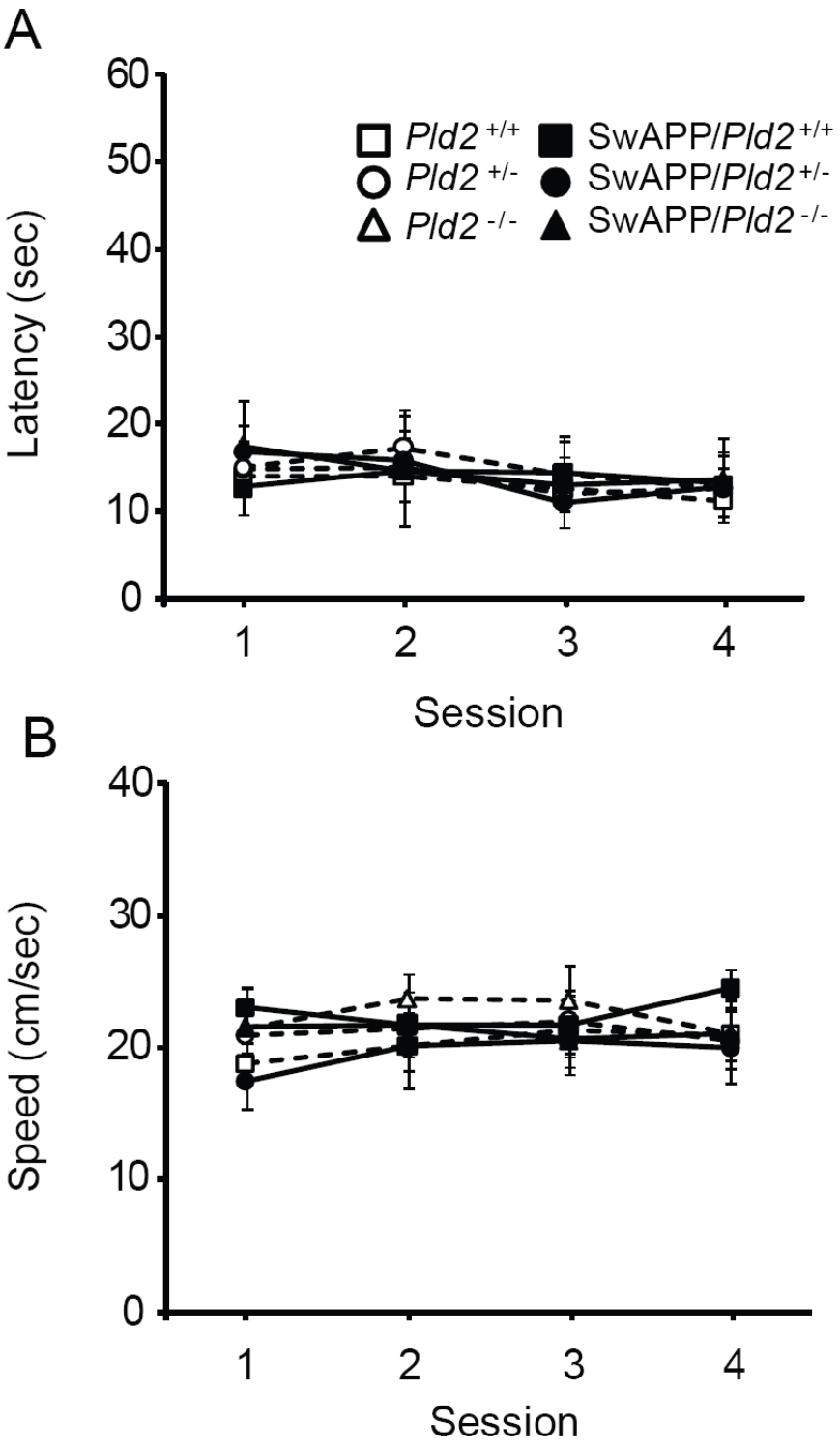




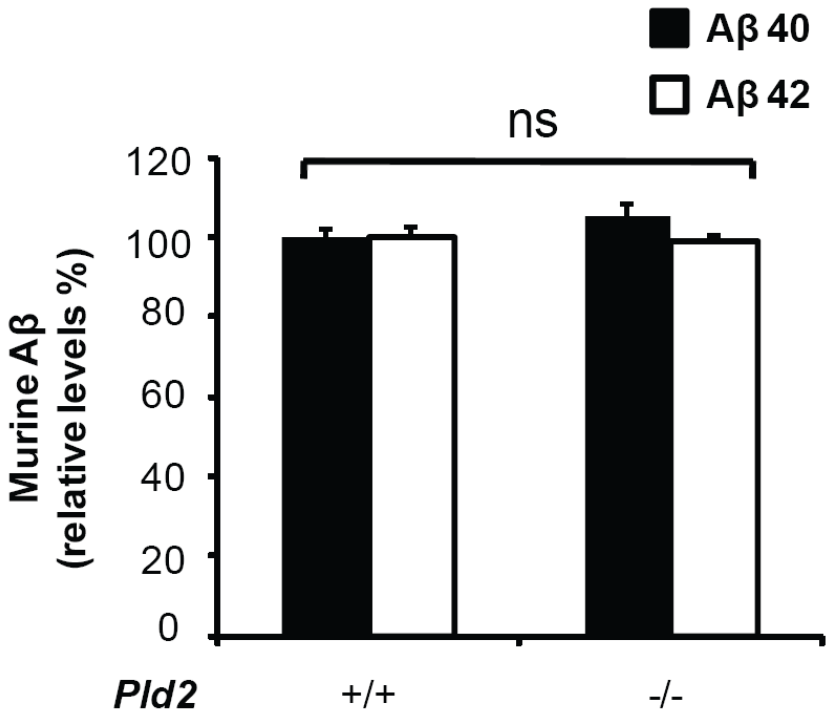
Supplementary Figure 1 (Oliveira et al)



Supplementary Figure 2 (Oliveira et al)



Supplementary Figure 3 (Oliveira et al)



Supplementary Figure 4 (Oliveira et al)

	Lipid Quantification						T-test,
Lipid Species	no SwAPP			SwAPP			*p* value
PEtOH 32:1	3.63E-04	±	1.85E-05	4.81E-04	±	2.89E-05	0.006
PEtOH 34:2	1.07E-03	±	4.98E-05	1.42E-03	±	8.48E-05	0.005
PEtOH 34:1	1.52E-02	±	9.37E-04	2.00E-02	±	7.45E-04	0.003
PEtOH 34:0	4.77E-04	±	1.20E-05	6.32E-04	±	2.13E-05	0.001
PEtOH 36:2	6.12E-03	±	8.32E-04	7.10E-03	±	4.11E-04	0.314
PEtOH 36:1	3.60E-03	±	1.29E-04	4.14E-03	±	2.64E-04	0.096
PEtOH 36:0	3.65E-04	±	2.03E-05	4.22E-04	±	2.62E-05	0.114
PEtOH 38:4	1.74E-03	±	9.06E-05	1.97E-03	±	1.28E-04	0.171
PEtOH 38:3	5.13E-05	±	5.89E-06	4.70E-05	±	2.76E-06	0.525
PEtOH 38:2	1.97E-04	±	1.99E-05	2.53E-04	±	1.81E-05	0.061

Supplementary Table 1 (Oliveira et al)

	Lipid Quantification				T-Test, *p* value				
Lipid Species	*Pld2*$^{+/+}$	*Pld2*$^{-/-}$	SwAPP/*Pld2*$^{+/+}$	SwAPP/*Pld2*$^{-/-}$	*Pld2*$^{+/+}$ vs *Pld2*$^{-/-}$	*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{+/+}$	*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{-/-}$	SwAPP/*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{-/-}$	*Pld2*$^{-/-}$ vs SwAPP/*Pld2*$^{-/-}$
PA 32:1	0.042 ± 0.004	0.022 ± 0.003	0.035 ± 0.002	0.026 ± 0.002	0.0021	0.2577	0.0092	0.0082	0.3056
PA 32:0	0.089 ± 0.005	0.111 ± 0.009	0.104 ± 0.005	0.141 ± 0.006	0.0486	0.0611	0.0001	0.0008	0.0197
PA 34:2	0.027 ± 0.002	0.024 ± 0.002	0.037 ± 0.002	0.028 ± 0.002	0.2168	0.0079	0.8187	0.0092	0.1117
PA 34:1	1.073 ± 0.069	0.894 ± 0.063	1.103 ± 0.044	0.963 ± 0.066	0.0761	0.7340	0.2775	0.1170	0.4608
PA 34:0	0.117 ± 0.008	0.097 ± 0.009	0.130 ± 0.008	0.096 ± 0.005	0.1353	0.2922	0.0556	0.0039	0.9680
PA 36:2	0.935 ± 0.056	0.936 ± 0.078	0.944 ± 0.050	0.999 ± 0.073	0.9854	0.9066	0.4897	0.5596	0.5699
PA 36:1	0.860 ± 0.048	0.848 ± 0.058	0.903 ± 0.040	0.871 ± 0.044	0.8810	0.5252	0.8694	0.6069	0.7663
PA 36:0	0.215 ± 0.011	0.211 ± 0.015	0.224 ± 0.012	0.220 ± 0.013	0.8463	0.6014	0.7638	0.8384	0.6624
PA 38:4	0.294 ± 0.014	0.183 ± 0.005	0.262 ± 0.019	0.164 ± 0.010	0.0001	0.1919	0.0001	0.0007	0.1239
PA 38:3	0.004 ± 0.000	0.004 ± 0.001	0.004 ± 0.001	0.005 ± 0.000	0.3911	0.9604	0.1935	0.3312	0.7462
PA 38:2	0.079 ± 0.009	0.104 ± 0.009	0.082 ± 0.007	0.097 ± 0.006	0.0710	0.8206	0.1686	0.1331	0.5110

Supplementary Table 2 (Oliveira et al)

Chapter 2.2.

Tiago Gil Oliveira, Robin B. Chan, Markus R. Wenk, Gilbert Di Paolo.

The impact of Phospholipase D2 ablation in the forebrain of Tg2576 mice

– mechanistic insights from a lipidomic study.

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Title: The impact of Phospholipase D2 ablation in the forebrain of Tg2576 mice – mechanistic insights from a lipidomic study.

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Abstract: 244 words
Introduction: 201 words
Discussion: 1146 words

Abstract

There is accumulating evidence that aberrant lipid signaling is implicated in Alzheimer's disease (AD). Phospholipase D (PLD) is a key lipid modifying enzyme that produces phosphatidic acid (PA), a bioactive lipid involved in multiple aspects of cell physiology, including signaling and membrane trafficking processes. There are two main PLD isozymes, PLD1 and PLD2, that in spite of catalyzing the same reaction differ in their structure and cellular localization. While PLD1 was shown to be involved in the trafficking and processing of amyloid precursor protein (APP), PLD2 was recently demonstrated to modulate the downstream effects of amyloid beta (Aβ). Moreover, *Pld2* ablation in a transgenic mouse model of AD (SwAPP), was shown to rescue memory deficits and confer synaptic protection in SwAPP mice despite a significant Aβ load. Initial mass spectrometry (MS)-based lipid analysis of *Pld2* mutant brains in the presence or absence of the SwAPP transgene unmasked striking crosstalks between different PA species. Here, we expand the MS-lipid analysis to a wide lipidomics study including other lipid classes than PA, such as phosphatidylcholine (PC), diacylglycerol (DAG), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin (SM), ceramides (Cer), glucosyl-ceramide (Glu-Cer), GM3 (a specific ganglioside class), cholesterol and cholesterol-ester. We found that overexpression of the SwAPP transgene leads to significant increase in the ganglioside, GM3. Remarkably, *Pld2* ablation rescues GM3 levels to normals in the SwAPP background. This lipidomic analysis uncovered interesting lipid signaling crosstalks that are modulated by PLD2 in the context of AD models.

Introduction

The accumulation of amyloid beta (Aβ) in the brain is one of the major hallmarks of Alzheimer's disease (AD) pathogenesis. Aβ is produced by the sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. There are two major forms of Aβ, Aβ40, the predominant cleavage product, and Aβ42, which is more cytotoxic and is more aggregate-prone (Small and Gandy 2006; Haass and Selkoe 2007; Vassar, Kovacs et al. 2009; De Strooper, Vassar et al. 2010). In the past few years the understanding of AD has advanced remarkably due to the development of animals models of the disease (Ashe and Zahs 2010). One of the major models used is the Tg2576 mouse line, which expresses human APP carrying the double mutation K670N and M671L found in a Swedish family with early onset of Alzheimer's disease (SwAPP), under the regulation of the hamster prion protein promoter (Hsiao, Chapman et al. 1996). This mouse model presents with age dependent accumulation of Aβ that correlates with memory behavioral deficits (Hsiao, Chapman et al. 1996).

It has been shown that signaling lipids regulate various processes involved in physiologic brain functions. Mounting evidence has implicated the dysregulation of lipid-based signaling pathways in a growing number of neurodegenerative disorders. Concerning AD, the majority of these studies have addressed the role of cholesterol (Puglielli, Tanzi et al. 2003). However, other classes of lipids, such as phospholipids, have also been implicated in the modulation of key pathological processes associated with AD (Landman, Jeong et al. 2006; Berman, Dall'Armi et al. 2008; Sanchez-Mejia, Newman et al. 2008; Tiago Gil Oliveira 2010). Given this amount of evidence demonstrating the importance of phospholipids and AD, lipid modifying enzymes, such as phospholipase D (PLD) are strong candidates to be involved in the pathogenesis of AD (Oliveira and Di Paolo 2010).

PLD-mediated hydrolysis of phosphatidylcholine (PC) produces phosphatidic acid (PA), a bioactive lipid involved in multiple aspects of cell physiology, including signaling and membrane trafficking processes. There are two main PLD isozymes, PLD1 and PLD2, which, differ in their structure and cellular localization, although they catalyze the same reaction. This lipid regulates membrane dynamics and signaling processes through its intrinsic physical properties (i.e., 'cone shape') and interaction with effector proteins (Jenkins and Frohman 2005; Stace and Ktistakis 2006; Haucke and Di Paolo 2007; Roth 2008; Raghu, Manifava et al. 2009). Localization studies suggest that PLD1 is predominantly localized in the Golgi complex as well as in secretory granules and endosomes (Jenkins and Frohman 2005; Roth 2008; Bader and Vitale 2009), while PLD2 is more concentrated at the plasma membrane (Du, Huang et al. 2004). The differential localization of the two PLD isozymes

has an impact on the way they modulate AD-related processes. While PLD1 was shown to regulate the trafficking and processing of amyloid precursor protein (APP) (Cai, Netzer et al. 2006; Cai, Zhong et al. 2006; Liu, Zhang et al. 2009), our recent study has shown that PLD2 modulates the downstream effects of Aβ (Tiago Gil Oliveira 2010). Moreover, the same report showed that *Pld2* ablation in SwAPP mice rescues memory deficits and confer synaptic protection, despite a significant Aβ load (Tiago Gil Oliveira 2010).

Although genomic and proteomic studies are nowadays commonly used in biomedical research, the field of lipidomics is now gaining increased attention, which was in part allowed by technological advances in liquid chromatography and electrospray ionization mass spectrometry (Wenk 2005; Piomelli, Astarita et al. 2007). Lipidomic studies allow the characterization of a vast array of lipid species from a given sample and can be used in the identification of potential lipid metabolic pathways, which are dysregulated in a certain disease, such as neurodegenerative disorders (Wenk 2005). In the case of AD, this approach is starting to be employed to identify new possible drug targets, using brain samples from human and mouse models (Sanchez-Mejia, Newman et al. 2008; Han 2010). Lipidomics can also be used to suggest potential mechanistic links between specific lipid alterations (*e.g.*, decreased production of PLD-derived PA) and the lipidome. Initial mass spectrometry (MS)-based lipid analysis of *Pld2* mutant brains in the presence or absence of the SwAPP transgene unmasked striking crosstalks between different PA species. Here, we expanded our MS-based lipid analysis to a more comprehensive lipidomic study that includes lipid classes other than PA. These include PC, diacylglycerol (DAG), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin (SM), ceramides (Cer), glucosyl-ceramide (Glu-Cer), GM3 (a specific ganglioside class), cholesterol and cholesterol-ester. Potential implications of alterations in the physiology of these lipid classes in the context of AD models are summarized in table 1. For instance, we found that overexpression of the SwAPP transgene leads to significant increase in the ganglioside, GM3 and in the total levels of PI. Remarkably, *Pld2* ablation rescues GM3 and PI levels to normal in the SwAPP background. This lipidomic analysis uncovered interesting lipid signaling crosstalks that are modulated by PLD2 in the context of AD models.

Materials and Methods

Mouse strains and breeding strategy. The genetic background of the *Pld2* mice is mixed (C57BL/6-129svj). *Pld2*$^{-/-}$ females were crossed with Tg2576 males (Taconic- mixed background C57BL/6-

SJL/N), which express human APP carrying the double mutation K670N and M671L found in a Swedish family with early onset of Alzheimer's disease, under the regulation of the hamster prion protein promoter (Hsiao, Chapman et al. 1996). From the F_1 hybrid generation we used as breeders *Pld2*$^{+/-}$/no tg females and *Pld2*$^{+/-}$/SwAPP males. For all our animal studies we used littermate mice (or in some cases, mice sharing at least one parent) derived from the F_2 generation. The survival rate of adult mice was >90% for all genotypes within the first 12 months of age (and thus the impact of *Pld2* deletion on the survival of SwAPP mice was not investigated further).

Lipidomics analysis. Lipid extracts were prepared from mice forebrain using a modified Bligh and Dyer method and analyzed by LC-MS. Polar glycerophospholipids and sphingolipids were separated via a Luna silica column (3µm, 2mmx150mm; Phenomenex) with a solvent gradient of 100% chloroform/methanol/water/ammonia solution (90:9.5:0.5:0.32, by vol.) changing to 100% chloroform/methanol/water/ammonia solution (50:48:2:0.32, by vol.) over 40min (Pettitt, McDermott et al. 2001). Non-polar neutral lipids were separated via a ZORBAX Eclipse XDB-C18 column (5 µm, 4.6mmx150mm, Agilent) with isocratic elution using chloroform:methanol:2% 0.1M Ammonium Acetate (100:100:4, by vol.) (Chan, Uchil et al. 2008). Lipid classes were quantified using a triple quadrupole instrument ABI 4000 Q-Trap (Applied Biosystems, Foster City, CA) operated in multiple reactions monitoring mode (MRM) using previously reported MRM transition pairs and instrument settings (Chan, Uchil et al. 2008). The signal levels of most glycerophospholipids and sphingolipids were converted to relative abundance levels by normalization to spiked internal standards. Neutral lipids were converted to relative abundance levels by normalization to total signal measured.

Statistics. Statistical analysis was performed using two-tailed equal variance and Student's *t* test, unless indicated otherwise. All the experiments were performed in blind to the genotypes.

Results

To gain insight into the molecular basis underlying AD pathogenesis as well as the protection conferred by PLD2 ablation, a lipidomics analysis of mutant animals covering approximately 140 lipids was conducted. Total forebrain lipids were extracted from 12 month-old *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$ mice in the presence or absence of the SwAPP transgene and analyzed using liquid chromatography-mass

spectrometry (LC-MS) (Chan, Uchil et al. 2008). Three classes of potential lipid alterations were investigated: (i) changes produced by the ablation of PLD2, with a strong emphasis on candidate PLD reaction products (i.e., PA, which were already published in (Tiago Gil Oliveira 2010)); (ii) changes occurring in response to the overexpression of SwAPP; and (iii) changes reflecting interactions between the SwAPP transgene and the *Pld2* genotypes. Total levels of each lipid class were analysed (**Fig. 1**). Also, results are expressed either in the form of molar percentages in a table (**Table 2**) or in the form of lipid heat maps whereby relative increases and decreases relative to control brains (*Pld2*$^{+/+}$, no SwAPP) are indicated in red and green colors of varying intensity, respectively (**Fig. 2**).

The data shows that ablation of PLD2 significantly decreases the levels of two molecular species of PA based on the different fatty acyl composition (see legend), namely, PA 32:1 (~50 %) and 38:4 (~40%), while a trend for a decrease (~20%) was observed for two other species, PA 34:1 and 34:0 (**Fig. 2; Table 2**). Surprisingly, the PA 32:0 and 38:2 species were upregulated (by 25% and ~30%, resp.), suggesting the occurrence of compensatory mechanisms in knockout brains. Indeed, the total amount of PA (i.e., all species measured) was overall unaffected by the *Pld2* genotype, thus denoting a tremendous plasticity in the metabolism of PA. While no changes were found in the total levels of DAG, PI, SM, GM3, Cer, Glu-Cer, cholesterol and cholesterol ester, the lack of PLD2 caused an increase in the total levels of PS and a decrease in PC, PE and PG (**Fig.1**). The analysis of the lipid classes by species shows that almost all the PS species were significantly increased and almost all PE species were decreased upon PLD2 ablation, denoting a widespread effect in these lipid classes. Interestingly, since certain species can be hydrolyzed by PLD, total PC levels are decreased, with significant decreases in PC 32:1, 32:0, 34:3, 34:1 and 34:0.

Additionally, expression of the SwAPP transgene had no significant effect in the total levels of PA, PC, DAG, PS, PE, SM, Glu-Cer, Cer and cholesterol. On the other hand, it was observed an increase in the total levels of PI, GM3 and cholesterol-esters. For both PI and GM3 the changes were observed in a widespread range of its lipid species, also denoting a widespread effect over these two lipid classes (**Fig. 1; Fig. 2; Table 2**).

When combined, SwAPP expression and PLD2 ablation, quite remarkably, lead to a normalization of both PI and GM3 total levels (**Fig. 1**).

Discussion

AD is a disorder whose pathophysiology has been shown to be associated with altered lipid metabolism. Here, we use LC-MS lipidomics to study the potential molecular basis of how PLD2 ablation confers protection in SwAPP mice. Besides the alterations in PA that were previously published and discussed in (Tiago Gil Oliveira 2010), here we covered other lipid groups, such as PC, DAG, PS, PI, PE, PG, SM, Cer, Glu-Cer, GM3, cholesterol and cholesterol-ester.

We found alterations in various lipid species induced by either PLD2 ablation or SwAPP overexpression, which are here reported and we believe these results will be important information for both the PLD and AD fields. However, we were particularly interested in the molecular alterations that could support the phenotypic observation that PLD2 ablation is protective in the SwAPP mouse model (Tiago Gil Oliveira 2010). Indeed, taken into account the total levels of the lipid classes, there was a significant increase in the levels in cholesterol-ester, PI and GM3, from which both PI and GM3 were rescued to normal levels in mice that both overexpressed SwAPP and were genetic ablated for PLD2. The lack of rescue of cholesterol-esters to normal levels, led us to speculate that the increase in cholesterol-esters upon SwAPP expression is not related with the memory deficits the mice present, but rather with another event does not change in both SwAPP/*Pld2*$^{+/+}$ and SwAPP/*Pld2*$^{-/-}$, such as the increased levels of Aβ, which are not affected by PLD2 ablation (Tiago Gil Oliveira 2010).

PI is important for membrane trafficking and for the biosynthesis of other phosphoinositides (Di Paolo and De Camilli 2006). Since it is a precursor of phosphoinositide synthesis, the dysregulation of its levels might be a consequence of the dysregulation of the phosphoinoside network. In fact, the link between phosphoinositides and AD has been addressed in the literature. In post-mortem lipid analysis of brain tissue from AD-affected individuals it was reported that anterior temporal cortex of brains from patients with AD had significantly lower levels of PI and trends for lower levels of PI phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P_2] (Stokes and Hawthorne 1987). Also, using ^{31}P Nuclear Magnetic Resonance to study AD brain preparations, a significant reduction in PI levels was observed (Pettegrew, Panchalingam et al. 2001). Even though these results are somewhat contradictory to what we observe, they support that there is an instability in the phosphoinositide metabolism. This has increased relevance in light of recent work that showed the importance of other phosphoinositides in the pathogenesis of AD, with a reported decrease in PI(4,5)P_2, which is dependent on the activation of PLC (Berman, Dall'Armi et al. 2008) and a reported increase in PI3-kinase activity

downstream of Aβ (Chiang, Wang et al. 2010). Finally, a mass spectrometry based lipidomic analysis in AD patients and AD mice (J20 mice that also produce high levels of Aβ) identified increased levels of the downstream products of the enzyme phospholipase A2 (PLA2), and that the ablation of PLA2-GIVA rescued synaptic and neurobehavioral dysfunction in the J20 mice (Sanchez-Mejia, Newman et al. 2008), in a similar way as observed for PLD2 (Tiago Gil Oliveira 2010). This further shows that rebalancing impaired lipid signaling pathways is a valid strategy to target in the context of AD. Here, this lipidomic analysis, due to technical reasons, did not cover the specific analysis of other classes of phosphoinositides, but this result is encouraging for future studies addressing the role of the different players in the phosphoinositide network in the context of AD.

Finally, the ganglioside GM3 was found to be overall increased in the SwAPP mice with a renormalization of its levels with PLD2 ablation. Due to technical reasons (i.e., different extraction conditions are required for the analysis of all the other species), other gangliosides were not measured. This finding was of particular interest, given the previously reported implication of gangliosides in the binding and aggregation of Aβ (Yanagisawa 2007; Ariga, McDonald et al. 2008)as well as in lysosomal storage disorders, such as Niemann-Pick Type C (NPC) (Walkley and Suzuki 2004). Additionally, expression of the SwAPP transgene produced a significant increase (~ 2-fold) in the levels of GM3, consistent with a previous study (Barrier, Ingrand et al. 2007) and expanding on the notion that NPC may share pathogenic mechanisms in common with AD (Nixon 2004). Further emphasizing some analogies between lipid storage disorders and AD, levels of cholesterol esters (but not cholesterol) were significantly increased in the SwAPP brains (~ 2-fold), consistent with current therapeutic efforts aiming at decreasing the activity of acyl-coenzyme A: cholesterol acyltransferase to reduce amyloidogenesis in AD patients (Puglielli, Tanzi et al. 2003). A prominent interaction between the SwAPP transgene and the *Pld2* genotype was in connection with ganglioside GM3, which accumulates in SwAPP forebrain. When combined, SwAPP expression and PLD2 ablation lead to a normalization of GM3 levels, suggesting that this phenomenon may be key to the pathogenicity associated with the AD model and its rescue in the *Pld2* null background. While future studies will address the relevance of such a hypothesis, our data suggest that it may not be directly connected to the metabolism of cholesterol esters, which accumulate in the SwAPP brains, yet, are not profoundly affected by the *Pld2* genotype.

Previous studies have shown that impairment of signaling lipids plays a key role in AD pathogenesis in animal models. Specifically, Aβ oligomers have been shown to dysregulate the PLC and PLA2 pathways with major implications for the signaling downstream of $PI(4,5)P_2$ and arachidonic

acid in the brain (Berman, Dall'Armi et al. 2008; Sanchez-Mejia, Newman et al. 2008). This study provides further support for the hypothesis that PLD2 signaling is altered in AD.

While a variety of previous studies have implicated the PLD pathway in AD pathogenesis either as a mediator of APP trafficking, presenilin regulation or downstream target of Aβ (Singh, McCartney et al. 1995; Cai, Netzer et al. 2006; Cai, Zhong et al. 2006; Brandenburg, Konrad et al. 2008; Tiago Gil Oliveira 2010), this study expands on the notion that PLD2, is pathophysiologically relevant in the context of mouse models of AD. Thus, our data is consistent with scenarios whereby PLD2 and its enzymatic product (i) mediate key signaling cascades downstream of Aβ; (ii) regulate the (cell surface) availability of putative Aβ receptors at synapses; or (iii) alter the capacity of Aβ to bind to its putative receptors. Results from the lipidomics analysis suggest that a dysregulation of ganglioside levels, likely stemming from an alteration of PLD2 function and thus PA metabolism, may be central to the pathogenicity associated with SwAPP overexpression and that inactivating PLD2 may counteract this mechanism. The recent identification of PLD2, and, to a lesser extent, PLD1, as positive regulators of cell surface levels of ganglioside GM1 and lipid raft-based signaling in antigen-stimulated mast cells (Lisboa, Peng et al. 2009) suggests that the interaction of Aβ with these lipid microdomains may be altered in PLD2-deficient neurons, with potentially important implications for Aβ oligomerization and signalling. Consistent with this idea, PLD isozymes have been reported to be raft-associated through palmitoylation of cysteine residues in their PH domain (Jenkins and Frohman 2005) .

Collectively, results presented in this study highlight PLD2 as an important player in AD pathogenesis. Moreover, this comprehensive brain lipidomics analysis of a widely used transgenic model of AD, may prove helpful as a clinical or diagnostic tool, particularly if some of the reported changes in lipid composition are also detected in blood cells or CSF.

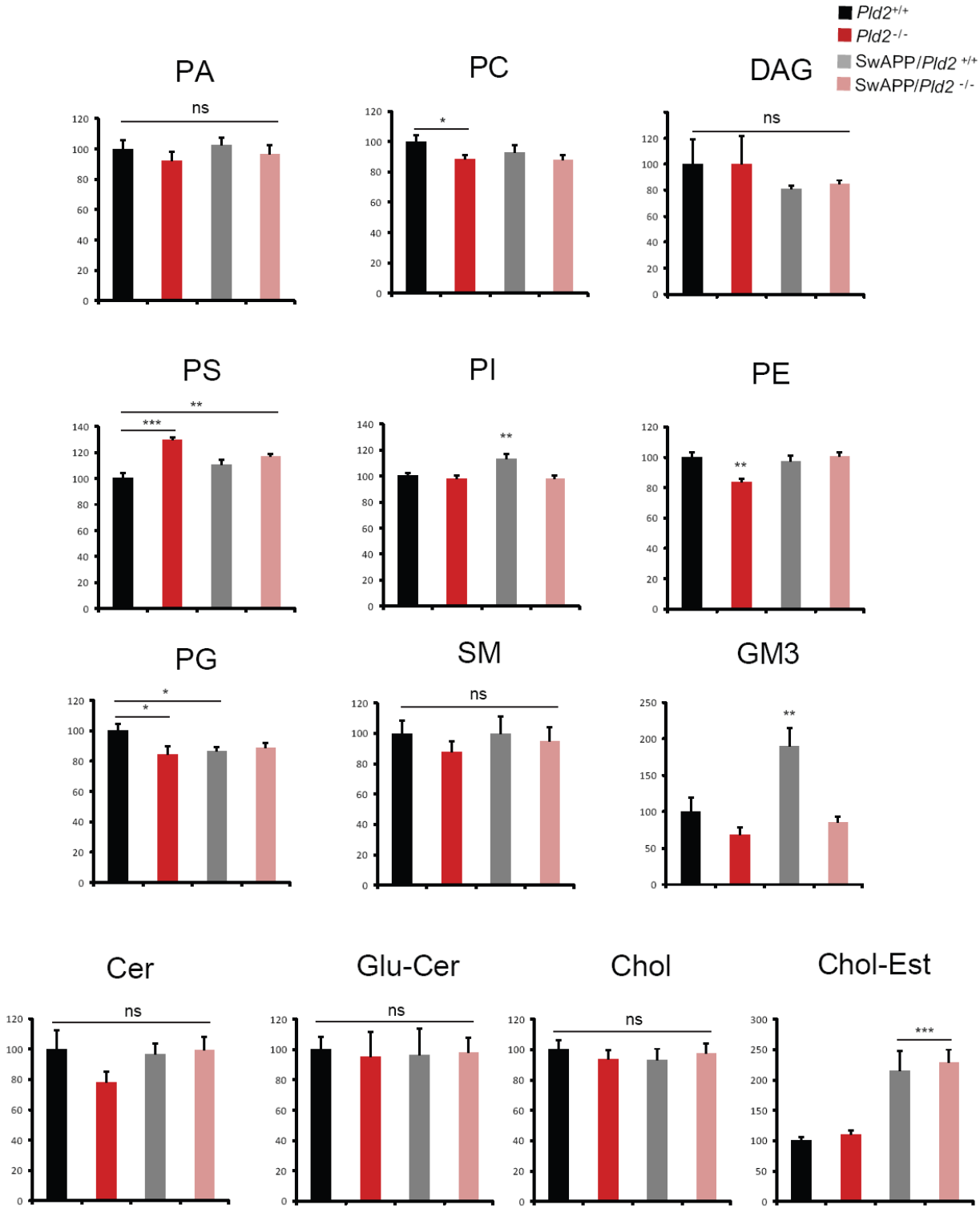

Figure 1. Effect of SwAPP overexpression and *Pld2* genotypes on forebrain lipid profile. Forebrain lipids were extracted from *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$mice with and without SwAPP transgene and subjected to LC-MS analysis. Total levels of different lipid classes. These include phosphatidic acid (PA), phosphatydilcholine (PC), diacylglycerol (DAG), phosphatidylserine (PS), phosphatidylinositol (PI),

phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin (SM), ceramides (Cer), glucosyl-ceramide (Glu-Cer), GM3 (a specific ganglioside class), cholesterol (Chol) and cholesterol-ester (Chol-Est). Y-axis is represented in % of relative levels to *Pld2*$^{+/+}$/no tg. n=6-8. Values denote means ± SEM. ns - non significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

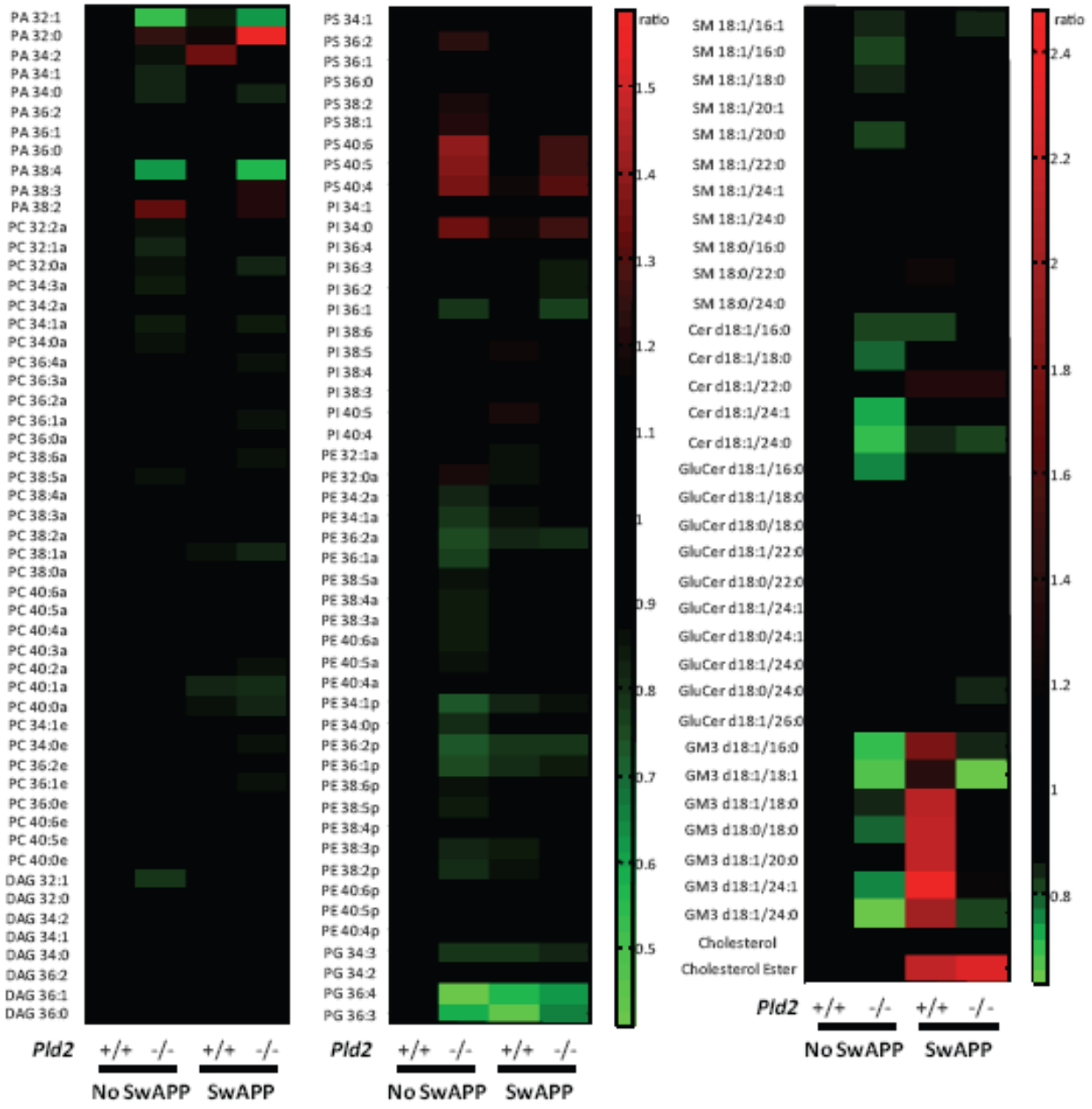

Figure 2. Effect of SwAPP overexpression and *Pld2* genotypes on forebrain lipid profile. Forebrain lipids were extracted from *Pld2*$^{+/+}$ and *Pld2*$^{-/-}$mice with and without SwAPP transgene and subjected to LC-MS analysis. Heat map of all lipids measured via LC-MS. The color bars, which are different for columns 1-2 and 3, represent the ratio of specific lipid species of mutant mice compared to that of control mice (*Pld2*$^{+/+}$, no SwAPP). The absolute levels of each lipid species are presented in Table 2. Phosphatidic Acid, PA; Phosphatidylcholine, PC; Diacylglycerol, DAG; Phosphatidylserine, PS; Phosphatidylinositol, PI; Phosphatidylethanolamine, PE (a – diacyl linkage; p – 1'-alkenyl-2-acyl

linkage); Phosphatidylglycerol, PG; Sphingomyelin, SM; Ceramide, Cer; Glucosylceramide, GluCer; Ganglioside, GM3. The nomenclature for phospholipids fatty acid composition are denoted as total chain length:number of unsaturated bonds while sphingolipids are denoted as sphingoid base residue/fatty acid residue.

Lipid class	Potential Implication
Cholesterol → Cholesterol Ester	Cholesterol homeostasis and lipid droplet formation
Cer	Apoptosis
Cer → GluCer → GM3	Aβ aggregation and raft signaling
PC→PA↔DAG	Phospholipase D signaling
PE	Mitochondria associated ER junction biosysnthesis
Plasmalogen PE	Peroxisome biosynthesis
PS	Apoptosis and phospholipid symmetry
PI	Phosphoinositide biosynthesis and membrane trafficking
PG	Mitochondria cardiolipin biosynthesis

Table 1. Potential molecular and biochemical pathways affected in Alzheimer disease models.

	Lipid Quantification				T-Test, *p* value				
Lipid Species	*Pld2* $^{+/+}$	*Pld2* $^{-/-}$	SwAPP/*Pld2* $^{+/+}$	SwAPP/*Pld2* $^{-/-}$	*Pld2* $^{+/+}$ vs *Pld2* $^{-/-}$	*Pld2* $^{+/+}$ vs SwAPP/*Pld2* $^{+/+}$	*Pld2* $^{+/+}$ vs SwAPP/*Pld2* $^{-/-}$	SwAPP/*Pld2* $^{+/+}$ vs SwAPP/*Pld2* $^{-/-}$	*Pld2* $^{-/-}$ vs SwAPP/*Pld2* $^{-/-}$
PA 32:1	0.042 ± 0.004	0.022 ± 0.003	0.035 ± 0.002	0.026 ± 0.002	0.0021	0.2577	0.0092	0.0082	0.3056
PA 32:0	0.089 ± 0.005	0.111 ± 0.009	0.104 ± 0.005	0.141 ± 0.006	0.0486	0.0611	0.0001	0.0008	0.0197
PA 34:2	0.027 ± 0.002	0.024 ± 0.002	0.037 ± 0.002	0.028 ± 0.002	0.2168	0.0079	0.8187	0.0092	0.1117
PA 34:1	1.073 ± 0.069	0.894 ± 0.063	1.103 ± 0.044	0.963 ± 0.066	0.0761	0.7340	0.2775	0.1170	0.4608
PA 34:0	0.117 ± 0.008	0.097 ± 0.009	0.130 ± 0.008	0.096 ± 0.005	0.1353	0.2922	0.0556	0.0039	0.9680
PA 36:2	0.935 ± 0.056	0.936 ± 0.078	0.944 ± 0.050	0.999 ± 0.073	0.9854	0.9066	0.4897	0.5596	0.5699
PA 36:1	0.860 ± 0.048	0.848 ± 0.058	0.903 ± 0.040	0.871 ± 0.044	0.8810	0.5252	0.8694	0.6069	0.7663
PA 36:0	0.215 ± 0.011	0.211 ± 0.015	0.224 ± 0.012	0.220 ± 0.013	0.8463	0.6014	0.7638	0.8384	0.6624
PA 38:4	0.294 ± 0.014	0.183 ± 0.005	0.262 ± 0.019	0.164 ± 0.010	0.0001	0.1919	0.0001	0.0007	0.1239
PA 38:3	0.004 ± 0.000	0.004 ± 0.001	0.004 ± 0.001	0.005 ± 0.000	0.3911	0.9604	0.1935	0.3312	0.7462
PA 38:2	0.079 ± 0.009	0.104 ± 0.009	0.082 ± 0.007	0.097 ± 0.006	0.0710	0.8206	0.1686	0.1331	0.5110
PC 32:2a	0.007 ± 0.000	0.006 ± 0.000	0.008 ± 0.001	0.007 ± 0.000	0.0615	0.7836	0.4463	0.3372	0.1460
PC 32:1a	0.509 ± 0.026	0.422 ± 0.016	0.507 ± 0.025	0.465 ± 0.016	0.0141	0.9534	0.1908	0.1711	0.0861
PC 32:0a	4.524 ± 0.161	3.894 ± 0.142	4.039 ± 0.215	3.768 ± 0.166	0.0108	0.0890	0.0062	0.3332	0.5730
PC 34:3a	0.024 ± 0.001	0.020 ± 0.001	0.024 ± 0.002	0.022 ± 0.001	0.0130	0.7363	0.3470	0.2533	0.0586
PC 34:2a	0.233 ± 0.014	0.208 ± 0.008	0.255 ± 0.010	0.257 ± 0.028	0.1406	0.2270	0.4234	0.9548	0.0952
PC 34:1a	5.518 ± 0.283	4.707 ± 0.157	5.024 ± 0.225	4.713 ± 0.158	0.0253	0.2204	0.0331	0.2722	0.9817
PC 34:0a	2.001 ± 0.100	1.745 ± 0.045	1.873 ± 0.082	1.771 ± 0.046	0.0349	0.3639	0.0683	0.2838	0.6935
PC 36:4a	2.462 ± 0.124	2.167 ± 0.124	2.222 ± 0.214	2.125 ± 0.142	0.1143	0.3240	0.0958	0.7051	0.8281
PC 36:3a	0.545 ± 0.027	0.490 ± 0.025	0.538 ± 0.049	0.533 ± 0.050	0.1654	0.9004	0.8289	0.9402	0.4449
PC 36:2a	1.323 ± 0.062	1.167 ± 0.056	1.218 ± 0.086	1.191 ± 0.076	0.0849	0.3344	0.2019	0.8168	0.7986
PC 36:1a	3.130 ± 0.141	2.777 ± 0.091	2.893 ± 0.120	2.703 ± 0.078	0.0540	0.2445	0.0241	0.1978	0.5525
PC 36:0a	0.373 ± 0.017	0.332 ± 0.010	0.348 ± 0.013	0.327 ± 0.007	0.0618	0.3071	0.0361	0.1667	0.7199
PC 38:6a	1.549 ± 0.083	1.369 ± 0.075	1.480 ± 0.108	1.341 ± 0.068	0.1291	0.6172	0.0790	0.2848	0.7925
PC 38:5a	0.994 ± 0.056	0.869 ± 0.046	0.935 ± 0.077	0.886 ± 0.051	0.1073	0.5339	0.1829	0.5963	0.8083
PC 38:4a	1.947 ± 0.098	1.760 ± 0.087	1.854 ± 0.133	1.768 ± 0.103	0.1751	0.5735	0.2310	0.6157	0.9500
PC 38:3a	0.363 ± 0.018	0.332 ± 0.015	0.364 ± 0.028	0.345 ± 0.023	0.2126	0.9767	0.5442	0.6060	0.6362
PC 38:2a	0.274 ± 0.019	0.250 ± 0.020	0.248 ± 0.026	0.242 ± 0.025	0.3929	0.4205	0.3166	0.8777	0.8019
PC 38:1a	0.261 ± 0.017	0.235 ± 0.018	0.227 ± 0.024	0.215 ± 0.020	0.3117	0.2551	0.0992	0.7195	0.4717
PC 38:0a	0.054 ± 0.003	0.052 ± 0.004	0.053 ± 0.005	0.052 ± 0.004	0.6474	0.8523	0.6149	0.8128	0.9516
PC 40:6a	1.594 ± 0.096	1.466 ± 0.089	1.530 ± 0.116	1.457 ± 0.100	0.3475	0.6797	0.3433	0.6384	0.9451
PC 40:5a	0.355 ± 0.021	0.327 ± 0.019	0.350 ± 0.028	0.330 ± 0.022	0.3585	0.8880	0.4390	0.5875	0.9338
PC 40:4a	0.197 ± 0.011	0.185 ± 0.010	0.192 ± 0.014	0.186 ± 0.013	0.4360	0.8043	0.5352	0.7524	0.9310
PC 40:3a	0.033 ± 0.002	0.030 ± 0.002	0.032 ± 0.002	0.031 ± 0.002	0.2648	0.8296	0.5891	0.7767	0.6505
PC 40:2a	0.064 ± 0.003	0.061 ± 0.004	0.057 ± 0.004	0.055 ± 0.003	0.5737	0.2006	0.0644	0.6249	0.2441
PC 40:1a	0.059 ± 0.003	0.057 ± 0.005	0.048 ± 0.002	0.048 ± 0.003	0.7090	0.0219	0.0238	0.8514	0.1289
PC 40:0a	0.015 ± 0.001	0.015 ± 0.001	0.013 ± 0.001	0.013 ± 0.001	0.8706	0.1280	0.0754	0.6057	0.1262
PC 34:1e	0.170 ± 0.010	0.153 ± 0.005	0.158 ± 0.008	0.152 ± 0.005	0.1617	0.4130	0.1557	0.5087	0.8726
PC 34:0e	0.082 ± 0.004	0.075 ± 0.003	0.078 ± 0.005	0.071 ± 0.002	0.1809	0.4742	0.0351	0.1878	0.2251
PC 36:2e	0.052 ± 0.003	0.048 ± 0.003	0.047 ± 0.003	0.047 ± 0.003	0.3324	0.2646	0.3189	0.9344	0.9248
PC 36:1e	0.109 ± 0.005	0.100 ± 0.003	0.101 ± 0.006	0.096 ± 0.003	0.1564	0.2853	0.0546	0.4592	0.3236
PC 36:0e	0.021 ± 0.001	0.020 ± 0.001	0.021 ± 0.001	0.020 ± 0.001	0.2837	0.8494	0.4445	0.6068	0.9867
PC 40:6e	0.037 ± 0.001	0.038 ± 0.002	0.040 ± 0.003	0.038 ± 0.002	0.6493	0.3197	0.7370	0.5629	0.9022
PC 40:5e	0.017 ± 0.001	0.018 ± 0.001	0.018 ± 0.001	0.017 ± 0.001	0.5727	0.6406	0.7955	0.8486	0.8239
PC 40:0e	0.797 ± 0.051	0.730 ± 0.049	0.759 ± 0.065	0.734 ± 0.049	0.3640	0.6496	0.3971	0.7672	0.9547
DAG 32:1	0.070 ± 0.009	0.055 ± 0.001	0.064 ± 0.006	0.067 ± 0.008	0.1322	0.6104	0.8075	0.7667	0.1439
DAG 32:0	0.107 ± 0.008	0.098 ± 0.002	0.101 ± 0.006	0.106 ± 0.008	0.3313	0.6119	0.9855	0.6235	0.3389
DAG 34:2	0.230 ± 0.011	0.226 ± 0.005	0.225 ± 0.008	0.237 ± 0.010	0.7047	0.7243	0.6716	0.3918	0.3319
DAG 34:1	0.340 ± 0.012	0.343 ± 0.008	0.345 ± 0.003	0.365 ± 0.006	0.8303	0.7086	0.0920	0.0171	0.0488
DAG 34:0	0.155 ± 0.008	0.145 ± 0.003	0.150 ± 0.005	0.157 ± 0.007	0.2501	0.5898	0.9053	0.4638	0.1511
DAG 36:2	0.343 ± 0.014	0.350 ± 0.008	0.347 ± 0.011	0.355 ± 0.011	0.6573	0.8317	0.5016	0.6068	0.7115
DAG 36:1	0.294 ± 0.013	0.293 ± 0.008	0.301 ± 0.013	0.312 ± 0.013	0.9356	0.6992	0.3512	0.5818	0.2307
DAG 36:0	0.090 ± 0.009	0.081 ± 0.001	0.086 ± 0.006	0.092 ± 0.009	0.3375	0.7439	0.8376	0.5787	0.2220

	Lipid Quantification				T-Test, *p* value				
Lipid Species	*Pld2*$^{+/+}$	*Pld2*$^{-/-}$	SwAPP/*Pld2*$^{+/+}$	SwAPP/*Pld2*$^{-/-}$	*Pld2*$^{+/+}$ vs *Pld2*$^{-/-}$	*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{+/+}$	*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{-/-}$	SwAPP/*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{-/-}$	*Pld2*$^{-/-}$ vs SwAPP/*Pld2*$^{-/-}$
PS 34:1	0.602 ± 0.026	0.629 ± 0.027	0.623 ± 0.027	0.587 ± 0.011	0.4831	0.5969	0.6121	0.2134	0.1926
PS 36:2	1.538 ± 0.054	1.898 ± 0.045	1.587 ± 0.073	1.618 ± 0.062	0.0001	0.5898	0.3445	0.7518	0.0024
PS 36:1	7.292 ± 0.317	8.275 ± 0.097	7.732 ± 0.283	7.344 ± 0.138	0.0103	0.3396	0.8894	0.2230	0.0001
PS 36:0	0.783 ± 0.035	0.898 ± 0.015	0.835 ± 0.029	0.792 ± 0.009	0.0088	0.2999	0.8141	0.1638	0.0001
PS 38:2	0.258 ± 0.014	0.310 ± 0.006	0.279 ± 0.017	0.278 ± 0.008	0.0037	0.3471	0.2406	0.9513	0.0056
PS 38:1	0.458 ± 0.025	0.561 ± 0.012	0.497 ± 0.030	0.472 ± 0.014	0.0026	0.3385	0.6515	0.4386	0.0003
PS 40:6	14.634 ± 0.552	20.376 ± 0.407	16.528 ± 0.763	18.400 ± 0.379	0.0001	0.0609	0.0001	0.0417	0.0038
PS 40:5	2.159 ± 0.085	2.954 ± 0.068	2.432 ± 0.112	2.744 ± 0.057	0.0001	0.0707	0.0001	0.0243	0.0365
PS 40:4	1.228 ± 0.063	1.664 ± 0.050	1.437 ± 0.076	1.612 ± 0.041	0.0001	0.0538	0.0003	0.0567	0.4476
PI 34:1	0.134 ± 0.008	0.128 ± 0.005	0.137 ± 0.009	0.131 ± 0.008	0.5929	0.7516	0.8460	0.6125	0.7500
PI 34:0	0.044 ± 0.002	0.059 ± 0.003	0.052 ± 0.003	0.056 ± 0.003	0.0017	0.0654	0.0073	0.3738	0.4798
PI 36:4	0.548 ± 0.016	0.535 ± 0.015	0.595 ± 0.018	0.485 ± 0.015	0.5707	0.0765	0.0143	0.0007	0.0336
PI 36:3	0.084 ± 0.003	0.077 ± 0.001	0.091 ± 0.003	0.071 ± 0.002	0.0322	0.1371	0.0028	0.0003	0.0465
PI 36:2	0.032 ± 0.002	0.028 ± 0.001	0.037 ± 0.002	0.027 ± 0.001	0.0970	0.0857	0.0233	0.0007	0.6408
PI 36:1	0.116 ± 0.003	0.091 ± 0.005	0.119 ± 0.007	0.089 ± 0.004	0.0018	0.6416	0.0001	0.0020	0.7023
PI 38:6	0.087 ± 0.003	0.085 ± 0.003	0.100 ± 0.005	0.084 ± 0.003	0.5445	0.0264	0.4518	0.0118	0.8881
PI 38:5	0.438 ± 0.011	0.428 ± 0.011	0.514 ± 0.019	0.461 ± 0.014	0.5354	0.0037	0.2246	0.0404	0.0859
PI 38:4	2.176 ± 0.040	2.134 ± 0.057	2.502 ± 0.081	2.144 ± 0.057	0.5545	0.0020	0.6450	0.0035	0.9037
PI 38:3	0.356 ± 0.007	0.341 ± 0.010	0.409 ± 0.012	0.362 ± 0.009	0.2507	0.0018	0.6085	0.0092	0.1494
PI 40:5	0.039 ± 0.001	0.040 ± 0.002	0.046 ± 0.002	0.042 ± 0.001	0.7078	0.0054	0.0953	0.1157	0.3736
PI 40:4	0.018 ± 0.001	0.019 ± 0.001	0.021 ± 0.001	0.020 ± 0.001	0.3940	0.0046	0.1486	0.3282	0.5850
PE 32:1a	0.006 ± 0.001	0.006 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.9404	0.5855	0.8390	0.6308	0.7061
PE 32:0a	0.015 ± 0.003	0.017 ± 0.001	0.012 ± 0.002	0.015 ± 0.001	0.3762	0.5937	0.9227	0.3124	0.1525
PE 34:2a	0.067 ± 0.003	0.055 ± 0.004	0.067 ± 0.006	0.069 ± 0.004	0.0294	0.9867	0.5930	0.7056	0.0159
PE 34:1a	0.636 ± 0.019	0.505 ± 0.011	0.551 ± 0.023	0.583 ± 0.024	0.0001	0.0140	0.1013	0.3566	0.0088
PE 36:2a	0.690 ± 0.023	0.523 ± 0.017	0.570 ± 0.032	0.553 ± 0.016	0.0001	0.0088	0.0004	0.6250	0.2245
PE 36:1a	0.837 ± 0.027	0.644 ± 0.016	0.750 ± 0.042	0.757 ± 0.018	0.0001	0.0938	0.0342	0.8736	0.0004
PE 38:5a	0.574 ± 0.026	0.492 ± 0.010	0.564 ± 0.026	0.582 ± 0.020	0.0102	0.7823	0.8145	0.5739	0.0010
PE 38:4a	2.393 ± 0.069	2.039 ± 0.063	2.305 ± 0.118	2.498 ± 0.079	0.0019	0.5035	0.3321	0.1887	0.0005
PE 38:3a	0.354 ± 0.016	0.297 ± 0.013	0.371 ± 0.017	0.381 ± 0.018	0.0152	0.5047	0.2918	0.6971	0.0022
PE 40:6a	3.838 ± 0.150	3.251 ± 0.098	3.874 ± 0.159	3.940 ± 0.093	0.0054	0.8751	0.5877	0.7180	0.0002
PE 40:5a	0.574 ± 0.023	0.500 ± 0.020	0.599 ± 0.025	0.605 ± 0.012	0.0295	0.4864	0.2719	0.8094	0.0010
PE 40:4a	0.386 ± 0.014	0.338 ± 0.012	0.395 ± 0.014	0.425 ± 0.015	0.0210	0.6764	0.0863	0.1790	0.0006
PE 34:1p	0.387 ± 0.022	0.284 ± 0.011	0.318 ± 0.019	0.337 ± 0.007	0.0010	0.0446	0.0633	0.3572	0.0020
PE 34:0p	0.075 ± 0.004	0.061 ± 0.004	0.069 ± 0.003	0.073 ± 0.003	0.0154	0.2119	0.6136	0.3341	0.0248
PE 36:2p	0.996 ± 0.048	0.737 ± 0.029	0.780 ± 0.061	0.792 ± 0.013	0.0004	0.0153	0.0019	0.8421	0.1254
PE 36:1p	0.601 ± 0.017	0.451 ± 0.018	0.481 ± 0.048	0.504 ± 0.010	0.0001	0.0214	0.0004	0.6088	0.0271
PE 38:6p	1.234 ± 0.050	1.056 ± 0.026	1.156 ± 0.049	1.213 ± 0.026	0.0071	0.2980	0.7260	0.3055	0.0009
PE 38:5p	0.809 ± 0.033	0.682 ± 0.021	0.728 ± 0.022	0.783 ± 0.021	0.0056	0.0820	0.5298	0.1006	0.0048
PE 38:4p	0.994 ± 0.034	0.871 ± 0.030	0.872 ± 0.036	0.992 ± 0.026	0.0178	0.0325	0.9687	0.0181	0.0102
PE 38:3p	0.204 ± 0.010	0.167 ± 0.007	0.172 ± 0.006	0.188 ± 0.006	0.0068	0.0214	0.2012	0.0903	0.0420
PE 38:2p	0.338 ± 0.016	0.272 ± 0.011	0.291 ± 0.019	0.302 ± 0.010	0.0044	0.0782	0.0867	0.6022	0.0746
PE 40:6p	2.024 ± 0.077	1.811 ± 0.059	1.911 ± 0.098	1.953 ± 0.028	0.0453	0.3777	0.4302	0.6689	0.0573
PE 40:5p	0.634 ± 0.025	0.564 ± 0.020	0.624 ± 0.024	0.643 ± 0.021	0.0437	0.7679	0.7963	0.5519	0.0165
PE 40:4p	0.410 ± 0.013	0.383 ± 0.014	0.400 ± 0.015	0.443 ± 0.016	0.1915	0.6620	0.1265	0.0818	0.0143
PG 34:3	0.009 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.007 ± 0.001	0.0610	0.0461	0.1223	0.7453	0.7691
PG 34:2	0.144 ± 0.007	0.126 ± 0.008	0.131 ± 0.004	0.132 ± 0.006	0.1081	0.1375	0.1858	0.9080	0.6046
PG 36:4	0.004 ± 0.001	0.002 ± 0.000	0.002 ± 0.001	0.002 ± 0.000	0.0019	0.0596	0.0644	0.7560	0.1242
PG 36:3	0.010 ± 0.001	0.006 ± 0.000	0.004 ± 0.001	0.007 ± 0.001	0.0001	0.0001	0.0085	0.0947	0.4553

Lipid Quantification					T-Test, *p* value			
Lipid Species	*Pld2*$^{+/+}$	*Pld2*$^{-/-}$	SwAPP/*Pld2*$^{+/+}$	SwAPP/*Pld2*$^{-/-}$	*Pld2*$^{+/+}$ vs *Pld2*$^{-/-}$	*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{+/+}$	*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{-/-}$	SwAPP/*Pld2*$^{+/+}$ vs SwAPP/*Pld2*$^{-/-}$	*Pld2*$^{-/-}$ vs SwAPP/*Pld2*$^{-/-}$
SM 18:1/18:0	3.361 ± 0.298	2.814 ± 0.179	3.090 ± 0.290	2.987 ± 0.239	0.1383	0.5379	0.3549	0.7865	0.5676
SM 18:1/20:1	0.109 ± 0.014	0.096 ± 0.011	0.111 ± 0.018	0.098 ± 0.013	0.4845	0.9161	0.6140	0.5764	0.8682
SM 18:1/20:0	0.841 ± 0.139	0.682 ± 0.046	0.846 ± 0.137	0.737 ± 0.075	0.2965	0.9786	0.5419	0.4820	0.5262
SM 18:1/22:0	0.539 ± 0.073	0.512 ± 0.071	0.615 ± 0.127	0.568 ± 0.097	0.7943	0.5919	0.8107	0.7734	0.6430
SM 18:1/24:1	1.699 ± 0.136	1.594 ± 0.148	1.843 ± 0.239	1.755 ± 0.228	0.6096	0.5669	0.8305	0.7957	0.5540
SM 18:1/24:0	0.531 ± 0.042	0.510 ± 0.049	0.573 ± 0.077	0.542 ± 0.059	0.7471	0.6162	0.8812	0.7498	0.6810
SM 18:0/16:0	0.075 ± 0.004	0.070 ± 0.004	0.071 ± 0.003	0.081 ± 0.005	0.3243	0.4614	0.3366	0.1434	0.0919
SM 18:0/22:0	0.100 ± 0.008	0.099 ± 0.007	0.120 ± 0.020	0.110 ± 0.013	0.9392	0.3173	0.4942	0.6870	0.4466
SM 18:0/24:0	0.062 ± 0.006	0.062 ± 0.005	0.066 ± 0.010	0.064 ± 0.007	0.9606	0.7066	0.8162	0.8682	0.8405
Cer d18:1/16:0	0.030 ± 0.005	0.024 ± 0.004	0.025 ± 0.004	0.026 ± 0.002	0.4197	0.4652	0.5208	0.7758	0.7483
Cer d18:1/18:0	1.188 ± 0.151	0.932 ± 0.100	1.125 ± 0.076	1.175 ± 0.097	0.1796	0.7426	0.9438	0.7012	0.1077
Cer d18:1/22:0	0.053 ± 0.003	0.052 ± 0.007	0.071 ± 0.008	0.071 ± 0.009	0.9163	0.0348	0.0668	0.9631	0.1158
Cer d18:1/24:1	0.235 ± 0.041	0.175 ± 0.009	0.227 ± 0.033	0.237 ± 0.025	0.1716	0.8843	0.9706	0.8113	0.0317
Cer d18:1/24:0	0.034 ± 0.010	0.023 ± 0.003	0.029 ± 0.004	0.028 ± 0.005	0.3128	0.6826	0.5912	0.8435	0.4539
GluCer d18:1/16:0	0.024 ± 0.002	0.018 ± 0.001	0.022 ± 0.002	0.022 ± 0.003	0.0272	0.5709	0.5981	0.9708	0.1451
GluCer d18:1/18:0	0.328 ± 0.024	0.298 ± 0.024	0.333 ± 0.021	0.322 ± 0.018	0.3928	0.8842	0.8626	0.7173	0.4435
GluCer d18:0/18:0	0.008 ± 0.001	0.007 ± 0.001	0.008 ± 0.000	0.008 ± 0.001	0.7250	0.7863	0.8091	0.4394	0.4734
GluCer d18:1/22:0	0.403 ± 0.032	0.392 ± 0.057	0.383 ± 0.055	0.402 ± 0.038	0.8794	0.7459	0.9874	0.7769	0.8977
GluCer d18:0/22:0	0.017 ± 0.002	0.016 ± 0.001	0.018 ± 0.002	0.017 ± 0.002	0.5101	0.8576	0.9432	0.8146	0.5723
GluCer d18:1/24:1	3.591 ± 0.308	3.390 ± 0.629	3.435 ± 0.673	3.494 ± 0.374	0.7776	0.8221	0.8433	0.9380	0.8924
GluCer d18:0/24:1	0.094 ± 0.008	0.086 ± 0.016	0.093 ± 0.018	0.089 ± 0.010	0.6514	0.9745	0.7064	0.8342	0.8678
GluCer d18:1/24:0	0.545 ± 0.043	0.547 ± 0.095	0.529 ± 0.098	0.537 ± 0.052	0.9853	0.8753	0.9041	0.9457	0.9292
GluCer d18:0/24:0	0.022 ± 0.002	0.023 ± 0.004	0.022 ± 0.004	0.019 ± 0.002	0.9124	0.9839	0.3524	0.5122	0.4805
GluCer d18:1/26:0	0.041 ± 0.003	0.041 ± 0.007	0.041 ± 0.008	0.045 ± 0.006	0.9880	0.9890	0.5587	0.7151	0.6849
GM3 d18:1/16:0	0.007 ± 0.001	0.005 ± 0.001	0.013 ± 0.001	0.006 ± 0.000	0.1293	0.0055	0.3527	0.0007	0.1361
GM3 d18:1/18:1	0.009 ± 0.001	0.006 ± 0.000	0.013 ± 0.002	0.006 ± 0.000	0.0906	0.1426	0.0422	0.0016	0.3403
GM3 d18:1/18:0	0.147 ± 0.030	0.125 ± 0.008	0.313 ± 0.026	0.134 ± 0.007	0.5396	0.0025	0.7128	0.0001	0.4155
GM3 d18:0/18:0	0.031 ± 0.007	0.025 ± 0.001	0.067 ± 0.005	0.027 ± 0.003	0.4553	0.0044	0.6426	0.0001	0.4531
GM3 d18:1/20:0	0.021 ± 0.003	0.018 ± 0.001	0.045 ± 0.002	0.021 ± 0.001	0.5182	0.0004	0.9121	0.0001	0.0664
GM3 d18:1/24:1	0.009 ± 0.002	0.007 ± 0.001	0.021 ± 0.002	0.010 ± 0.001	0.3198	0.0003	0.3795	0.0001	0.0010
GM3 d18:1/24:0	0.006 ± 0.001	0.004 ± 0.000	0.011 ± 0.000	0.005 ± 0.001	0.0961	0.0011	0.4271	0.0001	0.1181
Cholesterol	1.802 ± 0.116	1.681 ± 0.109	1.675 ± 0.136	1.751 ± 0.124	0.4577	0.4896	0.7686	0.6880	0.6757
Cholesterol Ester	0.759 ± 0.043	0.828 ± 0.063	1.628 ± 0.251	1.736 ± 0.158	0.3827	0.0020	0.0001	0.7139	0.0001

Table 2. Lipid composition of forebrain extracted from WT and *Pld2 KO* mice with and without SwAPP transgene. The relative abundance of individual lipid species are expressed as mean value ± S.E.M (n =

6-8). The statistical differences in relevant sample groups were analyzed using Student's t-test and the *p* values of these analyses are given.

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Chapter 3

Discussion, Conclusions and Future Perspectives

Discussion, conclusions and future perspectives

Besides being a major brain constituent, lipids are also involved in multiple aspects of brain physiology and cell signaling. Dysregulation of these lipid signaling pathways has been associated with a growing number of neurodegenerative disorders, including Alzheimer's disease (AD). In AD, most of the attention has been focused on cholesterol (Puglielli, Tanzi et al. 2003), however, growing evidence suggests that other classes of lipids, such as phospholipids, either mediate or modulate key pathological processes associated with AD (Landman, Jeong et al. 2006; Berman, Dall'Armi et al. 2008; Sanchez-Mejia, Newman et al. 2008).

Mounting evidence indicates that amyloid beta (Aβ) perturbs the metabolism of intracellular signaling lipids and that this phenomenon contributes to the pathogenic actions of this peptide (Hartmann, Kuchenbecker et al. 2007; Oliveira and Di Paolo 2010; Oster and Pillot 2010; Sanchez-Mejia and Mucke 2010). In particular, it was previously shown that Aβ leads to an activation of both phospholipase A2 (PLA2) (Sanchez-Mejia, Newman et al. 2008), phospholipase C (PLC) (Berman, Dall'Armi et al. 2008) and phospholipase D (PLD) (Singh, Sorrentino et al. 1998; Brandenburg, Konrad et al. 2008; Oliveira and Di Paolo 2010), although in the latter case the Aβ preparations used in the experimental setting were of questionable pathophysiological significance. It has been shown that both PLA2 (specifically the GIVA isoform) and PLC are activated by calcium. Also, a wealth of studies has implicated calcium dysregulation in AD pathogenesis (Querfurth and LaFerla 2010). Since PLD, and more specifically PLD2, is also activated by calcium (Kim, Lee et al. 1999), we hypothesized that it could be also involved in AD pathogenesis. In particular, a study from our laboratory indicated that Aβ treatment leads to a trend for an increase in phosphatidic acid (PA) levels in cultured neurons (Berman, Dall'Armi et al. 2008). Moreover, we reasoned that PLD2 was a strong candidate to mediate signaling processes at the plasma membrane in response to Aβ, due to the predominant localization of this isozyme at the cell surface (Du, Huang et al. 2004), as opposed to PLD1, which has a more intracellular localization. Because PA can be produced by PLD and based on the other studies linking phospholipase dysregulation to AD, we tested the involvement of PLD2 in AD pathogenesis and more specifically, in the synaptotoxic action of Aβ oligomers. In the sections below, we highlight how we addressed the three aims of our thesis proposal and discuss the new avenues our study has opened.

Aim 1. *To characterize the effects of Aβ on the PLD pathway.*

We tackled this issue by conducting our studies in cell culture models. To begin to address the role of PLD in Aβ pathogenesis and to determine which of the two isoforms is(are) stimulated by Aβ, we tested whether oligomeric Aβ signaling alters the subcellular localization of either PLD1 or PLD2. While no obvious phenotype was observed in GFP-PLD1 transfected cells, GFP-PLD2 was relocalized from the plasma membrane to the cytoplasm in a Ca^{2+} and cPLA2 dependent way. These results also highlight the crosstalk between different phospholipases, a question of critical relevance that will be explored in future studies. Moreover, this relocalization assay can now be used for automated drug screening assessing PLD2 localization. The identification of small molecules that affect PLD2 localization in response to Aβ42 may be beneficial for the treatment of AD (based on our studies), although these molecules would have to be validated in animal studies and assessed whether they effectively target PLD2 activity. Also, using primary cortical neurons we observed that while Aβ42 oligomers lead to an increase in total PLD activity, genetic ablation of one or two copies of *Pld2* blocks this increase in activity. The residual PLD activity in PLD2 KO (knock-out) cultures is likely dependent on PLD1, whose significance has yet to be determined. Studies with *Pld1* KO mice, recently developed in our lab (Dall'Armi and Di Paolo, unpublished observations) and by others (Elvers, Stegner et al. 2010), will clarify this question.

Aim 2. *To test the effects of PLD2 ablation in an AD mouse model.*

First, in order to understand the role of PLD2 activation in response to Aβ, we used an *ex vivo* preparation of the hippocampus to study long-term potentiation (LTP). This protocol was previously used in many studies showing that LTP is impaired by Aβ oligomers in CA1 after stimulation of the Schaeffer collaterals (Lambert, Barlow et al. 1998; Vitolo, Sant'Angelo et al. 2002; Haass and Selkoe 2007). This approach is used as an electrophysiological read-out of Aβ toxicity. We observe that while the *Pld2* wild type (WT) slices have impaired LTP after Aβ treatment, *Pld2* KO slices are resistant to the deleterious effects of Aβ. This result not only provides further evidence for a functional relationship between PLD2 and the Aβ signaling pathway, but it also suggests that the synaptotoxic actions of this peptide require PLD2.

This prompted us to address the role of *Pld2* ablation in an AD mouse model with the prediction (based on the LTP result) that it may be protective. We used the Tg2576 mouse line, which

overexpresses the Swedish amyloid precursor protein (SwAPP) transgene and presents with an age-dependent increase in the levels of Aβ. A key finding was that brains from the SwAPP mice are associated with increased *in vivo* PLD activity, suggesting that PLD (and likely PLD2) are *bona fide* targets of Aβ in a more pathophysiologically-relevant setting. When crossed with the PLD2 KO mice (which exhibit an approximately 50% decrease in total PLD activity), *Pld2* ablation had a protective effect in our behavioral analysis of fear contextual memory at 6 months and spatial working memory at 12 months.

Previously, we observed that the treatment with oligomeric Aβ led to a trend for increased levels of PA in primary neuronal cultures (Berman, Dall'Armi et al. 2008). In our mass spectrometry analysis of the SwAPP aged mice, the total levels of PA were not changed (Fig. 1 chapter 2.2). However, the analysis by specific species of PA, showed that only one species was significantly increased in the SwAPP mice, PA 34:2 (the nomenclature for phospholipids fatty acid composition are denoted as total chain length:number of unsaturated bonds), which was previously associated with a neurodegeneration phenotype (Raghu, Coessens et al. 2009). Importantly, *Pld2* ablation in the SwAPP background rescued the levels of PA 34:2 back to normal levels, supporting the idea that this PLD2-derived product may be a key factor involved in the toxic effects of Aβ. Our combined mass spectrometry analysis of PA and phosphatydilethanol (PEtOH) allows us to get a better understanding of PLD-derived PA. In the *in vivo* PLD activity assay in the SwAPP mice, four PEtOH species were increased, PEtOH 32:1, 34:2, 34:1 and 34:0 (32:0 was not analysed, due to technical reasons - it was chosen as an internal standard in the PEtOH analysis) (Fig. 3 – chapter 2.1). Curiously, the homologous PA species were shown to be somewhat affected by PLD2 ablation in our lipidomic study. Besides PA 34:2 (whose alterations were discussed in detail in chapter 2.1), PA 32:1, 34:1 and 34:0, had all the same trend for decreased levels upon PLD2 ablation, either in the presence or absence of the SwAPP transgene (Fig. 7 – chapter 2.1). While these referred species appear to be more plastic and sensitive to modulation, other species, such as PEtOH 36:2, 36:1 and 36:0, were not affected by SwAPP overexpression and its PA homologs were also not affected either by PLD2 ablation, SwAPP overexpression or both, supporting that these species are probably more stable. Finally, PA 38:4, was significantly decreased upon PLD2 ablation, independently of SwAPP expression (a result confirmed by the lack of differences in the PEtOH 38:4 upon SwAPP expression). Since, PA 38:4 is believed to be $PI(4,5)P_2$ derived, this analysis suggests that PLD2 might affect $PI(4,5)P_2$ metabolism in a Aβ independent way. This has increased interest since $PI(4,5)P_2$ levels were shown to be decreased in response to oligomeric Aβ applications in primary cortical neurons (Berman, Dall'Armi et al. 2008).

The work from Raghu et al was based on the overexpression of the PLD homolog in drosophila, showing that overexpression of PLD had a toxic effect in neurons (Raghu, Coessens et al. 2009). Moreover, a recent work from Gorbatyuk et al showed that specifically overexpression of PLD2, caused severe neurodegeneration in rats (Gorbatyuk, Li et al. 2010). This toxic effect of PLD overexpression, somehow resembles the increased PLD activity observed in SwAPP mice. To our knowledge the ablation of PLD2 has not been shown to have deleterious effects. In fact, in our study it has proven beneficial. This body of work seems to support a deleterious effect for PLD overexpression/increased activity and a protective effect for PLD2 ablation, in the context of neuronal functioning.

Overall, the concomitant mass spectrometry analysis of PEtOH and PA levels provides complementary information that deepens the understanding of PLD metabolism. Due to PLD's high affinity for primary alcohols, the analysis of PEtOH levels captures the flow of potential PA pool that sinks as PEtOH (which is less efficiently metabolized). The mass spectrometry analysis magnifies the specificity of this analysis by the quantification of each PEtOH species. On the other hand, the regular mass spectrometry method, in the absence of ethanol treatment, captures PA levels from many potential sources, PLD being only one of them, thus providing a snapshot of PA metabolism. We believe that our analytical method represents an important conceptual advance and should be highly valued in future studies in the PLD field **(Figure 1)**.

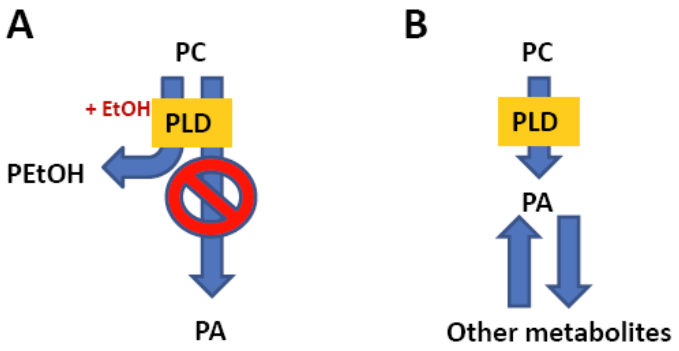

Figure 1. *A*. PLD-derived PA flow, which in the presence of ethanol, sinks as PEtOH. *B*. "Snapshot" of PA levels. PA – phosphatidic acid; PC – phosphatydilcholine; PLD – phospholipase D; EtOH – ethanol; PEtOH – phosphatydilethanol.

Aim 3. *To investigate the role of PLD2 in APP processing and Aβ generation.*

The rationale for this aim, was that there was a reported role of PLD1 in APP trafficking and processing (Cai, Netzer et al. 2006; Cai, Zhong et al. 2006; Liu, Zhang et al. 2009; Oliveira and Di Paolo 2010). Additionally, our behavioral results showed a protective effect of *Pld2* ablation in an AD mouse model. Thus, although our data supported a role for PLD2 downstream of Aβ signalling, another possibility was that PLD2 could also affect APP trafficking/processing and Aβ generation. We addressed this aim using biochemical analysis. First, no differences were found in murine Aβ (Supplementary Fig.4 - chapter 2.1) and APP levels (data not shown) in *Pld2* KO adult animals. We then evaluated APP processing in mice that expressed the SwAPP human transgene. Human Aβ and APP levels were found to have no differences upon *Pld2* ablation (Fig.8 - chapter 2.1). Although these results showed no role for PLD2 in APP processing and Aβ generation, the distribution or aggregation of Aβ in the brain may be affected by the lack of PLD2. As the lipidomic analysis uncovered, SwAPP/*Pld2*$^{+/+}$ mice had increased levels of GM3 compared with SwAPP/*Pld2*$^{-/-}$, and since gangliosides were found to interact with Aβ (Ariga, McDonald et al. 2008), this could be a possible mechanism for change in Aβ distribution and Aβ aggregation (discussed below). Logical experiments would be to assess Aβ distribution by immunohistochemical analysis and Aβ aggregation by measuring the levels of Aβ using conformation specific antibodies.

Future directions

Overall, the main conclusion from this work is the identification of PLD2 as a new player in AD pathogenesis. Specifically, we show that the genetic ablation of PLD2 has a protective effect in the context of Aβ synaptotoxic signaling and in a AD mouse model.

In search for a mechanism

One of the main questions that arises from this work is: "what is the exact mechanism through which PLD2 modulate Aβ effects?" Potentially, PLD2 may affect the various steps of Aβ oligomer pathogenic signaling, namely, its aggregation, interaction with membranes/receptors and Aβ intracellular downstream pathways. At the aggregation level as was already discussed (chapter 2.2) it can be potentially affected by the alterations observed in the ganglioside metabolism. Moreover, gangliosides, as a constituent of cellular membranes might also alter the interaction of Aβ oligomers

with membranes (Ariga, McDonald et al. 2008). Due to technical reasons, our lipidomic analysis only covered one ganglioside class, GM3. Importantly, our study reproduced the results from another group, which also observed an increase in GM3 levels in AD mouse models (Barrier, Ingrand et al. 2007). In fact, it has been shown that GM2 activator, a protein which activates β-hexosaminidase (the enzyme that catalyzes the reaction GM2→GM3), also interacts with PLD2 leading to increased PLD activity levels. It would be interesting to study the relationship of GM2 activator with Aβ signaling (Sarkar, Miwa et al. 2001). Moreover, a full ganglioside profile analysis will be of major importance in order to study both the impact of increased levels of Aβ and *Pld2* ablation on all different classes of gangliosides. Also, and in light of the ganglioside differences observed in our lipidomic analysis, it would be important to assess the extracellular versus intracellular location of Aβ (since our ELISA assays do not take into account that distinction), and within the intracellular pool, whether it is differentially distributed through the different cellular organelles. Since these results were observed in the context of a familial AD model, it would be interesting to study instead of SwAPP, mice expressing human WT APP, which would be more relevant for sporadic AD. Finally, similar studies should be performed with *Pld1* KO mice, which will probably be more fruitful in a APP processing point of view, due to the results observed by Cai et al showing decreased amyloidogenesis upon PLD1 overexpression in cell culture experimental models (Cai, Netzer et al. 2006; Cai, Zhong et al. 2006). If future experiments show no effects of PLD2 in APP processing, it will further highlight the differences between PLD1 and PLD2, curiously having different implications for the same disease.

One interesting finding we observed is that the SwAPP/*Pld2*$^{-/-}$ mice have preserved memory even in the presence of high levels of Aβ. Indeed, this situation was previously observed upon the ablation of other genes that encode for other proteins, such as, alpha7 nicotinic acetylcholine receptor (α7nAchR) (Wang, Lee et al. 2000), the prion protein (PrP) (Lauren, Gimbel et al. 2009; Gimbel, Nygaard et al. 2010), GIVA-PLA2 (Sanchez-Mejia, Newman et al. 2008) and tau (Roberson, Scearce-Levie et al. 2007; Ittner, Ke et al. 2010). One tentative thought is that all these players might be somehow inter-related in the modulation of Aβ effects. Interestingly, both α7nAchR and PrP are putative proteinaceous Aβ receptors. Since PLD2 has been proposed to modulate endocytic events (Roth 2008), one possibility is that PLD2 affects the surface availability of these Aβ receptors or the capability of Aβ to bind them. In fact, PLD has been shown to modulate the surface availability of a PrP related fragment (Brandenburg, Koch et al. 2007). Concerning GIVA-PLA2, we show in this thesis that it lies upstream of PLD2 in the Aβ signaling pathway, confirming previous results in leukocytes (Kim, Lee et al. 1999). This opens new lines of research, specifically in trying to understand how the different phospholipases regulate each

other. It would be interesting to study PLD metabolism upon PLA2 modulation and also the effects of PLD2 modulation in PLA2 metabolic pathways. The PLA2-Aβ-dependent pathway has been addressed and namely, PKC, p38, and MEK/ERK pathways were shown to be involved, and importantly, the anti-inflammatory inhibitors of cyclooxygenase-2 were shown to protect from Aβ toxic effects (Kriem, Sponne et al. 2005). Also, Mucke's group has shown that GIVA-PLA2 ablation protected from Aβ neurotoxicity, an effect that was shown to be mediated by AMPAR internalization (Sanchez-Mejia, Newman et al. 2008). As for the putative Aβ receptors and in light of the crosstalk between PLA2 and PLD2, PLD2 might also potentially mediate AMPAR internalization with potential impact in protecting from Aβ excitotoxic effects. Finally, the ablation of tau was shown to be protective in two different amyloidogenesis mouse models (Roberson, Scearce-Levie et al. 2007; Ittner, Ke et al. 2010). Tau ablation was shown to protect from excitotoxicity to which the AD mice were shown to be more susceptible. The molecular mechanism was further dissected and it was shown that tau binds Fyn and this interaction leads to stabilization of the NMDAR/PSD95 complex. In the absence of tau, the NMDAR/PSD95 interaction was less stable leading to excitability protection in the amyloidogenesis mouse models. It was further shown that a tau independent manipulation of the NMDAR/PSD95 interaction also led to a protective effect (Roberson, Scearce-Levie et al. 2007; Ittner, Ke et al. 2010). It is thus logical to speculate that PLD2 might modulate any of these molecular players: tau, Fyn, NMDAR or PSD95, or its molecular interactions. If this is the case, one important experiment to perform is to assess if *Pld2* KO mice are less susceptible to drug-induced excitotoxicity (as tau KO mice are). This would support a more basic role for PLD2 with an impact at the circuit/systems level and potential to be studied in the context of multiple neurological diseases.

In conclusion, in addition to fulfilling the initial aims proposed for this thesis, the body of work produced raised many more questions that are of the most interest to be addressed in the future. These are highlighted below:

1. An obvious future direction to pursue is to initiate drug trials with PLD2 specific inhibitors. Initial studies should be started with AD animal models and if the results are encouraging, experimentation should be expanded to clinical trials in humans.

2. The exact mechanisms through which PLD2 acts and Aβ leads to PLD activation are still elusive. The exhaustive characterization of these mechanisms will be key for the understanding of AD

pathophysiology and for a potential role of PLD2 in other neurodegenerative diseases.

3. The crosstalk of PLD2 with other signaling pathways (such as the PLC and PLA2 pathways) should also be addressed in more detail.

4. Our lipidomic analysis has produced a vast amount of data that can potentially lead to many more lines of research. An immediate one is the study of the potential role of gangliosides in AD.

5. Another important question is the study of the effect of *Pld1* ablation and the study of the impact of ablating both isoforms, *Pld* double KO. The characterization of the effects of pharmacologic agents that inhibit either PLD1, PLD2 or both will complement the genetic studies.

6. Finally, this work has provided major technical advances in the field of PLD, specifically in understanding PLD *in vivo* activity using mass spectrometry. Previous studies that have been performed using primary alcohols to inhibit PLD should be readdressed with the new genetic/pharmacological tools.

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Chapter 4

Annexes

Chapter 4.1.

Tiago Gil Oliveira and Gilbert Di Paolo.

Phosphopipase D in brain function and Alzheimer's disease.

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Phospholipase D in brain function and Alzheimer's disease

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ABSTRACT

Alzheimer's disease is the most common neurodegenerative disorder. Although lipids are major constituents of brain, their role in Alzheimer's disease pathogenesis is poorly understood. Much attention has been given to cholesterol, but growing evidence suggests that other lipids, such as phospholipids, might play an important role in this disorder. In this review, we will summarize the evidence linking phospholipase D, a phosphatidic acid-synthesizing enzyme, to multiple aspects of normal brain function and to Alzheimer's disease. The role of phospholipase D in signaling mechanisms downstream of beta-amyloid as well as in the trafficking and processing of amyloid precursor protein will be emphasized.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized clinically by progressive memory deficits, impaired cognitive function, altered and inappropriate behavior, and a decline in language function [1–3]. AD patients display cortical atrophy, loss of neurons and synapses, as well as extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). While senile plaques are largely composed of aggregated amyloid β-peptide (Aβ) [1,2], tangles consist of pairs of ~10 nm filaments wound into helices, also called paired helical filaments, and contain hyperphosphorylated forms of the microtubule-associated protein tau (MAPT). Growing evidence indicates that there is crosstalk between Aβ and tau pathogenesis [4]. Genetic studies of families with AD have identified several genes that are important in the pathogenesis of AD. The most widely studied mutations occur in three genes: β-amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2). APP is the precursor protein of Aβ, and the presenilins have been identified as components of the γ-secretase complex, which, alongside the β-secretase, are responsible for the generation of Aβ [1,2].

Aβ is derived from the sequential cleavage of type I transmembrane protein APP by membrane-bound proteases, β- and γ-secretase [1,5,6]. Beta-site APP cleavage enzyme 1 (BACE1) has been identified as the major β-secretase activity that mediates the first cleavage of APP in the β-amyloidogenic pathway [7,8]. BACE1-mediated cleavage leads to the release of the APP ectodomain sAPPβ into the extracellular space. The remaining COOH-terminal fragment (CTF), which can be either 99 or 89 amino acids in length (termed "C99" or "C89"), undergoes subsequent cleavage by γ-secretase to release Aβ and the APP intracellular COOH-terminal domain (AICD). The presenilins have been proposed to be the major catalytic component of the γ-secretase complex, whose sequential intramembrane cleavage of APP produces a spectrum of Aβ peptides varying in length by a few amino acids at the COOH-terminus [5,7]. The majority of Aβ normally ends at amino acid 40 (Aβ40), but the 42-amino acid variant (Aβ42) is more amyloidogenic, and has been hypothesized to nucleate senile plaque formation [1,5,7]. In the past few years it has been shown that soluble oligomeric Aβ40/42 in various assembly states better correlate with synaptic malfunction and cognitive impairment than neuritic plaques *in vivo*, consistent with mounting evidence that oligomeric Aβ is significantly synaptotoxic [1].

As mentioned above, a number of links exist between Aβ and tau pathologies. Importantly, in an AD mouse model associated with a significant Aβ burden, the genetic ablation of tau was neuroprotective for Aβ-induced deficits [9], supporting an intimate crosstalk between Aβ and tau. However, it is not clear whether the precise reasons for the protective role(s) of tau ablation are related directly to tau's pathogenic potential. Overall, this relationship between the two major pathogenic AD hallmarks remains poorly understood [4]. Nevertheless, development of an improved understanding of tau-related pathology may inform not only our knowledge of Aβ-related dysfunction in AD but the illness as a whole. While no familial genetic studies have shown a direct cause effect for tau mutations in AD, mutations in the *MAPT* gene were shown to cause a distinct neurodegenerative disorder, frontal temporal

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dementia, which is not associated with neuritic plaques. There is a single *MAPT* gene, which, through alternative splicing, can lead to the expression of six different isoforms in the human brain. MAP tau has a well-established known function of stabilizing microtubules. The microtubule-binding capacity of tau is known to be regulated by post-translational modifications. The most well established process is phosphorylation, mediated by a number of kinases including glygogen synthase kinase 3 (GSK3), cyclin-dependent kinase 5 (CDK5) and microtubule-affinity-regulating kinase (MARK). Additionally, oxidative stress has been suggested to play a role in tau modifications. The formation of NFTs is thought to be a sequential process that starts with impaired tau phosphorylation, detachment of tau from microtubules, accumulation of misfolded tau, tau aggregation in pretangles, formation of paired helical filaments and finally NFTs [10].

2. Phospholipid imbalance in Alzheimer's disease

Lipid-mediated signaling regulates a plethora of physiological processes, including multiple aspects of brain function. Dysregulation of lipid pathways has been involved in a growing number of neurodegenerative disorders. While much attention has been given to the sterol link to AD [11], growing evidence, suggests that other classes of lipids, such as phospholipids, either mediate or modulate key pathological processes associated with AD [12–14]. The first studies to address these questions were based on post-mortem lipid analysis of brain tissue from AD-affected individuals. For instance, it was reported that anterior temporal cortex of brains from patients with AD had significantly lower levels of phosphatidylinositol (PI) and trends for lower levels of PI phosphate (PIP) and PI-4,5-bisphosphate [$PI(4,5)P_2$] [15], a major signaling lipid regulating a variety of biological processes at the plasma membrane and whose normal balance is required for proper synaptic function [16]. Nitsch et al. [17] observed that brains of AD patients had decreased levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), proposing that AD may be associated with increased membrane phospholipid degradation. Furthermore, using ^{31}P Nuclear Magnetic Resonance to study AD brain preparations, a significant reduction in PE and PI levels was observed, with a trend for a decrease in PC, phosphatidic acid (PA) and cardiolipin and an increase in sphingomyelin and the plasmalogen derivative of PE [18].

The interaction of both tau and Aβ with cellular and artificial membranes has been extensively investigated. Given direct binding of tau to membrane phospholipids, it was hypothesized that this interaction may be relevant for the physiological function of tau as well as AD pathogenesis [19]. Similarly, Aβ can directly alter artificial lipid bilayers by forming pores that are permeable to various ions [20]. Moreover, an *in vitro* study showed that Aβ disrupts membranes containing acidic phospholipids in a pH-dependent way with more pronounced effects on membranes at acidic pH [21]. More recently, after the discovery of the synaptotoxic properties of soluble Aβ oligomers, studies showed that these oligomers destabilize membranes and trigger Ca^{2+} influxes through unknown mechanisms [22]. In part reflecting the pathophysiological relevance of oligomer-induced Ca^{2+} dyshomeostasis, acute and chronic treatments of neurons with soluble oligomers of Aβ were shown to disrupt the metabolism of $PI(4,5)P_2$ in a phenomenon requiring both extracellular Ca^{2+} and PLC activity [13]. Consistent with the view that $PI(4,5)P_2$ destabilization plays a key role in the synapse-impairing actions of Aβ oligomers, hippocampal slices derived from a genetic model lacking one copy of *Synj1* (i.e., a gene encoding the $PtdIns(4,5)P_2$ phosphatase synaptojanin) exhibit normal $PtdIns(4,5)P_2$ levels and long-term potentiation (LTP) despite the presence of Aβ42 oligomers [13]. Similarly, a recent study showed that dysregulation of the group IV A phospholipase A2 pathway mediates some aspects of synaptic and neurobehavioral dysfunction in a mouse model of AD [14]. These two studies provide further support to a prior hypothesis maintaining that phospholipases are highly dysregulated in AD [23]. FAD mutations of presenilins were also shown to cause an imbalance in the metabolism of $PI(4,5)P_2$ and that correcting this imbalance decreased amyloidogenesis [12].

Given the body of evidence presented above demonstrating the importance of phospholipids and AD, development of an improved understanding of phospholipid signaling is essential. Increasing evidence has pointed to one enzyme family especially in regulating biosynthesis and metabolism of phospholipids: phospholipase D (PLD). In this review, we will address the role of the PLD pathway in brain regulation as well as discuss its potential implication in AD.

3. Overview of PLD—structure, function and localization

The first evidence for PLD-like activity was reported in plants in 1947 [24], while a mammalian PLD activity was described for the first time in 1973 in rat brain extracts [25]. However, two decades passed until the purification [26] and cloning [27] of PLD from plants. This subsequently led to the identification of SPO14 as a gene that is essential for meiosis and sporulation in yeast and encodes an enzyme harboring PLD activity [28]. The availability of DNA sequences led to the cloning of two PLD isozymes in mammals, PLD1 [29] and PLD2 [30,31]. In mammals there are three validated isozymes of PLD: PLD1, PLD2 and the recently-identified mitochondrion-associated mitoPLD [32–34]. The majority of studies have so far focused on PLD1 and PLD2 (Fig. 1a), which share: (i) two HxKxxxxD (HKD) motifs that are essential for catalysis; (ii) a phox (PX) consensus sequence and (iii) a pleckstrin homology (PH) domain, which are phosphoinositide-binding modules that are required for proper targeting of PLD; and (iv) a PIP2-binding site, which is fundamental for the enzymatic activity. However, PLD1 differs from PLD2 by the presence of a loop region, which has been proposed to function as a negative regulatory element for catalysis (Fig. 1a) [32]. In the presence of water, both PLD1 and -2 hydrolyze phosphatidylcholine (PC) to generate phosphatidic acid (PA) and free choline. However in the presence of primary alcohols, such as ethanol and 1-butanol, PLD preferentially (~1000-fold) uses these nucleophiles over water for the transphosphatidylation reaction, thus leading to the formation of non-naturally occurring phospholipids, phosphatidylethanol (PtdEtOH) or phosphatidylbutanol (PtdBut), respectively [35] (Fig. 1c). This atypical property of the enzyme has been exploited in a myriad of studies either to block the production of bioactive lipid PA or to measure PLD activity in intact cells or tissues.

PA has unique bioactive properties and can modify both the physical and signalling properties of lipid bilayers. Structurally, it is composed of a three-carbon glycerol backbone, two fatty acid chains and a small phosphate headgroup, thus referred to as a "cone shape" lipid (i.e., a lipid with a small head groups relative to a large hydrophobic domain) [32,36]. This property not only confers PA a higher affinity for negative curvature within lipid bilayers, but it also reduces the energy barrier for bending membrane, thus acting as a fusogenic lipid. Somewhat reminiscent of phosphoinositides, PA also plays an important role at the membrane-cytosol interface through a direct interaction with effector proteins, such as PIP kinases, mTOR, SNARE proteins and sphingosine kinase [37]. However, unlike a variety of phosphoinositide-binding modules (e.g., pleckstrin homology or FYVE domains), PA-binding (poly)peptides generally do not consist of well defined three dimensional folds, but instead involve basic residues in unstructured parts of effector proteins. Additionally, PA can be metabolized to other lipids with potent bioactivity. For instance, PA can be converted to diacylglycerol (DAG) by PA phosphatases [38,39] and to lysophosphatidic acid (LPA), which has an inverted cone shape and thus prefers positive curvature. Finally, PA can serve as a precursor for other lipids in the biosynthetic pathway, where it is consumed for the generation of lipids such as PI via the CDP-DAG pathway. It should be noted that PLD is not the only source

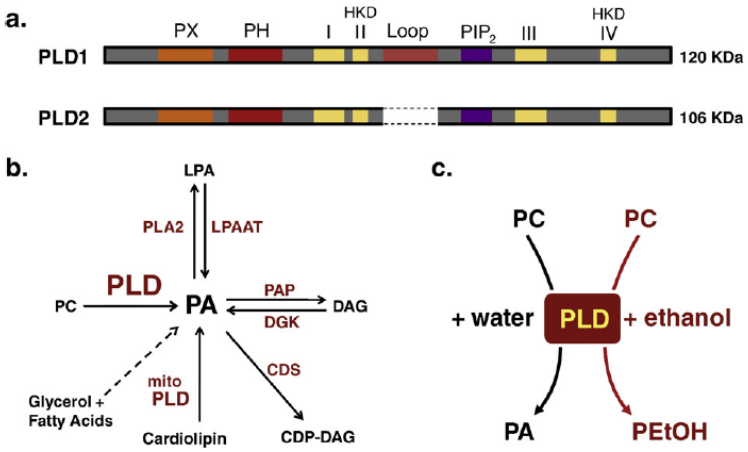

Fig. 1. PLD structure, PA metabolism and reactions catalyzed by PLD. (a) Structure of PLD isozymes. Structurally, the two isozymes differ by the presence of a loop domain in the PLD1 isozyme. (b) PA metabolism. Besides the PLD source, PA can be generated from other sources and further metabolized as represented in the figure. The dashed arrow represents the biosynthetic pathway. The enzymes catalyzing the respective reactions are shown in red. (c) PLD activity. In the presence of water, PLD produces PA. In the presence of primary alcohols, such as ethanol, PLD has a 1000-fold higher affinity for primary alcohols as nucleophiles, leading to a preferential generation of phosphatidylethanol (PEtOH). PLD, Phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; LPA, lyso-PA; DAG, diacylglycerol; CDP-DAG, cytidine diphosphate-DAG; PLA2, phospholipase A2; LPAAT, LPA acyltransferase; PAP, PA phosphatase; DGK, DAG kinase; CDS, CDP-DAG synthase; mitoPLD, mito-phospholipase D.

of PA, as it can be produced by DAG kinases, LPA acid acyltransferase, mitoPLD and other enzymes in the biosynthetic pathway [33,40] (Fig. 1c). However, the fatty acyl composition of PA pools varies, depending on the specific pathways mediating its production. For example, PLD-derived PA species harbor mainly saturated or monounsaturated fatty acids, rapidly giving rise to a pool of DAG with the same properties (i.e., a cone shape), although the negative charge of PA may confer distinct properties and allow for the binding to a different set of effector proteins compared to DAG. In contrast, the pool of DAG resulting from $PI(4,5)P_2$ cleavage by PLC predominantly harbors polyunsaturated fatty acids, likely achieving different physiological functions [41,42].

While PLD1 localizes to the Golgi complex, secretory granules and endosomes, PLD2 is concentrated at the plasma membrane, with smaller pools present in the Golgi apparatus, caveolae and in endosomes [43–46]. In part consistent with their subcellular localization, PLD1 has been implicated in the budding and fusion of *trans*-Golgi-derived secretory vesicles, whereas PLD2 mediates the internalization and recycling of a variety of receptors. Importantly, PLD1 translocates to the plasma membrane upon various stimuli and follows the endosomal internalization route [32,34,47]. Our unpublished studies show that PLD1 relocalizes to autophagosomes and modulates autophagy during nutrient deprivation (Claudia Dall'Armi and GDP, unpublished observations).

4. Role of the PLD pathway in brain function

Following the first description of mammalian brain-associated PLD activity in 1973 [25], the same team reported the formation of ethanol metabolite, PEtOH in rat brain synaptosomes through a PLD-mediated transphosphatidylation reaction [48]. This method was further used to measure PLD activity in homogenates of different brain regions, where the hippocampus, hypothalamus and cortex were associated with the highest PLD activity [49]. Both PLD1 and PLD2 are expressed throughout the brain during development and postnatal life, although levels of expression vary from one cell type to another, at least at the mRNA level [30] (see also reference [50]). In agreement with these expression patterns, both PLD1 and PLD2 have been implicated in neurite outgrowth [51–53].

PLD is likely to play a central role in neurophysiology based on its abundance in the brain and on its implication in multiple aspects of cell physiology including membrane trafficking, cytoskeleton regulation and signal transduction [53–55]. Although no genetic or *in vivo* studies support this idea at this time, several reports involving cell culture models suggest this may be the case, particularly for PLD2. For instance, a study utilizing both RNAi knockdown approach and primary alcohol application has shown that PLD2, likely in concert with small GTPase Ral, regulates the constitutive internalization of metabotropic glutamate receptors, mGluR1a and mGluR5a, as well as colocalizes with these receptors in the endocytic pathway [56]. While this study points to a role of PLD2 in the modulation of excitatory neurotransmission, growing evidence suggests broader involvement in internalization processes. Based on previous findings showing that opioid peptides stimulate PLD activity in chicken embryo cultures [57], more recent studies have shown that PLD regulates the trafficking of opioid receptors, such as the μ- and δ-opioid receptors, with potentially important implications for drug addiction [58,59]. In this case, however, PLD2 appears to be required for ligand-induced (rather than constitutive) endocytosis and to cooperate with the small GTPase Arf6 which plays an important role in modulating vesicle trafficking to and from the plasma membrane [60,61]. Interestingly, signalling processes downstream of PLD2 are beginning to be unravelled, as PLD2-activating opioids have been shown to lead to the production of reactive oxygen species (ROS) [62]. Collectively, these studies support a role for PLD2 in signalling processes downstream of the opioid receptors as well as in the regulation of their cell surface levels.

5. Links between PLD and Alzheimer's disease

The first study linking PLD to AD was published by Kanfer et al. [63], where a decrease in PLD activity, as measured by an *in vitro* choline release assay from radiolabeled PC, was first reported in AD brain homogenates relative to those from control patients. However, when PLD activity was re-investigated in AD brain homogenates relative to those from demented patients without AD, no difference was found [64]. The same group subsequently used PEtOH production as a read-out for PLD activity in ethanol-incubated brain homogenates and found an increase in PLD activity in AD brain extracts relative to

those from control subjects [65]. Perhaps related to this study, increased protein levels of PLD1 were found in mitochondrial fractions derived from AD brains, although the pathophysiological significance of this finding is unclear [66]. In the next sections, we will summarize more recent evidence connecting PLD to AD as well as to the function of proteins, such as presenilins, which are involved in this neurodegenerative disorder.

5.1. The role of PLD in the Aβ signaling pathway

Following the aforementioned *in vitro* studies with AD brain homogenates, cell culture models were used to assess the impact of APP overexpression and extracellular Aβ applications on PLD activity. Overexpression of the neuronal isoform of human wild type APP in P19 mouse embryonic cells caused an increase in PLD activity [67]. Aβ application experiments were used as well with various peptide preparation types. In LA-N-2 cells, a neuroblastoma cell line, an increase in PLD activity was observed after incubating cells with Aβ25–35, a peptide sequence whose pathophysiological significance has been questioned [68]. Additionally, the same group showed that indomethacin (a non-steroid anti-inflammatory drug), nordihydroguaiaretic acid (an anti-oxidant drug) and nicotine inhibited the increase in PLD activity produced by Aβ25–35 applications [69,70]. Furthermore, alanine substitution for the amino acids on the positions 29–34 of Aβ25–35 prevented the peptide from having an effect on PLD activity [71]. Finally, pre-treatment with Aβ25–35 desensitized the cells, which did not exhibit a PLD activity increase in response to a new Aβ25–35 treatment, thus prompting the authors to suggest that Aβ25–35 might mediate its effects on PLD upon receptor binding [70]. Aβ1–40 also produced an increase in PLD activity in rat hippocampal primary cultures, but this effect was seen with high concentrations of Aβ aged at 37 °C (likely reflecting a requirement for some aggregated state of Aβ for this phenomenon to occur) [72]. The increase in PLD activity induced by Aβ correlated with increased release of cytosolic protein lactate dehydrogenase, suggesting it is associated with Aβ-induced toxicity [72]. Besides neurons, Aβ1-42 used in the low micromolar range caused an increase in PLD activity in astrocytes and microglia in a process dependent upon formyl-peptide-receptor-like 1 (FPRL1). Specifically, Aβ1-42 was internalized alongside the FPRL1 receptor and stimulated a downstream signaling pathway involving the phosphorylation of extracellular signal-regulated kinase (ERK). Because both Aβ1-42 internalization and FPRL1-mediated signaling were abrogated by a primary alcohol, a role for PLD in this process was proposed [73]. Recently, our lab has addressed the effects of soluble oligomeric preparations of Aβ1–42 (oAβ1–42), which are highly synaptotoxic at submicromolar concentrations, on PLD activity. We found that oAβ42 at 200 nM leads to an increase in PLD activity in primary cortical cultures and that this effect is abolished in mouse neurons lacking PLD2. Strikingly, we also found that the genetic ablation of *Pld2* confers protection against the suppressive effects of oAβ1-42 on LTP and the memory-impairing action of SwAPP overexpression *in vivo* (TGO and GDP, unpublished observations).

5.2. The role of PLD on APP trafficking and Aβ generation

Because Aβ originates from the sequential cleavage of APP by β- and γ-secretases, tremendous effort has been put into attempting to understand the molecular mechanisms regulating the subcellular localization and intracellular sorting of APP and its cleaving enzymes, all of which are transmembrane proteins or protein complexes. Generally, an increasingly popular hypothesis in the field is that perturbation of the trafficking of APP, BACE1 and γ-secretase complex [including its catalytic components, presenilins (PS)] may profoundly affect amyloidogenesis and thus impact AD pathogenesis [74].

As with most type 1 transmembrane proteins, APP traffics through the secretory pathway toward the cell surface, where it can be re-internalized and reach the endosomal system. Additionally, there is evidence that APP traffics back to the *trans*-Golgi network (TGN) and it has been suggested that reducing this transport step results in increased amyloidogenesis. This may reflect the fact that the APP/BACE1 interaction is predominant in the endosomes and that the acidic pH of this compartment is optimal for BACE1-mediated proteolysis [74]. Despite the complexity in the intracellular trafficking pattern of APP, most studies addressing the role of PLD in this process have focused on the cell surface delivery of APP (and particularly, the Swedish mutant of APP) from the TGN. These studies were prompted by a large body of evidence implicating PLD1 as well as its product PA in the budding of secretory vesicles from the TGN [75–77]. Furthermore, while the bulk of the studies addressing the relationship between the γ-secretase complex and APP focus on the APP processing, some evidence indicates that presenilin can also regulate the trafficking of APP independently of its catalytic activity. Specifically, TGN-derived secretory vesicles produced from PS1-deficient cells contain higher levels of APP, thus resulting in increased cell surface delivery of APP. The converse phenomenon was observed in cells expressing an FAD mutant version of PS1 (i.e., ΔE9), suggesting that these findings may be relevant for the pathogenesis of AD [78]. Collectively, these results converged onto a transport pathway involving APP, PS and PLD1, although evidence for a *bona fide* crosstalk between these three molecules in the context of TGN-to-plasmalemma traffic emerged in subsequent studies.

Accordingly, PLD1 overexpression was shown to promote the formation of APP-containing secretory vesicles from TGN, thus mimicking the effect of *PSEN1* nullizygosity [79]. However, primary alcohols failed to rescue this phenotype in PS1 knockout cells, suggesting that the increased biogenesis of APP-containing vesicles observed in PS1-deficient cells is PLD-independent. While these data suggested that WT PS1 and PLD1 may regulate the traffic of APP through independent pathways, there is a clear functional link between these two proteins in the FAD mutant background (at least, for the PS1ΔE9 mutant). For instance, a subcellular fractionation experiment showed that expression of FAD PS1ΔE9 mutant in cells leads to an enrichment of PLD1 in the Golgi/TGN fraction at the expense of lighter fractions (which may in part represent endosomal structures) and that this redistribution was concomitant with a decrease in total PLD activity [79]. Importantly, overexpression of catalytically active PLD1 (but not the lipase-dead mutant) was found to rescue the defect in the budding of APP-containing vesicles from the TGN as well in the cell surface delivery of APP induced by the PS1 ΔE9 [79]. Whether PLD1 interacts with APP or not is a matter of debate as conflicting data were obtained in independent studies [66,80].

While the aforementioned study highlights the effects of PLD1 on APP trafficking, Cai et al. [81] also addressed the role of PLD1 in amyloidogenesis. First, it was shown that PLD1 (but not PLD2) physically interacts with the cytoplasmic loop region of PS1 and that through this binding PS1 mediates the recruitment of PLD1 to the Golgi complex. Furthermore, in N2a cells expressing the PS1 mutant ΔE9, overexpression of PLD1 decreased the levels of Aβ, whereas silencing PLD1 and expression of a lipase-dead PLD1 mutant produced the converse effect. Interestingly, co-precipitation experiments showed that PLD1 regulates the assembly of the γ-secretase complex through a direct effect on PS1 (but not the other components of the complex: Pen-2, nicastrin and APH1), although this phenomenon appears to be independent of the lipase activity of PLD1.

Since PLD1 regulates the traffic of APP and that APP, in turn, may affect the transport of PS1, it was hypothesized that PLD1 could regulate the trafficking of PS1 indirectly, through APP. However, recently published evidence has suggested that PLD1 positively regulates the delivery of PS1 to the cell surface in an APP-independent fashion [80]. Indeed, analysis of PS1 localization in mouse embryonic fibroblasts (MEFs) lacking both APP and its related family member APP-like-protein 2 (APLP2) (APP dKO) showed increased cell surface

delivery of PS1. This phenomenon was mimicked by the overexpression of catalytically-active PLD1, but it also occurred in APPdKO cells, thus suggesting that it is independent of an effect of PLD1 on APP transport [80].

In summary, there appears to be a significant crosstalk between PLD1, APP and PS1 with important implications for amyloidogenesis. However, it is unclear whether this crosstalk also occurs in vivo, which should be best addressed with genetic models. Importantly, PLD1 and PLD2 are likely to play distinct roles in AD pathogenesis, perhaps reflecting their differential subcellular localization, expression levels/profile, and regulation as well as their ability to control different aspects in the biology of the key proteins involved in AD.

6. Other potential links between PLD and neurodegeneration

There are at least four additional lines of research potentially linking PLD to degenerative processes. The first involves PLD and a potential link with α-synuclein; the second implicates PLD isozymes as substrates of caspases; the third points to how a perturbation of PA metabolism can alter membrane dynamics with consequences on cell viability; and the fourth involves the connection between PLD and mTOR in survival pathways.

6.1. PLD and Parkinson's disease

In addition to regulating the function of a variety of receptors, PLD has been implicated in brain-associated pathophysiology through an interaction with a disease-relevant cytoplasmic protein, α-synuclein. This aggregate-prone protein is the product of a gene that is mutated in familial cases of Parkinson's disease (PD) and also involved in AD pathology based on its presence in Lewy bodies and neuritic plaques, respectively [82]. The original study suggesting a functional link between α-synuclein and PLD2 demonstrated that α-synuclein inhibits the lipase activity of PLD2 *in vitro* [83]. Subsequent studies have corroborated this link through analyses of the direct physical interaction between these two proteins and of the effects of various PD-linked mutations on PLD activity both *in vitro* and in intact cells [84,85]. However the (patho)physiological relevance of this interaction has been questioned, since conflicting data has been obtained by others with respect to the inhibitory action of α-synuclein on PLD activity [86].

6.2. PLD and programmed cell death

Two recent studies have shown that caspases-8, -3 and -7 can cleave PLD isozymes *in vitro* [87] and that caspase-3 can also cleave these enzymes *in vivo* [88]. Both demonstrate that PLD1 is cleaved within its loop, which is a region that structurally distinguishes PLD1 from PLD2 and negatively regulates the lipase activity of PLD1. This caspase cleavage produces two fragments of approximately 60 (NH_2-terminus) and 55 kDa (at the COOH-terminus), respectively. In contrast, PLD2 is cleaved in proximity to the NH_2-terminus, resulting in a shift in PLD2's molecular weight from 106 to 102 kDa with a release of a small fragment and a larger one still encompassing the bulk of the PLD2 sequence. However, these studies present conflicting conclusions concerning the impact on the activity of the enzymes after caspase-induced cleavage and the role of the various proteolytic products in the modulation of apoptosis outcome [87,88]. For instance, the first study shows that caspase-cleaved PLD1 was no longer activated by PKC, but resulted in increased activity induced by small GTPases, and PLD2 activity did not change after caspase-3 induced cleavage [87]. However, the second study shows that apoptosis-induced PLD1 cleavage results in lower PLD1 activity levels and agrees with *in vitro* studies demonstrating that apoptosis-induced PLD2 cleavage had no effect on its activity [88]. Regardless of this discrepancy, caspase-mediated cleavage of PLD may be relevant for AD, as these proteases have been extensively implicated in this disorder (e.g., downstream of Aβ) [89] and both caspase-3 activity and PLD1 fragments matching the size of those generated by caspase-3 are upregulated in AD brain tissue [88].

6.3. PLD overexpression and cellular degeneration

A recent report by Raghu et al. [90] provided further insight into the role of PLD in neurodegeneration. In *Drosophila melanogaster* photoreceptors, PA levels were altered using distinct genetic manipulations targeting PA metabolism, such as (i) overexpression of the only *Pld gene*; (ii) partial inactivation of cytidine diphosphate (CDP)-DAG synthase (i.e., cds^1), which consumes PA for the *de novo* production of other phospholipids; and (iii) overexpression of *rdgA*, a gene encoding a fly DAG kinase. These three genetic manipulations produced major developmental defects in the photoreceptors, specifically with reduction in the size of rhabdomeres, a specialized structure in these photoreceptors where sensory transduction occurs. Remarkably, this phenotype correlated with increased levels of a single molecular species of PA (out of 17 analyzed) with a specific fatty acyl composition—34:2 (16:0/18:2 or 16:1/18:1) and was rescued by co-expression of a PA phosphatase. This study underscores the physiological importance of regulating metabolic pools of PA, and suggests that certain cells, tissues or organisms may not efficiently cope with increased PLD levels/activity, a situation proposed to occur in AD.

6.4. PLD and the mTOR pathway

In the past few years, several studies have highlighted a role for PLD in cell survival. For instance, increased PLD activity was correlated with enhanced survival rates in various human cancer tissues and neoplastic cell lines and also, numerous growth factors have been shown to lead to increased PLD activity [91]. These findings may be related to the observation that PLD can regulate mTOR (mammalian target of rapamycin), which is involved in cell growth and proliferation and has also been proposed to play a role in AD [91,92]. mTOR is present in two complexes, mTORC1 (when mTOR is bound to Raptor) and mTORC2 (when bound to Rictor). Rapamycin inhibits the formation of the mTORC1 complex through an interaction with FKBP12 (i.e., a modulator of the activity of various complexes in the cell). Interestingly, PA was found to compete with rapamycin for binding to the FRB domain of mTOR [93]. Moreover, PA was shown to be required for the stabilization of mTORC1 and 2 complexes, competing with rapamycin [94]. Additionally, PLD1, but not PLD2, was shown to be required for the activation of mTOR by Rheb [95]. Even though the majority of the data suggests a stronger role for PLD1, there is conflicting data concerning the specific role of how each PLD isozyme affects mTOR regulation [96].

7. Conclusions and future directions

Here we review an increasing number of reports showing the involvement of PLD in AD pathogenesis. Although PLD1 and PLD2 have similar protein structures (with the exception of the regulatory loop) and catalyze the same reaction, their different regulation and localization in the cell, implicate these proteins in different cellular processes. In the case of AD pathogenesis, several studies point to a role of PLD1 in the intracellular trafficking of APP and presenilin with important implications for amyloidogenesis. Although PLD2 has not been extensively studied in the context of AD, our unpublished work suggests it may regulate processes associated with Aβ signaling, perhaps due to its predominant localization at the cell surface, a main site of action of this cytotoxic peptide. Importantly, ablation of PLD2 appears to be protective in the context of a transgenic model of AD. Whether PLD has any role in AD-associated tau pathology remains to be discovered. However, the pleiotropic actions of PLD isozymes in the cell, including their implication in many signal transduction pathways,

make this scenario plausible. Indeed, a key factor in tau pathology appears to be hyperphosphorylation of microtubule-associated protein by such kinases as CDK5 and GSK3β, thus denoting a profound dysregulation of signaling pathways. Interestingly, a link between PLD and these two kinases has been recently published [97,98]. Furthermore, the implication of PLD in the mTOR pathway, combined with an increasing number of studies exploiting inhibitors of this pathway (e.g., rapamycin analogs) to treat proteinopathies as a result of their stimulatory actions on the macroautophagy pathway, suggests that a manipulation of PLD is likely to affect the clearance of protein aggregates.

PLD isozymes are ubiquitously expressed lipid enzymes implicated in multiple facets of cell physiology, consistent with the pleiotropic roles of their product, PA. Despite nearly two decades of research on these proteins, their physiological (*in vivo*) functions are still elusive, in part due to the lack of genetic tools in mammalian systems. While efforts along this line are ongoing in several laboratories (including our own), work in lower species suggests that PLD-encoding genes are largely non-essential, unless organisms are undergoing some stress conditions [99]. In this respect, while the discovery that PLD genes are non-essential in mammals contrasts the many studies implicating PLD1 and PLD2 in fundamental cellular processes, it suggests the exciting possibility that PLD isozymes may be used as potential drug targets for therapeutics in several areas, including AD and cancer. The recently generated pharmacological blockers of these enzymes may prove extremely helpful in this endeavor, particularly if they have different efficacies toward the two isozymes [100–102].

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