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The role of signaling lipids in the nervous system physiology and pathology: mechanistic insights of the phospholipase D pathway in neurodegenerative diseases
Ana Francisca Rodrigues Vaz Bravo
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Tese de Doutoramento em Envelhecimento e Doenças Crónicas

Trabalho efetuado sob a orientação do
Professor Doutor Tiago Gil Rodrigues Oliveira
e da
Professora Doutora Cláudia Guimas de Almeida Gomes

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Declaração

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Orientadores:

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"Thus, it become obvious that one must be wary in attributing scientific discovery wholly to any one person. Almost every discovery has a long and precarious history. Someone finds a bit here, another a bit there. A third step succeeds later and thus onward till a genius pieces the bits together and makes the decisive contribution. Science, like the Mississippi, begins in a tiny rivulet in the distant forest. Gradually other streams swell its volume. And the roaring river that bursts the dikes is formed from countless sources."

Abraham Flexner, *The usefulness of useless knowledge*
(1866-1959)

Abstract

Lipids are a major constituent of the brain and, more specifically, signaling lipids have been shown to regulate brain functioning. Moreover, lipid signaling modulation has been demonstrated to be a potential therapeutic option for neurological disorders, such as Alzheimer's disease (AD). AD is the most common neurodegenerative disease and currently there is no effective treatment to tackle its progression. Therefore, a detailed understanding of its pathogenesis is critical to envision new therapeutic strategies. The main hallmarks of AD are the accumulation of amyloid-beta (Aβ) and impaired phosphorylation of tau.

Growing evidence suggests that a group of enzymes called phospholipases, that modulate the metabolism of signaling lipids, have an impact in neuronal physiology, including membrane trafficking processes. The lipid modifying phospholipase D (PLD) isoenzymes, PLD1 and PLD2, were shown to affect endocytosis and membrane trafficking, as well as modulate AD-related signaling pathways. Moreover, Aβ was reported to increase both isoenzymes' activity in primary neuronal cultures and in an AD mouse model. Despite this, the precise role of PLD enzymes in AD pathology is poorly understood.

We envisioned a multidisciplinary approach using neuronal cell lines, nematode and AD mouse models for further characterization of the role of the PLD pathway in neurodegenerative diseases. While we found that ablation of PLD has no important effect in worm behavior, its ablation in an AD-like model that overexpresses mutated Aβ markedly improves various of its phenotypes. Additionally, we showed that PLD enzymes and tau interact and that PLD overexpression leads to decreased tau levels in a cell line model. Remarkably, PLD ablation in a *C. elegans* tauopathy-like model led to an increase in tau levels and marked improvement of various of its phenotypes. We also found that tau interacts with several phospholipids, such as phosphatidic acid, the product of PLD enzymatic activity. Collectively, our results support PLD as a downstream pathway of Aβ interaction with membranes, identifies the PLD pathway as a key regulator of tau physiology and pathology and confirms PLD as an important player in neurodegeneration in tauopathy disease models. To further address the role of PLD pathway as a key regulator in neurodegenerative diseases, future studies should be performed to dissect the mechanism underlying its neuroprotective effect.

Furthermore, it has been shown that AD patients are more susceptible to seizure-like activity and have a higher rate of epileptiform/sub-epileptic events. The use of AD mouse models (hAPP) mice, with Aβ overproduction, replicates many of the human disease hallmarks, such as memory deficits and susceptibility to pharmacological induced seizures. Concerning AD pathogenesis, it has been shown that specific lipid signatures have been identified both in human and mouse AD brain samples, showing that lipid metabolism is indeed dysregulated. We characterized the hippocampal lipidome of hAPP mice in the context of pharmacologically-induced seizures. With our unbiased lipidomic approach, we observed overall major lipid changes in hAPP mice in line with previous reports. Importantly, lipid profiling of the hAPP mouse brain hippocampus in the context of pharmacologically-induced seizures allowed for the identification of the dysregulated lipid pathways, revealing putative targets for pharmacological intervention that may potentially be used to protect from excitotoxicity in an AD context.

In summary, our work further suggests that PLD is a potential therapeutic target for neurodegenerative diseases and support the notion that lipid signaling is a promising target for pharmacological intervention in excitotoxicity pathological diseases.

Keywords: Alzheimer's Disease; Lipids; PLD; *C.elegans*

Resumo

Os lípidos são componentes essenciais do cérebro, e especificamente os lípidos de sinalização regulam o seu funcionamento. Estudos revelam que, a modulação da sinalização lipídica demonstrou ser uma potencial estratégia terapêutica para distúrbios neurológicos, como a doença de Alzheimer (DA). DA é a doença neurodegenerativa mais comum e, atualmente, não existem tratamentos eficazes para enfrentar o seu avanço clínico. Assim, uma compreensão detalhada da sua patogénese é fundamental para encontrar novos alvos terapêuticos. A DA é caracterizada patologicamente pela acumulação de amilóide-beta (Aβ) e hiperfosforilação da proteína tau.

Trabalhos anteriores sugerem que um grupo de enzimas chamadas fosfolipases, que modulam os lípidos de sinalização, têm um impacto na fisiologia neuronal, incluindo nos processos de tráfego membranar. As isoenzimas da fosfolipase D (PLD) que modificam lípidos, PLD1 e PLD2, afetam a endocitose e o tráfico membranar e modulam as vias de sinalização relacionadas a DA. Curiosamente, foi demostrado que a Aβ aumenta a atividade de ambas as isoenzimas em culturas primárias de modelos de ratinho, que expressam o gene humano que codifica a proteína precursora da amilóide (APP). Contudo, o papel preciso das enzimas PLD na patologia da DA continua mal compreendido.

Neste trabalho de investigação optámos por uma estratégia holística que envolveu o uso de linhas celulares neuronais, nemátodes e modelos de ratinho com DA para uma caracterização adicional do papel da via da PLD em doenças neurodegenerativas. Embora a ablação da PLD não tenha um efeito notório no comportamento dos nemátodes, a sua ablação num modelo "DA-like" que sobre-expressa Aβ melhora marcadamente vários fenótipos. Adicionalmente, observámos que ambas as enzimas PLD interagem fisicamente com a tau e que a sobre-expressão da PLD leva a uma forte diminuição dos níveis de tau em células. O nosso estudo também revelou que a ablação da PLD num modelo de tauopatia em *C. elegans* leva a um aumento nos níveis de tau e marcadamente a uma melhoria de vários fenótipos. Também observámos que, supreendentemente a tau interage com os fosfolípidos como por exemplo, o ácido fosfatídico, produto da actividade enzimática da PLD. Coletivamente, os nossos resultados suportam a PLD como uma via "downstream" da interação Aβ com membranas, identificámos a via PLD como um regulador chave da fisiologia e patologia da tau e corroborámos os estudos anteriores que apontam a PLD como crucial na neurodegeneração em modelos de tauopatia. Estudos futuros necessitam de ser realizados para

abordar com maior detalhe o papel da via PLD como regulador chave em doenças neurodegenerativas.

Estudos anteriores revelaram que, os doentes com DA são mais susceptíveis a convulsões e têm uma maior taxa de eventos epileptiformes/subepilépticos. O uso de modelos de ratinho DA (hAPP), que sobre-expressam amyloid-beta (Aβ) replicam muitas das características da doença humana, como défices de memória e susceptibilidade excitotóxica. No que diz respeito à patogénese da DA, verificou-se que assinaturas metabólicas lipídicas específicas foram identificadas tanto em amostras de cérebro de DA humanas quanto em ratinhos, demonstrando que o metabolismo lipídico está realmente desregulado. Caracterizámos o lipidoma do hipocampo de ratinhos hAPP no contexto de convulsões induzidas farmacologicamente. Esta análise lipídica mostra alterações globais de lípidos em ratinhos hAPP. De realçar que o perfil lipídico do hipocampo do cérebro do ratinho hAPP no contexto de convulsões induzidas pelo pentylenetetrazole (PTZ) permitiu a identificação de vias lipídicas desreguladas, revelando potenciais alvos terapêuticos que podem ser utilizados para proteger da excitotoxicidade num contexto de DA.

Palavras Chave: Doença de Alzheimer; Lípidos; PLD; *C.elegans*

Contents

Abbreviations

Aβ	Amyloid Beta
AcylPG	Acyl Phosphatidylglycerol
AD	Alzheimer's Disease
AGD	Agyrophilic grain disease
AICD	APP intracellular COOH-terminal domain
APP	Amyloid precursor protein
ASO	Antisense oligonucleotides
BACE	β-site APP-cleaving enzyme
BIN1	Bridging integrator 1
BSA	Bovine serum albumin
BMP	Bis(monoacylglycero)phosphate
C83	Transmembrane 83-residues long C-terminal fragment
CBD	Cortical basal degeneration
CD2AP	CD2-associated protein
CDK5	Cyclin-dependent kinase 5
CE	Cholesterol ester
Cer	Ceramides
CGC	Caenorhabditis Genetics Center
CNS	Central nervous system
CSF	Cerebrospinal fluid
dhCer	Dihydroceramide
dhSM	Dihydrosphingomyelin
DG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMP	Defecation Motor Program
ER	Endoplasmic reticulum
ERDF	European Regional Development Fund

FBS	Fetal bovine serum
FDA	Food and Drug administration
FTDP-17	Frontotemporal Dementia with Parkinsonism-17
GFAP	Glial Fibrillar Acidic Protein
GM3	Monosialodihexosylganglioside;
GSK3β	Glycogen Synthase Kinase 3β
GWAS	Genome wide association studies
HD	Huntington disease
HPLC	High profile liquid chromatography
JNK	JUN N-terminal kinase
LDs	Lipid droplets
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPCe	Ether lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPEp	Plasmalogen lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
LPS	Lysophosphatidylserine
LysoPC	Lysophosphatidylcholine
LTP	Long-term potentiation
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein tau
MhCer	Monohexosylceramide
MRM	Multiple reactions mode
MS	Mass spectrometry
MSA	Multiple systems atrophy
mTOR	Mechanistic target of rapamycin
NAPS	N-acyl Phosphatidylserine

NFTs	Neurofibrillary Tangels
NGM	Nematode growth media
NMDAR	N-methyl-D-aspartate receptor
Nser	N-acyl Serine.
PA	Phosphatidic Acid
PAP	Phosphatidic Acid Phosphatases
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCe	Ether Phosphatidylcholine
PE	Phosphatidylethanolamine
PEp	Plasmalogen Phosphatidylethanolamine
PH	Pleckstrin Homology
PG	Phosphatidylglycerol
PHF	Paired Helical Filaments
PI	Phosphatidylinositol
PI3P	Phosphatidylinositol 3 phosphate
PI(4,5)P2	Phosphoinositol-4,5-biphosphate
PICALM	Phosphatidylinositol Binding Clathrin Assembly Protein
PLA	Proximity Ligation Assay
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PP2A	Phosphatase 2A
PKA	Protein Kinase
PS1	Presenilin 1
PS2	Presenilin 2
PS	Phosphatidylserine

PSD95	Post synaptic density 95
PtdEtOH	Phosphatidylethanol
PSP	Progressive supranuclear palsy
RT	Room temperature
SM	Sphingomyelin
SNARE	Soluble NSF attachment receptor
SORL1	Sortilin-related receptor 1
SREBP	Sterol regulatory element binding protein
Sulf	Sulfatides
SwAPP	Swedish APP
Thr	Threonine
Tyr	Tryptophan
TGN	Trans-Golgi network

Chapter 1

Introduction

Introduction

The role of lipids in the brain

Lipids are fundamental components of the brain, are involved in essential processes such as vesicle synthesis, regulation of membrane assembly, neurotransmitter release, and signaling propagation (Piomelli, Astarita et al. 2007). While genomic and proteomic studies are nowadays extensively explored, the field of lipidomics is now having increased attention due to the advances in analytical techniques. Specifically, mass spectrometry (MS) based techniques, currently allow the characterization of the lipid profile from cells to tissues or whole organisms (Wenk 2010). Lipidomic studies are an essential tool to understand the role of lipids in neuronal physiology and pathology processes, including essential aspects of brain function and dysfunction (Wenk 2010) and allow the identification of lipid signatures associated with a disease state (Miranda and Oliveira 2015). Piomelli et al. highlight that approximately half of the brain dry weight are represented by lipids and this is in accordance with its role as key players in disease physiology (Piomelli, Astarita et al. 2007).

Lipids have been defined as biological molecules soluble in organic solvents and which are generally hydrophobic in nature (Fahy, Subramaniam et al. 2005). They are amphipathic molecules with a hydrophobic domain or polar end and a hydrophilic domain, which interacts with water (Simons and Sampaio 2011). They are chemically diverse in their structures which confers a plethora of different biological functions (Vattulainen and Rog 2011). The polar head groups can differ in shape and charge, while the hydrocarbon chain domain can diverge in length, saturation, and hydroxylation (Fahy, Subramaniam et al. 2005). In mammalian cells, the main lipid categories are glycerophospholipids, sphingolipids and sterols, namely cholesterol (Fahy, Subramaniam et al. 2005) (Figure 1). The most cholesterol-rich organ in the body is the brain and contains 25% of total body cholesterol. It is an essential component of cell membranes and it is also present in myelin sheaths (Hartmann, Kuchenbecker et al. 2007). This lipid plays a central role on the physical properties of membranes, affecting fluidity, thickness and permeability (Miranda and Oliveira 2015). Glycerophospholipids are the most abundant lipids in the cells and act as membrane components and signaling molecules. Their structure is defined by the presence of a polar head phosphate group at the position sn3 esterified to one of the glycerol hydroxyl groups and by one or

two fatty acids present at sn1 and sn2 positions, which differ in length and degree of saturation (Fahy, Subramaniam et al. 2005). Also, the sphingolipids have the propensity to create diversity by the substitution of the glycerol backbone by sphingosine. They can be divided into sphingosine derivates, ceramides (Cer), and more complex sphingolipids with 500 different carbohydrate structures, which make up the head groups of the glycosphingolipids (Fahy, Subramaniam et al. 2005). In addition, it has been proposed that sphingolipids have a cylindrical shape and this feature allows the assembly of specific membrane domains, such as lipid rafts, which are also enriched in cholesterol. These structures of higher complexity have been shown to be extremely dynamic, altering the local diffusion and fluidity properties of membranes, with implications for lipid-lipid, protein-lipid and protein-protein interactions (Simons and Sampaio 2011).

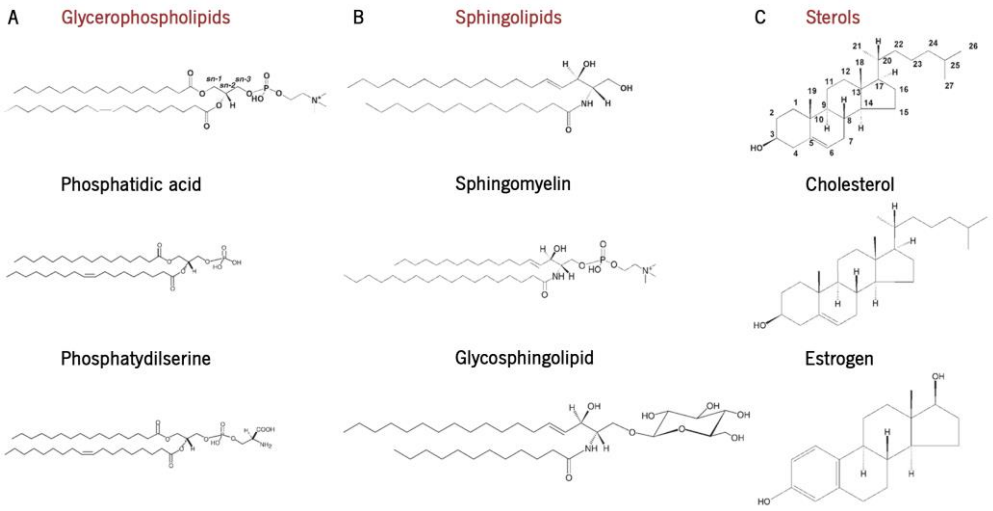

Figure 1. Examples of representative classes of the major lipid categories in mammalian cells. (A) Glycerophospholipids are divided in classes according to the nature of the head group linked to the glycerol backbone. Representative structures for glycerophospholipids (examples: phosphatidic acid and phosphatidylserine). (B) Sphingolipids contain a sphingoid backbone, which is acylated to form a ceramide. Representative structures for sphingolipids (sphingomyelin and glycosphingolipids). (C) Cholesterol is the most abundant sterol in mammals. It contains an inflexible four-ring core that confers high hydrophobicity and interferes with acyl packing in membranes, promoting the assembly of lipid microdomains (adapted from Miranda and Oliveira 2015).

The lipid composition allows cells to organize their internal constituents in specific organelles with individual identities and functions. Of particular interest, the diversity of lipid composition of plasma membrane is to assure a stable and impermeable barrier, even when physiological or pathological

events change locally the composition, osmolarity, or pH. On the other hand, the membrane of endoplasmic reticulum (ER), has a flexible nature, important to biogenic functions, such as the synthesis and transport of lipids as part of the secretory vesicles (Holthuis and Menon 2014). Despite the fact that lipids are mostly interconvertible molecules, the lipid modifying enzymes are able to breakdown structural membrane components, creating large numbers of small lipid-soluble second messenger molecules, which can diffuse easily throughout the membrane (Piomelli, Astarita et al. 2007).

All these structural and functional features of membranes results from a complex and dynamic network biochemical interconnection of a large spectrum of lipids (Miranda and Oliveira 2015). Although in the past few years important insights have emerged in this area, we are only now starting to understand the role of lipid compositional complexity in essential aspects of brain function and dysfunction.

Glycerophospholipids and Phospholipases

The major components of all biological membranes are a specific category of lipids named, glycerophospholipids. Structurally they present four components: fatty acyl chains, a glycerol backbone to which the fatty acyl chains are attached, a phosphate head group, and an alcohol attached radical group that defines the specific classes of phospholipids (Fahy, Subramaniam et al. 2005) (Figure 2).

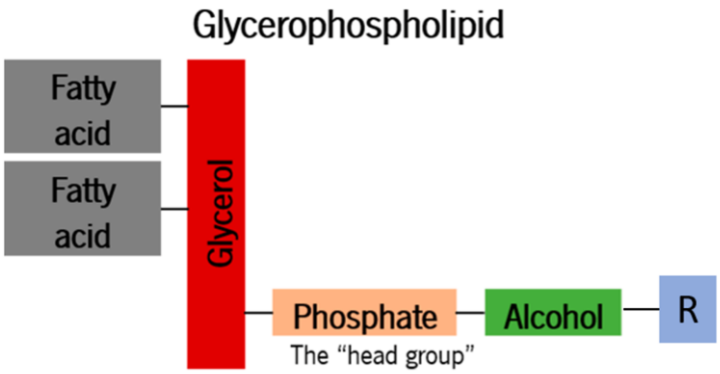

Figure 2. Schematic figure representing the main structure of glycerophospholipids. The structural bone of glycerophospholipids is composed of a phosphate group attached to a glycerol-based (glycerophospholipids) with two long fatty acid chain(s), (adapted from Fahy and Dennis 2010).

Examples of different subclasses of glycerophospholipids are phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI), which differ among themselves by the presence of a phosphocholine, phosphoserine, or a phosphoinositol ring as head groups, respectively. More complex phospholipids, such as bismonoacylglycerophosphate and cardiolipin can be synthesized by adding more than two glycerol units (Miranda and Oliveira 2015). Table 1 summarizes the different glycerophospholipids obtained by the addition of distinct head groups.

Table 1. Examples of glycerophospholipids found in nature and their correspondent head groups.

Glycerophospholipids	Head group
Phosphatidic acid (PA)	Hydroxyl
Phosphatidylethanolamine (PE)	Ethanolamine
Phosphatidylcholine (PC)	Choline
Phosphatidylserine (PS)	Serine
Phosphatidylglycerol (PG)	Glycerol
Cardiolipin (CL)	Phosphatidylglycerol
Phosphatidylinositol (PI)	Inositol
Phosphatidylinositol phosphate (PIP)[1]	Inositol-phosphate
Phosphatidylinositol bisphosphate (PIP_2)[2]	Inositol-biphosphate
Phosphatidylinositol triphosphate (PIP_3)[3]	Inositol-triphosphate

[1] PI(3),PI(4) or PI(5)P

[2] PI(4,5)P2, PI(3,4)P2 or PI(3,5)P2

[3] $PI(3,4,5)P_3$

As mentioned before, the diversity of lipids translates into the different shapes and biochemical behaviors when assembled together in membranes. Considering the geometry of their polar heads, lipids can assume cylindrical or conical shapes. This lipid feature predicts the form of membranes, inducing planar or curved membrane structures, respectively. It is also possible for a membrane to assume different properties through the modulation of its lipids, namely thickness, fluidity and microdomain assembly, such as lipid rafts. Interestingly, a cylindrical phospholipid such as PC can be converted into conical lipids, such as diacylglycerol (DG) by phospholipase C (PLC), or phosphatidic acid (PA) by phospholipase D (PLD). It can also be converted into the inverted cone-shaped lysophosphatidylcholine (lysoPC) with the release of a fatty acyl chain by phospholipase A (PLA) (Miranda and Oliveira 2015) (Figure 3).

The various bonds in phospholipids can be cleaved through hydrolysis by specific enzymes called, phospholipases. Enzymatic processing of phospholipids by phospholipases converts these molecules into lipid mediators or second messengers (such as arachidonic acid, PA and DG) that play key roles in membrane trafficking, signal transduction, cell proliferation and apoptosis. These enzymes act in different sites on the phospholipid structure and are generally grouped into four classes: A, B, C and D (Figure 3). Thus, dysregulation of phospholipases contributes to several human diseases and these phospholipases have been identified as therapeutic targets for prevention and treatment of diseases (Aloulou, Ali et al. 2012). In light of this, the pharmaceutical industry has demonstrated an interest in developing selective and potent inhibitors of these enzymes, mainly because lipid metabolism has been implicated in the basic cell physiology and consequently in the pathogenesis of various diseases (Klose, Surma et al. 2013).

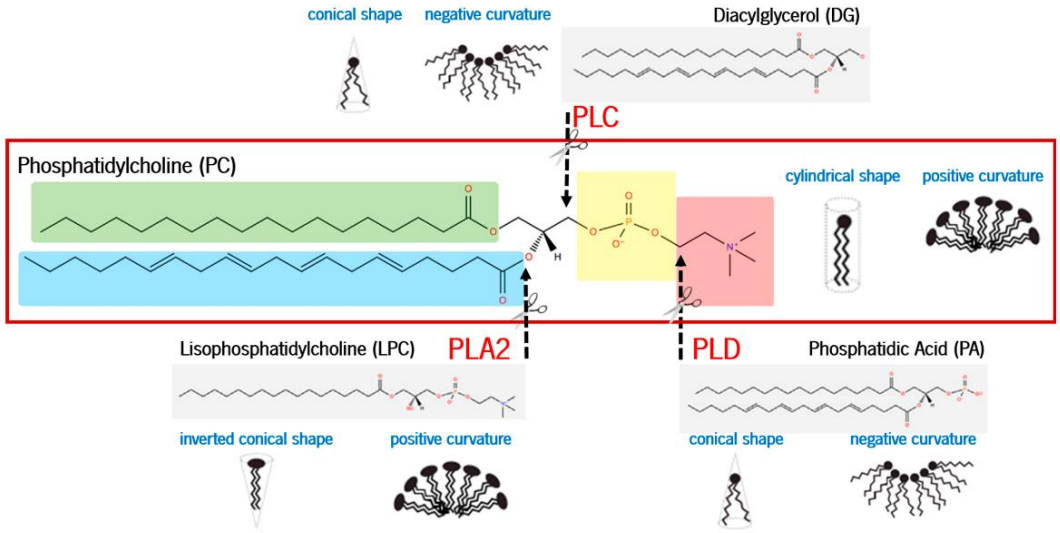

Figure 3. The specificities of phospholipases. PLA2 cleave the second (R2) acyl chains of phospholipids; PLC cleaves phospholipids just before the phosphate group and PLD cleaves after the phosphate group. The respective products contribute to changes in membrane properties, such as induction of curvature (lysoPC and PA) or act as intracellular signaling molecules (fatty acyls and DG). PLA2 – phospholipase A2, PLC – phospholipase C, PLD – phospholipase D (adapted from (Miranda and Oliveira 2015) and (Simons and Sampaio 2011).

Phospholipase D – Structure, Function and Localization

While both the PLC and phospholipase A2 (PLA2) pathways have been widely studied, less is known about the PLD pathway. The first evidence for PLD activity was first described in carrot extracts in 1947 (Hanahan and Chaikoff 1947). In a mammalian system it was first demonstrated in 1973 after Saito and Kanfer reported the presence of PLD activity in rat brain extracts (Saito and Kanfer 1973). Later, the purification of PLD led to the cloning of PLD from plants (Wang, Dyer et al. 1993, Wang, Xu et al. 1994). Afterwards, the SPO14 gene was identified as essential for meiosis and sporulation in yeast and was shown to exhibit PLD activity (Rose, Rudge et al. 1995).

The majority of studies have focused on two mammalian PLD isoenzymes, PLD1 (Hammond, Altshuller et al. 1995) and PLD2 (Colley, Sung et al. 1997) with 120 KDa and 106 KDa, respectively (Figure 4A). They are structurally similar, both catalyzing the same enzymatic reaction, but differ in their cellular location and regulation. While PLD1 localizes to the Golgi complex, secretory granules and endosomes (Brown, Thompson et al. 1998, Freyberg, Sweeney et al. 2001), PLD2 is concentrated in the vicinity of the plasma membrane, with smaller pools present in the Golgi apparatus, caveolae and endosomes. PLD1 also differs from PLD2 by the presence of a loop region, which has been proposed to function as a negative regulatory element for catalysis (Colley, Sung et al. 1997, Freyberg, Bourgoin et al. 2002). The two isoforms are well characterized and have been described to be involved in different signaling cascades that affect membrane trafficking, cytoskeletal reorganization, receptor endocytosis, exocytosis, cell migration, and regulation of the cell cycle (Jenkins and Frohman 2005). For instance, PLD1 is translocated to the plasma membrane, following the endosomal route upon cell stimulation (Jenkins and Frohman 2005, Roth 2008, Donaldson 2009). Dall'Armi and Di Paolo reported that PLD1 localizes in autophagosomes and modulates autophagy during nutrient deprivation (Dall'Armi, Hurtado-Lorenzo et al. 2010). On the other hand, PLD2 mediates the internalization and recycling of a variety of receptors (Jenkins and Frohman 2005, Roth 2008).

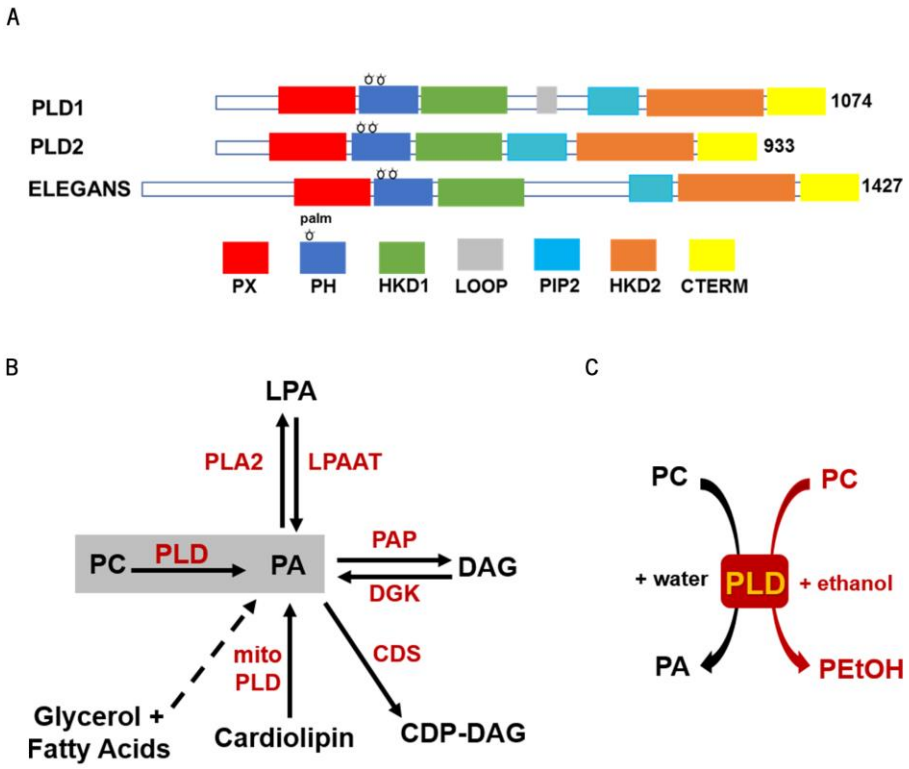

Figure 4. PLD structure, PA metabolism and reaction catalyzed by PLD. (A) Domain structure of human PLD1, PLD2 and PLD ortholog in *Caenorhabditis elegans*. The domains are drawn to scale. The number at the end represents the total number of amino acids for each protein. They both have binding sites for phosphatydilinositol 4,5-biphosphate and palmitoylation (adapted from Raghu, 2009). (B) PA metabolism and reactions catalyzed by PLD. Besides the PLD source, PA can also be obtained from DG by a reaction catalyzed by DGK, or from LPA as a result of LPAAT activity. PA can also be dephosphorylated by PAP to generate DG or hydrolyzed by PLA1 or PLA2 to generate LPA. (C) Graphical representation of the synthesis reaction of phosphatidylethanol (PEtOH) in vivo. PLD has an increased affinity to primary alcohols, leading to the generation of PA in the presence of water, or PEtOH if in the presence of ethanol. This reaction is the only source of PEtOH and can be used as a measure of PLD activity. PLD, Phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; LPA, lyso-PA; DG, diacylglycerol; CDP-DG, cytidine diphosphate-DG; PLA2, phospholipase A2; LPAAT, LPA acyltransferase; PAP, PA phosphatase; DGK, DG kinase; CDS, CDP-DG synthase; mitoPLD, mito phospholipase D (adapted from Oliveira and Di Paolo, 2010).

PLD enzymes share (i) two HxKxxxxD (HKD) motifs that are essential for catalysis, where H corresponds to histine, K for lysine, D for aspartic acid and x for any amino acid; (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 phosphoinositol-4,5-biphosphate (PI(4,5)P2)-binding site, which is fundamental for the enzymatic activity (Frohman, Sung et al. 1999). Although additional proteins with an identified HKD structure have been identified such as PLD3 (Munck, Bohm et al. 2005, Osisami, Ali et al. 2012), PLD4 (Otani,

Yamaguchi et al. 2011), PLD5 (Anney, Klei et al. 2010), and PLD6 (also known as mitoPLD)(Choi, Huang et al. 2006) until now, PLD1 and PLD2 are the only members that were shown to convert the neutral lipid PC into free choline and PA. Importantly, mitoPLD also presents an enzymatic activity, producing PA from cardiolipin, which occurs mainly in the mitochondria (Frohman 2015) (Figure 4B). In *Caenorhabditis elegans* there is only one PLD enzyme, which is structurally similar to its mammalian ortholog sharing 6 of its 7 domains (Figure 4A).

Interestingly, in the presence of primary alcohols, such as ethanol, PLD preferentially (approximately 1000-fold more) uses it as a substrate, producing a specific lipid, phosphatydilethanol (PEtOH), which is often used to measure PLD activity in intact cells or tissues (Oliveira and Di Paolo 2010) (Figure 4C).

PA has unique bioactive properties, being implicated in the biosynthesis of most other phospholipids and triaglycerols, and is a crucial player in lipid signaling pathways (English 1996, Wang, Devaiah et al. 2006). Structurally, it is composed by a three-carbon glycerol backbone, two fatty acyl chains and a small phosphate headgroup, which is responsible for its typical cone-shape that drives membrane to undergo negative curvature (Kooijman, Chupin et al. 2003). As a result of this feature, PA acts as a fusogenic lipid and reduces the energy barrier for bending membranes (Oliveira and Di Paolo 2010). Indeed, this signaling lipid plays a key role at the membrane-cytosol interface through a direct interaction with effector proteins, such as PIP kinases, mechanistic target of rapamycin (mTOR), soluble NSF attachment receptor (SNARE) proteins and sphingosine kinase (Stace and Ktistakis 2006). Additionally, PA can be converted in other lipids. For instance, PA can generate DG and lysophosphatidic acid (LPA) by the result of the activity of phosphatidic acid phosphatases (PAP) and PLA, respectively (Sonoda, Aoki et al. 2002) (Figure 4B). Finally, in the biosynthetic pathway, PA can be consumed for the generation of lipids such as PI via the CDS – DG pathway. Moreover, PLD is not the only source of PA. Other sources of PA include DG kinases, LPA acid acyltransferase, mitoPLD and other enzymes implicated in the biosynthetic pathway (Choi, Huang et al. 2006, Haucke and Di Paolo 2007)(Figure 4B).

Phospholipase D and phosphatidic acid signalling

PLD1 and PLD2 are both expressed throughout the brain during development and postnatal life (Zhao, Berse et al. 1998, Peng and Rhodes 2000), although the expression levels of mRNA vary

from one cell type to another (Kim, Moon et al. 2010). Kobayashi et al. reported that, the PLD activity is lower in adult rats medulla/pons and cerebellum than in the hippocampus, hypothalamus and cerebral cortex (Kobayashi, McCartney et al. 1988).

Based on the PLD abundance in the brain and on its implication in multiple aspects of cell physiology including membrane trafficking, cytoskeleton regulation and signal transduction, it is expected that PLD could have significant roles in neurophysiology (Klein, Chalifa et al. 1995, Klein 2005, Kanaho, Funakoshi et al. 2009). Accordingly, both PLD1 and PLD2 have been implicated in neurite outgrowth (Zhang, Huang et al. 2004, Kanaho, Funakoshi et al. 2009). Despite some contradictory studies, PLD1 has been proposed to be involved in neuronal dendritic arborization (Zhu, Kang et al. 2012, Ammar, Humeau et al. 2013). A study using cultured hippocampal neurons from rat embryos with PLD1 downregulated by short interfering RNA (siRNA), showed a negative control by PLD1 in dendritic branching showing an increase in dendritic arborization length and complexity when PLD1 is reduced (Zhu, Kang et al. 2012). However, Ammar and colleagues described the opposite effect, a decrease of the dendritic arborization in cultures of embryonic cortical neurons from PLD1 knockout (KO) mice (Ammar, Humeau et al. 2013). Another study using KO animal models demonstrated that PLD has also been implicated in crucial cellular processes, such as macroautophagy which depends on the activity of phosphatydilinositol 3-kinase (PI3K), being Vps34 an example of a PI3K, and consequently on the production of phosphatydilinositol 3-phosphate (PI3P). Both PLD and PI3K have a PX domain, and their interaction seems to activate the latter, since PLD inhibition impairs autophagy and leads to the accumulation of toxic proteins in the cell (Dall'Armi, Hurtado-Lorenzo et al. 2010). In fact, PLD1 has been shown to be colocalized with the microtubule-associated protein 1A/1B-light chain 3 (LC3), an essential protein for autophagosome formation (Dall'Armi, Hurtado-Lorenzo et al. 2010). An increasing number of studies have associated both PLD1 and PLD2 (as well as PLD3) with some neuronal pathological conditions, particularly Alzheimer's disease (AD) (Cai, Netzer et al. 2006, Oliveira, Chan et al. 2010, Cruchaga, Karch et al. 2014). Specifically, Aβ (amyloid beta), a toxic peptide that accumulates in AD, has been shown to increase PLD activity (Oliveira, Chan et al. 2010). Moreover, it was shown that the genetic ablation of PLD2 protected from the deleterious effects of Aβ (Oliveira, Chan et al. 2010). Interestingly, Aβ was also shown to decrease the levels of PI(4,5)P2 (Berman, Dall'Armi et al. 2008). These results highlight that PLD, and potentially its enzymatic product, PA, could be related with PI(4,5)P2 metabolism in a disease process (Di Paolo and Kim 2011).

Alzheimer's Disease

Presently, one of the major biomedical challenges is finding therapeutical strategies to tackle neurodegenerative diseases (Haass and Selkoe 2007). AD is a neurodegenerative disorder and the most common form of dementia in the elderly (De Strooper and Karran 2016). AD is characterized by progressive memory deficits, impaired cognitive function and is associated with a decline in language function. In 1907, Alois Alzheimer first described the pathological presentation of diseased brains, which are characterized by the presence of senile plaques, intracellular neurofibrillary tangles (NFTs), astrogliosis, neuronal dystrophy, neuronal loss and vascular alterations (Huang and Mucke , Takahashi, Almeida et al. 2004, De Strooper and Karran 2016). While senile plaques are composed of aggregates of a small peptide (4.2 kDa) with 40 or 42 amino acids known as Aβ, NFTs consist of ≈10 nm filaments wound into helices, also called paired helical filaments, and contain hyperphosphorylated forms of the microtubule-associated protein tau (MAPT) (Haass and Selkoe 2007, LaFerla, Green et al. 2007, O'Brien and Wong 2011). The identification of the precise pathways that are involved in AD is yet to be fully characterized. Genetic studies have identified several genes associated with increased risk for sporadic AD (Bertram, McQueen et al. 2007) namely, apolipoprotein E (APOE), sortilin-related receptor 1 (SORL1) (Rogaeva, Meng et al. 2007), phosphatidylinositol binding clathrin assembly protein (PICALM) (Harold, Abraham et al. 2009), bridging integrator 1 (BIN1) (Pant, Sharma et al. 2009), and CD2-associated protein (CD2AP) (Naj, Jun et al. 2011), among others. Importantly, many of these genes have been implicated in lipid metabolism and membrane trafficking regulation. The study of familial forms of the disease has led to the identification of causal mutations, associated with familial forms of AD, that occur in three genes: β-amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2), involved in γ-secretase complex. Interestingly, APP is cleaved by β secretase and γ-secretase to generate Aβ (Haass and Selkoe 2007). The mechanism that leads to Aβ accumulation when causal genetic mutations are not present is less well understood. This presentation of the disease, sporadic AD, is characterized by an older age of presentation of cognitive dysfunction symptoms, but pathologically it also presents Aβ plaques and NFTs.

In the past decades, efforts have been performed, exploring disease-modifying therapies which could block the progression of the disease and drugs have been designed to target specific molecular pathways (Kumar, Singh et al. 2015). Currently, the only approved treatments by the Food and Drug administration (FDA), include five drugs such as AChEIs-rivastigmine (Exelon), galantamine (Razadyne, Reminyl), tracine (Cognex), and donepezil (Aricept) and NMDA receptor

antagonist – memantine (Namenda) that modestly slow the progression of cognitive symptoms and reduce problematic behaviors in some people (Kumar, Singh et al. 2015).

The Aβ peptide is produced by the sequential cleavage of type I transmembrane APP. APP is a single pass transmembrane protein with an extracellular domain at N-terminus (the bigger domain) and an intracellular domain (cytoplasmic domain) at C-terminus (Turner, O'Connor et al. 2003, O'Brien and Wong 2011). Under physiological conditions, APP is ubiquitously expressed and present different isoforms, which arise from alternative splicing of the gene. In the central nervous system (CNS), the isoform with 695 amino acids is expressed exclusively in neurons, whereas the other two isoforms are ubiquitously expressed (Turner, O'Connor et al. 2003, O'Brien and Wong 2011). The physiological function of APP is not yet well defined; however, it seems that is closely involved in axonal growth and pruning, neuronal migration, repair via interaction with extracellular matrix proteins (Musardo, Saraceno et al. 2013), synaptic activity and long-term potentiation (LTP) mechanisms (Koo, Sisodia et al. 1990, Roch, Masliah et al. 1994, Allinquant, Hantraye et al. 1995, Yamazaki, Koo et al. 1997, Soba, Eggert et al. 2005, Nikolaev, McLaughlin et al. 2009, Hong, Beja-Glasser et al. 2016). Although the downstream signaling pathway and molecular targets of APP are still poorly understood, these findings shed some light into the function of this protein in the brain.

The processing of APP can occur in different sites by different enzymes and can be divided into nonamyloidogenic and amyloidogenic pathways. The processing of APP can be initially mediated by two distinct secretases: (1) α-secretase with the cleavage site within the Aβ peptide region; (2) β-secretase, also called β-site APP-cleaving enzyme (BACE), with the cleavage site in the N-terminus of Aβ peptide. In the nonamyloidogenic pathway, APP at cell surface is cleaved by α-secretase producing a large soluble ectodomain at N-terminus (612 amino acids), named sAPPα, that is secreted to the extracellular space and the formation of a transmembrane 83-residues long C-terminal fragment (C83). C83 is subsequently cleaved by the γ-secretase complex that includes PS1 and PS2 leading to the formation of 2 fragments, a small one called p3 that is released predominantly to the extracellular space, which has no propensity for aggregation, and the C-terminal fragment that remains inside the cell, called APP intracellular COOH-terminal domain (AICD) (Turner, O'Connor et al. 2003, LaFerla, Green et al. 2007, O'Brien and Wong 2011). Concerning the amyloidogenic pathway, APP is internalized by clathrin-mediated endocytosis into endosomes containing BACE-1 and γ-secretase. The first cleavage by β-secretase results in the release of sAPPβ, a large soluble ectodomain at N-terminus (595 amino acids) that is

predominantly secreted to the extracellular space. The remaining membrane retained the β-carboxyl-terminal fragment (β-CTF), which can vary in size, being the major form, a fragment containing 99 amino acids (C99) that includes at the N-terminus the first amino acid of Aβ peptide. C99 is subsequently cleaved by the γ-secretase to release Aβ, either to the extracellular space or to remain in endosomes, and the AICD that is released to the cytosol. The presenilins have been proposed to be the major catalytic component of the γ-secretase complex and to contribute to the sequential cleavage of APP with the production of Aβ peptides with different sizes such as Aβ40 and Aβ42, that vary by a few amino acids at the COOH-terminus (Landman and Kim , Haass and Selkoe 2007). The majority of Aβ normally ends at amino acid 40 (Aβ40), but the 42-amino acid variant (Aβ42) has been shown to be more amyloidogenic and the main form present in Aβ plaques in AD (LaFerla, Green et al. 2007). Noticeably, increasing evidence shows that the amyloidogenic processing of APP and the oligomerization of Aβ40/42 peptides in the brain are contributors to global neuronal malfunctioning and cognitive impairment (Haass and Selkoe 2007).

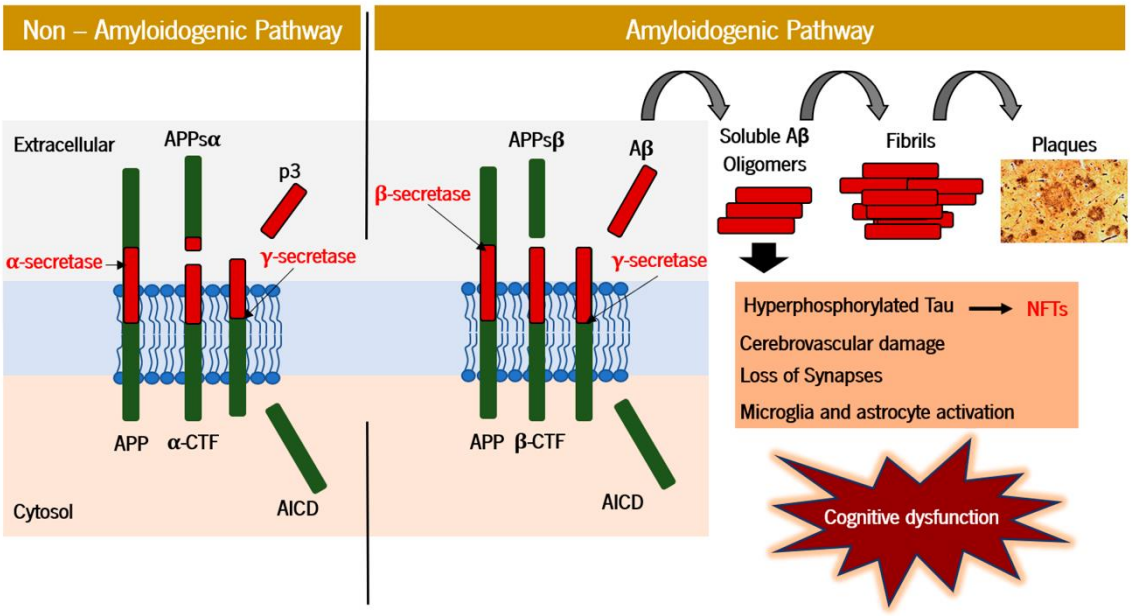

Figure 5. APP proteolysis. On nonamyloidogenic pathway, APP at cell surface is cleaved by α-secretase producing sAPPα and the α-CTF, containing 83 amino acids (C83). C83 is subsequently cleaved by the γ-secretase leading to the formation of p3 and a 9 C-terminal fragment, named APP intracellular domain (AICD). On amyloidogenic pathway, APP at cell surface is cleaved by β-secretase (BACE-1), releasing an ectodomain (sAPPβ), and retaining the last 99 amino acids of APP (known as C99) within the membrane. The first amino acid of C99 is the first amino acid of Aβ. C99 is subsequently cleaved by the γ-secretase complex leading to the formation of Aβ peptide that is released to the extracellular space, and C-terminal fragment, named APP intracellular

domain (AICD). This cleavage predominantly produces Aβ1–40, and more amyloidogenic Aβ1–42 at a ratio of 10:1 (adapted from Kumar and Ekavali, 2015 and O'Brien and Wong, 2011).

For over 20 years, the ''amyloid cascade hypothesis'' has provided the main theoretical construct for AD (Hardy and Selkoe 2002) and is based in a neuron-centric, linear cascade initiated by Aβ and leading to dementia. Several therapeutics that were purported to reduce amyloid-β production or aggregation have failed in Phase III clinical testing and many others are in various stages of development (Karran, Mercken et al. 2011). The linearity of the cascade remains very controversial and the neuron-centric view must be expanded to one that includes the contribution of different cell types, their interactions with each other, and the gradual evolution of the disease (De Strooper and Karran 2016). Strooper and Karran claim that AD only starts when a pathological cellular reaction is initiated and that the lower amounts of Aβ could be pathogenic if the Aβ produced is longer than Aβ40/42. Thus, the pathological effects of Aβ production could be viewed as qualitative and not only necessarily quantitative (Kuperstein, Broersen et al. 2010, Szaruga, Veugelen et al. 2015). Moreover, Palop and Mucke described that the different conformations of Aβ and tau exert proteopathic stress on different cells of the brain and they also interact with proteins such as APP, tau and lipid membranes which disturb the normal activities of these proteins in neurotransmission (Palop and Mucke 2010). It has also been reported that a reduction in endogenous levels of tau can ameliorate some of the behavioral and other deficits that are mediated by Aβ in an APP transgenic mouse model, placing tau downstream of Aβ (Roberson, Scearce-Levie et al. 2007). Importantly, the accumulation of Aβ and tau is a sign of increased proteopathic stress, but the additional cellular factors are determinative in the evolution toward dementia (De Strooper and Karran 2016). In fact, the proteopathic stress results in the view of a "cellular phase" of AD, but the "clinical phase" of disease is only initiated when the homeostasis in the proteostatic network is affected. The majority of sporadic AD cases are characterized also by a defective clearance of Aβ and tau and it is now clear that the normal Aβ turnover depends on bulk flow via the perivascular circulation and the glymphatic system in the brain (Tarasoff-Conway, Carare et al. 2015). Increasing neuronal activity rapidly increased the steady-state levels of extracellular tau *in vivo* and some secreted tau can also be cleared by the glymphatic flow (Yamada, Holth et al. 2014). However, the role of the vasculature in AD is poorly understood. Multiple studies by Palop and Mucke suggest that the increases of Aβ in AD leads to an aberrant excitatory network activity and compensatory inhibitory responses involving learning and memory associated circuits

(Palop and Mucke 2010). Moreover, considering the role of astrocytes in synapse formation and synaptic strength regulation, the ablation of astrocytes in these specific domains of the mouse brain causes neurodegeneration. Burda and Sofroniew noticed that reactive astrogliosis is characterized by astrocytic hypertrophy, proliferation, and augmented expression of intermediate filaments (glial fibrillar acidic protein [GFAP], vimentin, and nestin) (Burda and Sofroniew 2014). Until now, the relationship between amyloidosis, tau pathology, and astrogliosis remains enigmatic. The microglia are the phagocytic cells of the CNS and are also involved in the "cellular phase" of AD. These cells have ramified processes and a dynamic structure that interacts with neurons (Li, Du et al. 2012). Several studies show complement activation, increases in inflammatory cytokines in the cerebrospinal fluid (CSF), increased levels of reactive oxygen species, as other pathogenic mechanisms involved in AD (Hong, Beja-Glasser et al. 2016). Recent findings regarding the "inflammasome" activation led to connect it to the seeding and spreading of Aβ in patients with AD (Venegas, Kumar et al. 2017). The oligodendrocytes are the largest group of non-neuronal cells in the brain (Pelvig, Pakkenberg et al. 2008) and evidences supports also their role in the "cellular phase" of AD (Ettle, Schlachetzki et al. 2016). They produce myelin, facilitate neuronal transmission and importantly are dramatically decreased in number in the aging brain (Pelvig, Pakkenberg et al. 2008), which was supported by Bartzokis et al. with a cellular loss associated with myelin breakdown in magnetic resonance imaging scans of patients from age 50 (Bartzokis 2011). A more recent study by Heneka et al. is in line with the concept that the seeding and spreading of Aβ pathology in patients with AD are linked with the "inflammasome" activation, showing that that intrahippocampal injection of ASC specks in transgenic double mutant mice ($APP_{Swe}PSEN1_{dE9}$) leads to a spreading of Aβ pathology (Venegas, Kumar et al. 2017). Novel ideas will emerge, and an integrated conceptual framework will shed light in new potential therapeutical strategies that address different elements of the AD in a stage dependent manner.

Tau in physiology and pathology

The microtuble-associated protein tau was discovered in 1975 and since then it was shown to have a significant role in microtubule assembly and stabilization. A wide range of neurodegenerative diseases known as tauopathies, that include AD, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), agyrophilic grain disease (AGD), Pick disease (PiD), Huntington disease (HD) and frontotemporal dementia with parkinsonism-17 (FTDP-17) are characterized by

the aggregation of tau into paired helical filaments (PHFs) and NFTs (Wang and Mandelkow 2016). For many years, researchers made an effort to understand the role of tau in physiology and disease (Lee, Goedert et al. 2001). First, as mentioned before, Roberson et al. found that tau is necessary for Aβ induced toxicity (Roberson, Scearce-Levie et al. 2007). Second, the use of tau KO mice as research models has allowed the identification of novel physiological and pathological functions of tau in neurodegenerative diseases. Third, growing evidence suggests that tau pathology can propagate between neurons (Holmes and Diamond 2014).

The protein tau is mainly expressed in neurons, but is also present at low levels in glia. Importantly, tau has been described to be released into the extracellular space using models of cultured cells *in vitro* and by neurons in mouse brains *in vivo* (Pooler, Phillips et al. 2013). The microtubule-associated protein tau gene, *MAPT*, which comprises 16 exons and is located in chromosome 17, encodes human protein tau. The alternative splicing of exon 2, 3 and 10, yields six main isoforms. These isoforms differ by the absence (0N) or presence of one (1N) or two (2N) amino-terminal inserts of 29 amino acids each at the N-Terminal and can be categorized depending on whether they contain three (3R) or four (4R) carboxy-terminal repeat domains (Wang and Mandelkow 2016) (Figure 6). Interestingly, tauopathies can be divided into three groups based on the tau isoforms found in aggregates: 4R tauopathies (including PSP, CBD and AGD), 3R tauopathies (for example, PiD) and 3R + 4R tauopathies (for example, AD) (Dickson, Kouri et al. 2011).

Tau is a hydrophilic protein, stable under acidic conditions and in high temperatures. Its longest isoform (2N4R) contains 80 serine (Ser) or threonine (Thr) residues, 56 negative (aspartate or glutamate) residues, 58 positive (lysine or arginine) residues and 8 aromatic (5 tyrosine and 3 phenylalanine, but no tryptophan (Tyr)) residues. In the longest tau isoform (2N4R), there are as many as 85 potential phosphorylation sites (80 Ser Thr, and 5 Tyr) but only, 45 of these phosphorylation sites have been observed experimentally. The structure of tau can be subdivided into two major domains: the C-terminal assembly domain that comprises the repeat domains and flanking regions and is responsible for binding to microtubules and for tau aggregation; and the N-terminal projection domain which is the amino-terminal section that projects away from microtubules. The proline (Pro)-rich region, that links the two domains, contains multiple threonine-proline (Thr-Pro) or serine-proline (Ser-Pro) motifs that are targets of Pro-directed kinases, such as glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (CDK5), mitogen-activated protein kinase (MAPK) and JUN N-terminal kinase (JNK). In AD brain and other tauopathies, these

and other motifs can be hyperphosphorylated and be recognized by tau phosphorylation-dependent antibodies (for example, AT8, AT180, AT100, 12E8 and PHF1). Normal tau contains 2-3 mol phosphate/mol of the protein, the level of phosphorylation for its optimal activity (Iqbal, Alonso Adel et al. 2005). The main regulator of tau phosphorylation is phosphatase 2A (PP2A). This enzyme accounts for ≈70% of the total tau phosphatase activity in the human brain (Liu, Grundke-Iqbal et al. 2005). PP2A regulates the phosphorylation of tau by regulating the activities of several tau protein kinases, including calcium-calmodulin-dependent kinase II (CaMKII), protein kinase (PKA), CDK5 and GSK-3β (Iqbal, Alonso Adel et al. 2005).

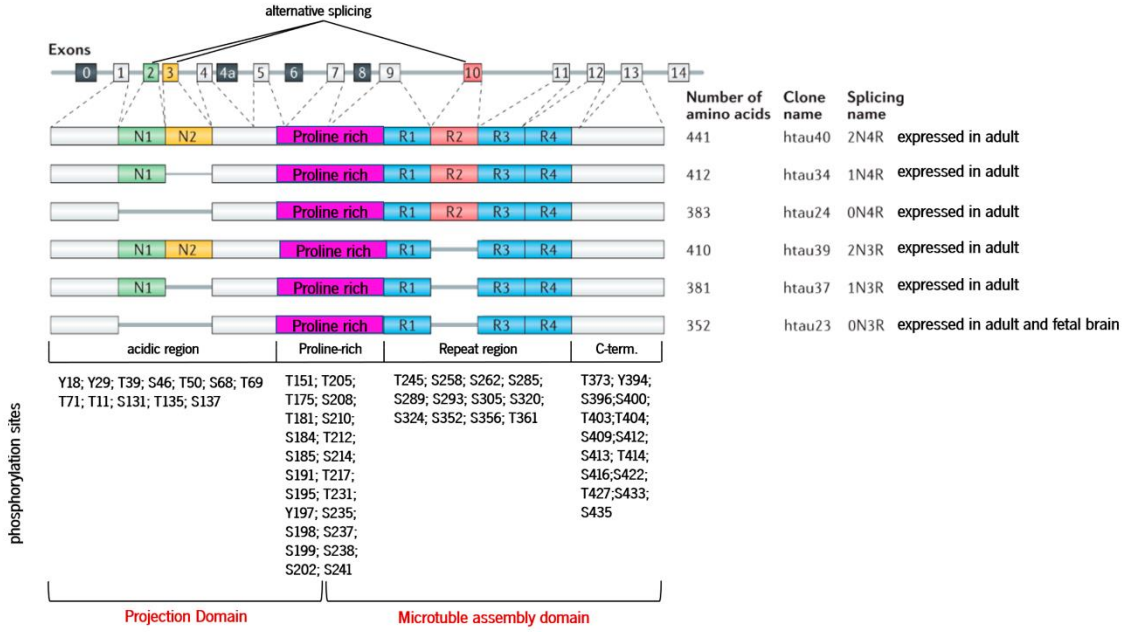

Figure 6. The human MAPT gene and the splice isoforms of tau in the human brain. The human tau gene is located on chromosome 17q21 and contains 16 exons. Alternative splicing of E2, E3, and E10 produces the six tau isoforms. tau isoforms differ by the absence or presence of one or two 29 amino acid inserts encoded by exon 2 (green) and 3 (yellow) in the N-terminal region, in combination with either three (R1, R3 and R4) or four (R1-R4) repeat regions in the C-terminal part. The R2 repeat is encoded by exon 10 (pink). The longest 2N4R adult tau isoform has 441 amino acids (aa) and the shortest 0N3R isoform of 352 aa. Sites in the acidic, proline-rich, repeat and C-terminal regions of tau were reported to be phosphorylated in vivo or in vitro (adapted from Wang and Mandelkow 2016).

The main function of tau protein at the cellular level is to promote the assembly of the microtubule protein subunit tubulin into microtubules and stabilize their structure promoting the maintenance of the overall neuronal cytoskeleton and cytoarchitecture (Drubin and Kirschner 1986). In addition, the tau protein has also been shown to be involved in neurotransmission through the Fyn pathway

(Lee, Thangavel et al. 2004). The microtubule assembly by tau is regulated by its degree of phosphorylation and hyperphosphorylation has been reported to suppresses this activity (Lindwall and Cole 1984). In AD, and other tauopathies, tau is abnormally hyperphosphorylated and is accumulated as intraneuronal tangles of PHF (Iqbal, Alonso Adel et al. 2005). Importantly, a recent report by Ittner et al. has added complexity to this matter, showing that specific tau phosphorylation sites have actually a protective effect (Ittner, Chua et al. 2016).

In a polarized neuron, tau is present in axons, with minor amounts found in the dendrites and nuclei, where it might play a part in maintaining the integrity of genomic DNA (Papasozomenos and Binder 1987, Sultan, Nesslany et al. 2011). Several factors may contribute to the axonal localization of tau, including: a faster turnover of tau in the somatodendritic compartment than in axons; more affinity of tau to microtubules (MTs) in axons than in dendrites (Hirokawa, Funakoshi et al. 1996); fast axonal transport of tau soon after its synthesis in the cell body (Kosik, Crandall et al. 1989); and an axonal initial barrier against the retrograde propagation of tau into dendrites (Li, Kumar et al. 2011). Importantly, one of the early signs of neurodegeneration is the missorting of tau into dendrites (Hoover, Reed et al.).

In familial tauopathies, the mutations of the *MAPT* gene seem to be the cause of tau aggregation, but in sporadic tauopathies (such as the majority of cases of AD), the trigger of tau pathology is unclear. The loss of function, toxic gain of function and mislocalization have been implicated in tau-mediated neurotoxicity. More than 80 mutations in human *MAPT* have been identified and linked not only to FTDP-17, but also to other tauopathies, such as CBD and PSP. They can be classified as missense or as splicing mutations. In missense mutations, the sequence of tau is altered, unlike in splicing mutations, where only changes in the relative ratio of different tau isoforms are present. In pathological conditions, tau may undergo post-translational modifications – for instance, hyperphosphorylation, acetylation, ubiquitylation or truncation, which could lead to the detachment of tau from microtubules, resulting in microtubule disassembly in axons. Detached tau may mislocalize into presynaptic terminals and induce synaptic dysfunction, causing a reduction in the number of synaptic vesicles in presynaptic terminals and synaptic loss (Decker, Krüger et al. 2015). Finally, tau may form aggregates that may be released into the extracellular space and be taken up again by other neurons, leading to the spread of tau pathology throughout the brain. The mechanism controlling neuronal transmission of tau pathology remains unclear, however it is believed to occur through endocytosis of tau oligomers by neurons in close proximity to affected neurons (Sanders, Kaufman et al. 2014), by synaptic dysfunction (Khan, Liu et al. 2014), and/or

by synaptic transmission from affected neurons to connecting naïve neurons (Liu, Drouet et al. 2012). Treatment with Aβ oligomers induces dendritic missorting of tau in primary neuronal cultures, which results in the translocation of tubulin tyrosine ligase-like enzyme 6 (TTLL6) into dendrites and subsequent severing of microtubules by spastin (Zempel, Luedtke et al. 2013). Furthermore, dendritic tau could serve as a protein scaffold to deliver the kinase FYN to postsynaptic sites. On the other hand, FYN phosphorylates the subunit 2 of the NMDA receptor (NR2B), allowing the stabilization of the interaction of this receptor with postsynaptic density protein 95 (PSD95) that potentiates glutamatergic signaling and enhances Aβ toxicity (Ittner, Ke et al. 2010). Despite the lack of knowledge concerning the mechanisms underlying tau-mediated neurotoxicity, multiple therapeutic approaches have been proposed based on tau function or dysfunction. However, the success and progress of therapeutics will depend on the availability of suitable disease animal models to test these hypotheses.

Role of lipids in Neurodegeneration

An increasing number of genome wide association studies (GWAS) have identified various genes associated with late onset AD (LOAD), also referred to as sporadic AD, that have been linked directly or indirectly to lipid metabolism or cellular membrane dynamics (Hollingworth, Harold et al. 2011, Naj, Jun et al. 2011). Some of these are clusterin/Apolipoprotein J, cholesteryl ester transfer protein and other gene risk factors for AD above mentioned. Taking into account what is known about AD pathogenesis, the following reasons, among others, further support this connection between lipids and AD: (1) neuronal lipid composition regulates the proteolytic activity of membrane-bound proteins, including APP, BACE1 and presenilins, controlling Aβ levels (Di Paolo and Kim 2011); (2) Aβ exerts is cytotoxic effects by modulating the activity of phospholipases, such as PLA2 (Sanchez-Mejia, Newman et al. 2008), PLC (Berman, Dall'Armi et al. 2008), and PLD2 (Oliveira, Chan et al. 2010); (3) Aβ forms aggregates with the ganglioside GM1, enhancing its cytotoxic effects (Matsuzaki, Kato et al. 2010).

Remarkably, the major genetic LOAD risk factor according to multiple GWAS is APOE, namely its ε4 isoform, a protein involved in cholesterol transport. Its role in amyloid pathology is supported by evidence that it binds Aβ and modulates the aggregation and clearance of Aβ (Bu 2009, Kim, Basak et al. 2009). The majority of cholesterol in the brain is derived from the endoplasmic reticulum, even though small amounts can be delivered to the brain from periphery, via high density

lipoproteins (HDL)(Di Paolo and Kim 2011). Previous studies showed that the APOE containing HDL-like particles inhibit the aggregation of Aβ, while free APOE promote Aβ aggregation in vitro (Hirsch-Reinshagen, Maia et al. 2005). The balance between free cholesterol and cholesterol esters is fundamental to control amyloidogenesis, although the molecular basis is not well understood.

It has been suggested that amyloidogenic processing of APP occurs in lipid rafts because active pools of BACE1 and γ-secretase are present in these microdomains. Cholesterol depletion decreases the pool of BACE1 localized in lipid rafts, which leads to a decrease in amyloidogenic processing of APP (Ehehalt, Keller et al. 2003). Finally, components of the γ-secretase complex, are also associated with lipid rafts. Therefore, inhibition of γ-secretase complex activity induces the accumulation of APP COOH-terminal products in lipid rafts (Vetrivel and Thinakaran 2010). Moreover, sphingolipids, including Cer, sphingomyelin (SM) and glycosphingolipids (GSLs), are essential components of lipid rafts, playing a central role in these cellular processes. Several *in vitro* studies point out that sphingolipids modulate the activity of γ-secretase and BACE1 as well as the localization of APP/Aβ, although more studies are needed to unravel the underlying mechanistic details. Studies in another category of lipids called glycerophospholipids, such as phosphoinositides and PA, have implicated these lipids in processes involving Aβ generation, namely the trafficking of APP and presenilin, and Aβ signaling (Chan, Oliveira et al. 2012).

Previous studies indicate that Aβ exerts its toxic effect by perturbing the integrity and signaling properties of cellular membranes. Thus, gangliosides, which are an abundant glycosphingolipid in neuronal membranes, promote the membrane-bound assembly (or aggregate) of Aβ. The most abundant gangliosides in the brain are GM1, GD1a (a-series) and GD1b and GT1b (b-series). Interestingly, gangliosides, such as GM1 act as a "seed" for Aβ aggregation in neurons and this feature increases with the aging process (Yanagisawa 2007). Genetic evidence shows that the ablation of GM2 synthase leads to an accumulation of GM3 and a loss of GM1 in a transgenic model of AD. The consequence is the accumulation of Aβ in brain parenchyma and in vascular smooth muscle tissue (Yanagisawa 2007). Previous research has highlighted the potential benefits of therapies based in gangliosides in transgenic AD models, in which injections of GM1 were shown to decrease Aβ burden by promoting the degradation of Aβ in the periphery (Matsuoka, Saito et al. 2003).

Taking into account that synapses are major sites of Aβ release, the synaptic activity could be affected by enhanced Aβ release (Cirrito, Yamada et al. 2005). Moreover, the synaptic dysfunction

leads to a cognitive impairment in the early stage of AD, which is crucial to understand the role of lipid changes in mediating Aβ synaptotoxic signaling pathway (Palop and Mucke 2010). Until now important studies have shown that Aβ can modulate cell surface levels of AMPA and NMDA receptors and affect calcium homeostasis, suggesting that Aβ can alter the signaling properties and permeability of synaptic membranes by forming pores that are permeable to various ions (Arispe, Rojas et al. 1993). The Aβ peptide has a central action in lipid metabolism by causing dysregulation of calcium-sensitive phospholipid-metabolizing enzymes such as PLC, cytosolic phospholipase A2 (cPLA2, also known as GIVA-PLA2) and PLD2 (Green and LaFerla 2008).

Despite the fact Aβ is probably a key initiator in AD pathogenesis, it has been shown that aggregation and accumulation is poorly correlated with disease symptoms or tissue loss (Josephs, Whitwell et al. 2008). However, there is strong evidence showing that the accumulation of tau in AD brains and in other tauopathies is correlated with clinical signs and neurodegeneration. A recent study on this topic found that APOE affects tau pathogenesis and regulates tau-mediated neurodegeneration independently of Aβ (Shi, Yamada et al. 2017). The absence of APOE attenuates the neuronal loss and brain atrophy observed in a P301S tauopathy mouse model (Shi, Yamada et al. 2017). This further highlights the role of tau in AD pathogenesis, not only downstream of Aβ, but also as player in APOE dependent disease mechanisms, which are highly relevant for sporadic AD.

Phospholipase D and AD

Several studies have addressed the link between PLD and AD. Kanfer et al. found an increase in PLD activity in AD brain extracts comparatively with control subjects (Kanfer, Singh et al. 1996). Oliveira and Di Paolo observed that Aβ42 oligomers at 200nM enhance total PLD activity in primary cortical neurons and that this increase is abolished in mouse cultured neurons lacking PLD2. They also found that the genetic ablation of PLD2 confers protection against the cytotoxic effects of Aβ42 on LTP and protected from the memory impairment action of SwAPP overexpression *in vivo* (Oliveira, Chan et al. 2010).

The molecular mechanism underlying the subcellular localization and intracellular sorting of APP and its cleaving enzymes remains unclear. It has been demonstrated that a perturbation of the trafficking of APP, BACE1 and γ-secretase complex could affect amyloidogenesis with an impact in

AD pathogenesis (Small and Gandy 2006). APP, as a transmembrane protein, has the ability to traffic through the secretory pathway toward the cell surface, where it can reach the endosomal system. Moreover, it has been suggested that reducing the APP trafficking back to the trans-Golgi network (TGN) results in an increase of amyloidogenesis. This suggests that the acidic pH of endosomes is optimal to APP/BACE1 interaction and towards proteolysis mediated by BACE-1 (Small and Gandy 2006). Importantly, several studies addressing the role of PLD in intracellular trafficking of APP have implicated PLD1, as well as its product PA, in the budding of secretory vesicles from the TGN (Chen, Siddhanta et al. 1997). Furthermore, it was shown that presenilins can regulate the trafficking of APP independently of its catalytic activity. The cells expressing an FAD mutant version of PS1 contain higher levels of APP, thus resulting in increased cell surface delivery of APP. Indeed, the overexpression of PLD1 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 mutant ΔE9 (Chen, Siddhanta et al. 1997).

Cai et al. also addressed the role of PLD1 in amyloidogenesis. First, it was shown that PLD1 physically interacts with PS1 and cause the recruitment of PLD1 to the Golgi complex. Coimmunoprecipitation studies showed that PLD1 regulates the assembly of the γ-secretase complex through a direct interaction with PS1 (Cai, Netzer et al. 2006). This indicates that while overexpression of PLD1 decreases the catalytic activity and the association of γ-secretase subunits, the down-regulation of PLD1 increases it (Cai, Netzer et al. 2006).

Interestingly, phosphoinositides are the major signalling phospholipids in cellular membranes and particularly, PI(4,5)P2 and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) are very relevant for processes occurring at the cell surface. Berman and Di Paolo found that Aβ-induced synaptic dysfunction can be ameliorated by genetic manipulations that maintain the normal PI(4,5)P2 balance in the brain, showing for instance that this was partially dependent on PLC increased activity induced by Aβ (Berman, Dall'Armi et al. 2008). Since PA is a precursor of PI(4,5)P2 synthesis it is still unclear if PLD1 or PLD2 could affect specific pools of PI(4,5)P2 that could affect Aβ signalling.

Finally, studies addressing PLD3 have identified it as a risk factor for late-onset AD (LOAD) (Cruchaga, Karch et al. 2014). PLD3 presents high expression in cortex and hippocampus and is expressed lower levels in neurons from the brains of patients with AD than in control brains, but further characterization of this new isoform is required to address its potential role in lipid signaling

(Wang, Yu et al. 2015). However, until now, no lipid modulating activity has been shown for PLD3, suggesting that its role in AD could be independent of lipid signalling regulation. Cruchaga et al. observed that the overexpression of PLD3 leads to a decrease of APP levels and extracellular Aβ, on the other hand the PLD3 knockdown has an opposite effect (Cruchaga, Karch et al. 2014). However, Fazzari et al. studies observed no alterations in APP levels under milder PLD3 overexpression (Fazzari, Horre et al. 2017). Immunocytochemistry studies, shown that PLD3 is a lysosomal protein that becomes proteolytically cleaved in acidic compartments (Gonzalez, Schweizer et al. 2018). Interestingly, the inhibition of intracellular trafficking or lysosomal acidification stopped this processing and supports its lysosomal localization (Gonzalez, Schweizer et al. 2018). Moreover Gonzalez et al. proposed that the biosynthetic route of PLD3 depends on the endosomal sorting complex required for transport machinery to reach lysosomes in mammalian cells (Gonzalez, Schweizer et al. 2018).

Importantly, despite the different subcellular localization, expression levels/profile, and regulation of PLD1, PLD2 and PLD3, they have the ability to control several aspects in the biology of key proteins involved in AD and future studies are needed to understand how these enzymes regulate each other.

Model systems to study neurodegenerative diseases – from *in vitro* models to rodent and nematode models.

To better understand the cellular and molecular mechanisms underlying the pathological progression in AD, it can be relevant to use a holistic approach using different models. Specifically, *in vitro* models, such as neuroblastoma cultured cells that have synaptic structures, functional axonal vesicle transport and express neurospecific proteins, are widely used as a model for the study of nerve cells since it develops neuronal like processes.

Furthermore, animal models have been developed using various genetic, biochemical, or dietary manipulations to approximate full-blown symptoms of the disease. Transgenic mouse models of AD continue to be refined and to make tremendous contributions to the knowledge about mechanisms relating to Aβ production, deposition, and clearance and in terms of engendering strategies for therapeutics. There are mouse models of amyloid pathology that address this issue inserting transgenes encoding human mutations associated with familial forms of AD, targeting

APP and PS1. Two examples are the B6.129-Tg(APPSw)40Btla/Mmjax with Swedish mutation, the APP/PS1ΔE9 line, that combines APP and PS1 mutations to generate a model that displays extensive amyloid pathology, and the B6. Cg-Tg (PDGFB-APPSwInd)20Lms/2J/Mmjax (J20) mice with two mutations linked to familial AD (the Swedish and Indiana mutations). Mouse models of Tau pathology, tauP301L, with the P301L mutation that is also associated with a familial form of frontotemporal dementia, generates hyperphosphorilated tau tangles. Mouse models of both amyloid and tau pathology like the 3xTgAD line that combines $MAPT^{P301L}$ and $APP^{K670N/M671L}$ transgenes by the simultaneous embryonic microinjection into mouse embryos carrying a PS1 mutant knock-in (Platt, Reeves et al. 2013) are another example where both tau pathology and amyloidogenesis is artificially modeled in a transgenic mouse. However, the great variety of AD-relevant mouse models should be used appropriately and in conditions that are relevant for the scientific questions that are being addressed concerning AD pathogenesis.

In order to dissect other specific functions of Aβ/APP and tau, alternative models, such as the simpler animal model, *C. elegans* emerged as a meaningful tool to provide complementary approaches. In 1963, Sydney Brenner introduced the nematode *C. elegans* as a model to study development and neurobiology (Brenner 1974). *C. elegans* is a highly advantageous model organism due to their transparency, which makes it easy for manipulation and observation and easiness to maintain in a laboratory. It presents a rapid reproduction cycle in only 3 days developing from egg to an adult worm, it has a small size (about 1 mm in length) and the fact that all 302 neurons have been mapped and described provides a unique neuroanatomical simplicity that can be specifically targeted. *C. elegans* are more than 99% self-fertilizing hermaphrodites and 1% males. When hermaphrodites mate with males, 50% of the progeny will be males and 50% will be hermaphrodites. In the laboratory, self-fertilization of hermaphrodites or crossing with males can be manipulated to produce progeny with desired genotypes that are especially useful for genetic studies. The genome size of *C. elegans* is about a hundred million base pairs. This is approximately 20 times bigger than that of *Escherichia coli* and about 1/30 of that of human. Moreover, as its genome is surprisingly similar to that of humans (40% homologous), *C. elegans* becomes an attractive organism in the study of human diseases (Kenyon 1988). Despite the fact that the nervous system of this model is small, these animals display complex behaviours that address the neuronal function and dysfunction (Bargmann and Kaplan 1998). For instance, a simple and complex behavioural analysis of *C. elegans* with laser-ablated neurons was performed, including chemotaxis, thermotaxis, several responses to touch, male-specific mating rituals, social feeding,

and both associative and non-associative learning, and allowed the identification of the function of small groups and even individual neurons.

Taking into account the potential of using *C. elegans*, this model has been employed to study a number of neurodegenerative disorders, including AD. The existence of APP, PS and tau homologs in worms has been essential in the identification of the biological function of these proteins. The goal of engineering transgenic *C. elegans* has not been to generate "senile" worms but to underlie molecular and cellular processes of AD pathology. Indeed, the absence of endogenous Aβ production in *C. elegans* presents an opportunity to disentangle the role of multiple processes involved in AD. Link and colleagues developed transgenic *C. elegans* models that have inducible expression of Aβ in different cell types. One of these models presents pan-neuronal expression of Aβ (strain CL2355) and it is useful to study the toxic effects of the Aβ peptide, while evaluating behaviors that rely in the whole animal homeostasis (Link 2005). Furthermore, Kraemer et al generated a *MAPT* transgenic (Tg) *C. elegans* model of FTDP-17 in order to identify the critical steps in tau-induced neuronal-like processes.

In summary, the neuroblastoma cell line is an optimal model to access protein-protein and lipid - protein interactions. *C. elegans* offers advantages in terms of the high degree of experimental control and the relatively short life span of the organism. Transgenic AD mouse models confer the possibility to identify lipid signaling pathways relevant in AD brain pathogenesis, since its brain lipidomic composition is closer to the human lipidome composition. Also, these mouse models allow the comparison with relevant behaviors that are affected in a similar way in humans, such as memory-related and seizure-like behaviors. Using multiple and diverse approaches spanning from cell lines, nematode and mouse animal models, we shed here some light in potential mechanisms involved in neurodegeneration in AD.

Aims of the Study

The main goal of this thesis project is to understand the role of the signaling lipids in the nervous system physiology and pathology and gain mechanistic insights concerning the role of the PLD pathway in neurodegenerative diseases.

The specific aims of this project are:

1. To test the effects of PLD ablation in an AD *C. elegans* model.
2. To investigate the role of the PLD pathway in the regulation of tau.
3. To characterize the effects of seizure severity on the hippocampus lipidome in an AD mouse model.

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Chapter 2

Experimental work

Chapter 2.1

Bravo FV, da Silva JD , Chan RB, Di Paolo G, Teixeira-Castro A, Oliveira TG

Phospholipase D functional ablation has a protective effect in an Alzheimer's disease *Caenorhabditis elegans* model

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Phospholipase D functional ablation has a protective effect in an Alzheimer's disease *Caenorhabditis elegans* model

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Phospholipase D (PLD) is a key player in the modulation of multiple aspects of cell physiology and has been proposed as a therapeutic target for Alzheimer's disease (AD). Here, we characterize a PLD mutant, *pld-1*, using the *Caenorhabditis elegans* animal model. We show that *pld-1* animals present decreased phosphatidic acid levels, that PLD is the only source of total PLD activity and that *pld-1* animals are more sensitive to the acute effects of ethanol. We further show that PLD is not essential for survival or for the normal performance in a battery of behavioral tests. Interestingly, *pld-1* animals present both increased size and lipid stores levels. While ablation of PLD has no important effect in worm behavior, its ablation in an AD-like model that overexpresses amyloid-beta (Aβ), markedly improves various phenotypes such as motor tasks, prevents susceptibility to a proconvulsivant drug, has a protective effect upon serotonin treatment and reverts the biometric changes in the Aβ animals, leading to the normalization of the worm body size. Overall, this work proposes the *C. elegans* model as a relevant tool to study the functions of PLD and further supports the notion that PLD has a significant role in neurodegeneration.

Alzheimer's disease (AD) is the most common form of late-onset dementia. One of the main pathological hallmarks of AD is the accumulation of amyloid-beta (Aβ) plaques in the brain, derived from the sequential cleavage of the amyloid precursor protein (APP) by beta and gamma secretases[1]. Presently, there are no effective therapeutical options for AD and one potential strategy being pursued is to block Aβ pathological signaling. Remarkably, using amyloidogenesis AD mouse models, it was shown that the genetic ablation of a myriad of putative Aβ signaling downstream players, such as tau[2], PrP[3], GIVA-phospholipase A2 (GIVA-PLA2)[4] or phospholipase D2 (PLD2)[5] ameliorates rodent behavioral cognitive deficits, independently of APP processing or Aβ levels modulation.

Lipids are a major constituent of the brain and specifically signaling lipids have been shown to regulate brain functioning and to modulate various neurodegenerative processes[6]. Indeed, Aβ has been shown to activate a group of lipid modulating enzymes, such as PLC[7], GIVA-PLA2[4] and PLD[8]. While the PLC and PLA2 pathways are well studied, less is known about the PLD pathway. In mammals, six members of the PLD superfamily have been identified[9]. From these, there are two canonical PLD isoenzymes, PLD1 and PLD2, which are structurally similar and enzymatically both convert phosphatidylcholine (PC) to phosphatidic acid (PA), but differ in their intracellular localization and mechanisms of regulation[8,10]. Interestingly, in the presence of primary alcohols, such as ethanol, PLD preferentially uses it as a substrate, producing a specific lipid, phosphatidylethanol (PEtOH), which is often used to measure PLD activity[8]. Even though PLD2 has been shown to be involved in Aβ signaling[5], PLD1 has been proposed to modulate APP trafficking and processing[11,12]. As an approach to understand the role of the PLD pathway in physiology and in a pathological context, the study of PLD mutant associated phenotypes

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in several model organisms, such as nematodes, drosophila[13,14] and mice[5,15], can give key insights. Importantly, while in mice there are two PLD isoenzymes, in drosophila and in nematodes there is only one PLD enzyme[16].

The study of neurodegenerative diseases in simple organisms, such as *Caenorhabditis elegans*, when appropriately adapted to the nematode physiology, provides a powerful tool in the identification of relevant pathological pathways[17]. For instance, the strain CL2355, which overexpresses human Aβ in neurons and presents multiple aberrant behaviors[18–20], has been proposed to be an effective model to study Aβ pathological signaling.

Here, we studied the impact of PLD genetic ablation in *C. elegans* in a physiological context and upon crossing it with an AD-like model. We showed that PLD ablation leads to a decrease in PA levels and that PLD is the only source of PEtOH upon ethanol treatment. While we found no major behavioral deficits, we observed a small increase in the worm volume. Remarkably, PLD ablation restored not only worm volume in an AD-like model, but also had a protective effect in motor behaviors and in sensitivity to serotonin and pharmacologically-induced seizures, suggesting a disease-modifying role for PLD in *C. elegans*.

Results

pld-1 worms present decreased PA levels and PLD activity. In order to address the role of PLD in *C. elegans* lipid metabolism (Supplementary Fig. S1), we performed a lipidomic analysis to biochemically characterize the *pld-1 C. elegans* model. Since PLD converts PC to PA, we measured the levels of PA in N2 and *pld-1* worms, using liquid chromatography-mass spectrometry (LC-MS). We found that PLD mutants present a ~50% decrease in total PA levels (Fig. 1A). Specifically, six molecular species of PA (based on their different fatty acyl composition), namely PA 32:1, PA 34:1, PA 36:1, PA 38:1, PA 38:4 and PA 40:6 were diminished, while a trend for a decrease was observed for the other species (Fig. 1B). Since PLD is not the only source of PA, we developed an *in vivo* PLD activity assay, relying on the incubation of worms for 1 hour with 1% ethanol and subsequent measurement of phosphatidylethanol (PEtOH), a lipid uniquely produced by PLD. We found a major decrease in multiple PEtOH species in *pld-1* comparing with N2 worms (Fig. 1D), which indicates that PLD is the only source of PEtOH in *C. elegans*. Next, we performed an ethanol susceptibility assay, since PLD uses ethanol as a substrate to produce PEtOH. Animals were exposed to different doses of ethanol and their mean speed was evaluated. An aldehyde dehydrogenase mutant (*alh-6*) was used as an ethanol-sensitive control strain and we observed that in two doses (100 and 200 mM), *pld-1* worms were more susceptible than N2 animals to the acute ethanol effects, similarly to *alh-6* animals (Fig. 1F). Moreover, in order to test if the fraction of ethanol metabolized by PLD would be significant in the context of total levels of ethanol, we compared total ethanol levels in N2 animals and with *pld-1* animals after one hour of treatment and we observed no significant differences (1.00 ± 0.10 and 0.98 ± 0.08, in N2 vs. *pld-1*, resp. as relative levels to control, from three independent experiments n = 250 animals per experiment). Taken together, these results show that PLD ablation in worms leads to decreased PA levels, decreased total PLD activity and impacts the sensitivity to ethanol acute effects.

PLD ablation causes no gross phenotypes in _C. elegans_. In order to perform a characterization of *pld-1* animals, we ran a battery of tests. We first found that the number of progeny (Fig. 2A) and subsequent development (Fig. 2B), assessed by the percentage of adult worms in the total population, was unchanged. Additionally, motor behavior, which was evaluated by assessing locomotion defects, was not affected in *pld-1* worms (Fig. 2C). Considering that *C. elegans* has different types of sensory neurons, we performed chemotaxis assays to evaluate the ability of *pld-1* worms to approach or avoid a compound, and we observed no differences in the chemotactic responses for all tested chemicals (Fig. 2D and E). Moreover, we performed an associative learning task and no deficits were observed in *pld-1* worms (Fig. 2F). In order to evaluate the effect of PLD ablation on survival, we performed a lifespan assay. No differences were observed in the median lifespan of *pld-1* worms when compared to N2 (Fig. 2G) (Supplementary Table S2). Furthermore, in order to explore the role of PLD in PA-mediated cell signaling, we performed a dopamine susceptibility assay. Dopamine binds to dopaminergic receptors coupled to a G-protein, activating several effectors, including PLC. The activation of PLC leads to an increase in diacylglycerol (DAG) that could then be converted to PA. As previously described, DAG kinase 1 mutant worms (*dgk-1*) were shown to be resistant to dopamine[21]. However, even though DGK and PLD are both sources of PA, *pld-1* animals presented no differences in the susceptibility to dopamine induced locomotion impairment (Fig. 2H). Thus, our observations suggest that PLD ablation has no impact in worm lifespan and causes no gross behavioral alterations.

PLD modulates the body size of _C. elegans_. As part of our characterization of PLD mutants, we performed a biometric analysis and interestingly we observed that *pld-1* animals consistently presented an increase (~10%) volume of the body when compared to N2 worms (Fig. 3A and B). Importantly, we saw no differences in the defecation cycles (Fig. 3C) and the pharyngeal pumping rates (Fig. 3D) of well-fed mutants. Using Nile red fluorescence, an indicator of neutral lipid content, we showed that *pld-1* animals presented an increase (~30%) in lipid accumulation (Fig. 3E and F). It was previously shown that cholesterol deprivation leads to developmentally-induced volume reduction in the F2 generation[22,23]. We evaluated the impact of PLD ablation in this lipid induced developmental volume deficit and we observed that *pld-1* animals had no volume differences compared to N2 in cholesterol-deprived F2 generation animals (Fig. 3G). These results suggest that PLD regulates worm body size and lipid stores.

PLD functional ablation ameliorates Aβ phenotypes in an AD-like model. We had previously observed that the CL2355 strain (Aβ), which has a pan-neuronal expression of Aβ after induction by temperature up-shift to 23 °C, presented a major decrease (~40%) in worm volume (Fig. 4A). We then crossed the *pld-1* strain with the Aβ strain to test the impact of PLD functional ablation in an AD-like model. Remarkably, *pld-1*; Aβ animals presented significantly higher volumes than Aβ worms (Fig. 4B). Taking into account our previous

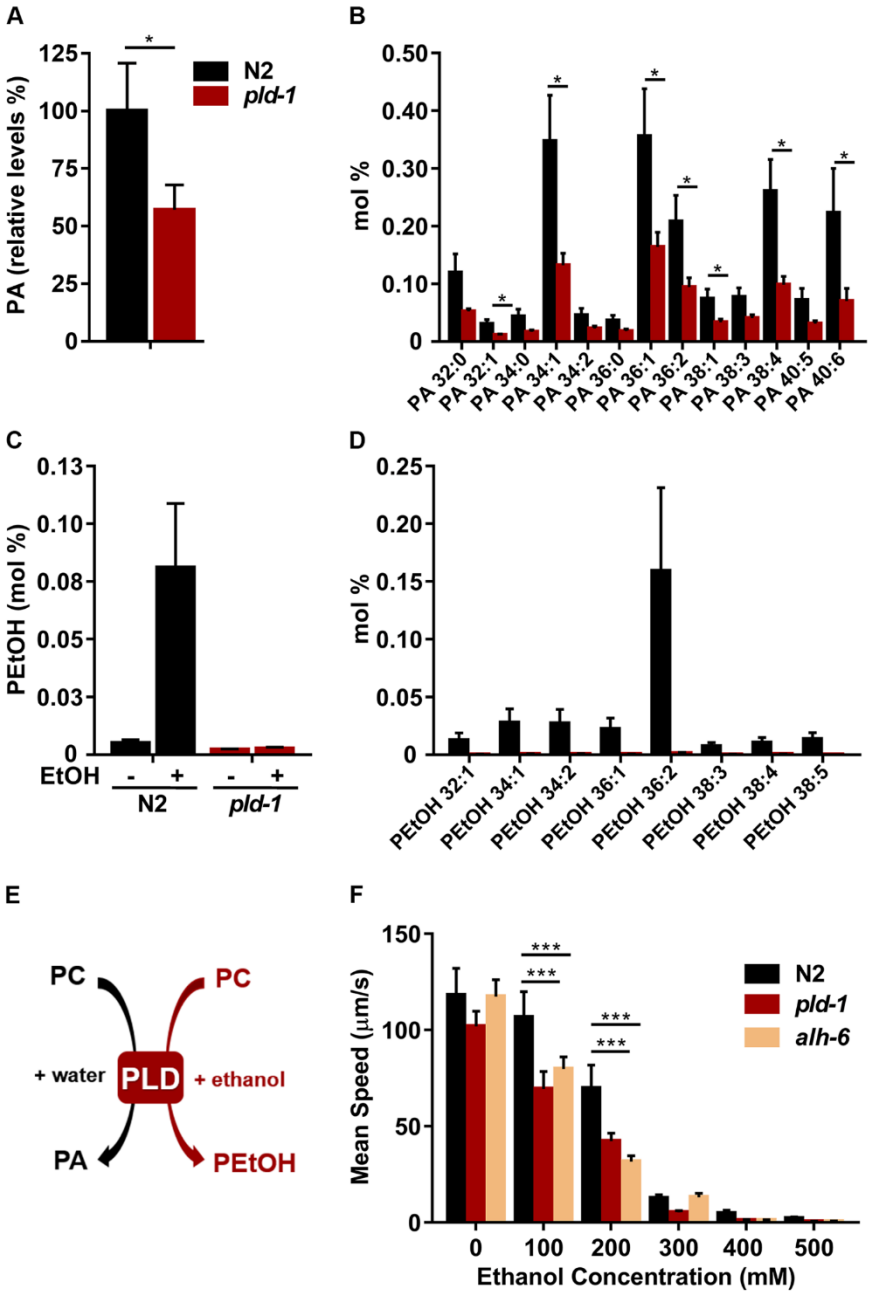

Figure 1. *pld-1* animals show decreased PA levels and PLD activity. (**A**) *pld-1* ablation causes a 50% reduction on total phosphatidic acid (PA) relative levels in *C. elegans*. (**B**) Relative amounts of the different PA species measured in *pld-1* and WT animals. Levels of PA were quantified by Liquid Chromatography Mass Spectrometry (LC-MS) analysis (n = 6). (**C**) Relative amounts of total PEtOH (**D**) and of the different PEtOH species measured in *pld-1* and WT animals (n = 6) and quantified by LC-MS. (**E**) Graphical representation of the synthesis reaction of phosphatidylethanol (PEtOH) *in vivo*. PLD has an increased affinity to primary alcohols, leading to the generation of PA in the presence of water, or PEtOH if in the presence of ethanol. This reaction is the only source of PEtOH and can be used as a measure of PLD activity. (**F**) *pld-1* animals are more susceptible than WT to the acute effects of ethanol (at 100 and 200 mM), similarly to the ethanol-susceptible strain *alh-6*, as shown by the decrease in the mean speed of the animals (n = 6). Values denote mean ± SEM (*$p < 0.05$, ***$p < 0.001$). The nomenclature for PA and PEtOH fatty acid species composition is expressed as total chain length: number of unsaturated bonds.

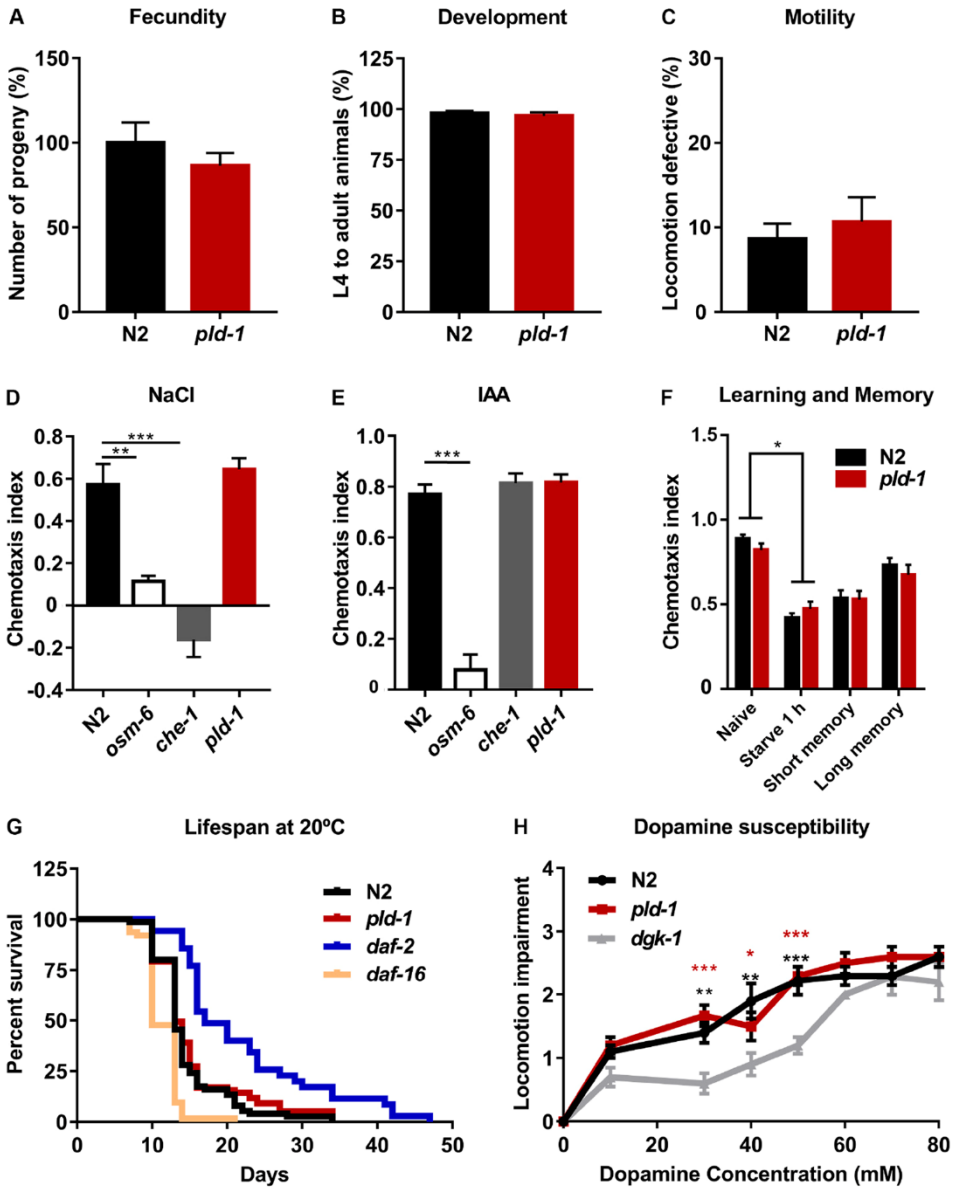

Figure 2. *pld-1* animals show no major phenotypic alterations. (**A**–**C**) Effect of PLD ablation on *C. elegans* egg-laying, development and locomotion behavior. (**A**) Number of progeny of adult hermaphrodite N2 and *pld-1* animals for 5 h. The progeny of 10 worms was counted per experiment (n = 3). (**B**) Percentage of L4 to adult N2 and *pld-1* animals after 48 h post egg laying. At least 150 animals were averaged per dataset (n = 3). (**C**) The percentage of locomotion defective age-synchronized adult animals (day 3 post-hatching) was measured by scoring the animals that remain inside a 10 mm circle, 1 min after being placed on its center. The number of animals used per trial was 10 (n = 3). (**D**,**E**) *pld-1* worms have a normal chemotaxis response. Chemotaxis response to NaCl and isoamyl alcohol (IAA) was evaluated in N2, *pld-1*, *che-1* and *osm-6* animals. Mutant strains unable to detect NaCl (*osm-6* and *che-1*) and IAA (*osm-6*) were used as negative controls. Three independent experiments were performed (n = 200 worms per assay). (**F**) *pld-1* animals have no deficit in an associative learning task. N2 and *pld-1* short-term and long-term associative memory profile after 1 h and 24 h of conditioning. Representative example of three independent experiments is shown (n = approximately 100 worms per chemotaxis assay plate). (**G**) *pld-1* worms have a normal lifespan. Kaplan-Meier survival curve of *pld-1* worms, show no difference in the median lifespan. Survival rate was scored everyday and is expressed as percentage of survival. *daf-2* and *daf-16* strains were used as long and short-lived controls, respectively. The data results from the analysis of 100 worms per strain in 3 independent experiments. (**H**) Dose-response curves measuring paralysis induced by exogenous dopamine. Locomotion impairment of animals moving 30 min after being placed on agar plates containing the indicated concentrations of dopamine is shown. N2 and *pld-1*

animals show no differences to N2 in sensitivity to dopamine, whereas *dgk-1* animals exhibit resistance to paralysis induced by exogenous dopamine. The data for three independent experiments is represented (n = 10 animals for each dopamine concentration). Values denote means ± SEM, ns-non-significant. (**$p \leq 0.01$, ***$p \leq 0.001$).

work showing that *Pld2* genetic ablation had a protective effect in synaptic and behavioral deficits in an AD amyloidogenesis mouse model[5], we tested if the functional ablation of PLD in worms had not only an effect in the volume of Aβ worms but also in other phenotypes. First, we performed a lifespan assay, conducted at 23 °C, in order to evaluate the effect of neuronal Aβ expression on overall survival. We observed a significant decrease in the median life span of Aβ worms compared to N2 and interestingly *pld-1*; Aβ animals presented increased median survival relative to Aβ worms (Fig. 4C) (Supplementary Table S3). Moreover, while we observed diminished egg-laying in Aβ animals (Fig. 4D), the ablation of PLD in the Aβ background resulted in an increase in the number of laid eggs (Fig. 4D) (Supplementary Fig. S3). Egg viability was not affected by either Aβ or PLD ablation (Fig. 4E). Concerning motor task assessment, both crawling and swimming were shown to be impaired in Aβ worms and, again, PLD ablation partially restored these deficits in *pld-1*; Aβ worms (Fig. 4F and G). We also evaluated the sensitivity of animals to serotonin, since it was previously shown that Aβ transgenic worms are more sensitive to this neurotransmitter[18]. Notably, *pld-1*; Aβ worms presented reduced sensitivity to serotonin-induced impairments when comparing with Aβ worms (Fig. 4H). Finally, we performed a pharmacologically-induced pro-excitatory assay using pentylenetetrazol (PTZ), a GABA receptor antagonist that increases neuronal excitability by disrupting the normal excitatory/inhibitory balance[24]. We exposed worms to different doses of PTZ and measured seizure severity. We showed that while Aβ transgenic worms have increased susceptibility to the effects of PTZ, PLD functional ablation confers a protective effect in PTZ-susceptibility induced by Aβ expression (Fig. 4I) (Supplementary Table S5). Taken together, these observations indicate that PLD ablation protects from Aβ-induced deficits.

Discussion

PLD is a lipid modulatory enzyme involved in multiple aspects of cell physiology, such as signaling and membrane trafficking processes. Furthermore, it has been implicated in pathologic conditions like cancer and neurodegenerative diseases[8,25]. To understand the role of PLD, mutant models have been developed using different organisms, such as mice[5,15] and flies[13,14]. While in mammals there are two canonical isoenzymes, PLD1 and PLD2, in flies and nematodes there is only one PLD ortholog[16].

Here, we present an extensive characterization of the effects of PLD ablation using a *C. elegans* model. We show that PLD mutants, *pld-1*, have decreased levels of PA (Fig. 1A) and that PLD is the only source of PEtOH (a specific product of PLD activity) (Fig. 1C). This is in accordance with the results from another model organism, showing that with the genetic ablation of PLD in drosophila, PLD was the only source of PLD activity[13]. In mammals, the contribution to total PLD activity by either PLD1 or PLD2 depends on the tissue or cell type. For instance, it was previously shown in the brain that while PLD2 ablation leads to decreased total PLD activity[5], no changes are observed in total PA levels[5,26]. In another study, it was observed that PLD1 ablation led to decreased PLD activity in the liver and while there were no differences in total PA levels, PA supplementation restored autophagy deficits in PLD1 knock-out cells[27]. Moreover, we show that PLD metabolizes ethanol, producing PEtOH, and that *pld-1* animals are more sensitive to ethanol-induced slowing (Fig. 1F). While PEtOH measurements provide direct evidence that PLD metabolizes ethanol acutely, we observed no differences in total ethanol levels after one hour treatment, suggesting, at least in *C. elegans*, PLD has a minor role for ethanol elimination. The increased sensitivity of *pld-1* animals to acute ethanol effects could be explained alternatively by a diversion of PA synthesis or due to potential protective effects by PEtOH. Accordingly, previous reports showed that, not only chronic ethanol exposure, with associated production of PEtOH[28,29], but also PEtOH itself[30], induce resistance to acute ethanol effects on membranes, which suggests that PEtOH could have a protective role in acute ethanol exposure. Since in mammals there are two PLDs, the specific role of either PLD1 or PLD2 in ethanol-induced toxicity should be differentially studied.

Our results show that PLD is not essential for the survival or for the normal functioning of various behavioral tasks in a *C. elegans* model (Fig. 2). In mice, both PLD1 and PLD2 knock-out animals are viable[5,15]. Even though it was reported that PLD1 or PLD2 ablation led to decreased juvenile brain volume and to social and object recognition deficits[31], no major behavioral deficits were observed by other research groups in PLD2 knock-out adult mice[8,26], apart from olfaction deficits in aged animals[26].

Concerning biometric and metabolic parameters, deletion of either of the PLD enzymes led to elevated body weight and increased adipose tissue content in aged animals[32]. However, while others did not observe elevated body weight in PLD2 knock-out animals[33], PLD1 knock-out animals presented not only increased hepatic weight, but also increased triacylglycerol levels and increased cholesterol levels[27]. Here, in a *C. elegans* model, we observe that *pld-1* animals present both increased size and lipid stores (Fig. 3). We show that PLD is a main source of PA in *C. elegans* (Fig. 1A), so it is possible that the effects of PLD perturbation could be due to altered PA metabolism. Therefore, other enzymes, which modulate PA levels, such as lipin (*i.e.*, a phosphatic acid phosphatase), could potentially be involved in the regulation of convergent physiologic mechanisms. Curiously, it was previously shown that silencing of the *C. elegans* homolog of lipin (*lpin-1*) leads to reduced body size and defects in lipid storage[34]. It was also observed in *C. elegans* that depletion of PC synthesis enzymes stimulates sterol regulatory element binding protein (SREBP) transcription factors, increases both fat-7 levels and lipid stores[35] and in a follow-up study lpin-1/Lipin-1 knock-down reduced the effects of PC-depleted conditions in a PA dependent way[36]. Since lipin-1 converts PA to DAG, our data raises the possibility that in *C. elegans*, the PA species derived

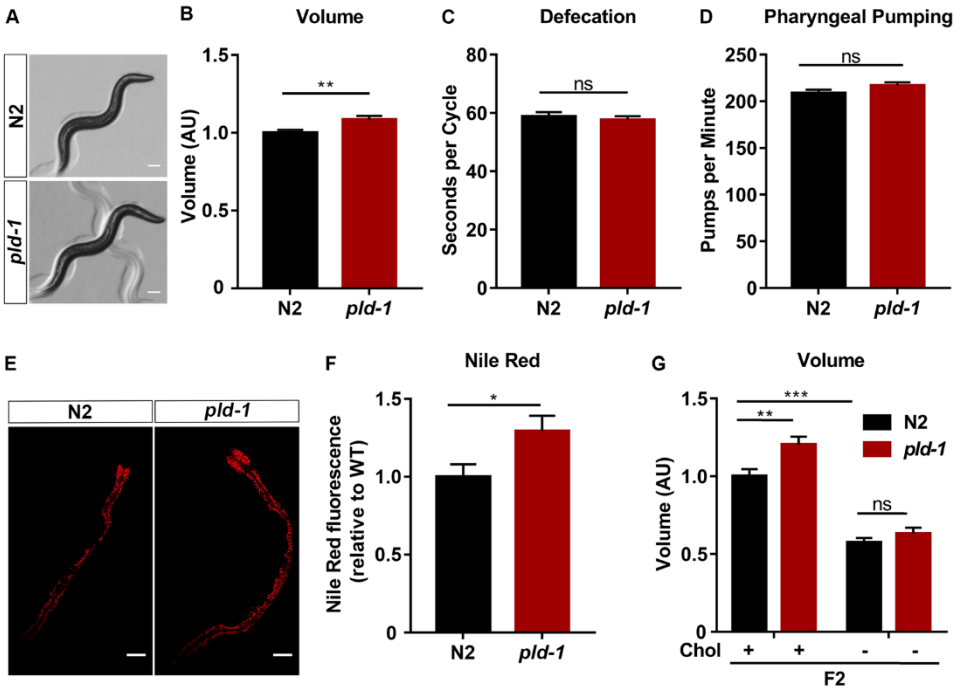

Figure 3. PLD modulates *C. elegans* volume. (**A**,**B**) *pld-1* worms have an increase in body volume. Representative photos (**A**) and graphic analysis of volume (**B**) of N2 and *pld-1* animals at day 3 after egg laying. The scale bar represents 100 µm. (**B**) 40 animals were analyzed per strain in 3 independent experiments. (**C**,**D**) *pld-1* worms have no alterations in physiological parameters, namely (**C**) defecation cycle length and (**D**) pharyngeal pumping rate (n = 3, 10 animals per assay). (**E**,**F**) *pld-1* worms have changes in lipid balance. Nile Red staining representative photos (**E**) and quantification of Nile Red fluorescence of N2 and *pld-1* worms at day three post-hatching. Photographs were taken at 60 × magnification. Scale bar represents 100 µm. (**G**) *pld-1* and N2 animals present similar volumes upon cholesterol depletion. Volume analysis of F2 generation (N2 and *pld-1*) animals at 72 h after egg laying, in the presence or absence of cholesterol. The data results from the analysis of 45 worms per strain in 4 independent experiments. Values denote means ± SEM. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

from PLD, could be involved in the mechanism of lipid stores regulation observed upon *lpin-1* knock-down. Moreover, lipid stores have been previously shown to be consumed through a specialized form of macroautophagy called macrolipophagy[37]. Since, previous reports have shown a role for PLD1 in macroautophagy[38] and in the regulation of lipid stores[27] we hypothesize that this could be an alternate explanation for this phenotype.

To understand the impact of PLD ablation in a neurodegenerative disease *C. elegans* model, we crossed *pld-1* animals with the CL2355 strain, which overexpresses human Aβ in neurons. This Aβ strain was shown to present multiple behavioral deficits and decreased survival[18–20]. We had previously observed that *C. elegans* expressing Aβ presented decreased worm size, and remarkably, *pld-1*; Aβ animals had a significant recovery in animal volume (Fig. 4B), with a major impact in worm survival (Fig. 4C). Additionally, PLD ablation in *pld-1*; Aβ animals had a protective effect in motor behaviors (Fig. 4F and G) and in the defective responses to serotonin (Fig. 4H) and PTZ (Fig. 4I). This is in line with previous results showing that Aβ leads to increased total PLD activity and that PLD2 genetic ablation ameliorates synaptic and behavioral deficits in an amyloidogenesis AD mouse model, independently of an effect on APP processing[5]. Interestingly, in an unbiased gene expression study, it was observed in another *C. elegans* strain expressing human Aβ that the toxic peptide leads to gene expression changes that overlap with changes induced by the membrane pore-inducing toxin, Cry5B[39], suggesting that membrane damage mechanisms could be important pathways induced by Aβ. In fact, PLD has been shown to be involved in membrane damage pathways[40]. Moreover, fly PLD was shown to be required to support rhabdomere volume during illumination, a task that relies on active membrane turnover[13]. Previously it had been shown that PLD overexpression also has a deleterious impact, leading to degeneration of rhabdomeres[14,16]. These reports in drosophila, show that either a decrease or increase in PLD levels can have a functional impact in fly rhabdomeres, which highlights the importance of tightly regulating PLD activity in physiologic processes with high membrane turnover.

To our knowledge, this is the first report evaluating the impact of PLD ablation in *C. elegans*. We further present observations that support PLD as a downstream pathway of Aβ's interaction with membranes or a putative membrane receptor. Overall, we observe multiple phenotypes that somewhat phenocopy previous observations in other PLD genetic models. Future studies will be able to benefit from *C. elegans* PLD models as relevant tools to study the role of PLD in physiologic and pathologic mechanisms.

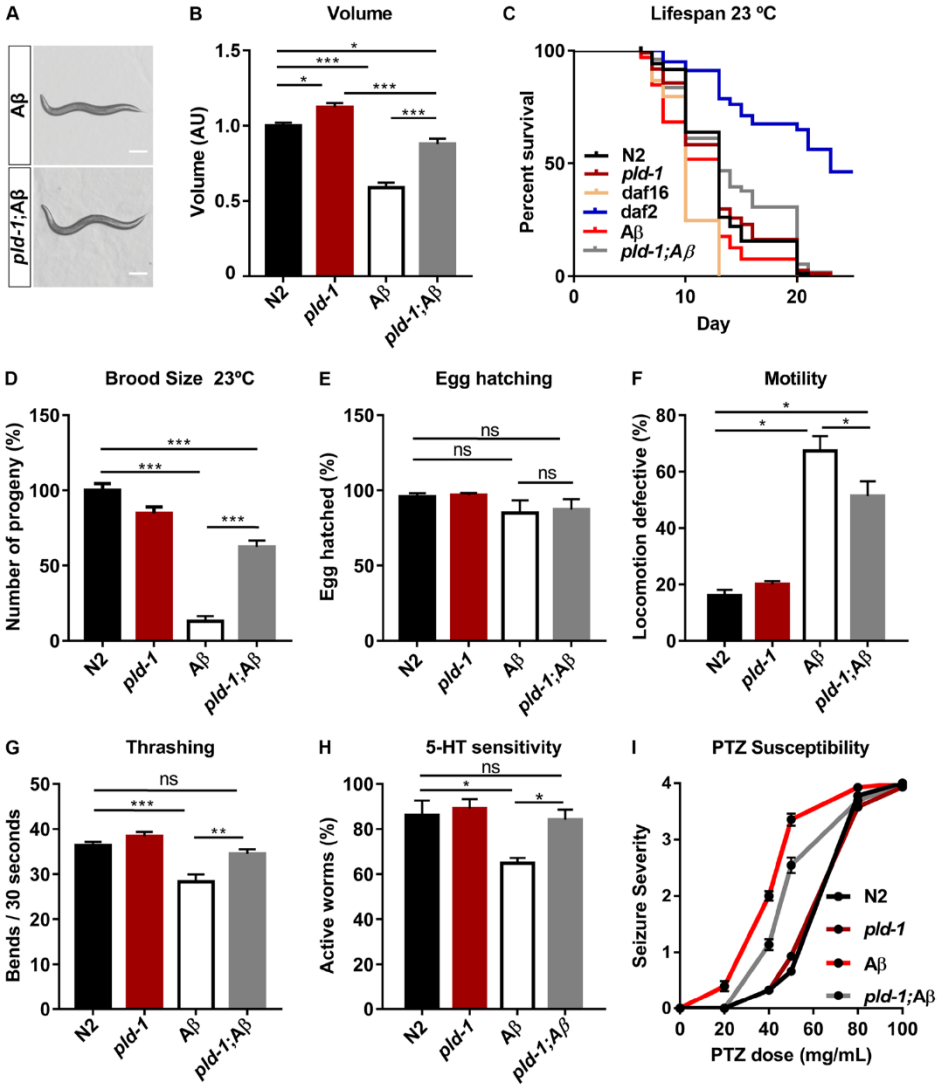

Figure 4. PLD functional ablation ameliorates Aβ induced phenotypes. (**A**) Representative photos of Aβ and *pld-1*;Aβ and volume (**B**) of N2, *pld-1*, Aβ and *pld-1*;Aβ animals at day 3 after egg laying. The scale bar represents 100 µm. (**B**) Aβ transgenic worms have a decrease in body size, which is partially renormalized upon PLD ablation. The data results from the analysis of 45 worms per strain in 4 independent experiments. Values denote means ± SEM. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). (**C**) Effects of neuronal Aβ expression on adult lifespan at 23 °C. The experiment was conducted twice with 100 worms per strain. Representative example of two independent experiments is shown. Kaplan-Meier survival curve of Aβ and *pld-1*; Aβ worms, showing differences in the median lifespan compared with N2 and *pld-1* animals. The mean adult lifespan of *pld-1*; Aβ animals was significantly longer than Aβ transgenic animals. (**D–I**) Neuronal expression of Aβ in *C. elegans* leads to a defect in brood size(**D**), locomotion (**F**), decrease in thrashes over time (**G**), serotonin sensitivity (**H**) and pentylenetetrazol (PTZ) susceptibility (**I**), when compared to the N2 and *pld-1* animals. (**D**) Improvement in brood size due to ablation of PLD in Aβ animals. Total number of progeny of adult hermaphrodite N2, *pld-1*, Aβ and *pld-1*; Aβ worms for 8 days. The progeny of 15 worms was counted per experiment (n = 2). (**E**) Egg viability was not significantly affected by neuronal expression of Aβ (n = 3). (**F**) Aβ worms have a motility defect partially recovered by PLD ablation. Percentage of uncoordinated N2, *pld-1*, Aβ and *pld-1*; Aβ adult hermaphrodite animals was measured by scoring the animals that remain inside 10 mm circle after 1 min (n = 4 experiments, 10 worms per strain and experiment). (**G**) The number of body thrashes per 30 seconds was partially rescued by PLD ablation in Aβ transgenic animals (n = 4 experiments, 10 worms per strain and experiment). (**H**) PLD ablation in Aβ animals leads to a decrease in hypersensitivity to 1 mM serotonin caused by Aβ (n = 4 experiments, 60 worms per strain and experiment). (**I**) PLD ablation reduces seizure-severity induced by PTZ in Aβ transgenic worms. PTZ susceptibility of worms were scored after exposure to the concentrations of 20, 40, 50, 80 and 100 mg/mL of PTZ (n = 4 experiments, 15 animals per dose/strain/experiment). Values denote means ± SEM. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Methods

Nematode Strains and culture conditions. Strains used in this work were acquired from the *Caenorhabditis* Genetics Center, namely Bristol N2; RB1737, *pld-1*(*ok2222*) II; CB1370, *daf-2*(*e1370*) III; CF1038, *daf-16*(*mu86*) I; PS2627, *dgk-1* (*sy428*) X; PR811, *osm-6* (*p811*) V; PR696, *che-1* (*p696*) I, and CL2355, *smg-1*(*cc546*) *dvIs50* I, *dvIs50* [pCL45 (*snb-1*: Abeta 1–42::3' UTR(long) + mtl-2::GFP]. The CL2355 strain has a pan-neuronal expression of $A\beta_{1-42}$, which is inducible by temperature up-shift to 23 °C. The transgenic strain is referred to as neuronal Aβ strain. All the strains were backcrossed to Bristol strain N2 eight times. Strain CL2355 was crossed with strain RB1737 using standard procedures, generating *pld-1;* Aβ animals. Worms were grown in agar plates with nematode growth media (NGM) at 20 °C as previously described[41]. In the experiments where the Aβ strain was used, the animals grew 36 h at 16 °C followed by temperature upshift to 23 °C. Synchronized cultures were used for all assays and obtained through egg laying, by collecting embryos laid by adult animals during 3 h or using a bleaching procedure, by treating animals with an alkaline hypochlorite solution (0.5 M NaOH, 2.6% NaClO) for 7 min[42].

Lipid analysis. N2 and *pld-1* animals were incubated in the presence or absence of ethanol (1%) for 1 h and all samples were immediately collected, frozen in liquid nitrogen and stored at −80 °C until further processing. Approximately 100 animals were used per sample. Lipids were subsequently extracted by a chloroform/methanol extraction, as previously described[43,44]. Lipid species were analyzed using a 6490 Triple Quadrupole LC/MS system (Agilent Technologies, Santa Clara, CA) operated in multiple reactions mode (MRM). PA and PEtOH levels were quantified by comparing to spiked internal standards diC17-PA and diC16-PEtOH (Avanti Polar Lipids). Lipid concentration was normalized by molar concentration across all species for each sample, and the final data is presented as the mean mol %[43,44].

Ethanol susceptibility assay. Plates (60 mm) with 8,5 mL NGM and 1 mL of ethanol at final concentrations ranging from 100 to 500 mM (adjusted to the volume of the agar) were freshly prepared. Plates were then sealed for 2 h at room temperature (RT) and copper rings (10 mm diameter) were melted onto the agar surface. Day three synchronized worms were placed in plates in the absence of food for 30 min prior to the assay, and then transferred to the ethanol plates. After 20 min of exposure, one-min videos were taken with an Olympus PD72 digital camera attached to an Olympus SZX16 stereomicroscope. Mean worm speed was quantified using the dVision software (Delta Informatika ZRt, Budapest, Hungary)[45].

Ethanol Assay Kit. Worms were synchronized by egg laying and grown at 20 °C until they reached the adult stage (day three post-hatching). Ethanol levels were assayed using the ethanol assay kit (MAK076, Sigma). Briefly, nematodes were washed in M9 buffer and 50 worms were placed per well to a final volume of 50 µL with the ethanol assay buffer. The master reaction mix was prepared according to the specifications of kit. To each well, 50 µL of the master reaction mix were added, and incubated for 60 min at room temperature, after which the absorbance at 570 nm (A_{570}) was measured. The concentration of ethanol was determined based on the A570 of standards provided in the kit.

Fecundity and Egg Viability Assay. Animals were synchronized by egg-laying. At day three, animals were individually transferred into 30 mm plates (10 animals per strain) with a bacterial lawn of 10 mm of diameter. After 5 h, all worms were removed and the total number of eggs in each plate was counted. The plates were maintained at 4 °C in order to delay egg hatching, while counting them. Egg viability was determined as the percentage of eggs that were able to hatch over the following 24 h.

Brood Size. Brood size evaluation was performed as previously described[46]. Briefly, 15 L4 animals, per strain, were kept at 23 °C in individual plates (30 mm) with a bacterial lawn of 10 mm of diameter and allowed to lay eggs. Animals were transferred to fresh plates daily and total progeny counted every day for 8 days.

Development. Synchronized worms through hypochlorite treatment were placed in a freshly seeded NGM plate (50 animals per strain). After 48 h, the percentage of animals which were in the L4 to adult stage was scored.

Motility assay. The motility assay was performed as previously described[47] at RT (~20 °C), using day three synchronized animals grown at 20 °C. Five animals were placed simultaneously in the middle of a freshly seeded plate, equilibrated at 20 °C. Animals remaining inside a 10 mm circle after 1 min were scored as locomotion-defective. At least 150 animals were scored for each strain in three independent assays.

Chemotaxis assay. Chemotaxis assays were performed based on the assays previously developed[48,49]. Well-fed, synchronized adult day three animals (through bleaching) were collected and washed with S-Basal buffer three times to remove all the food. The assay plates (20 g/L agar-agar; 5 mM KH_2PO_4; 1 mM $CaCl_2$; 1 mM $MgSO_4$) were prepared by adding 1 µL of 5 M NaCl or 0.1 M isoamyl alcohol (IAA) 10 mm from the center of the plate on one side. On the opposite side of the plate, a 1 µL drop of water or of 100% ethanol was added. Afterwards, 1 µL of 1 M sodium azide was additionally added to the preexisting spots to paralyze the animals. Worms (~100–200) were quickly transferred to the center of the plate and the excess of liquid removed with a filter paper. The assay plates were incubated at 20 °C for 60 min and the chemotaxis index was scored as the (number of animals at attractant - number of animals at counter-attractant)/Total number of animals in assay. Three to four independent assays were conducted with at 100 to 150 animals per assay per plate. For each strain, two to three plates were tested per assay.

Short-term and Long-term Associative Memory Assays. A *C. elegans* odorant preference assay protocol was adapted from previous reports[50]. A chemotaxis assay using 1 M diacetyl (Sigma-Aldrich) was performed

as described above in order to confirm that the animals' genotype did not affect the chemotaxis index to diacetyl (naive group). To assess the 1× associative learning, well-fed day three animals were starved for 1 h in the presence of diacetyl and placed on the lid of the plates. Right after the starvation period, the chemotaxis index was scored again to assess learning (represented as starv 1 h in the graph). After conditioning the worms with diacetyl, worms were placed on NGM plates with food for 1 h. To test for short-term associative memory of the food-diacetyl association, the chemotaxis index was again evaluated (short memory). The long term associative memory was performed 24 h later (long memory). As a control, the same conditions were tested in fed animals to test for habituation.

Lifespan. Synchronized adult animals were placed on 60 mm NGM plates at 20 °C, examined every day and scored as dead if no mechanical response was obtained after gentle touch with a platinum wire. Animals were transferred to fresh plates every 2 days to avoid starvation and progeny contamination. Animals were censored from the analysis if lost, desiccated on the edge of plates, if showing extruded gonad or suffered internal progeny hatching. Evaluations ended after all animals were dead or censored. The lifespan evaluation at 23 °C was performed as described above. Experiments were performed blindly.

Dopamine susceptibility assay. Worms were synchronized by egg laying and grown at 20 °C until they reached the adult stage (day three post-hatching). Different solutions of dopamine hydrochloride (Sigma-Aldrich) (concentrations ranging from 100 to 800 mM) were prepared and 1 mL of the solution was added to NGM plates (60 mm) without OP50 and allowed to dry for 60 min. The adult worms were placed in each plate (10 animals per strain) and after 30 min their motor phenotype was assessed according to the following scores: 0 for normal locomotion, 1 for sluggish/slower movement, 2 for semi-paralysis (body bends without moving), 3 for paralysis (only the head moves after mechanical stimulation) and 4 for death[21].

Biometric analysis. Biometric analysis was performed at 72 h after egg laying. Length and diameter measurements were calculated using ImageJ software®, and volume was determined by treating worms as cylinders ($v = \pi * r^2 * l$)[51].Biometric analysis of F2 animals was performed using the progeny of synchronized worms grown in NGM plates with or without cholesterol. Pictures were acquired 72 h after hatching. Worms were photographed using an Olympus PD72 digital camera attached to an Olympus SZX16 stereomicroscope.

Nile Red Staining. The Nile red staining protocol was adapted from previously described protocols[52]. Nile red (Molecular Probes) was dissolved in a 0.5 mg/mL acetone stock solution. On the day of the assay, the stock solution was freshly diluted in 1× PBS to a final concentration of 1 µg/mL. Egg laying synchronized worms were washed 3 times with M9 and transferred to a conical tube containing Nile Red. Worms were incubated at 20 °C for 2 h and washed 3 times to remove the excess of dye before imaging[52].

Confocal Imaging. For confocal dynamic imaging and quantification of Nile Red staining, live animals were paralyzed with 3 mM levamisole (Sigma-Aldrich) and mounted on a 3% agarose pad. All images were acquired on an Olympus FV1000 (Japan) confocal microscope, under a 60× oil objective and resolution of 640 × 640. A z-series image was acquired for all treated worms using a 594 nm laser. The pinhole was adjusted to 1.0 Airy unit. The images were analyzed and processed using ImageJ software®.

Thrashing analysis. Single synchronized adult animals were transferred to a 10 µL drop of M9 buffer. After 1 min animals were filmed at a rate of 15 frames-per-second, in a total of 600 frames, using an Olympus PD72 digital camera attached to an Olympus SZX16 stereomicroscope. The number of total body bends per 30 seconds was then quantified using ImageJ software® with the wrMTrck plugin[53].

Defecation Motor Program (DMP). Adult well-fed synchronized worms (10 per strain) were placed on NGM plates (90 mm), freshly seeded with OP50, for 10 min. Each animal was individually evaluated for exactly 10 min and the total number of DPMs was counted in this interval. The results were expressed as the average time (in seconds) between each successive cycle.

Pharyngeal Pumping. Synchronized day three worms were placed on plates (90 mm) freshly seeded with OP50. After 1 h, worms present in the border of the OP50 were selected and recorded with an Olympus PD72 digital camera attached to an Olympus SZX16 stereomicroscope (10 worms per strain). Each movie was recorded for 30s and the total number of pharyngeal contractions was counted in this interval.

Serotonin Sensitivity Assay. The serotonin assay was performed as previously described[18]. Serotonin (creatine sulfate salt, Sigma-Aldrich) was dissolved in M9 buffer to 1 mM. Synchronized three-day worms underwent temperature upshift to 23 °C to activate transgene expression of the Aβ strain, prior to the assay. Worms were washed with M9 buffer and placed in 200 µL of serotonin 1 mM in a 96-well plate. The animals were scored as active or paralyzed in each well after 5 min.

PTZ susceptibility Assay. The PTZ susceptibility assay was adapted from others[24]. Plates (30 mm) were prepared with 3 mL NGM each (without food). A stock solution of 100 mg/mL PTZ (Sigma-Aldrich) and the respective dilutions (20, 40, 50, and 80 mg/mL) were prepared. To each plate, 250 µL of PTZ was added. The plates were allowed to dry for 90 min in a flow chamber. Afterwards, 5 times concentrated OP50 was added to the center of each plate. The susceptibility assay was evaluated by placing worms in the bacterial lawn in each plate. After 30 min, each worm's phenotype was evaluated using the following score: 0 for no major decrease in worm movement, 1 for sluggish/slower movement, 2 for semi-paralysis (body bends without moving), 3 for paralysis (only the head moves after mechanical stimulation) and 4 for death.

Statistical analysis. A confidence interval of 95% was assumed for all statistical tests. Normality was tested using the Kolmogorov-Smirnov test, and was assumed for all tested variables. In all experiments comparing two variables, the data was analyzed with Student's t-test with the Levene's test for equality of variances. When more than two variables were analyzed, a one-way analysis of variance with the Levene's test for equality of variances and a post-hoc Tukey test for multiple comparisons was performed. The ethanol and dopamine susceptibility assays were analyzed using a two-way analysis of variance with the Levene's test for equality of variances and a post-hoc Tukey test for multiple comparisons. The PTZ susceptibility assay was analyzed using a repeated measures analysis of variance with the Mauchly's test for sphericity and a post-hoc Games-Howell test for multiple comparisons. Lifespan was evaluated by the log-rank (Mantel-Cox) test and the Hazard Ratio obtained from a Cox regression model, using the strain as a categorical covariate and a simple contrasts analysis. Statistical analysis was performed using GraphPad Prism 6.01 software® and SPSS 22.0 (SPSS Inc.)

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Author Contributions

T.G.O. conceived the idea. F.V.B., A.T.C. and T.G.O. designed and planned the experiments. F.V.B. and J.D.S. performed the experiments. R.B.C. and G.D.P. performed the lipidomic analysis. F.V.B., J.D.S., A.T.C. and T.G.O. analyzed the data. F.V.B. and T.G.O. wrote the paper and all authors reviewed and corrected the manuscript.

Additional Information

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Competing Interests: G.D.P. is a full time employee of Denali Therapeutics Inc. G.D.P. and T.G.O. are inventors on the patent number WO2010138869A1 entitled "Modulation of phospholipase D for the treatment of neurodegene-rative disorders". R.B.C., G.D.P. and T.G.O. are inventors on the patent number US20120302604A1 entitled "Modulation of phospholipase D for the treatment of the acute and chronic effects of ethanol".

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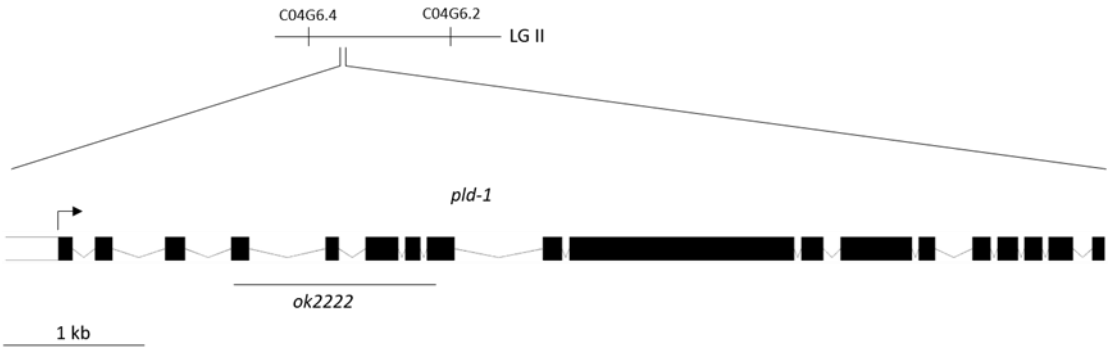
Phospholipase D functional ablation has a protective effect in an Alzheimer's disease *Caenorhabditis elegans* model

Francisca Vaz Bravo, Jorge Da Silva, Robin Barry Chan, Gilbert Di Paolo, Andreia Teixeira-Castro, Tiago Gil Oliveira

Supplementary Data



Supplementary Figure S1: *pld-1* encodes the *C. elegans* ortholog of phospholipase D. Genetic map of the *pld-1* region of LG II. Intron–exon structure of *pld-1* (RB1737), inferred from cDNA sequences. Boxes, Coding regions; lines, untranslated regions, arrow, direction of transcription. The *pld-1* open reading frame is 4285 bp within a 4659 bp cDNA. The extent of the 1430 bp deletion is depicted.

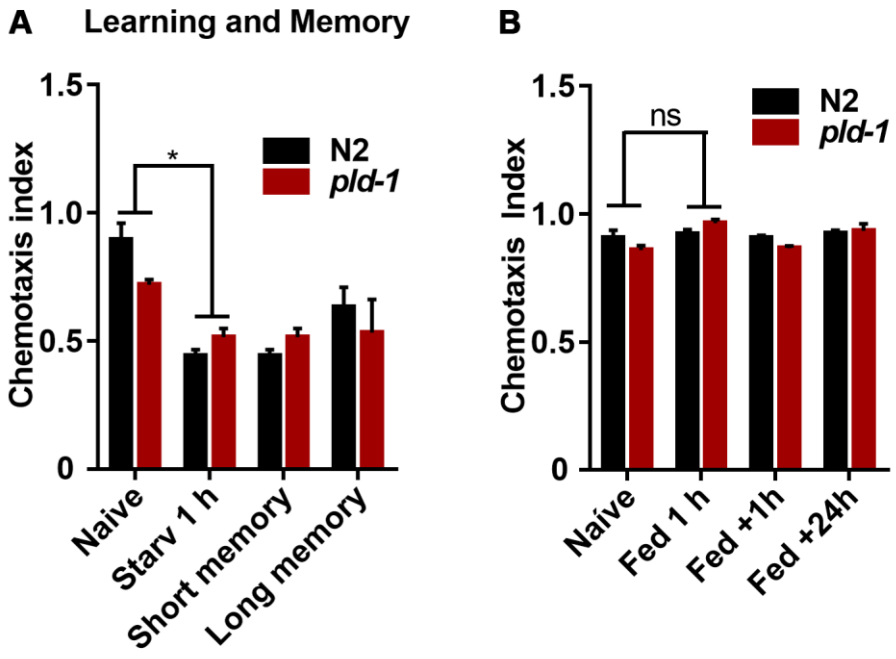
	N2	Assay 1		Assay 2		Assay 3		*pld-1*	Assay 1		Assay 2		Assay 3	
		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6
Naïve	Diacetyl	45	65	130	125	145	94	Diacetyl	36	50	120	110	130	147
	EtOH	1	0	12	3	5	3	EtOH	4	4	2	8	2	7
	Out	7	3	8	2	6	3	Out	6	8	7	8	5	13
	Total	53	68	150	130	156	100	Total	46	62	129	126	137	167
	CI	0,83	0,96	0,79	0,94	0,90	0,91	CI	0,70	0,74	0,91	0,81	0,93	0,84

	N2	Assay 1		Assay 2		Assay 3			*pld-1*	Assay 1		Assay 2		Assay 3		
		Plate 1	Plate 2	Plate 4	Plate 5	Plate 6	Plate 7			Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7
Starve 1 h	Diacetyl	85	79	75	77	60	40		Diacetyl	50	35	57	52	67	70	62
	EtOH	26	35	20	18	25	20		EtOH	18	5	14	8	11	13	14
	Out	11	3	23	27	4	4		Out	6	13	50	83	14	22	20
	Total	122	117	118	122	89	64		Total	74	53	121	143	92	105	96
	CI	0,48	0,38	0,47	0,48	0,39	0,31		CI	0,43	0,57	0,36	0,31	0,61	0,54	0,50

	N2	Assay 1		Assay 2		Assay 3			*pld-1*	Assay 1		Assay 2		Assay 3		
		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7
Short term	Diacetyl	72	75	85	70	98	70	65	Diacetyl	75	73	40	90	80	87	123
	EtOH	28	26	26	25	23	9	10	EtOH	20	22	15	1	23	19	35
	Out	5	4	9	21	7	7	5	Out	5	11	35	35	6	2	8
	Total	105	105	120	116	128	86	80	Total	100	106	90	126	109	108	166
	CI	0,42	0,47	0,49	0,39	0,59	0,71	0,69	CI	0,55	0,48	0,28	0,71	0,52	0,63	0,53

	N2	Assay 1		Assay 2		Assay 3		*pld-1*	Assay 1		Assay 2		Assay 3	
		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6		Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6
Long Term	Diacetyl	148	140	140	125	30	30	Diacetyl	63	25	147	140	65	105
	EtOH	25	39	13	7	2	4	EtOH	10	8	18	16	12	8
	Out	0	3	4	12	3	3	Out	7	9	4	7	3	8
	Total	173	182	157	144	35	37	Total	80	42	169	163	80	121
	CI	0,71	0,55	0,81	0,82	0,80	0,70	CI	0,66	0,40	0,76	0,76	0,66	0,80

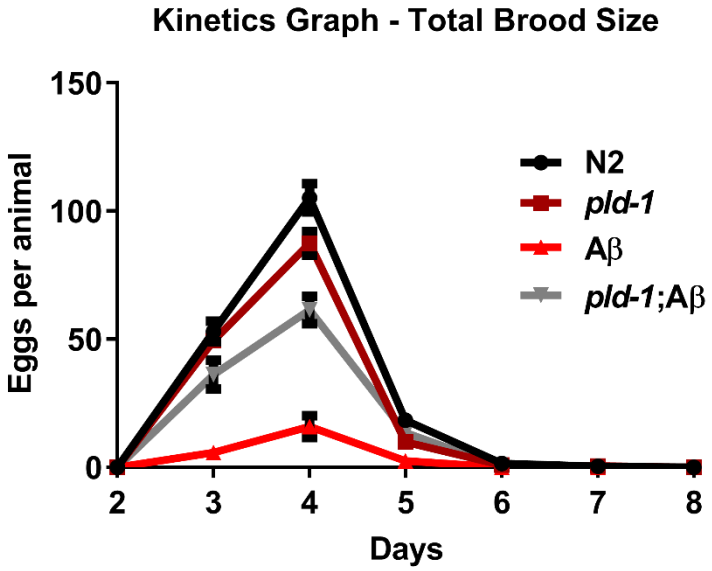
Supplementary Table S1: Raw data from learning and memory assay represented in Fig. 2F. The chemotaxis index was scored to assess the naïve, learning (starve 1 h), short term and long term memory Three independent experiments were performed and the number of animals used in each assay are scored in the table.



Supplementary Figure S2. (A) Additional representative experiment of learning and memory independent assay *pld-1* animals have no deficit in an associative learning task. N2 and *pld-1* short-term and long-term associative memory profile after 1 h and 24 h of conditioning. Three independent experiments were performed (n = approximately 100 worms per chemotaxis assay plate). **(B) *pld-1* animals in fed conditions and in the presence of diacetyl have a normal chemotaxis upon repetitive exposure.** N2 and *pld-1* chemotaxis index profile after 1 h and 24 h the fed period. Representative figure of two independent experiments performed (3 chemotaxis assay plates per strain, n=100 worms per chemotaxis assay plate) experiment).

Strain (n)*	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	Control stain (n)	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	*P* value (Log Rank,Mantel-Cox)
***daf-16* (63)**	10	0.381	11.413	0.291	**N2 (75)**	13	0.442	15.364	0.566	< 0.0001
***daf-2* (75)**	17	1.137	22.086	1.641	**N2 (75)**	13	0.442	15.364	0.567	< 0.0001
***pld-1* (77)**	13	0.566	15.364	0.687	**N2 (75)**	13	0.442	15.363	0.566	0.326

Supplementary Table S2. *pld-1* animals have a normal lifespan. Survival statistics analysis of N2, *pld-1*, *daf-16* and *daf-2* animals at 20°C.



Supplementary Figure S3. PLD function ablation increases the number of progeny in Aβ animals. Number of eggs layed in adult hermaphrodite N2, *pld-1,* Aβ and *pld-1*; Aβ worms was counted for 8 days. The progeny of 15 worms per strain was evaluated per day. Representative figure of two independent experiments performed.

A

Strain (n)*	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	Control strain (n)	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	*P* value (Log Rank,Mantel-Cox)	Hazard Ratios (HR)
***daf-16* (62)**	10	0.188	10	0.227	**N2 (79)**	13	0.395	13	0.410	< 0.0001	1.948
***daf-2* (80)**	23	1.189	24	1.025	**N2 (79)**	13	0.395	13	0.410	< 0.0001	0.189
***pld-1* (78)**	13	0.544	13	0.459	**N2 (79)**	13	0.395	13	0.410	0.961	1.002
Aβ (80)	13	0.424	11	0.402	**N2 (79)**	13	0.395	13	0.410	0.011	1.371
***pld-1* ; Aβ (65)**	13	1.129	14	0.569	**N2 (79)**	13	0.395	13	0.410	0.134	0.831
***pld-1* ; Aβ (65)**	13	1.129	14	0.569	**Aβ (80)**	13	0.424	11	0.402	< 0.0001	0.606

B

Strain (n)*	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	Control strain (n)	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	*P* value (Log Rank,Mantel-Cox)	Hazard Ratios (HR)
***daf-16* (62)**	9	0.227	9	0.185	**N2 (71)**	11	0.412	12,6	0.397	< 0.0001	3.270
***daf-2* (80)**	19	1.574	18	0.686	**N2 (71)**	11	0.412	12,6	0.397	< 0.0001	0.313
***pld-1* (78)**	11	0.412	12	0.379	**N2 (71)**	11	0.412	12,6	0.397	0.052	1.340
Aβ (80)	10	0.383	11	0.298	**N2 (71)**	11	0.412	12,6	0.397	< 0.01	1.563
***pld-1* ; Aβ (65)**	12	0.344	13	0.403	**N2 (71)**	11	0.412	12,6	0.397	0.589	0.951
***pld-1* ; Aβ (65)**	12	0.344	13	0.403	**Aβ (80)**	10	0.383	11	0.298	< 0.0001	0.609

Supplementary Table S3. PLD ablation restores survival of Aβ transgenic animals. (A) Survival statistics analysis of experiment #1. **(B)** Survival statistic analysis of experiment #2 of N2, *pld-1*, Aβ, *pld-1;* Aβ, *daf-16* and *daf-2* animals at 23°C.

% Bagging animals					
N2	*pld-1*	Aβ	*pld-1* ;Aβ	daf 16	daf 2
3%	4%	1%	3%	2%	8%

Supplementary Table S4. Percentage of bagging animals present in survival experiment of N2, *pld-1*, Aβ, *pld-1;* Aβ, *daf-16* and *daf-2* animals at 23°C.

Control Strain	Strain	Mean Difference	s. e. m	*P* value
N2	***pld-1***	0.06	0.047	0.573
N2	**Aβ**	-0.75	0.059	< 0.0001
N2	***pld-1; Aβ***	-0.36	0.055	< 0.0001
Aβ	***pld-1; Aβ***	0.39	0.062	< 0.0001

Supplementary Table S5: Repeated measures analysis of variance of the PTZ susceptible assay in *C. elegans.* $F(3, 3.335) = 3309.545$, $p < 0.0001$.

Chapter 2.2

Bravo FV, Magalhães R, Da Silva JD, Rosa R, Teixeira-Castro A, Oliveira TG

Regulation of tau by the Phospholipase D pathway

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Regulation of tau by the Phospholipase D pathway

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Abstract

Alzheimer's disease (AD) is the most common form of late-onset dementia. It is characterized by the accumulation of amyloid-beta (Aβ) and impaired phosphorylation of tau. The lipid modifying phospholipase D (PLD) isoenzymes, PLD1 and PLD2, were shown to affect endocytosis and membrane trafficking and modulate AD-related signaling pathways. Moreover, Aβ was reported to increase total PLD activity in primary neuronal cultures and in an AD mouse model. Despite this, the precise role of PLD enzymes in AD pathology is poorly understood. Several studies indicate that Aβ pathological signaling is connected with tau pathology, and that tau ablation protected from the deleterious effects of Aβ. Interestingly, there are several evidences that point to a potential crosstalk between Aβ and tau at the lipid raft interface. Here, we show that PLD enzymes and tau physically interact and that particularly PLD2 overexpression leads to a strong decrease in tau levels in a cell line model. We further show that PLD ablation in a *C. elegans* tauopathy-like model leads to increased tau levels and markedly improves various phenotypes such as motor tasks, survival, thermotolerance and associative learning. Finally, we found that tau interacts with phospholipids, especially PA, and re-localizes to the vicinity of the plasmatic membrane in the presence of the PA binding probe, Spo20. Overall, this work sheds new light on the role of PLD in neurodegeneration, particularly in the regulation of pathological tau events, and supports the use of nematode models as a powerful tool to study neurodegenerative diseases.

Introduction

Alzheimer's disease (AD) is the most common form of late-onset dementia. It is characterized by the accumulation of amyloid-beta (Aβ) and formation of neurofibrillary tangles (NFTs) (Querfurth and LaFerla 2010). Additionally, endocytosis and membrane trafficking are known to be affected in AD pathogenesis. The lipid modifying phospholipase D (PLD) isoenzymes, PLD1 and PLD2, were shown to modulate AD-related signaling pathways (Cai, Zhong et al. 2006, Oliveira, Chan et al. 2010). PLD is responsible for the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA), a signaling lipid with a cone-shaped conformation which alters membrane curvature (Oliveira, Chan et al. 2016). Consequently, the modulation of its levels can affect endocytosis and membrane trafficking, potentially altering synaptic properties and impacting neuronal functioning (Oliveira, Chan et al. 2016). Therefore, lipid-modifying enzymes are potential candidates for modulation of pathological alterations in AD. Although having similar catalytical functions, PLD1 and PLD2 have different subcellular localizations and impact AD in different manners: while PLD1 was shown to modulate amyloid precursor protein (APP) trafficking (Cai, Zhong et al. 2006), PLD2 ablation prevents behavioral deficits in AD mouse models (Oliveira, Chan et al. 2010). Moreover, Aβ was reported to increase PLD activity both in primary neuronal cultures and in an AD mouse model (Oliveira, Chan et al. 2010). Even though these reports indicate PLD as a modulator of AD pathogenesis, its precise mechanism of action is still elusive. Importantly, several studies indicate that Aβ pathological signaling is connected with tau pathology, and that tau ablation protected from the deleterious effects of Aβ (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010). Tau is a MAP mostly expressed in neurons and predominantly localized in axons (Binder, Frankfurter et al. 1985). The fibrillary aggregation of tau is characteristic of several neurodegenerative diseases, termed as tauopathies. These tauopathies include AD, progressive supranuclear palsy (PSP), cortical basal degeneration (CBD), multiple systems atrophy (MSA), genetic forms of Pick's disease (PiD) and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hauw, Verny et al. 1990, Hof, Bouras et al. 1994, Hutton, Lendon et al. 1998). Biochemical studies indicate that the tau pathology associated with these different tauopathies arises from distinct tau isoforms (Sergeant, David et al. 1997). The six isoforms combinations of tau gene resulting from alternative splicing of exons 2, 3 and 10. Tau isoforms differ on the number of 29 amino acid inserts (0, 1 or 2 inserts are known as 0N, 1N and 2N) encoded by exon 2 and 3 in the N-terminal

region, in combination with either three (R1, R3 and R4) or four (R1-R4) repeat regions in the C-terminal part. The R2 repeat is encoded by exon 10 (Wang and Mandelkow 2016). The longest 2N4R adult tau isoform has 441 amino acids (aa) and the shortest 0N3R isoform of 352 aa. It has been shown that 4R tau isoforms that contain exon 10 are more efficient at promoting microtubule assembly than 3R tau isoforms that lack exon 10 (Goedert and Jakes 1990, Gustke, Trinczek et al. 1994). Taking into account that a pool of hyperphosphorylated tau is present in lipid rafts, as well as beta-secretase 1 (BACE1), γ-secretase complex, APP metabolites (including dimeric Aβ) and ApoE (whose E4 polymorphism is the main genetic risk factor for AD), there are several evidences that point to a significant crosstalk between Aβ and tau at the lipid raft interface (Kawarabayashi, Shoji et al. 2004).

Although the relationship between lipid dysregulation and tau pathology remains unclear in AD, we hypothesized that a protein complex comprising PLD and tau could play a relevant role in AD pathogenesis. Here, we demonstrate that both PLD enzymes and tau physically interact. Moreover, we observe that PLD2 overexpression, to a more extent than PLD1, leads to decreased tau levels in a cell line model. To study the role of lipids in tau pathology, we used a nematode model of parkinsonism chromosome 17 type (FTDP-17). The pan neuronal expression of FTDP-17 mutant *MAPT*, the gene encoding tau, resulted in behavior deficits such as uncoordinated movement, accumulation of insoluble phosphorylated tau, age dependent neuronal loss and structural damage of axonal tracks (Kraemer, Zhang et al. 2003). Here, we studied the impact of PLD genetic ablation in *C. elegans* upon crossing it with a tauopathy-like model. We observed that even though PLD ablation leads to an increase in levels tau levels, surprisingly it had a protective effect in motor behaviors, survival, thermotolerance and in an associative learning task. Interestingly, it was found in AD brains an association between paired helical filaments (PHFs) and membrane glycolipids (Jones, Dubey et al. 2012). Thus, the amino-terminal projection domain of tau has been reported to indirectly interact with the plasma membrane (Brandt R 1995) and it is found in membrane microdomains resistant to detergent (Williamson, Usardi et al. 2008). At present, the interaction between lipid membranes and tau remains poorly understood, particularly the effects of interaction on structural perturbations in both tau and the membrane. The current study also investigates the interactions between tau with phospholipids which are abundant in plasma membranes. Furthermore, to investigate the effects of these interaction on tau localization, a spatial distribution analysis using a PA probe (Spo20) was performed. Spo20 is localized in the nucleus and translocated to the plasma membrane, where it binds to PA, once the sporulation cascade begins

(Ganesan, Shabits et al. 2015). We found that tau interacts with phospholipids, particularly with PA and this interaction may contribute to a re-localization of tau in membranes. The specificity of the phenotypes in our characterization sheds light in potential mechanisms contributing to the understanding of tau pathology, such as the molecular pathways involved in cellular size regulation, autophagy and neuroplasticity.

Materials and Methods

Cell culture. Wild type mouse neuroblastoma-2a (N2a) cells were a kind gift from Claudia Almeida (Nova Medical School, Lisbon). The cells were cultured in a medium containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (10 000 Units/ mL of penicillin; 10 000 μg/ml of streptomycin) (GibcoTM, Life technologies) in 5% CO_2 at 37°C. The tau 352 and 441 WT constructs were a kind gift by Roland Brandt (University of Osnabrück, Germany). The plasmids PLD1-GFP, PLD2-GFP, Tau 352 WT and Tau 441 WT were prepared using a plasmid midiprep kit (Thermo Fisher Scientific) and transfected into N2a cells using LipofectamineTM 3000 (Invitrogen). The amounts and volumes are given on a per well basis. One day before transfection, N2a cells (5 x 10^4 cells) were seeded in coverslips in a 24-well format. On the transfection day, the cellular confluence was about 90%–95%. The cell culture media was removed and 250 μL complete growth medium without antibiotics was added. For each transfection, two separate mixes were prepared, one with 0.5 μg DNA, 1 μL P300 reagent in 25 μL of Opti-MEM medium and the other with 1 μL LipofectamineTM 3000 in 25 μL of Opti-MEM medium were and incubated for 5 min at room temperature. Then, the DNAmix and the LipofectamineTM 3000 mix were combined gently and incubated for another 10 min at room temperature. The mixture was then added to each well and incubated with the cells at 37°C in a CO_2 incubator for 48 h prior to determination of the expression of transfected genes (Wang, Zhang et al. 2005).

Immunofluorescence. Cells cultured on coated coverslips were carefully removed. After two rinses in phosphate buffered saline (PBS), the cells were fixed in a freshly prepared solution of 4% paraformaldehyde for 20 min. After two more rinses in PBS, the cells were permeabilized in 0.1% saponin in PBS for 20 min. Then, cells were incubated in 2% FBS/1% BSA (bovine serum albumin)

in PBS for 1 h and incubated with the respective primary antibody at 20°C at room temperature (RT). After three rinses with PBS to remove the excess of primary antibody, cells were incubated in the respective secondary antibody (1:500) for 1 h at RT. After three more rinses in PBS, coverslips were mounted with Fluoromount-G (SouthernBiotech)) on glass slides (Wang, Zhang et al. 2005).

Antibodies. The following antibodies were purchased from commercial sources: anti-β-actin (AM4302, Ms, Ambion, 1:5000 in WB), anti-MAPT, clone Tau-5 (MAB1832, Abnova, 1:1000 in WB, 1:500 in IF), anti-GAPDH (D16H11, Cell Signaling, 1:1000 in WB), anti-GFP (G1544, Sigma, 1:1000 in WB, 1:500 in IF), anti-FLAG (F2555, Sigma, 1:1000 WB, 1:500 IF).

Proximity Ligation Assay (PLA). PLA was carried out with Duolink® In Situ Detection Reagents Orange (#DUO92007, Sigma Aldrich). Additional reagents Duolink® In Situ PLA® Probe Anti-Rabbit PLUS/ MINUS (#DUO92002/DUO92005, Sigma Aldrich) and Duolink® In Situ PLA® Probe Anti-Mouse PLUS/MINUS (#DUO92001/DUO92004, Sigma Aldrich) were used. PLA uses one pair of primary antibodies. Those primary antibodies target proteins of interest, for instance two distinct proteins to study its proximity (Tau and PLD). In this case, primary antibodies are raised in rabbit and mouse species and are detected with secondary antibodies conjugated to short DNA oligonucleotides. If the oligonucleotides are in close proximity (less than 40 nm) the DNA strands hybridize and participate in rolling circle DNA synthesis. These DNA copies can further be detected through hybridization of fluorescent-labeled oligonucleotides. The resulting high concentration of fluorescence is easily visualized under a microscope and quantified.

Proximity Ligation Assay (PLA) Analysis. Quantification of interaction puncta of the PLA was performed using BlobFinder 3.2 (Centre for Image Analysis, Swedish University of Agricultural Sciences/Uppsala University). Analysis settings were the same for all evaluated photographs, namely a puncta threshold of 15, minimum nucleus area of 100 squared pixels, minimum cytoplasm radius of 50 pixels and puncta size of 3 x 3 pixels. Red puncta not surrounding a cell's nucleus (DAPI staining) were considered as background and not taken in account in the analysis. Average intensity per cell was determined as a measure of the number of puncta per cell, and the relative intensity of each individual one.

Confocal Microscopy. All images were acquired on an Olympus FV1000 (Japan) confocal microscope, under a 100x oil objective and resolution of 640 x 640 pixels. A z-series image was acquired for all images using a 594 nm and/or 488 nm laser. The pinhole was adjusted to 1.0

Airy unit. The images were analyzed and processed using ImageJ software®. Colocalization was calculated with the JACoP plugin of ImageJ and is expressed as Pearson coefficient.

Spatial distribution analysis. The analysis of the spatial distribution of protein expression was done using in-house built scripts running on Matlab (v9.0, www.mathworks.com). The process was divided in two steps: i) the isolation and identification of individual cells expressing the proteins; ii) the interpolation of continuous surfaces from the peri-nuclear space until the cell surface and quantification of the signal. For i) the algorithm, first the channels were isolated into the signal expected to represent signal of the nucleus (blue channel) and outside the nucleus (red and green channels). Each channel was cleaned of small components to remove noise (clusters of pixels with size lower than 6000 pixels, with 26 connectivity criteria in three dimensions) and surfaces were closed through dilation followed by erosion. Next, both channels were combined into a single mask and the different isolated components (using connectivity criteria of 26) identified. Each component will represent an isolated cell or an aggregation of connected cells. For each component the spatial frame is restricted to the area where signal is expressed and saved individually (Supplementary Fig.S1b). Through visual inspection the components representing a correctly isolated cell expressing the desired protein are identified. The components identified as correctly isolated were used as input for ii) where the masks for the nucleus and extra-nuclear space were rebuilt and refined (Supplementary Fig.S1c) to build a uniform edge for the nucleus and for the cell wall. Once these surfaces were defined the area interior to these edges was filled to avoid the existence of empty spaces in intra-cellular places where no protein was expressed. The next step aimed at dividing the perinuclear/intracellular space into layers of different distance from the nucleus to the cell surface, to allow the quantification of protein expression in function of this distance. To do this, the nucleus signal was attributed a value of 1 and the extra-cellular area a value of 100, and the intermediate space an unknown value to be interpolated. Matlab's meshgrid function using a linear interpolation algorithm was used to estimate the unknown values, building a gradient of increasing values with increased distance from the nucleus. The totality of interpolated values was divided into 8 ranges and each range attributed a value from 1 to 8, allowing us to build 8 intermediate areas with the same size between the nucleus and the extracellular surface (Supplementary Fig.S1d)). This interpolation is done individually for each 2D frame of the image. The final step defines the local pixel-wise expression of the chosen protein as a fraction of the total intra-cellular signal and sum the fraction of the expression over each layer, thus defining the total fraction

expressed over each layer (Supplementary Fig.S1e). The total values and each frame were saved individually and visually inspected to choose the frames representing the midplane of each cell as well as verify the correct construction of the layers.

Phosphoinositide-binding assay. Phosphoinositide-binding assay using lipid strips supplied by Molecular Probes, Inc. (Leiden, Netherland) was performed following the specifications of the kit. The membranes were blocked with 3% fatty-acid free BSA (Sigma # A-5253) in tris buffered saline with tween® 20 (TBS-T) for 1 h at RT. After discarding the blocking solution membranes were incubated with 0.5 μg /mL of the desired protein (endogenous Tau, Tau 352 WT or Tau 441 WT) in TBS-T + 3% fatty acid free BSA for 2 hours, at RT. Then, the protein solution was discarded, and membranes were washed with TBS-T + 3% fatty acid free BSA, three times 10 minutes each. Protein binding was detected by immunoblot analysis with polyclonal FLAG (F2555, Sigma, 1:1000) antibody as the primary and anti-mouse IgG (A27033, Thermo Fisher Scientific, 1:5000) as the secondary antibody.

Immunoblotting Assay. Cells were washed in PBS and scraped in lysis buffer (150 mM NaCl, 1% NP-40, 0,5% sodium deoxycholate, 0,1% SDS, 50 mM Trizma base, pH 8.0) supplemented with protease and phosphatase inhibitor cocktail (Roche). Homogenates were centrifuged for 20 min at 13,000 × g at 4°C and the supernatant collected. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and was set to a final concentration of 1–2 μg/μL in all experimental conditions. SDS–PAGE was carried out per manufacturer's protocol (Thermo Fisher Scientific). Samples (20–40 μg protein) were prepared with NuPage LDS sample buffer and NuPage reducing reagent and loaded in NuPAGE 4–12% Bis–Tris gels; separation was carried out using MES SDS running buffer (Thermo Fisher Scientific). Wet transfer was performed on 0.22 μm nitrocellulose membranes (Amersham) at 100 V for 2h at 4 °C using transfer buffer (50% running buffer, 20% methanol and 30% water). Membranes were blocked with 5% BSA for 1h at room temperature. Primary antibodies were diluted in blocking buffer (5% milk in TBS-T) and incubated overnight at 4°C; horseradish peroxidase-coupled (HRP) conjugated secondary antibodies were incubated for 60 min at room temperature. For signal detection, chemiluminescent signal was used (ECL western-blotting detecting reagents, Amersham Pharmacia). Western blot quantifications were performed using Chemidoc XRS Software with ImageLab Software (Bio-Rad). For analysis of *C. elegans* protein, nematodes were collected, washed with M9 buffer and frozen in liquid nitrogen. Worm pellets were sonicated for ≈2 min in lysis buffer (150 mM NaCl, 1% NP-

40, 0,5% sodium deoxycholate, 0,1% SDS, 50 mM trizma base, pH 8.0) supplemented with protease and phosphatase inhibitor cocktail (Roche), followed by placing them in ice for 2 min. The previous step was repeated 3 more times. The samples were centrifuged at 13000 × g at 4°C and the supernatant collected.

Nematode Strains and culture conditions. Strains used in this work were acquired from the Caenorhabditis Genetics Center, namely Bristol N2; RB1737, *pld-1*(ok2222) II*;* CB1370*, daf-2*(e1370) III*;* CF1038*, daf-16*(mu86) I*;* tau (V337M); PR811, *osm-6* (p811) V. The Tau (V337M) strain was kindly provided by Brian Kraemer. The Tau (V337M) strain has an intestinal expression of GFP as a transgenic marker. The transgenic strain is referred to as tau strain. All the strains were backcrossed to Bristol strain N2 eight times. Strain Tau (V337M) was crossed with strain RB1737 using standard procedures, generating *pld-1*; tau animals. Worms were grown in agar plates with nematode growth media (NGM) at 20°C as previously described (Brenner 1974). Synchronized cultures were used for all assays and obtained through egg laying, by collecting embryos laid by adult animals during 3 h or using a bleaching procedure, by treating animals with an alkaline hypochlorite solution (0.5 M NaOH, 2.6% NaClO) for 7 min (Stiernagle 2006).

Biometric analysis. Biometric analysis was performed at 72 h after egg laying. Length and diameter measurements were calculated using ImageJ software®, and volume was determined by treating worms as cylinders ($v=\pi*r^2*l$) (McCulloch and Gems 2003). Biometric analysis of F2 animals was performed using the progeny of synchronized worms grown in NGM plates with cholesterol. Pictures were acquired 72 h after hatching. Worms were photographed using an Olympus PD72 digital camera attached to an Olympus SZX16 stereomicroscope.

Motility assay. The motility assay was performed as previously described (Gidalevitz, Ben-Zvi et al. 2006) at RT (≈20°C), using day three synchronized animals grown at 20°C. Five animals were placed simultaneously in the middle of a freshly seeded plate, equilibrated at 20°C. Animals remaining inside a 10 mm circle after 1 min were scored as locomotion-defective. At least 150 animals were scored for each strain in three independent assays.

Thrashing analysis. Single synchronized adult animals were transferred to a 10 µL drop of M9 buffer. After 1 min animals were filmed at a rate of 15 frames-per-second, in a total of 600 frames, using an Olympus PD72 digital camera attached to an Olympus SZX16 stereomicroscope. The number of total body bends per 30 seconds was then quantified using ImageJ software® with the wrMTrck plugin (Nussbaum-Krammer, Neto et al. 2015).

Lifespan. Synchronized adult animals were placed on 60 mm NGM plates at 20°C, examined every day and scored as dead if no mechanical response was obtained after gentle touch with a platinum wire. Animals were transferred to fresh plates every 2 days to avoid starvation and progeny contamination. Animals were censored from the analysis if lost, desiccated on the edge of plates, if showing extruded gonad or suffered internal progeny hatching. Evaluations ended after all animals were dead or censored.

Thermotolerance. Animals were synchronized by egg laying and 50 adults of each strain were selected. The *C. elegans* were transferred from a chamber at 20°C to an incubator at 35°C, with thermal shock occurring. The survival of the *C. elegans* was analyzed every 90 minutes for 9 h (540 minutes).

PTZ susceptibility assay. The PTZ susceptibility assay was adapted from others (Locke, Berry et al. 2008). Plates (30 mm) were prepared with 3 mL NGM each (without food). A stock solution of 100 mg/mL PTZ (Sigma-Aldrich) and the respective dilutions (20, 40, 50, and 80 mg/mL) were prepared. To each plate, 250 µL of PTZ was added. The plates were allowed to dry for 90 min in a flow chamber. Afterwards, 5 times concentrated OP50 was added to the center of each plate. The susceptibility assay was evaluated by placing worms in the bacterial lawn in each plate. After 30 min, each worm's phenotype was evaluated using the following score: 0 for no major decrease in worm movement, 1 for sluggish/slower movement, 2 for semi-paralysis (body bends without moving), 3 for paralysis (only the head moves after mechanical stimulation) and 4 for death.

Diacetyl Preference Assay. Well-fed, synchronized adult day three animals (through bleaching) were collected and washed with S-Basal buffer three times to remove all the food. A *C. elegans* diacetyl preference assay protocol was adapted from previous reports (Pereira and van der Kooy 2012). A chemotaxis assay using 1 M diacetyl (Sigma-Aldrich) was performed in order to confirm that the animals' genotype did not affect the chemotaxis index to diacetyl (naive group). The assay plates (20 g/L agar-agar; 5 mM KH_2PO_4; 1 mM $CaCl_2$; 1 mM $MgSO_4$) were prepared by adding 1 µL of 1 M diacetyl from the center of the plate on one side. On the opposite side of the plate, a 1 µL drop of 100% ethanol was added. Afterwards, 1 µL of 1M sodium azide was additionally added to the preexisting spots to paralyze the animals. Worms (≈100-200) were quickly transferred to the center of the plate and the excess of liquid removed with a filter paper. The assay plates were incubated at 20°C for 60 min and the chemotaxis index was scored as the [number of animals at attractant - number of animals at counter-attractant]/Total number of animals in assay. To condition the

worms, well-fed day three animals were starved for 1 h in the presence of diacetyl and placed on the lid of the plates. Right after the starvation period, the chemotaxis index was scored again (represented as conditioned in the graph).

Statistical analysis. A confidence interval of 95% was assumed for all statistical tests. Normality was tested using the Kolmogorov-Smirnov test and was assumed for all tested variables. In all experiments comparing two variables, the data was analyzed with Student's t-test with the Levene's test for equality of variances. When more than two variables were analyzed, a one-way analysis of variance with the Levene's test for equality of variances and a post-hoc Tukey test for multiple comparisons was performed. The PTZ susceptibility assay was analyzed using a repeated measures analysis of variance with the Mauchly's test for sphericity and a post-hoc Games-Howell test for multiple comparisons. Lifespan was evaluated by the log-rank (Mantel-Cox) test. Statistical analysis was performed using GraphPad Prism 6.01 software® and SPSS 22.0 (SPSS Inc.).

Results

PLD1 and PLD2 co-localize with tau

In order to address the hypothesis that PLD enzymes interact with tau, we performed an immunofluorescence assay using neuronal cell lines (N2a cells) (Fig. 1A). PLD1 or PLD2-GFP-expressing constructs were co-transfected with tau constructs (tau 352 and tau 441 WT), and its cellular localization was evaluated. Confocal microscopy analysis using JACoP plugin of the ImageJ software showed a mild overlap between PLD1 and both tau constructs (tau 352 WT and tau 441 WT). The linear correlation coefficient is r=0.54 and r=0.49 respectively. On the other hand, a strong co-localization between PLD2 and both tau constructs was observed since the pearson correlation coefficient is r= 0.71 to tau 352 WT and r= 0.59 to tau 441 WT (Fig. 1B).

PLD1 and PLD2 interact with tau

To further characterize a possible association between these proteins, a proximity ligation assay was performed, as a quantitative measure of interaction (Fig. 2A and C). The average intensity per cell was determined as a measure of the number of red puncta per cell. Interestingly, we observed that both PLD isoforms have a strong interaction (≈600k of intensity per cell) with tau 441 WT (Fig. 2B and D). Overall, our results show that PLD1 and PLD2 physically interact with endogenous tau and both tau 352 and 441 constructs.

PLD1 and PLD2 overexpression reduces endogenous tau, human tau 352 and tau 441 levels

N2a cells were transfected with either PLD1 or PLD2, and their effect on endogenous tau levels was measured (Fig. 3A). PLD1 or PLD2 were also co-transfected with overexpressing tau constructs, and the respective levels were also evaluated (Fig. 3C). Immunoblots show a decrease in the levels of tau (endogenous and the co-expressed constructs), upon PLD1-GFP and PLD2-GFP expression (Fig. 3B, D and E). Although changes in total tau levels were found after PLD1/2 overexpression, it is noteworthy that the overexpression of PLD2 causes a more marked decrease in tau levels. These results highlight a connection between PLD enzymes and tau, suggesting that these interactions could be relevant to the pathogenesis of AD and other tauopathies.

PLD functional ablation increases tau levels in a tauopathy *C. elegans* model

In order to study the impact of the functional ablation of PLD, we used a tauopathy *C. elegans* model that expresses tau with frontotemporal dementia with parkinsonism chromosome 17 type (FTDP-17) mutations V337^{M} (337^{M-1} and 337^{M-2}). The transgenic (Tg) line expresses GFP in the pharynx. We analyzed the effect of PLD ablation on tau levels by western blot using total nematode protein extracts (Fig. 4A). We observed a significant increase in the tau levels of *pld-1; tau* compared to tau animals (Fig. 4B). Thus, our observations further suggest that PLD ablation has an impact in the total protein levels of tau.

PLD functional ablation has a protective effect in a tauopathy *C. elegans* model

As part of our characterization of *pld-1; tau* transgenic worms, we performed a biometric analysis and, interestingly, we observed that the ablation of PLD in a transgenic tau strain leads to a significant decrease (≈10%) volume of the body when compared to remaining strains (Fig.5A and B). Taking into account our previous work showing that the genetic ablation of PLD2 had a protective effect in synaptic and behavioral deficits in an AD amyloidogenesis mouse model (Oliveira, Chan et al. 2010), we tested if the functional ablation of PLD in worms had not only an effect in the volume of tau worms but also in other phenotypes. First, we performed a lifespan assay, conducted at 20°C, in order to evaluate the effect of mutant tau expression on overall survival. We observed a significant decrease in the median life span of tau worms compared to N2 and, interestingly, *pld-1; tau* animals presented increased median survival when compared to tau worms (Fig. 5C) (Supplemental Table S1). Moreover, we exposed worms to a lethal heat shock at 35°C and we observed that *pld-1; tau* animals exhibited a significant higher rate of survival compared to tau animals (Fig. 5F) (Supplemental Table S2). Concerning motor task assessment, both crawling and swimming were shown to be impaired in tau worms and, again, PLD ablation partially restored these deficits in *pld-1; tau* worms (Fig. 5D and E). Finally, we performed a pharmacological induced pro-excitatory assay using PTZ, a GABA receptor antagonist that increases neuronal excitability by disrupting the normal excitatory/inhibitory balance (Locke, Berry et al. 2008). We exposed worms to increasing doses of PTZ and measured seizure severity. We showed that the tau transgenic worms have increased susceptibility to the effects of PTZ and that PLD functional ablation does not confer a protective effect in PTZ-susceptibility induced by tau expression (Fig. 5G) (Supplemental Table S3). Finally, we also evaluated an associative learning task and we observed that tau transgenic worms display a basal affinity for the diacetyl but an impaired associative learning. Remarkably, PLD ablation restores the normal performance in associative learning in tau animals (Fig.5H). Taken together, these observations indicate that PLD ablation protects from mutant tau deficits.

Endogenous tau, tau 352 and 441 WT bind to phospholipids

In order to study the role of signaling lipids and tau as key modulators of neurodegeneration, we extended our studies to examine the interaction of tau with phospholipids, using pre-prepared strips to evaluate protein-lipid interactions. Our results show that protein preparations were endogenous

tau binding to the strips was probed with a tau antibody, reactivity was shown in phosphatidylinositol monophosphate (PI), phosphatidylinositol 3 phosphate (PI3P), phosphatidylinositol 4 phosphate (PI4P), phosphatidylinositol 5 phosphate (PI5P), PA and phosphatidylserine (PS) (Fig.6B). To further investigate the interactions of tau with lipids, and to increase the specificity of our analysis, we additionally tested this binding assay using two overexpressed isoforms of tau (tau 352 and tau 441 WT) in our protein extracts. We observed a strong interaction between both constructs with PA and PS (Fig.6C and D). Importantly, a mild interaction between PI3P and tau 352 WT was observed (Fig.6C). These results suggest that tau binds to lipids, especially tau 352 WT to PA, which is a product of the enzymatic activity of PLD, via the hydrolysis of PC.

Spo20 leads to tau cellular relocalization

We observed here that 352 WT tau isoform presented a relevant interaction with PA. Taking into account that Spo20 contains a regulatory amphipathic motif that has been suggested to recognize PA (Horchani, de Saint-Jean et al. 2014), we co-transfected Spo20 with tau 352 WT in N2a cells to evaluate the cellular distribution of tau by a spatial distribution analysis assay. Our rational was that Spo20, as PA binding probe could potentially compete with tau for PA binding, leading to its relocalization. Thus, the spatial distribution analysis assay allowed us to evaluate the subcellular distribution of tau 352 WT individually (Fig.7A) and upon co-transfection with Spo20 (Fig.7C). To perform a precise quantification of the protein expression, the extra-cellular space was divided into layers with different distance from the nucleus (Fig. 7B) (detailed in the methods section). Remarkably, we observed that Spo20 co-expression leads to a relocalization of tau from locations more internal in the cell to more to a more peripheral cellular localization (Fig.7 C1 and C2).

Discussion

Lipids are involved in multiple aspects of brain physiology and cell signaling (Di Paolo and Kim 2011). Furthermore, the dysregulation of lipid metabolism has been implicated in a growing number of neurodegenerative diseases, including AD (Di Paolo and Kim 2011). Several studies

indicate that the cytotoxic action of amyloid fibers formed by the Aβ peptide is associated with membrane processes (Fernandez and Berry 2003, Bravo FV 2018). Importantly, there is evidence that Aβ is upstream of tau in AD pathogenesis and triggers the conversion of tau from a normal to a toxic state (Hurtado, Molina-Porcel et al. 2010). On the other hand, the Aβ peptide leads to an activation of PLD (Oliveira, Chan et al. 2010) that curiously, mediates membrane and signaling processes due to its intrinsic physical properties (Haucke and Di Paolo 2007). Since, PLD2 and tau genetic ablation were both shown to be protective in AD mouse models (Roberson, Scearce-Levie et al. 2007, Oliveira, Chan et al. 2010), with no major impact on APP processing and Aβ levels, it is thus plausible that the protective cellular mechanism could be shared. Firstly, we co-transfected N2a cells, with either PLD1 or PLD2 and with both tau isoforms (352 and 441). We evaluated the colocalization and cellular distribution of these proteins using confocal microscopy and we observed an overlap between the PLD enzymes and both tau constructs (Fig. 1). Even though both PLD enzymes colocalize with tau, PLD2-GFP demonstrated a stronger colocalization effect (Fig. 1D). To further evaluate the physical interaction of PLD1/PLD2 and tau, we performed a proximity ligation assay (PLA) that allows an *in situ* validation of the molecular proximity of two proteins, a relevant approach to establish comprehensive interactome maps in basic research and for clinical diagnosis. The results show that both PLD enzymes interact with either endogenous tau or tau constructs (Fig. 2B and D). This is in line with what was previously observed in the colocalization study (Fig. 1). More studies must be performed to investigate if tau and PLD directly interacts or potentially form a complex with other proteins. Importantly, genome-wide association studies (GWAS) revealed bridging integrator 1 (Bin1) also known as amphiphysin 2 (Querfurth and LaFerla 2010), a protein involved in endocytosis and membrane trafficking (Tan, Yu et al. 2013), as the second most important genetic risk factor for AD (Querfurth and LaFerla 2010). Although the precise role of Bin1 in the disease is still unclear, it was shown that its mRNA levels are increased in patients (Querfurth and LaFerla 2010). Interestingly, Bin1 was shown to colocalize and physically interact with tau (Chapuis, Hansmannel et al. 2013) and also with PLD enzymes (Lee, Kim et al. 2000) in independent studies. Our results provide further evidence that a protein complex comprising PLD, Bin1, and tau could play an important role in AD pathogenesis, possibly unravelling new therapeutical targets. Future co-immunoprecipitation (Co-IP) studies will be able to identify potential physiologically relevant protein-protein interactions.

To evaluate the impact of individual overexpression of either PLD1 or PLD2 in tau levels, we measured protein levels through western blot (total levels of tau). Interestingly, we observed that

PLD1-GFP and PLD2-GFP overexpression reduces the levels of endogenous tau (Fig. 3B), human tau 352 (Fig.3D) and 441 (Fig.3E), a shorter and longer isoform of tau, respectively. Once again, PLD2 overexpression produces a strong impact in tau by reducing its protein levels. Previous work showed that PLD2 ablation was shown to be protective in an AD mouse model, decreasing the synaptotoxicity of Aβ (Oliveira, Chan et al. 2010) in a similar way as the ablation of tau (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010). A recent paper from our group supports the notion that PLD has a significant role in neurodegeneration (Bravo FV 2018). The ablation of PLD in a *C. elegans* AD-like model that overexpresses Aβ, improves various phenotypes (Bravo FV 2018), which is in line with previous observations showing PLD as a downstream pathway of Aβ's interaction with membranes (Sanchez-Mejia, Newman et al. 2008, Oliveira, Chan et al. 2010). To better understand the link between PLD and tau, we evaluated the role of the PLD pathway in a pathological context, upon crossing PLD mutant animals with a nematode tauopathy-like model. We used tau (V337^{M}) strain with pan-neuronal expression of FTDP-17 mutant MAPT. In accordance with our cell-line models, we observed that PLD ablation had the opposite effect to the one observed with overexpressing PLD1/2 in N2a cells, leading to an increase in tau levels (≈4-fold increase) in *pld-1*; tau animals (Fig. 4A and B). We had also previously observed that tau animals presented a normal worm size, and remarkably, *pld-1*; tau animals had a significant decrease in animal volume (Fig. 5A and B). Importantly, it has been reported that the clearance of tau is related with the ubiquitin-proteasome and the autophagy lysosome systems (ALS) (Wang and Mandelkow 2012). Notably, with aging the impairment of the protein-degradation systems has been shown to lead to A*β* and tau accumulation and subsequent aggregation (Wang and Mandelkow 2012). Moreover, previous reports have shown that the catalytic activity of PLD may regulate the clearance of protein aggregates, such as p62 and tau, with strong implications in neurodegenerative disorders, including tauopathies (Dall'Armi, Hurtado-Lorenzo et al. 2010). Thus, taking into consideration that tau levels are increased and that worm volume is decreased we hypothesize that autophagy related mechanisms could partly explain this phenotype.

To better understand the impact of PLD ablation in a disease context, we performed a multitude of behavior tests. Our findings demonstrate that PLD ablation in *pld-1*; tau animals has a major impact in worm survival (Fig. 5C). Concerning motor behaviors, thermotolerance, and associative learning tasks, once again, the ablation of PLD had also a protective effect. This is in line with a previous report that points PLD as an emerging key player in the modulation of phospholipid metabolism, and a valuable therapeutic target for AD and other brain disorders as a downstream effector of Aβ,

another potential toxic protein (Oliveira, Chan et al. 2010, Bravo FV 2018). Overall, these results indicate that PLD ablation may have a protective effect in the context of tauopathies. However, there are still many issues related to the importance of PLD in neurodegenerative diseases, namely the cellular protection mechanism, if this effect is specific for the V337M mutation (present in our study model) or is a global neuroprotective effect.

It is indeed puzzling that even though PLD ablation leads to an increase in the total levels of tau, it is decreasing its toxic effects in the worm organism. Considering previous reports that show that tau neuronal relocalization to dendrites and spines is necessary for its toxic effects (Hoover, Reed et al.) and that tau partly interacts with membranes (Brandt R 1995), we raised the hypothesis that tau could interact with specific lipids and this interaction could be relevant for tau dependent toxic effects. In this situation tau cellular/neuronal localization would be the key determinant of its toxic effects, more so than its total levels. To address that question we used a protein-lipid overlay assay and we demonstrated that tau presented a strong interaction with PA, PS and PI3P. Taking into account that PA is a product of PLD's enzymatic activity, this supports our initial findings about a potential interaction between tau and PLD enzymes, since it is reminiscent of a common mechanism in lipid biology that a lipid modulating enzyme binds to its own substrate (Di Paolo and Kim 2011). Interestingly, a key molecule described in endolysosomal function and autophagy is the class III phosphatidylinositol-3-kinase (PI3KIII), Vps34, which generates PI3P by the phosphorylation of PI on the 3' position of the inositol ring. Interestingly, the dysregulation of this pathway is associated with endolysosomal perturbation leading to neurodegeneration (Morel, Chamoun et al. 2013), increased tau levels and a decrease of Aβ secretion (Miranda, Lasiecka et al. 2018). Moreover, it was previously shown that decreasing Vps34 levels leads to decreased PLD1 levels (Dall'Armi, Hurtado-Lorenzo et al. 2010), showing that both these pathways could be interconnected.

Finally, to further understand the role of PLD in the regulation of tau, we used a genetically encoded probe for PA, Spo20 (Ganesan, Shabits et al. 2015). It was previously shown that the expression of specific lipid probes, such as the pleckstrin homology (PH) domain from general receptor for phosphoinositides (GRP1), which is experimentally used as a PI(3,4,5)P3 probe, competes for the lipid binding site and disrupts functions relying on its lipid-protein interactions (Arendt, Royo et al. 2010). We tested the impact of overexpressing this PA lipid probe, Spo20, on tau localization. Roland Brandt et al. showed that tau is partly localized in membrane fractions, showing that it potentially interacts with specific lipids (Brandt R 1995). In line with our observations that tau

interacts with PA, we observed that upon disruption this interaction, with Spo20 co-expression, we observed a relocalization of intracellular tau pools from an internal localization to a more peripheral localization in the cell (Fig. 7). Hoover et al. reported that the mislocalization of tau to dendritic spines mediates synaptic dysfunction (Hoover, Reed et al. 2010). Future studies, concerning the localization of the potential tau-PLD complex will be able to further characterize the impact of PLD1, PLD2 or PA in mediating the excitotoxicity effects of tau in neuronal degeneration. A possible approach would be to perform subcellular fractionation studies and imaging studies addressing tau localization in neurons upon disrupting its connection with PLD and or PA.

Overall, our results indicate that PLD pathway is a key regulator of tau physiology and pathology. Since the PLD pathway was shown to have a role in autophagy, endo-lysosomal regulations and in both APP processing and Aβ signaling, this further connection with tau is of high relevance in the design of therapeutical applications that target PLD.

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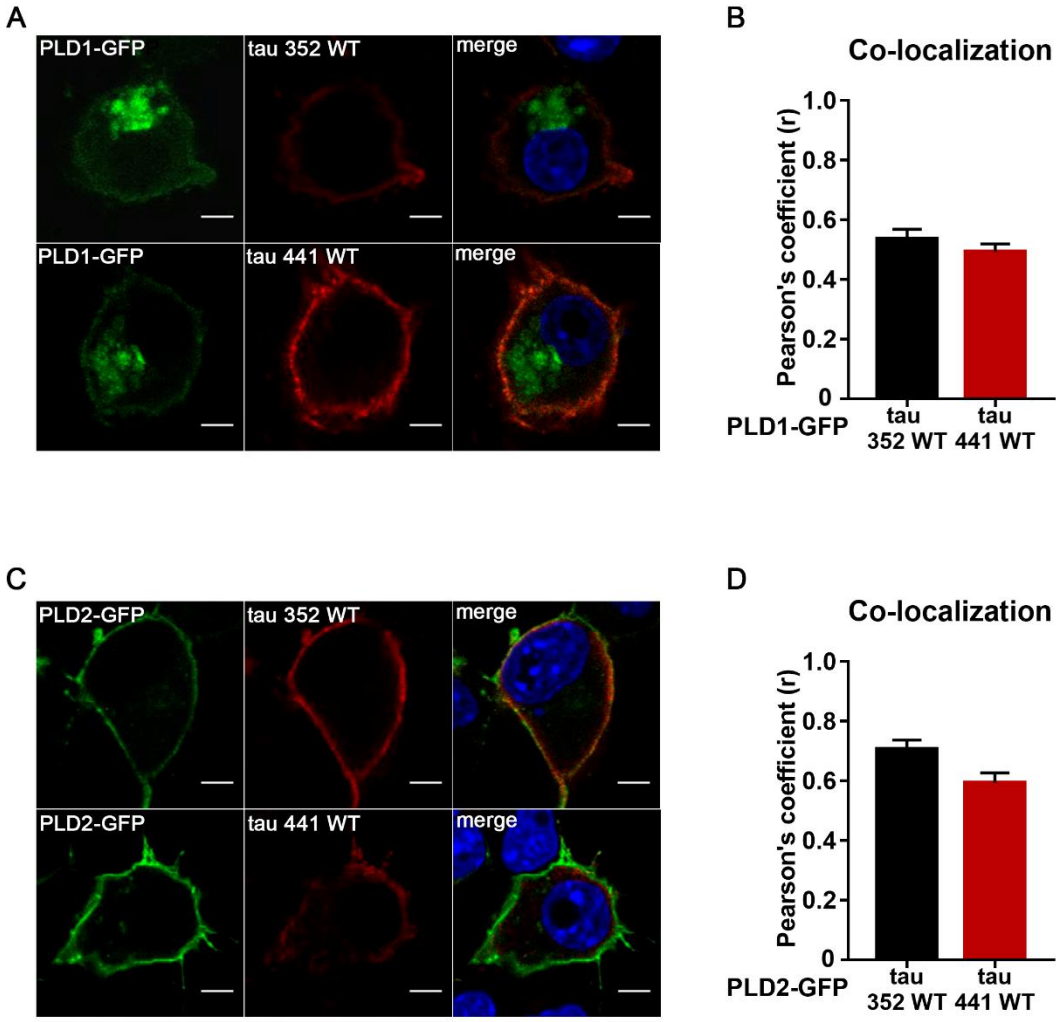


Figure 1. PLD1-GFP and PLD2-GFP co-localizes with tau 352 and 441 WT. (A) Representative confocal images of N2a cells transiently co-transfected with PLD1-GFP with FLAG tau 352 WT or FLAG tau 441 WT. (B) Quantification of co-localization by JACoP plugin in ImageJ using Pearson's correlation coefficient ($r_{PLD1\text{-}GFP/tau\ 352\ WT}$=0.54; $r_{PLD1\text{-}GFP/tau}$ 441 WT=0.49). (C) Representative confocal images of N2a cells transiently co-transfected with PLD2-GFP with FLAG tau 352 WT or FLAG tau 441 WT. (D) Quantification of co-localization by JACoP plugin in ImageJ using Pearson's correlation coefficient ($r_{PLD2\text{-}GFP/tau\ 352\ WT}$=0.71; $r_{PLD2\text{-}GFP/tau\ 441\ WT}$=0.59). Photographs were taken at 100x magnification. Scale bar represents 5 μm. The data results from the analysis of 10 cells, from three independent experiments. Values denote means ± SEM.

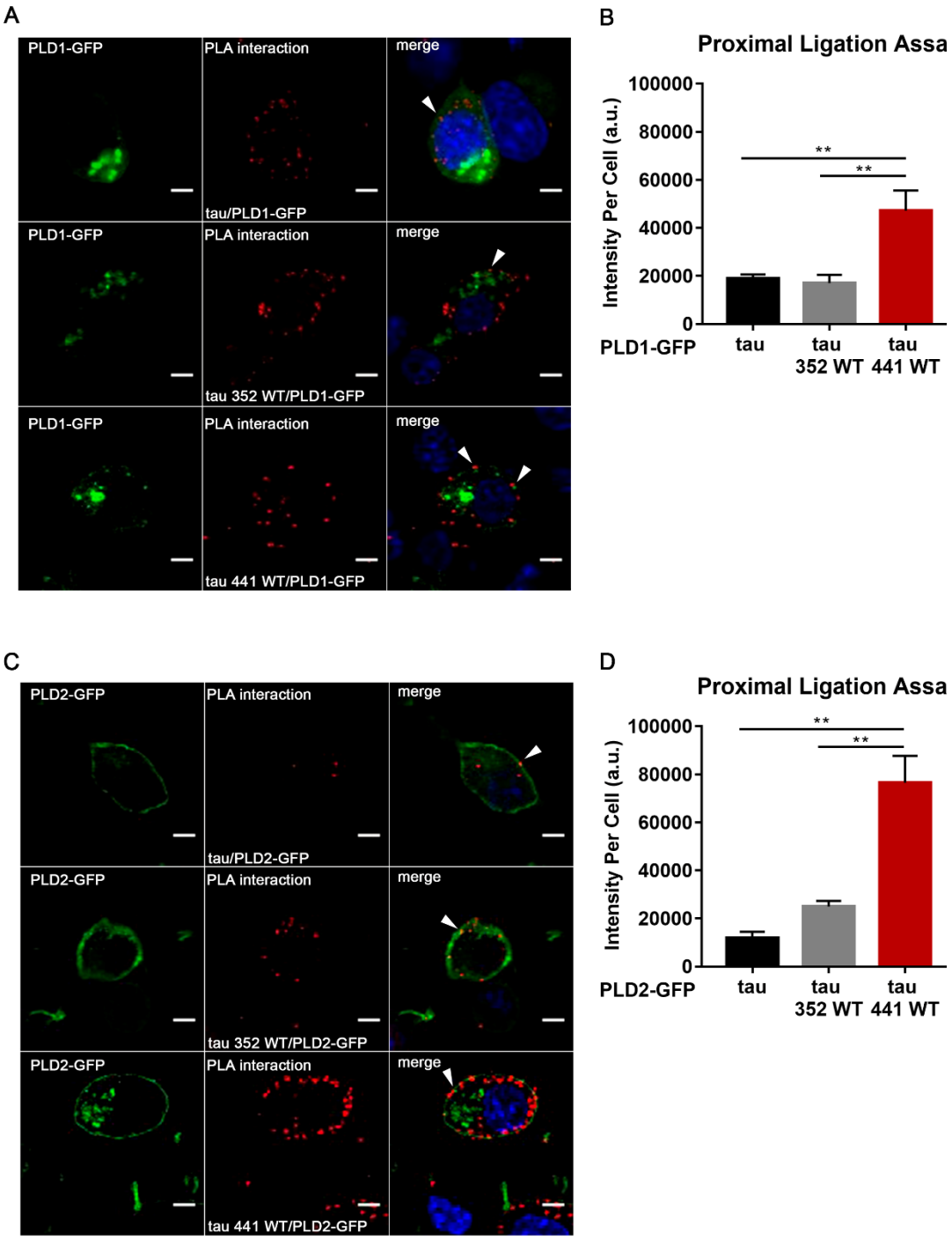


Figure 2. PLD1-GFP and PLD2-GFP interacts with tau 352 and 441 WT. (A) Representative confocal images showing PLA fluorescence (tau endogenous/PLD1-GFP, tau 352 WT/PLD1-GFP and tau 441 WT/PLD1-GFP interaction, red, center column), PLD1-GFP (green, left column), and merged with DAPI (right column) in N2a cells. (B) Quantification of the PLA signal (dots plots) on N2a cells transiently co-transfected with PLD1-GFP with tau endogenous, tau 352 or tau 441 WT. PLA signals showing the tau/PLD-1 interaction. (B) Representative confocal images

showing PLA fluorescence (tau endogenous/PLD2-GFP, tau 352 WT/PLD2-GFP and tau 441 WT/PLD2-GFP interaction, red, center column), PLD2-GFP (green, left column), and merged with DAPI (right column) in N2a cells. (B) Quantification of the PLA signal (dots plots) on N2a cells transiently co-transfected with PLD2-GFP with tau endogenous, tau 352 or tau 441 WT. PLA signals showing the tau (endogenous, tau 352 and 441 WT)/PLD-2 interaction. Photographs were taken at 100x magnification. Scale bar represents 5 μm. The data results from the analysis of 30 cells, from three independent experiments. Values denote means ± SEM (**p ≤ 0.01).

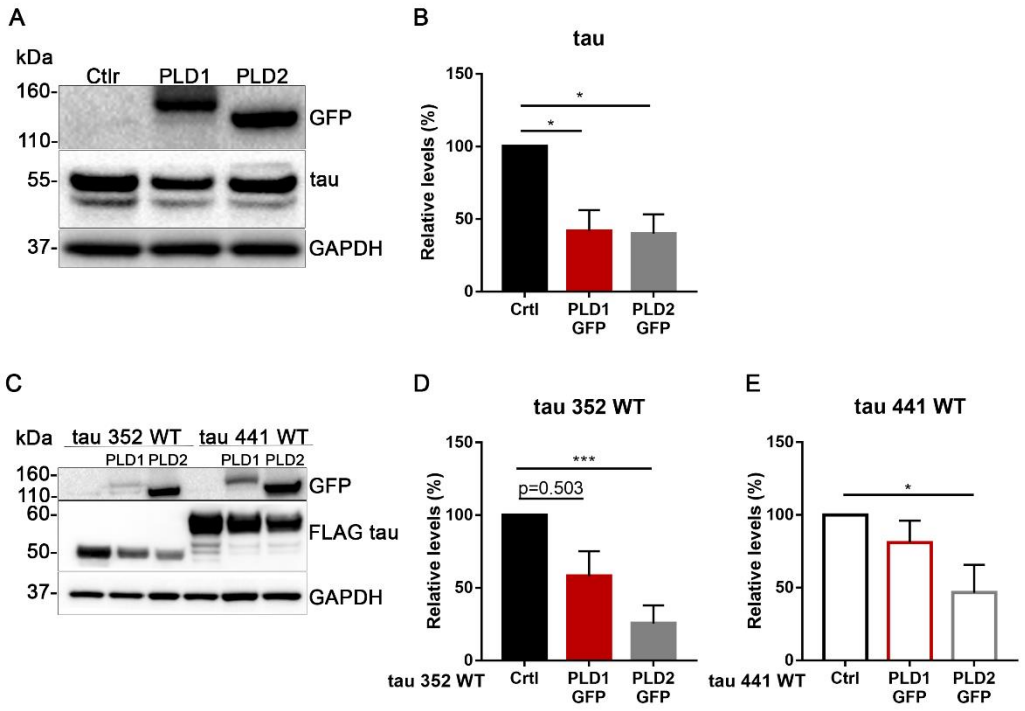


Figure 3. PLD1-GFP and PLD2-GFP reduces endogenous tau, human tau 352 and tau 441 WT in N2a cells. (A) N2a cells were transiently transfected with PLD1 and PLD2 GFP for 48h. Western blot analysis of cell lysates from N2a cells overexpressing PLD1 and PLD2 GFP with antibodies against GFP, tau and GAPDH. (B) Quantification of band intensities of tau of the western blot shown in A, normalized by GAPDH. (C) N2a cells were transiently co-transfected with PLD1/PLD2 GFP and tau 352/tau 441 WT for 48h. Western Blot analysis of cell lysates from N2a cells overexpressing PLD1 and PLD2 GFP with antibodies against GFP, FLAG and GAPDH. Quantification of band intensities of tau 352 (D) and tau 441 (E) of the western blot shown in C, normalized by GAPDH. Bar graph denotes average protein levels normalized to control. The data for five independent experiments is represented. Values denote means ± SEM. (* p ≤0.05, *** p ≤ 0.001).

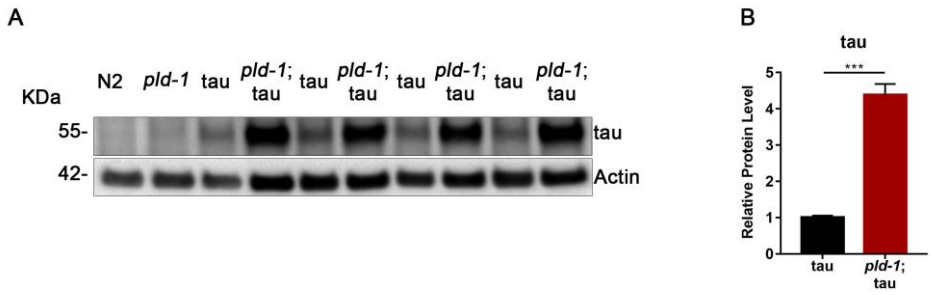


Figure 4. PLD functional ablation increases tau levels in a tauopathy *C. elegans* model. (A) Representative western blot analysis of nematodes lysates from adults N2, *pld-1*, tau and *pld-1*;tau animals with antibodies against tau and actin. (B) Quantification of band intensities of tau of the western blot shown in A, normalized by actin. The data for three independent experiments is represented (N=4). Values denote means ± SEM (*** $p \leq 0.001$).

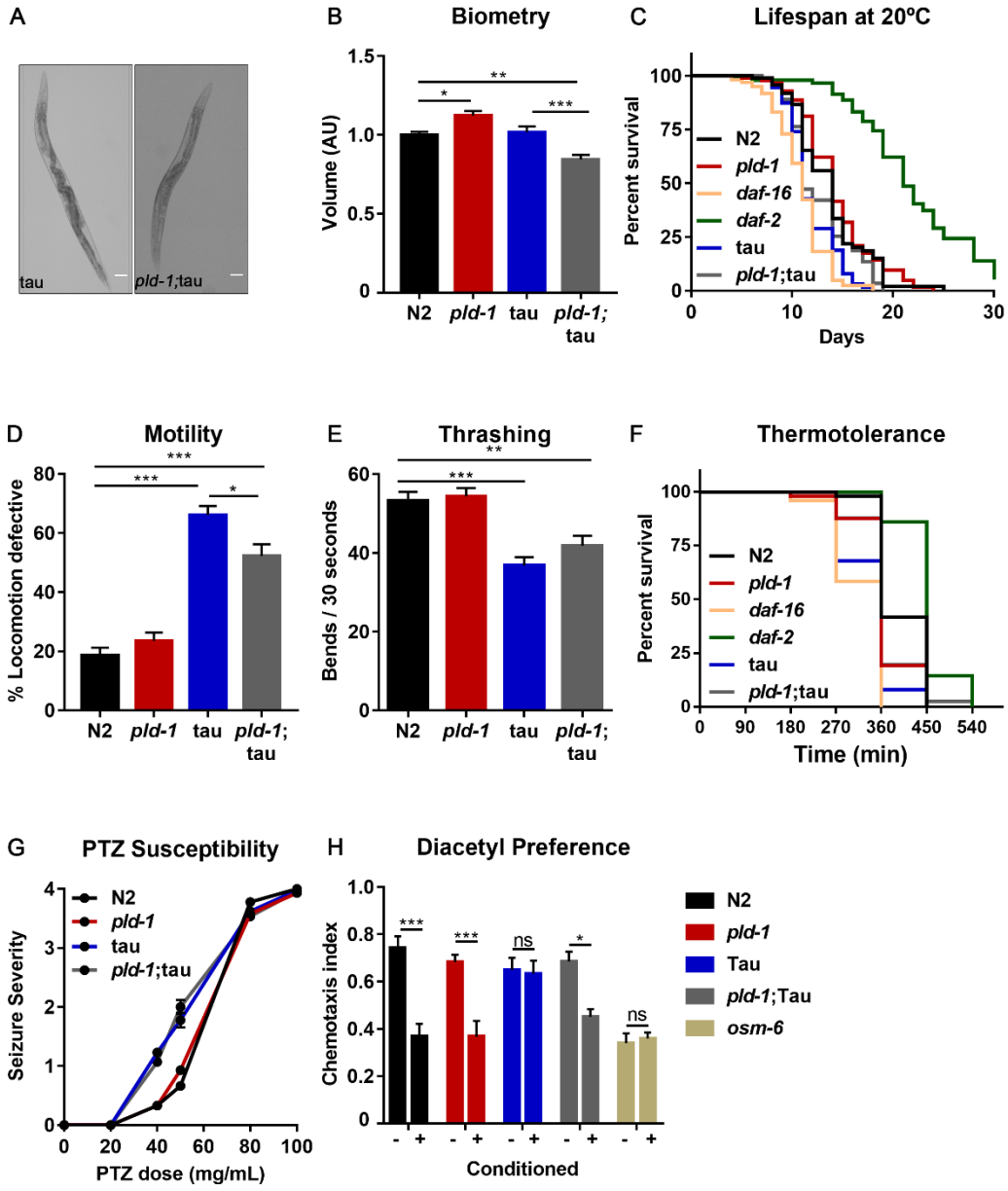


Figure 5. PLD functional ablation has a behavioral protective effect in a tauopathy *C. elegans* model. (A) Representative photos of tau and *pld-1*;tau and graphic analysis of volume (B) of N2, *pld-1,* tau and *pld-1*;tau animals at day 3 after egg laying. The scale bar represents 100 µm. (B) 40 animals were analyzed per strain in 3 independent experiments. Values denote means ± SEM (* $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$). (C) Effects of mutant tau expression on adult lifespan at 20°C. The experiment was conducted twice with 100 worms per strain. Kaplan-Meier survival curve of tau and *pld-1*; tau worms, showing differences in the median lifespan compared with N2 and *pld-1* animals. The mean adult lifespan of *pld-1*; tau animals was significantly longer than tau transgenic animals. (D) tau worms have a motility defect partially recovered by PLD ablation. Percentage of uncoordinated N2, *pld-1*, tau and *pld-1*; tau

adult hermaphrodite animals was measured by scoring the animals that remain inside 10 mm circle after 1 min (n=4 experiments, 10 worms per strain and experiment). (E) The number of body thrashes per 30 seconds was partially rescued by PLD ablation in tau transgenic animals (n=4 experiments, 10 worms per strain and experiment). (F) Effects of mutant tau expression on thermotolerance. The experiment was conducted three times with 100 worms per strain. Kaplan-Meier survival curve of tau and *pld-1*; tau worms under acute thermal stress at 35°C, showing differences in the median thermotolerance compared with N2 and pld-1 animals. The mean adult thermotolerance of *pld-1*; tau animals were significantly longer than tau transgenic animals. (G) tau transgenic and *pld-1*; tau animals are more susceptible to seizure-severity induced by PTZ compared with N2 and *pld-1* animals. PTZ susceptibility of worms were scored after exposure to the concentrations of 20, 40, 50, 80 and 100 mg/mL of PTZ. (N=4 experiments, 15 animals per dose/strain/experiment). (H) Associative learning in transgenic tau neuronal expressing *C. elegans.* Worms were starved for 2 h either in the presence (conditioned) or absence (naive) of the odorant diacetyl and subsequently subjected to a chemotaxis assay. A mutant strain unable to detect diacetyl (*osm-6*) was used as a negative control. Tau transgenic worms display a basal affinity for the diacetyl but an impaired associative learning. PLD ablation leads to an increase in associative learning. Three independent experiments were performed (n = 200 worms per assay). Values denote means ± SEM. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

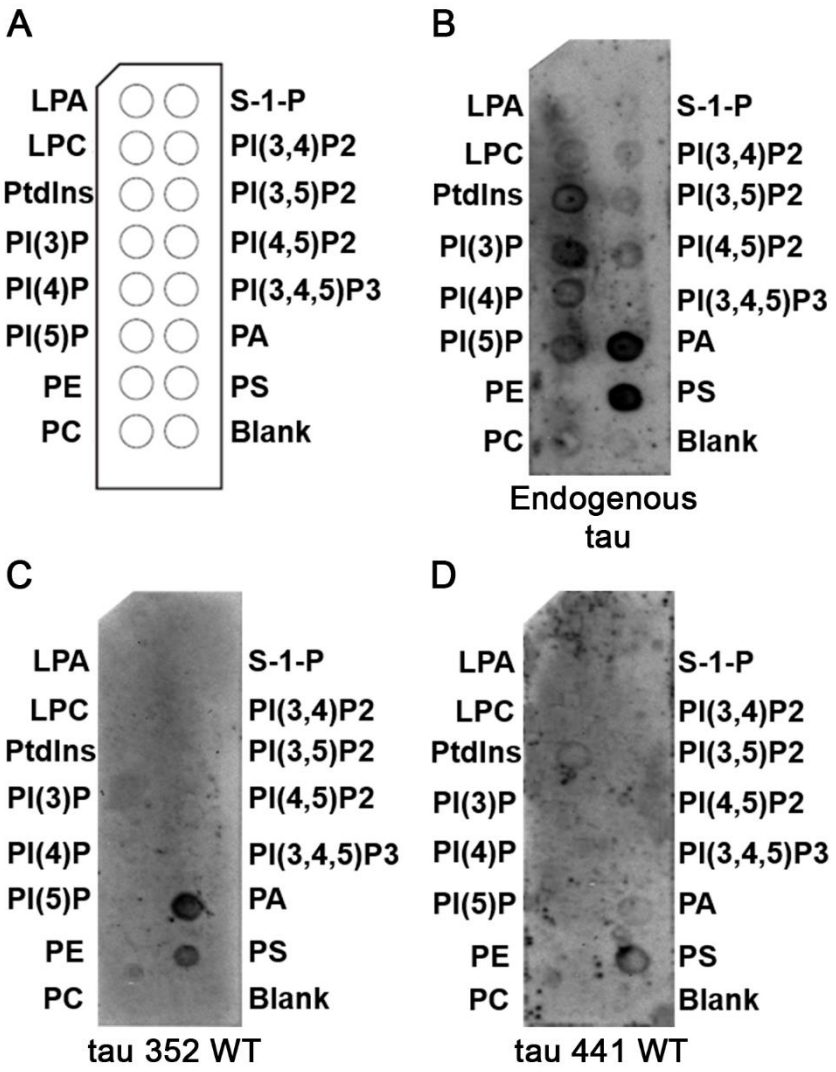


Figure 6. Endogenous tau, tau 352 and 441 WT bind to phospholipids. (A) Schematic representation of PIP strip indicating the phospholipid present in each dot. Nomenclature of different phospholipids spotted on membrane are Lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), phosphatidylinositol (PtdIns), PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, phosphatidylethanolamine (PE), 8-phosphatidylcholine (PC), sphingosine 1-phosphate, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2, PtdIns(3,4,5)P3, phosphatidic acid (PA), phosphatidylserine (PS), blank. (B-D) Comparison of lipid binding affinity of tau, tau 352 WT and tau 441 WT cell lysates (0.5 μg /mL) respectively. Lipids strips were incubated with polyclonal primary antibody and the lipid-bound protein was detected with anti-mouse IgG as secondary antibody. The data of three independent experiments is represented.

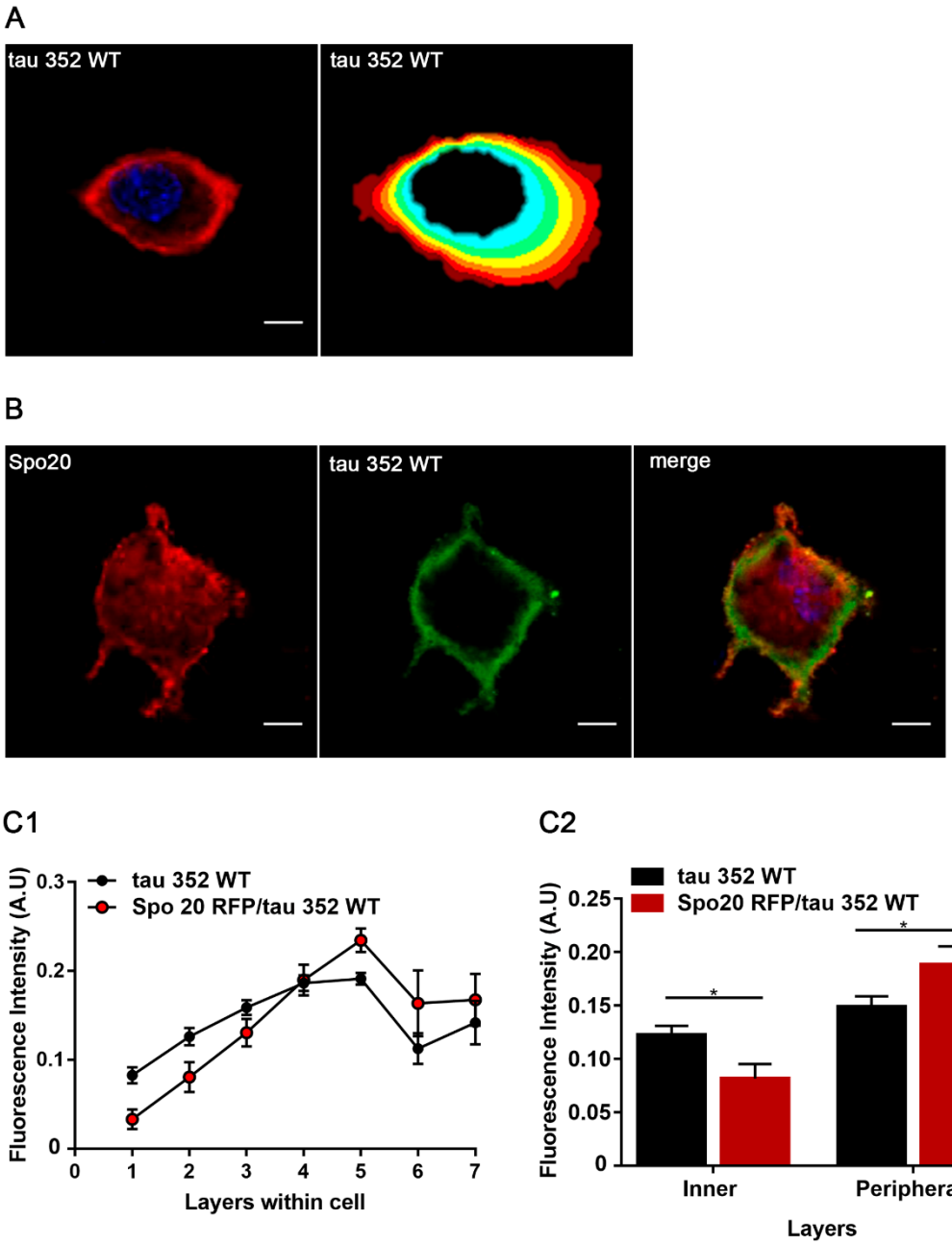


Figure 7. Spo20 leads to tau relocalization. (A) Representative confocal images of N2a cells transiently transfected with tau 352 WT. Representative image of the extra-cellular space divided into layers with different distance from the nucleus. (B) Representative confocal images of N2a cells transiently co-transfected with Spo20 RFP and tau 352 WT. (C1-C2) Quantification of the fluorescence intensity on N2a cells transiently transfected with Spo20 RFP and co-transfected with Spo20 RFP and tau 352 WT. Photographs were taken at 100x magnification. Scale bar represents 5 μm. The data results from the analysis of 20 cells. Values denote means ± SEM (*$p \leq 0.05$).

Regulation of tau by the Phospholipase D pathway

Francisca Vaz Bravo, Ricardo Magalhães, Jorge Da Silva, Ricardo Rosa, Tiago Gil Oliveira

Supplementary Data

Strain (n)*	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	Control stain (n)	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	*P* value (Log Rank,Mantel-Cox)
***daf-16* (48)**	360	0	340	7.64	**N2(65)**	360	11.38	399	8	<0.0001
***daf-2* (41)**	450	6.30	462	9.54	**N2(65)**	360	11.38	399	8	<0.0001
pld-1 (47)	360	7.58	350	8.33	**N2(65)**	360	11.38	399	8	<0.0001
tau (47)	360	5.76	351	6.68	**N2(65)**	360	11.38	399	8	<0.0001
***pld-1* ;tau (46)**	360	7.79	382	10.91	**N2(65)**	360	11.38	399	8	0.311
***pld-1* ;tau (46)**	360	7.79	382	10.91	***pld-1* (65)**	360	7.58	350	8.33	0.008

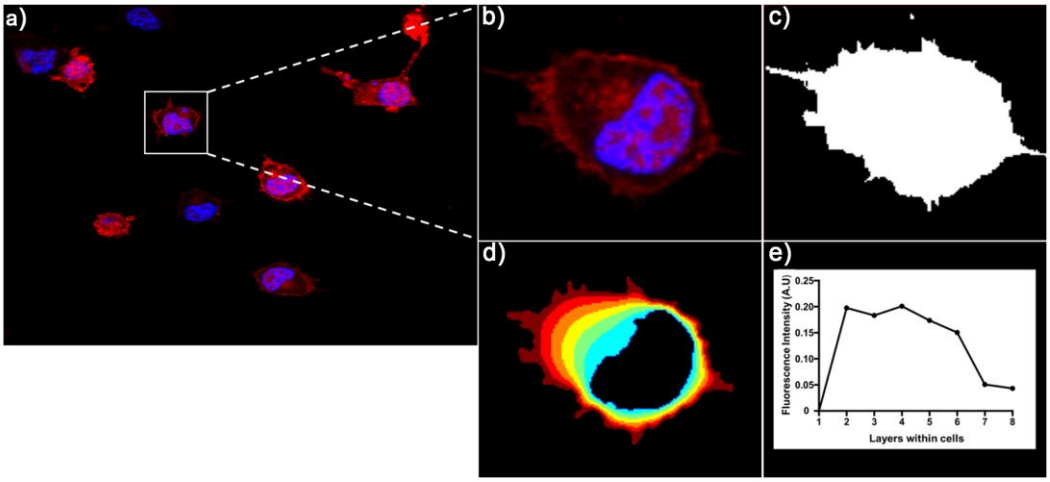
Supplementary Table S1. PLD ablation partially restores survival of tau transgenic animals. Survival statistic analysis of experiment of N2, pld-1, tau, pld-1; tau, daf-16 and daf-2 animals at 20°C.

Strain (n)*	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	Control stain (n)	Median lifespan (days)	s.e.m (days)	Mean lifespan (days)	s.e.m (days)	*P* value (Log Rank,Mantel-Cox)
***daf-16* (48)**	360	0	340	7.64	**N2(65)**	360	11.38	399	8	<0.0001
***daf-2* (41)**	450	6.30	462	9.54	**N2(65)**	360	11.38	399	8	<0.0001
pld-1 (47)	360	7.58	350	8.33	**N2(65)**	360	11.38	399	8	<0.0001
tau (47)	360	5.76	351	6.68	**N2(65)**	360	11.38	399	8	<0.0001
***pld-1* ;tau (46)**	360	7.79	382	10.91	**N2(65)**	360	11.38	399	8	0.311
***pld-1* ;tau (46)**	360	7.79	382	10.91	***pld-1* (65)**	360	7.58	350	8.33	0.008

Supplementary Table S2. PLD ablation increases the resistance to lethal heat shock (35°C) of tau transgenic animals. Survival statistic analysis of experiment of N2, pld-1, *tau*, pld-1; *tau*, *daf-16* and *daf-2* animals at 35°C.

Control Strain	Strain	Mean Difference	s. e. m	*P* value
N2	***pld-1***	0.06	0.047	0.529
N2	**tau**	-0.24	0.053	< 0.0001
N2	***pld-1;* tau**	-0.22	0.051	< 0.0001
tau	***pld-1;* tau**	0.01	0.053	0.993

Supplementary Table S3. Repeated measures analysis of variance of the PTZ susceptible assay in *C. elegans*. $F(3, 2.498) = 3803.934$, $p < 0.0001$.



Supplementary Figure S1. Spatial distribution of protein assay. a) identification if individual cells expressing the desire proteins; b) cell identified as correctly isolate; c) representative image of the masks for the nucleus and extra-cellular space; d) extra-cellular space divided into layers of different distance from the nucleus; e) quantification of protein fluorescence intensity in function of this distance.

Chapter 2.3

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Lipidomic determinants of seizure severity in AD mouse model

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Lipidomic determinants of seizure severity in AD mouse model

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Abstract

Alzheimer's disease (AD) is the most common neurodegenerative disease and currently, there are no effective treatments to tackle the advancement of its clinical course. Therefore, a detailed understanding of its pathogenesis is critical to envision new therapeutical strategies. It has been shown that AD patients are more susceptible to seizure-like activity and have a higher rate of epileptiform/sub-epileptic events. The use of hAPP mice, replicates many of the human disease hallmarks, such as the accumulation of amyloid-beta (Aβ) and excitotoxic susceptibility.

Since lipids are the main constituent of the brain, they are central candidates to be modulating brain physiology and pathology. Concerning AD pathogenesis, it has been shown that specific lipid metabolic signatures have been identified both in human and mouse AD brain samples, showing that lipid metabolism is indeed dysregulated. Moreover, members of our team, and others, showed that mice genetically ablated with either phospholipase D2 (PLD2) or phospholipase A2 (PLA2) are cognitively protected when crossed with hAPP mice.

In this study, we propose to characterize the hippocampal lipidome of susceptible animals to epileptic activity, using hAPP mice. With our unbiased lipidomic approach, we observed that the mouse brain hippocampus hAPP presented significant lipid composition. Moreover, we found that specific lipid species of brain hippocampus are correlated with susceptibility to PTZ-induced seizures. Finally, we found changes in the saturation and length of glycerophospholipids and sphingolipids fatty acyl composition in hAPP, with specific signatures that correlate with pharmacological induced seizure severity.

In summary, the lipidomic profiling of the hAPP mouse brain hippocampus in the context of pharmacological induced seizures allowed for the identification of dysregulated lipid pathways, revealing putative targets for pharmacological intervention that may potentially be used to protect from excitotoxicity associated events.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and is characterized by a progressive loss of cognitive function. Brain regions affected in AD are typically characterized by the deposition of amyloid plaques and neurofibrillary tangles, which are constituted by amyloid-beta (Aβ) and hyperphosphorylated tau, respectively. Aβ is a peptide produced by the sequential cleavage of amyloid precursor protein (APP), by BACE1 and γ-secretase. Although recent major advances have been achieved in the understanding of AD pathogenesis, there are no effective treatments to tackle the advancement of AD clinical course (Huang and Mucke 2012).

Despite the late onset development of amyloid plaques, Aβ soluble species are thought to impair synaptic neuronal functioning at earlier stages (Palop and Mucke 2009, Palop and Mucke 2016). In fact, the risk of epileptic activity is higher in AD patients, with an 87-fold increase in seizure incidence when compared to age-matched patients (Amatniek, Hauser et al. 2006, Palop, Chin et al. 2007, Vossel, Tartaglia et al. 2017). This abnormal epileptiform activity is associated with transient amnesia and disorientation in AD (Rabinowicz, Starkstein et al. 2000), not always perceivable by care providers, eliciting the question whether this subclinical epileptic activity is not clinically underdiagnosed and an early manifestation of AD onset. Interestingly, this was also observed in AD mouse models (which express human amyloid precursor protein with familial AD mutations and that overproduce Aβ – hAPP mice), with a higher rate of sub-epileptic events and increased susceptibility to pharmacologically-induced seizures (Palop, Chin et al. 2007). Moreover, treatment with the anti-epileptic drug levetiracetam had a cognitive protective effect, both in mice and humans, suggesting neuronal excitotoxicity induced by Aβ to be a central pathogenic mechanism in AD (Bakker, Krauss et al. 2012, Sanchez, Zhu et al. 2012).

Although current evidence suggest Aβ to have a pivotal role in AD, other factors are also likely to have a pathological function in the development of the disease (Palop and Mucke 2010). Since the main neuropathological hallmarks of AD are constituted of Aβ and tau, many researchers have addressed the potential signaling link between these two key molecular players. Importantly, while mutations affecting APP and its processing have been found to lead to familial forms of AD (FAD), mutations affecting tau lead to other non-AD neurodegenerative diseases (Huang and Mucke 2012). Moreover, it was shown in human neural stem cells from FAD patients cultured in a three-

dimensional environment recapitulating the classical hallmarks of Aβ accumulation and neurofibrillary tangle formation, that decreasing Aβ production or its downstream signaling effectors, prevented the accumulation of the toxic tangles (Choi, Kim et al. 2014). This favours the possibility that tau may be acting as a modulator of Aβ pathogenic pathway. Indeed, tau knockout (KO) mice and tau reduction with antisense oligonucleotides (ASO) leads to resistance to Aβ-induced seizures and excitotoxicity (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010, DeVos, Goncharoff et al. 2013). Therefore, there is an increasing interest in defining the cellular and molecular mechanisms regulating tau's function in AD.

Lipid signaling is fundamental in the regulation of key cellular processes such as membrane trafficking, ion channel regulation and protein-lipid interactions and many research groups have been studying the role of lipid signaling in neuronal excitability homeostasis (Di Paolo and De Camilli 2006). Accordingly, repeated events of pharmacological-induced seizures have been shown to induce changes in brain lipid composition (Guan, He et al. 2006) suggesting that lipid-modifying enzymes are potentially involved in seizure-associated neuronal impairment. However, to date, the role of lipid signaling in seizure-associated pathology and the lipid determinants of seizure-resistance or susceptibility have not been addressed in detail. This broad-perspective analysis is now possible with the advancement of techniques such as high profile liquid chromatography (HPLC) and LC-mass spectrometry (LC-MS), which currently allow to study in an unbiased way, the lipid composition of a given biological sample (Wenk 2005).

Since lipids are a major brain constituent, its role in AD pathogenesis has been increasingly studied in AD pathogenesis. In fact, the E4 allele of the apolipoprotein E, a lipid transporting protein, was found to be the strongest genetic risk factor in AD (Di Paolo and Kim 2011). Also, the use of new techniques to study lipids, such as LC-MS lipidomic analysis, led to the identification of various lipid signaling pathways to be altered in both human and rodent brain samples (Oliveira, Chan et al. 2010, Chan, Oliveira et al. 2012, Morel, Chamoun et al. 2013). Specifically, it was found that Aβ leads to increased activity levels in phospholipase C (PLC) (Berman, Dall'Armi et al. 2008), PLA2 GIVa (Sanchez-Mejia, Newman et al. 2008) and PLD (Oliveira, Chan et al. 2010), in a calcium dependent way, and that the genetic ablation of the lipid modulating enzymes PLD2 (Oliveira, Chan et al. 2010) and PLA2 GIVa (Sanchez-Mejia, Newman et al. 2008) had a protective cognitive effect in AD mouse models. Curiously, a major excitotoxic mechanism relies on the overactivation of N-methyl-D-aspartate receptor (NMDAR), leading to increased intraneuronal toxic calcium levels

(Ittner, Ke et al. 2010). It is thus plausible that lipid signaling pathways impairment in Aβ-downstream-signaling could be connected with excitotoxicity. Moreover, these studies highlight the possibility that lipid-signaling modulation can be an effective therapeutical strategy in AD.

In this study, we propose to characterize the hippocampal lipidome of susceptible animals to epileptic activity, using hAPP. With our unbiased lipidomic approach, we expect to better understand the underlying changes in lipid composition of the hippocampus, an area affected by excitotoxicity and fundamental for cognitive function. Our overall aim is to find new therapeutical targets for excitotoxicity protection modulating lipid signaling pathways.

Materials and methods

Animals. Male hemizygous B6.Cg-Tg(PDGFB-APPSwInd)20Lms/2J/Mmjax (J20) mice, which expresses an hAPP minigene with the Swedish (K670M/N671L) and Indiana (V717F) familial Alzheimer's disease (AD) mutations under control of the PDGF promoter, in a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA), and were previous developed by Professor Lennart Mucke, of The J. David Gladstone Institutes (San Francisco, CA, USA). Mice were kept on a 12 h light/12 h dark cycle and access to food and water. Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and National Institutes of Health guidelines on animal care and experimentation. All experiments were performed blinded to the genotypes (in the presence or absence of the hAPP transgene).

Seizure Severity Score. Seizure Severity Score was assessed as previously described (Roberson, Scearce-Levie et al. 2007). A stock solution of 5mg/mL pentylenetetrazole (PTZ, Sigma, St. Louis, MO) and a dose of 40 mg/kg were used. PTZ was dissolved in phosphate-buffered saline (PBS) and delivered by intraperitoneal injection (i.p.). After IP administration, each mouse was placed in a cage and recording for 20 min. The PTZ susceptibility of each mouse, was evaluated using the following score: 0 = normal behavior; 1 = immobility; 2 = generalized spasm, tremble, or twitch; 3 = tail extension; 4 = forelimb clonus; 5 = generalized clonic activity; 6 = bouncing or running seizures; 7 = full tonic extension; 8 = death. According to previous published scales (Loscher,

Honack et al. 1991), an investigator blinded to genotypes quantified the time course and severity of seizures.

Analysis of Lipids Using High Performance Liquid Chromatography-Mass Spectrometry. Upon 20 min post-injection, in case the animals did not die upon pharmacological induced seizure assessment, the animals were sacrificed, and the hippocampus was dissected and prepared for lipid extraction. Lipids were subsequently extracted by a chloroform/methanol extraction, as previously described (Chan, Uchil et al. 2008, Fei, Shui et al. 2008). Lipid species were analyzed using a 6490 Triple Quadrupole LC/MS system (Agilent Technologies, Santa Clara, CA) operated in multiple reactions mode (MRM). Individual lipid species were measured by MRM transitions and lipid concentration was calculated by referencing to appropriate internal standards. Lipid concentration was normalized by molar concentration across all species for each sample, and the final data is presented as the mean mol %.

Statistical analysis. A confidence interval of 95% was assumed for all statistical tests. Normality was tested using the Kolmogorov-Smirnov test, and was assumed for all tested variables. In all experiments comparing two variables, the data was analyzed with Student's t-test with the Levene's test for equality of variances. Correlation data analysis was performed using the linear regression model and coefficient of determination and values expressed as R^2. Statistical analysis was performed using GraphPad Prism 6.01 software®.

Results

hAPP mice are more susceptible to pharmacological-induced seizures.

We performed a pharmacological induced pro-excitatory assessment using pentylenetetrazol (PTZ), a GABA receptor antagonist that increases neuronal excitability by disrupting the normal excitatory/inhibitory balance (Locke, Berry et al. 2008). We used hAPP mice, which have increased brain Aβ levels, with age matched control animals, and acute i.p. PTZ injections (40 mg/kg) were performed. The seizure severity score was evaluated through video analysis by an independent researcher. As shown by others (Palop, Chin et al. 2007), we observed that hAPP mice have increased susceptibility to the effects of PTZ when comparing with non-transgenic mice (Fig. 1A).

hAPP mice present altered mouse hippocampal lipid composition.

We characterized the hippocampus lipid composition of non-transgenic and hAPP mice with similar average age. Considering the major lipid species, we observed that free cholesterol is the most abundant lipid in mouse brain hippocampus both in non-transgenic and hAPP animals, in accordance with previous reports(Oliveira, Chan et al. 2016). Moreover, we found that hAPP expression induced a sphingolipid imbalance with a decrease in sulfatide (Sulf) and monohexosylceramide (MhCer) levels and an increase in sphingomyelin (SM) and dihydrosphingomyelin (dhSM) levels. Other lipid changes, such as an increase in cholesterol ester (CE), monoacylglycerol (MG), diacylglycerol (DG) and a decrease in bis(monoacylglycero)phosphate (BMP) levels were also observed (Fig. 2A and B). A more profound lipid species analysis of the major altered lipid subclasses (PS, DG, MhCer and CE) in the mouse brain hippocampus revealed that there was an overall effect on the majority of the analyzed lipid species within these lipid classes (Fig. 3).

Specific lipid species are correlated with pharmacological-induced seizure severity.

To test which lipid species were associated with seizure severity scores, we performed a full scale unbiased correlation analysis between the seizure severity score after acute treatment with PTZ and the levels of the 650-lipid species in mouse brain hippocampus region covered in our lipidomic analysis. We defined a cut-off correlation index of 0,4 (R^2) in order to limit the hits in our analysis (Fig. 4A). Curiously, we observed that in the seizure severity score ranging from 1 to 5 the majority of phospholipids, GM3, CE, LPC and LPE have a negative correlation, which inverted to an overall positive correlation in scores 6 to 8 (Fig. 4A). Moreover, other classes of lipids such as PA, TG, MhCer and Sulf present an overall positive correlation with latency-time in all scores evaluated (Fig. 4A). Particularly, we found that PTZ-induced seizures were directly correlated with total levels of MhCer (Fig. 4B) and various specific lipid species, such as TG 54:1 (Fig. 4C), PS 34:2 (Fig. 4D), PA 40:7 (Fig. 4E), CE 20:4 (Fig. 4F) and LPC 20:4 (Fig. 4G) species. On supplementary data we also present other lipid species, which its levels are directly correlated with PTZ-induced seizures. (Supplementary Fig. S1).

Specific fatty-acyl lipid composition is correlated with pharmacological-induced seizure severity.

The perturbation of brain function homeostasis of cellular membranes, could partially explain the neuronal deficits observed in several neurological conditions. The membrane biophysical properties of membrane lipids are determined by both carbon chain length and the degree of saturation of the fatty acyls. In fact, neuronal functioning such as altered function of membrane-bound proteins, can be affected by modulation of the composition of its fatty acid chains (Wenk 2010, Carta, Lanore et al. 2014). In our study we performed a deep analysis focusing in the fatty acyl chain profile of glycerophospholipids/DG and sphingolipids. First, our data shows that hAPP overexpression leads to a significant alteration in the fatty acyl chain profile of glycerophospholipids and DG, with decreased levels of long chain fatty acyls with 36 carbons (36C) and an increase in polyunsaturated fatty acyls with 4 double bonds and a decrease in polyunsaturated fatty acyls with 1 and 2 double bonds (Fig. 5A and B). Interestingly, concerning the effects of hAPP overexpression in sphingolipids composition, we observed an increase in short fatty acyls (18C), a decrease in medium length (22C) and in long length (24C) fatty acyl composition (Fig. 5C). Moreover, to test if the degree of saturation and fatty acyls chain length were associated with seizure severity, we performed an unbiased correlation analysis between the seizure severity score latency time after acute treatment with PTZ and the fatty acyls chain length and degree of saturation of glycerophospholipids/DG and sphingolipids in the mouse brain hippocampus (Supplementary Fig. S2). We found that PTZ-induced seizures were directly correlated with degree of saturation 2 of glycerophospholipids/DG (Fig. 5B1) and sphingolipid fatty acyl chain length 24 and 22 (Fig. 5C1 and C2).

Discussion

In the present study we used a MS lipidomic approach to identify lipid signatures of seizure severity in AD mouse models. It has been shown by others that targeting lipid metabolism is a promising strategy to treat neurodegenerative diseases, such as AD (Berman, Dall'Armi et al. 2008, Sanchez-Mejia, Newman et al. 2008, Oliveira, Chan et al. 2010). Thus, it is fundamental to understand the lipid changes associated with both neurotoxic and protective states, in order to devise new therapeutic targets. In this specific study, we characterized the hippocampal lipidome of an AD mouse model that overexpresses hAPP (the J20 mouse line) upon acute treatment with

pentylenetetrazol (PTZ). We chose the hippocampus as a read-out brain area, since it is (i) fundamental for learning and memory, (ii) its functioning is majorly affected in the J20 mice in an Aβ-dependent way, and (iii) it is a major target for excitotoxicity.

Our analysis replicated the observations by Palop et al. which have shown that hAPP mice had increased overall seizure severity upon treatment with a $GABA_A$ antagonist compared with non-transgenic mice (Palop, Chin et al. 2007) (Fig. 1A). The cognitive deficits observed in hAPP mice, which mimics the effects in humans, may result from the aberrant increases in network excitability and development of compensatory inhibitory mechanisms in the hippocampus (Palop, Chin et al. 2007, Palop and Mucke 2009). In hAPP mice, Aβ-induced epileptic activity was associated with alterations in calcium levels, synaptic activity, and in the regulation of granule cells proteins including calbindin, Fos, and Arc (Palop, Jones et al. 2003, Palop, Chin et al. 2005, Cheng, Scearce-Levie et al. 2007, Palop, Chin et al. 2007).

A possible key factor in the onset and progression of nervous system disorders is the lipid dyshomeostasis and ensuing signaling imbalance. For instance, it has been previously reported that the inhibition of PLA2 and cyclooxygenase-2 after excitotoxicity induced by kainate, a glutamate analog, was shown to prevent neuronal damage (Thwin, Ong et al. 2003). The elucidation of the molecular mechanism of neurotoxicity is fundamental to understand the impact of changes of lipid metabolism in neurodegenerative diseases. Here, we used a comprehensive lipidomic approach to determine the changes of hippocampal lipids during PTZ-induced seizures.

Our analysis of the lipidome of hAPP mice points to changes in several lipid species, with decrease levels of Sulf, BMP and MhCer and elevated levels of CE, MG, DG and SM, when comparing to control mice. Curiously, BMP plays an important role in the biogenesis of multivesicular bodies (Falguieres, Luyet et al. 2009) and its reduction may explain the dysfunction of late endosomes/ multivesicular bodies reported in AD (Cataldo, Mathews et al. 2008). Indeed, the accumulation of SM and CE, normally found in lipid droplets, supports the endolysosomal dysfunction hypothesis linked to AD since these lipids impair the lysosomal degradation of APP-CTFs, implicated in amyloidogenesis (Kobayashi, Stang et al. 1998). Moreover, it has been reported that the dysregulation of sphingolipids in the hAPP hippocampus may contribute to the alterations of synaptic vesicles and trafficking of NMDA receptors(Haughey, Bandaru et al. 2010). In fact, we observed several changes in the levels of Sulf and MhCer in hAPP mice that are in line with previous observations in AD mice models (Chan, Oliveira et al. 2012). Of relevance, we observed changes

in a diversity of individual lipid species, suggesting an important role for the length and saturation of the fatty acyl carbon chains of glycerophospholipids/DG and sphingolipids in AD pathogenesis. We found an accumulation of polyunsaturated DG 38:4, a primary metabolite of PI(4,5)P2, that has been shown to lead to the dysregulation of dendritic spine morphology, plasticity, and neurotransmitter release (Kim, Yang et al. 2010). Indeed, a reduction in levels of PS 36:2, a lipid involved in memory enhancement (Zhang, Yang et al. 2015), is in accordance with what was observed by Guan et al., namely, an alteration in this lipid species upon kainate treatment. In summary, DG 38:4 and PS 36:2 lipid species emerge here as important lipid signatures of excitability dysregulation.

Since hAPP mice are susceptible to PTZ-induced seizures, we determined the changes of hippocampal lipids associated with different seizure severity states. Firstly, we identified which lipid species had the highest degree of correlation with the susceptibility to pharmacological-induced seizures. We found that PTZ-induced seizures were directly correlated in the hAPP mouse hippocampus with total MhCer levels, multiple TG, PS and PA species levels, and inversely correlated with CE and LPC species levels. Interestingly, we found that, specifically, LPC 20:4 was found as a negative correlation hit with seizure severity score in our analysis and it was previously shown that it can promote inflammatory events that are related with AD (Riederer, Ojala et al. 2010). Remarkably, since LPC 20:4 was also observed to be directly correlated with corticosterone upon exposure to a chronic unpredictable stress model in the prefrontal cortex, hippocampus and amygdala, it furthers highlights the importance of this specific lipid species in neuronal dysregulation. Moreover, PA 40:7 levels was directly correlated with PTZ-induced seizures, which suggests that enzymes that modulate PA levels, such as phospholipase D (PLD, could also play an important role in excitotoxicity events. In fact, the PLD pathway has been extensively reported as a modulator of AD pathogenesis (Oliveira, Chan et al. 2010, Oliveira and Di Paolo 2010).

Besides comparing the individual species, we also evaluated the length and degree of saturation of both glycerophospholipids/DG and sphingolipids. These are important determinants of many membrane features such as thickness, fluidity, local curvature and regulation of membrane-bound enzymes (Spector and Yorek 1985). In summary, we found that hAPP overexpression leads to a decrease in C36 fatty acyl phospholipids/DG levels, as well as mono and polyunsaturated phospholipids/DG levels in the hAPP mouse hippocampus. We also observed an increase in 4 double bonds polyunsaturated phospholipids/DG levels and in C18 fatty acyl sphingolipids levels. Interestingly, an association between dietary fat and plasma cholesterol levels has been observed,

probably linked to the increased risk of developing AD by specific genetic polymorphisms of apoliprotein E, a protein involved in cholesterol transport. Experimental animal studies found that diets rich in unsaturated fat lead to a superior learning and memory in rats (Greenwood and Winocur 1996). On the other hand, diets rich in cholesterol induce an increase in Aβ deposition in the brain (Sparks, Scheff et al. 1994, Sparks, Kuo et al. 2000). Furthermore, docosahexaenoic acid (DHA, 22:6n-3), the main omega-3 polyunsaturated fatty acid, one of the fatty acids that make up phospholipids, has been implicated as having a protective role in AD. Animal studies observed that a diet enriched with omega-3 fatty acid results in improved regulation of neuronal membrane excitability (McGahon, Martin et al. 1999), better neuronal transmission(Delion, Chalon et al. 1996), and reduced oxidative injury (Kubo, Saito et al. 1998), which translate in a protective role in AD. Therefore, the knowledge of the detailed membrane lipid composition of specific brain regions, both in physiological or pathological states, can direct therapies that target the modulation of lipid brain composition either through drugs or customized diets.

In summary, we identified lipid signatures of seizure severity in an AD mouse model. Our data provides the identification of dysregulated lipid pathways that can be targeted for pharmacological intervention to protect from excitotoxicity related processes in an AD context. Alternatively, nutrition may have an important role in the development of AD and novel therapeutic strategies based on dietary changes which may contribute to prevent the deleterious effects of neurodegenerative disorders by altering in a predetermined way the composition of the brain lipidome.

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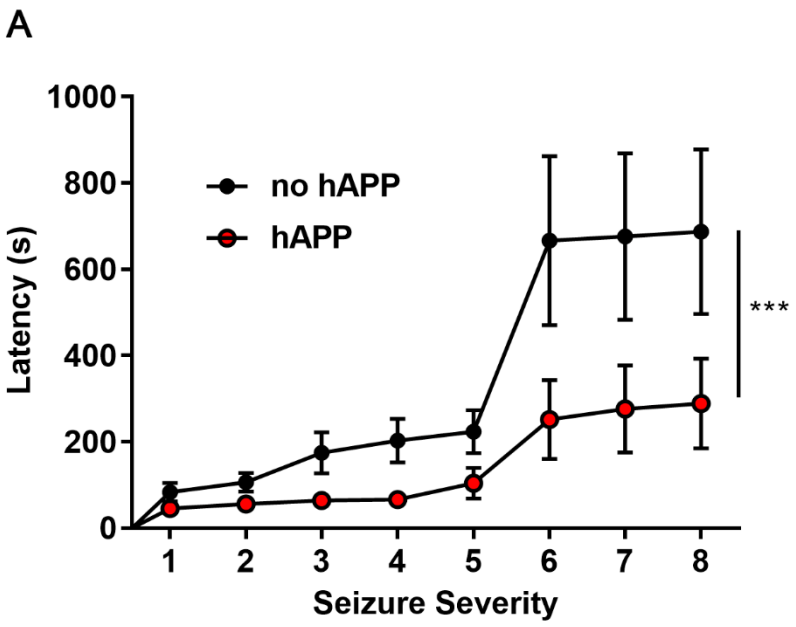

Figure 1. hAPP mice (J20 line) are more susceptible to pharmacological induced seizures. hAPP mice and control non-transgenic animals were submitted to i.p. injections with pentylenetetrazole (40mg/Kg) and seizure score was evaluated through video analysis and scored as, 0 = normal behavior; 1 = immobility; 2 = generalized spasm, tremble, or twitch; 3 = tail extension; 4 = forelimb clonus; 5 = generalized clonic activity; 6 = bouncing or running seizures; 7 = full tonic extension; 8 = death. The mean values of 8 animals per genotype are presented. Values denote means ± SEM (*** $p \leq 0.001$).

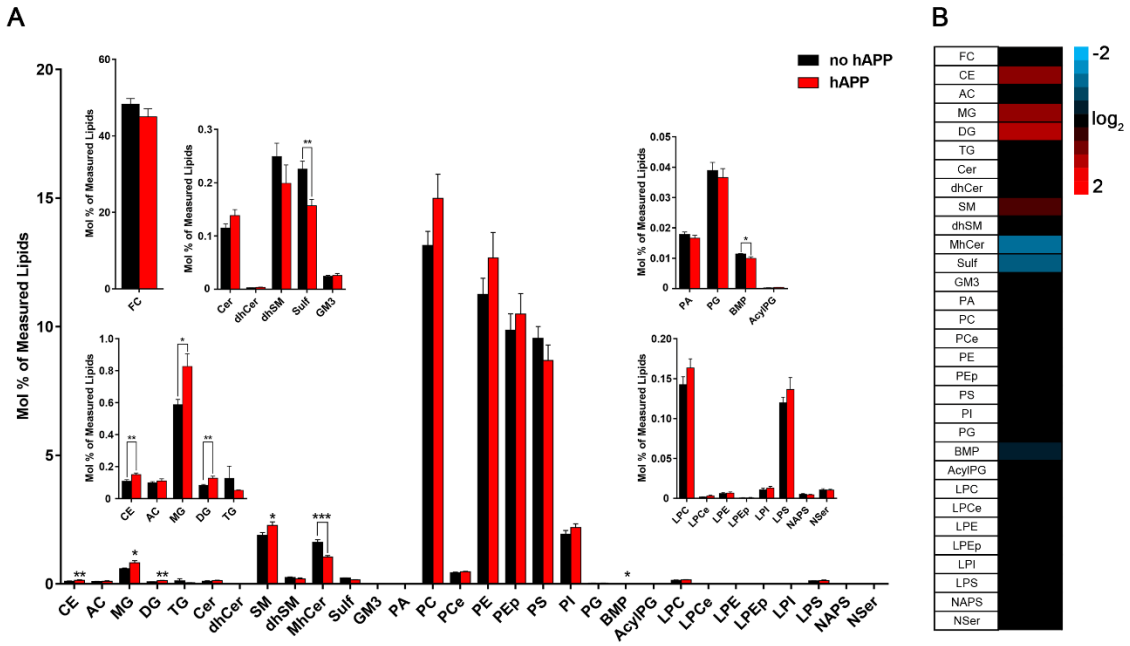

Figure 2. Lipid composition of hAPP (J20 line) mouse brain hippocampus. (A) Values shown are normalized to measured molar lipid concentration. Values denote means ± SEM. (B) Ratio of lipid levels of hippocampal hAPP mice compared with non transgenic animals. The data is expressed as log2-normalized values relative to each control group (red - higher values; blue - lower values). Only significant changes, i.e., $p < 0.05$, are shown. FC, free cholesterol; CE, cholesteryl ester; AC, acyl carnitine; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; Cer, ceramide; dhCer, dihydroceramide; SM, sphingomyelin; dhSM, dihydrosphingomyelin; MhCer, monohexosylceramide; Sulf, sulfatides; GM3, monosialodihexosylganglioside; PA, phosphatidic acid; PC, phosphatidylcholine; PCe, ether phosphatidylcholine; PE, phosphatidylethanolamine; PEp, plasmalogen phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; BMP, bis(monoacylglycero)phosphate; AcylPG, acyl phosphatidylglycerol; LPC, lysophosphatidylcholine; LPCe, ether lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPEp, plasmalogen lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; NAPS, n-acyl phosphatidylserine; Nser, n-acyl serine. The data results from the analysis of 8 animals per genotype. Values denote means ± SEM (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

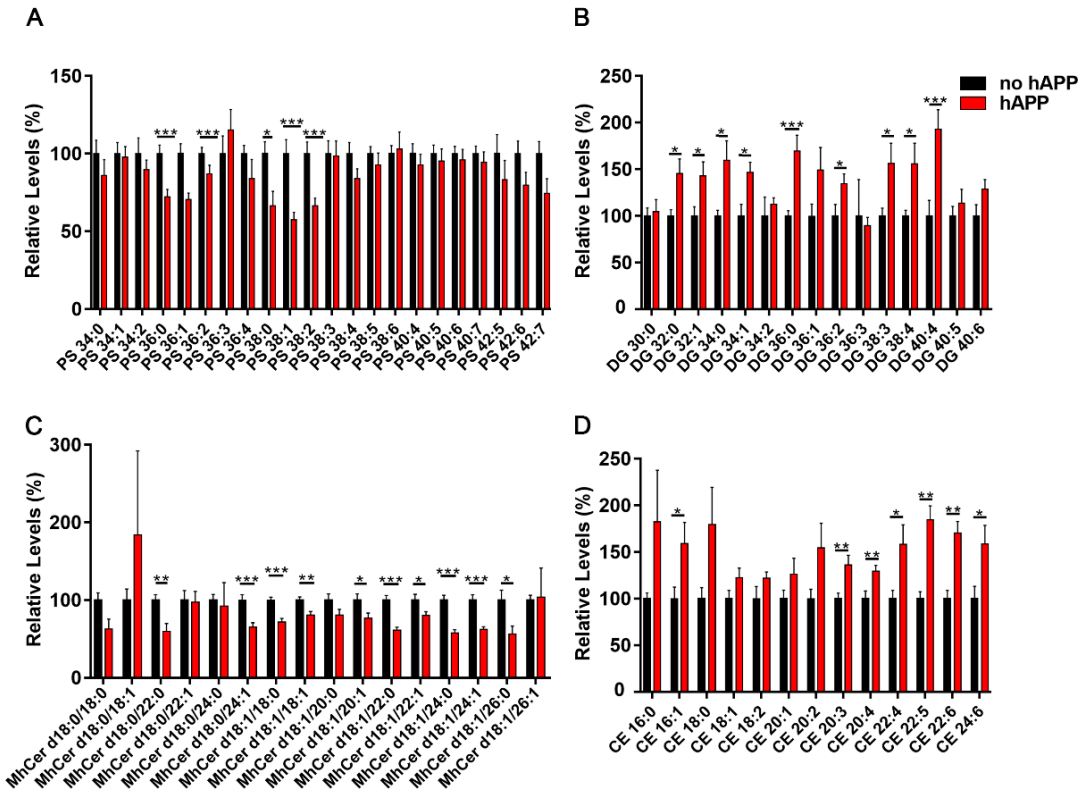

Figure 3. Lipid species composition of hAPP (J20 line) mouse brain hippocampus. Analysis showed decrease in phosphatidylserine (A) and many monohexosylceramide (C) species and increases in diacylglycerol (B) and cholesterol ester (D) species and. y values expressed as relative levels to control animals. CE, cholesterol ester; DG, diacylglycerol; MhCer, monohexosylceramide; PS, phosphatidylserine. The data results from the analysis of 8 animals per genotype. Values denote means ± SEM (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

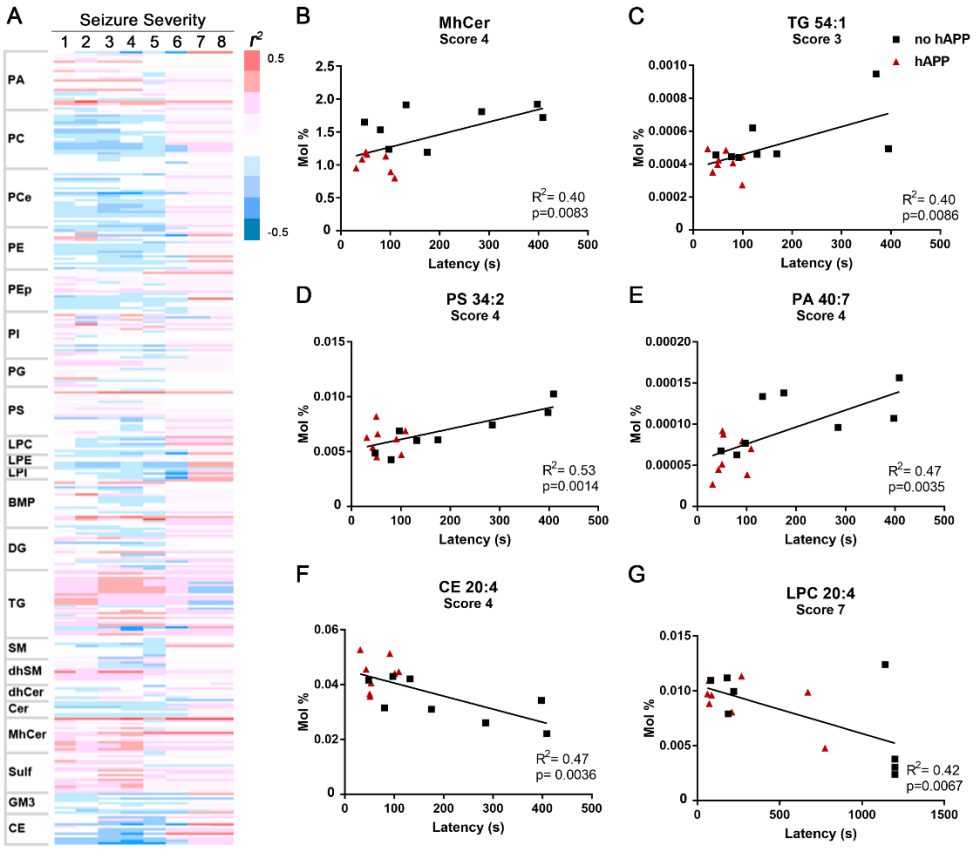

Figure 4. Specific lipid species brain hippocampus levels are directly correlated with susceptibility to pharmacological-induced seizures. (A) Correlation levels between all lipid species analyzed and the susceptibility to pentylenetetrazol (PTZ) seizures. Lipid species are grouped per lipid class and the different color dots represents the R2 levels (red - higher positive correlation; blue - lower negative correlation). (B-G) The major positive lipid species PTZ- correlation hits are represented. Not all positive hits are represented in the figure. CE, cholesterol ester; MhCer, monohexosylceramide; TG, triacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; LPC, lysophosphatidylcholine. The data results from the analysis of 8 animals per genotype.

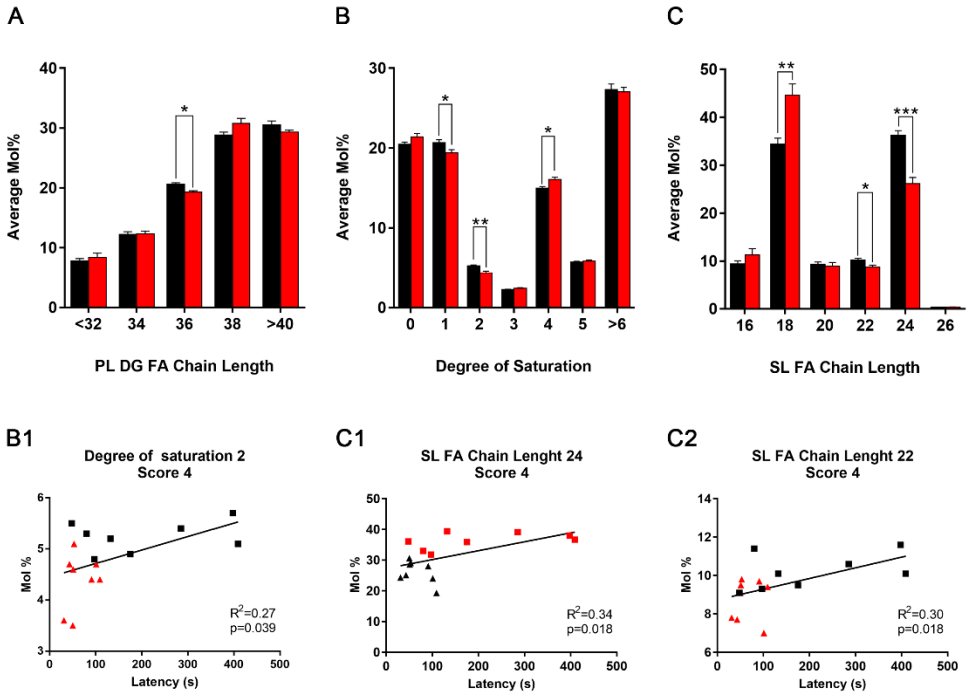

Figure 5. Altered fatty acid composition of hAPP (J20 line) mouse brain hippocampus. Analysis of fatty acyl composition of diacyl-glycerophospholipids and DG species by total carbon chain length (A), total degree of saturation (B) and sphingolipid N-acyl chain lengths (C). Values are expressed as mol % + SEM of included lipid species. (B1) Correlation levels between the degree of saturation and the susceptibility to pentylenetetrazol (PTZ) seizures. (C1-C2) Correlation levels between the sphingolipid N-acyl chain lengths and the susceptibility to pentylenetetrazol (PTZ) seizures. The data results from the analysis of 8 animals per genotype. Values denote means ± SEM (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Lipidomic determinants of seizure severity in AD mouse model

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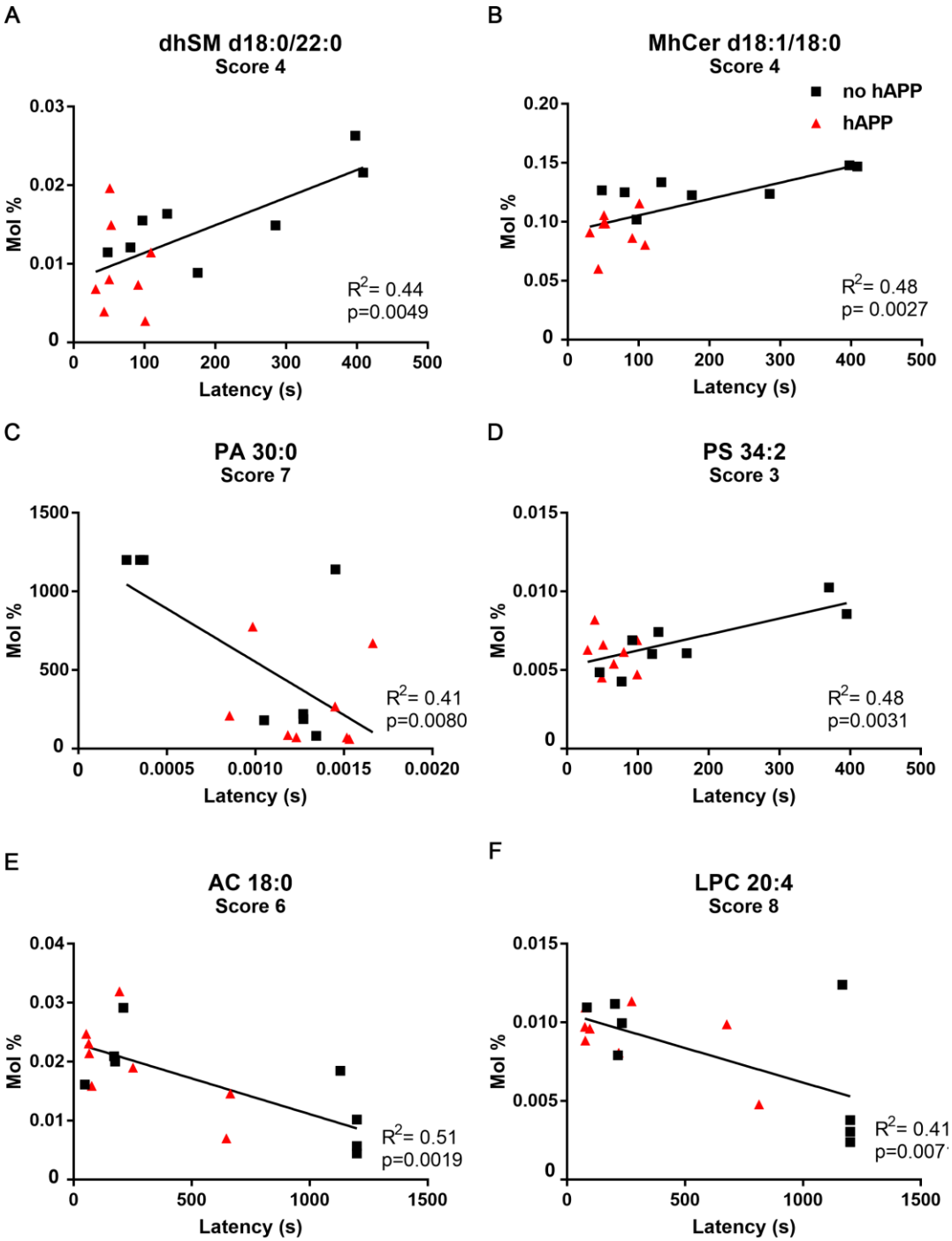
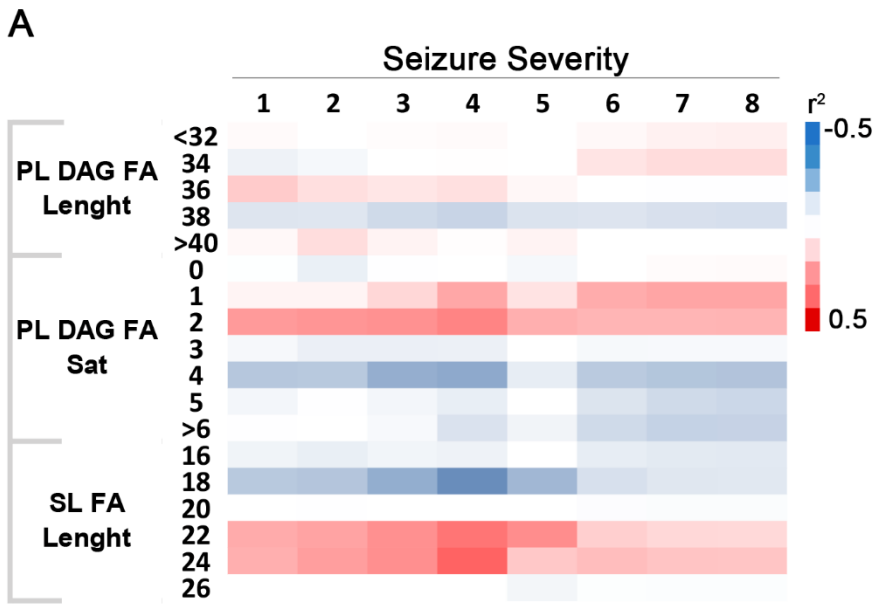
Supplementary Data



Supplementary Figure S1. Specific lipid species brain hippocampus levels are directly correlated with susceptibility to pharmacological-induced seizures. (A-F) The major positive lipid species PTZ- correlation hits are represented. dhSM, sphingomyelin; MhCer, monohexosylceramide; PA, phosphatidic acid; PS, phosphatidylserine; AC, acyl carnitine and LPC, lysophosphatidylcholine.



Supplementary Figure S2. Altered fatty acid composition of hAPP (J20 line) mouse brain hippocampus. (A) Correlation levels between the phospholipids fatty acid chain length, degree of saturation, N-acyl fatty acid chain length and the susceptibility to pentylenetetrazol (PTZ) seizures. Lipid species are grouped per lipid class and the different color dots represents the R2 levels (red - higher positive correlation; blue - lower negative correlation)

Chapter 3

Discussion and Future Perspectives

Discussion

Lipids are a main constituent of the brain and are involved in multiple aspects of brain physiology and cell signalling. State of the art lipidomic studies currently allow the identification of lipid signatures associated with essential aspects of brain function and dysfunction. Mounting evidence indicates that the dysregulation of lipid signalling pathways has been associated with neurodegenerative disorders, namely, Alzheimer's disease (AD). Among others, an important evidence that supports the key role of lipids in the modulation of AD pathological processes is that amyloid beta (Aβ) exerts is cytotoxic effects by modulating the metabolism of both phospholipase A2 (PLA2) (Sanchez-Mejia, Newman et al. 2008), phospholipase C (PLC) (Berman, Dall'Armi et al. 2008) and Phospholipase D (PLD) (Oliveira, Chan et al. 2010, Bravo, Da Silva et al. 2018), in a calcium dependent way. Oliveira and Di Paolo identified PLD2 as key player in AD pathogenesis showing that the genetic ablation of PLD2 has a protective effect in an AD mouse model (Oliveira, Chan et al. 2010). Despite the potential role of PLD in Aβ signaling, no differences were found in Aβ and APP levels in PLD2 knock-out animals (Oliveira, Chan et al. 2010). Additionally, it was reported in a follow-up study that the protective effect of PLD2 ablation in an AD-like model could be due to the modulation of a specific group of gangliosides, GM3 (Chan, Oliveira et al. 2012). In fact, gangliosides were shown to interact with Aβ (Ariga, McDonald et al. 2008) and curiously, PLD2 ablation normalizes GM3 levels in an APP model (Chan, Oliveira et al. 2012). Thus, these findings support a role for PLD2 as downstream effector of Aβ signaling. Since, the exact mechanism through which PLD modulates Aβ effects remained poorly understood, we envisioned here a multidisciplinary approach using neuronal cell lines, nematode models and AD mouse models for further characterization of the role of the PLD pathway in neurodegenerative diseases. Overall, the findings presented here (Chapters 2.1, 2.2 and 2.3) further add to the idea that lipids are important mediators of AD pathogenesis and specifically, the PLD pathway presents as key player in neurodegenerative diseases, especially in AD related mechanisms.

In the sections bellow we discuss how we addressed the three specific aims of this PhD thesis regarding the research questions and explore new avenues our work has opened.

Aim1. *To test the effects of PLD ablation in an AD Caenorhabditis elegans model*.

To better understand the role of the PLD pathway in physiology and in a pathological context, we studied the impact of PLD ablation in a nematode model and these findings gave us key insights into some of the basic PLD functions (chapter 2.1). We took advantage of the nematode model, *C. elegans*. This is a powerful tool that allows the identification of relevant pathways for organismal functioning through the use of specific mutants. To our knowledge, we performed here the first study addressing the impact of PLD ablation in a *C. elegans*. We observed that PLD was the only source of PLD activity in nematodes and this matches well with the results from another fly model organism (Thakur, Panda et al. 2016). While no obvious phenotypes were observed in a PLD mutant nematode model, it was reported that PLD1 or PLD2 knock-out animals have decreased juvenile brain volume and social and object recognition deficits (Burkhardt, Stegner et al. 2014). Interestingly, no major behavioral deficits were observed by others (Oliveira, Chan et al. 2010, Vermeren, Zhang et al. 2016), showing some discrepancy in the PLD literature concerning the effects of PLD ablation in brain physiology. Concerning our studies studying PLD ablation in a nematode model, we observed an increase in body size and lipid stores in *pld-1* animals. Since, we observed that PLD is a main source of PA in *C. elegans,* we hypothesized that the effects of PLD perturbation could be due to altered PA metabolism. In fact, previous reports highlighted that other enzymes that modulate PA levels, such as lipin (i.e., a phosphatidic acid phosphatase, PAP) leads to reduced body size and defects in lipid storage in a *C. elegans* model where lipin was silenced (*lpin-1*) (Golden, Liu et al. 2009). Remarkably, our data indicates that the mechanism of lipid stores regulation observed upon *lpin-1* knock down could be explained by the involvement of PA species that could potentially be derived from PLD, knowing that PAP/*lpin-1* converts PA to diacylglycerol (DG). On the other hand, the role of PLD1 in macroautophagy and in the regulation of lipid stores could be an alternative explanation for this phenotype since lipid stores could be consumed through a specialized form of macroautophagy (Singh, Kaushik et al. 2009). Additionally, to further analyze the impact of PLD ablation in a neurodegenerative disease *C. elegans* model, we crossed *pld-1* animals with a *C. elegans* AD-like model, based on the overexpressesion of human Aβ in neurons. Remarkably, PLD ablation was protective in multiple behavioral deficits observed in Aβ strains, such as overall survival, motor tasks, defective response to serotonin and susceptibility to pentylenetetrazol (PTZ). These findings are in line with previous observations showing that Aβ leads to increased total PLD activity and that PLD2 genetic ablation

ameliorates synaptic dysfunction and cognitive deficits associated to AD mouse model (Oliveira, Chan et al. 2010). Therefore, these observations could be viewed in the context of Aβ signaling, which is also associated with excitotoxicity downstream effects. Moreover, Aβ signaling is also associated with an increase in susceptibility to PTZ, which occurs not only in nematodes overexpressing Aβ (Chapter 2.1), but also in mouse models that overproduce Aβ (Palop, Chin et al. 2007). Since PLD ablation blocks this deleterious effect of Aβ in nematodes, these findings support a potential role for PLD in regulation of neuronal activity with a potential relevant impact at the circuit/systems level. Moreover, PLD has been shown to be involved in membrane damage pathways (Arun, Xie et al. 2013) and curiously, a study with a drosophila model showed that either a decrease or an increase in PLD levels can have a functional impact in fly rhabdomeres volume during illumination, a task that relies on active membrane turnover. This highlighted the potential importance of PLD activity in physiologic processes involved in membrane turnover (LaLonde, Janssens et al. 2005, Raghu, Manifava et al. 2009). Finally, future studies should be performed to support *C. elegans* PLD models as relevant tools to address the role of PLD in physiologic and pathologic mechanisms. Also, these findings guide further experiments that could be pursued in mouse animals with increased levels of Aβ, where the exact mechanism of PLD2 ablation protective mechanism is still elusive.

Aim.2 *To investigate the role of the PLD pathway in the regulation of tau.*

Importantly, the ablation of tau was shown to be protective in two different AD mouse models (Roberson, Scearce-Levie et al. 2007, Ittner, Ke et al. 2010) suggesting that Aβ is upstream tau. Since PLD2 and tau knock-out models were both shown to be protective in an AD mouse model, with no major impact on APP processing and Aβ levels it is thus plausible that the protective cellular mechanism could be at least partially shared. In fact, we observed that PLD physically interacts with tau and regulates tau pathological events (chapter 2.2). Furthermore, we observed that PLD2 overexpression produces a strong impact in tau by reducing its protein levels. Our findings highlight a potential interaction between tau and PLD and led us to further evaluate the role of the PLD pathway in a pathological context, using again a nematode model. We crossed the *pld-1* animals with a tauopathy-like *C. elegans* model (*pld-1*; tau animals) and somewhat accordingly, the PLD ablation leads to an increase in total tau levels. Considering that the activity of PLD could regulate the clearance of protein aggregates, such as tau (Dall'Armi, Hurtado-Lorenzo et al. 2010), one

possibility that should be considered is that autophagy related mechanisms could partly explain this phenotype. In fact, Dall'Armi et al. demonstrated that after nutrient starvation, PLD1 relocalizes to the outer membrane of autophagosome-like structures. The phosphatidylinositol 3-kinase inhibitor, wortmannin led to the alteration of PLD1 localization and PLD activity is increased upon nutrient deprivation (Dall'Armi, Hurtado-Lorenzo et al. 2010), suggesting that PLD1 is downstream of Class III PI-3 kinase (Vps34), which generates the phosphatidylinositol 3-phosphate (PI3P) (Dall'Armi, Hurtado-Lorenzo et al. 2010). Finally, in that study it was also found that PLD inhibition in organotypic brain slices from hTau mice leads to increased levels of soluble and insoluble tau (Dall'Armi, Hurtado-Lorenzo et al. 2010), which is in accordance with our observations. What was still completely unknown was the functional impact of PLD modulation in tau dependent phenotypes at the organismal level. We tested that by studying the behavior of *pld-1*; tau animals. We found that the ablation of PLD in a tauopathy-like model had remarkably a protective effect in several phenotypes. This was in a way somewhat surprising, since the overall literature in neurodegeneration associates increased tau levels with also a deleterious effect. It should be pointed out that actually, a major fraction of tau related studies show that tau ablation leads to a protective state in the context of Aβ overexpression (Roberson, Scearce-Levie et al. 2007) . Future studies should also address the possibility that PLD ablation confers a state of neuronal protection, for instance blocking calcium deleterious downstream effects, since it is known that PLD is activated by calcium (Oliveira, Chan et al. 2010). Finally, even though we observe increased total tau levels upon PLD ablation, we do not know where are these tau levels distributed within the organism. Therefore, a mechanism of tau relocalization could also potentially play a role.

Taking into account that the neuronal relocalization of tau to dendrites and spines (Hoover, Reed et al.) is responsible for its toxic effects and tau partly interacts with membranes (Brandt R 1995), we proposed that tau could potentially interact with specific lipids. Remarkably, we observed using a protein-lipid overlay assay a strong interaction between tau and phosphatidic acid (PA), phosphatidylserine (PS) and PI3P. The physicochemical properties of PA (i.e., a cone-shape lipid), a product of PLD's enzyme activity may contribute to the role of PLD in membrane trafficking (Roth 2008, Bader and Vitale 2009). Indeed, the dysregulation of the pathway that leads to the generation of PI3P by VpS34 is associated with endolysosomal perturbation (Morel, Chamoun et al. 2013), increased tau levels and decrease of Aβ secretion (Miranda, Lasiecka et al. 2018). Interestingly, Vps34 decreased levels leads to decreased PLD1 levels, suggesting a potential connection between both these pathways. To further question the role of tau interaction with specific lipids, we tested

what would happen if we disturbed the interaction between tau 352 and PA. To do that, we used a probe for PA (Spo20) that competes for the lipid binding site, thereby potentially disrupting the interaction between tau and PA. Remarkably, we observed a relocalization of intracellular tau from more internal/perinuclear localization to a more peripheral localization in the cell. Our data supports the notion that tau interacts with PLD and certain lipids although it is not clear whether it is a direct or indirect link with the contribution of other protein interactores in a multiprotein complex. Future questions should focus on understanding the connection with other potential elements that interact with both tau and PLD, such as the AD risk factor bridging integrator 1 (Bin1). The disruption of these complexes could have major implications in tau neuronal distribution, which could affect neuronal susceptibility and/or resistance to excitotocity exposures.

Aim.3 *To characterize the effects of seizure severity on the hippocampus lipidome in an AD mouse model.*

A 650-full scale lipidomic study was performed to identify potential lipid signatures associated with seizure severity in an AD mouse model (chapter 2.3). As previously shown, we observed that AD mice were more susceptible to pharmacological-induced seizures (Palop, Chin et al. 2007). Concerning the mechanism that can modulate this susceptibility to seizures driven by Aβ increased levels, it was previously reported that the ablation of tau confers resistance to excitotoxicity associated phenotype (Roberson, Scearce-Levie et al. 2007). Moreover, other research groups studied this protective effect conferred by tau reduction in hAPP mice and found that tau binds to Fyn, leading to the stabilization of the N-methyl-D-aspartate receptor/ postsynaptic density protein 95 (NMDAR/PSD95) complex (Ittner, Ke et al. 2010). Curiously, some of these synaptic complex proteins interact with lipid modulating enzymes, such as PLD. In fact, phosphorylation through the Src kinases, Fyn and Fgr, was shown to regulate PLD2 activation and degranulation in mast cells (Choi, Hiragun et al. 2004) and through our own results presented here we observe that PLD interacts with tau and modulates its levels and cellular localization (Chapter 2.2). Therefore, there are a number of observations that point to a common mechanism in hAPP susceptibility to seizures, that can involve lipid metabolism and lipid modulating enzymes, being PLD one the candidates.
Previously, it has been shown that specific lipid metabolic signatures have been identified both in human and mouse AD brain samples, showing that lipid metabolism is indeed dysregulated. Besides this, many research groups have been studying the role of lipid signaling in neuronal

excitability homeostasis (Di Paolo and De Camilli 2006) and it has been proposed that pharmacological-induced seizures induce changes in brain lipid composition (Guan, He et al. 2006). Moreover, it is thus plausible that lipid impairment that occurs downstream of Aβ signaling could be connected with excitotoxicity linked mechanisms. It is thus fundamental to determine the molecular mechanisms of neurotoxicity to understand the impact of changes in lipid metabolism in neurodegenerative diseases. Here, we took advantage of our optimized MS lipidomic approach to determine the changes in hippocampal lipids during PTZ-induced seizures using an AD mouse model. We observed overall major lipid changes in hAPP mice and particularly there was an accumulation of SM and CE, which are normally found in lipid droplets. In fact, these lipids were shown to impair the lysosomal degradation of APP-CTFs with a strong implication in amyloidogenesis processes (Kobayashi, Stang et al. 1998). Curiously, previous observations (chapter 2.1 and (Oliveira, Chan et al. 2010)) showed that PLD ablation has a protective role in AD-like models, is one of the main sources of PA and leads to an increase in lipid droplets suggesting that the effects of PLD perturbation could somehow be also related with lipid droplet modulation. Future studies should be performed to identify the lipid species specifically altered in lipid droplets. This could propel studies where the role of these species could be evaluated both in AD pathogenesis and lipid droplet physiology. Moreover, we found a dysregulation of sphingolipids, such as sulfatide (Sulf) and monohexosylceramide (MhCer), in hAPP hippocampus that may contribute to the alterations of synaptic vesicles and trafficking of NMDA receptors (Haughey, Bandaru et al. 2010). One interesting finding we also observed is an accumulation of DG 38:4 species, a primary metabolite of $PI(4,5)P_2$ that was shown by Berman et al. to be decreased by Ca^{2+} dyshomeostasis in primary cortical neurons treated with Aβ (Berman, Dall'Armi et al. 2008). Moreover, lysophosphatidylcholine (LPC) 20:4 was found as a negative correlation hit with the degree of seizure severity, and curiously presented an opposed effect with a positive correlation with endogenous corticosterone (CORT) levels in a chronic stress rat model (Oliveira, Chan et al. 2016). Importantly, this lipid is generated by PLA2 which is responsible for the cleavage of membrane phospholipids into lysoglycerophopholipids and fatty acids (such as arachidonic acid, AA), being associated with inflammatory processes (Malcher-Lopes, Franco et al. 2008). This is in line with the findings reported that chronic stress enhances the sensitivity to inflammatory processes of the brain areas affected in AD (Guo, Yu et al. 2002, Espinosa-Oliva, de Pablos et al. 2011) and that PLA2 could play a role in AD pathophysiology (Sanchez-Mejia, Newman et al. 2008). On the other hand, PA 40:7 is directly correlated with PTZ-induced seizures which supports

the hypothesis that the PLD pathway is involved in excitability-related processes as previous demonstrated in chapter 2.1. In fact, the ablation of PLD in a *C. elegans* AD-like model induces less susceptibility to pharmacological induced seizures in a model with Aβ overproduction, which further supports PLD as having a role in excitotoxicity related mechanisms. Finally, the evaluation of the length and degree of saturation of both phospholipids/DG and sphingolipids were of critical relevance for evaluation of membrane biophysical properties altered in hAPP mice and as signatures that could predispose to PTZ-induced seizures. These findings could lead to the development of specifically designed therapies, either with drugs or diets, which could target specific lipid pathways or induce specific lipid alterations in the brain lipidome.

In summary, our holistic approach supports PLD as a downstream pathway of Aβ interaction with membranes, identifies the PLD pathway as a key regulator of tau physiology and pathology and confirms PLD as an important player in neurodegeneration in tauopathy like models. Finally, our results further contribute to the notion that lipid signaling is a promising target for pharmacological intervention in excitotoxicity pathological processes (Figure 1).

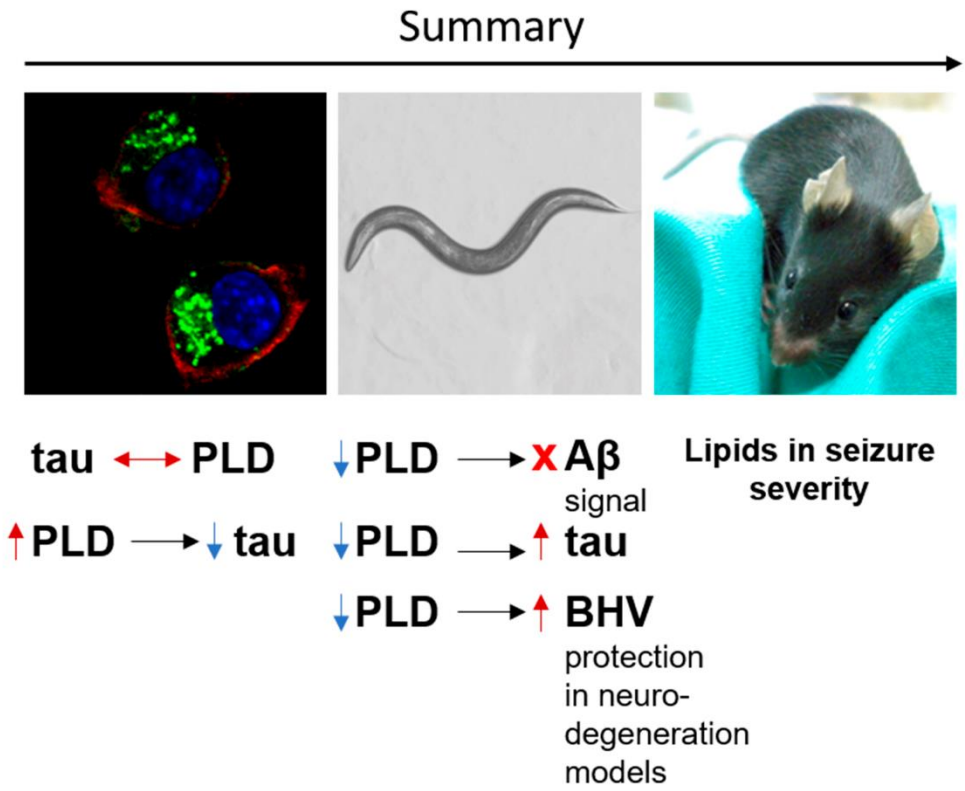


Figure 1. Multidisciplinary approach using neuronal cell lines, nematode and AD mouse models. Neuronal cell lines: Tau interacts with PLD; PLD overexpression leads to a decrease of tau levels. *C. elegans*: PLD as a downstream pathway of Aβ interaction with membranes; ablation of PLD in an tauopathy model leads to an increase in tau levels; ablation of PLD confers behavioral protection in neurodegeneration models. AD mice models: lipid signaling is a promising target for pharmacological intervention in excitotoxicity pathological processes.

Future Perspectives

To further address the role of PLD pathway as a key regulator in neurodegenerative diseases, future studies should be performed taking into account the questions raised in this work:

1. Unravelling the mechanism underling LDs increase in *pld-1* animals. Using cell lines derived from PLD1/2 knockout animals could be useful to understand the molecular lipid droplet dynamics mechanism. Considering LDs interacts with mitochondria, lysosomes,

and the plasma membrane (Saka and Valdivia 2012), PLD could potentially contribute to a homeostatic regulation of organellar functioning.

Previous studies, demonstrated that PLD and the formation of phosphatidic acid are important in the assembly of low density lipoproteins, an important step in the lipid droplet formation (Olofsson, Stillemark-Billton et al. 2000). Moreover, the release of LDs is inhibited by 1-butanol suggesting, PLD and the formation of PA essential components in this process (Marchesan, Rutberg et al. 2003). These studies with primary alcohols should be readdressed not only with the recently generated PLD mutant mouse models, but also with isoform specific PLD inhibitors.

2. We intend to explore the synaptic protein levels of PLD1 and PLD2 mice and perform electrophysiological studies to investigate the role of PLD in neuroplasticity.

Regarding the previous studies implicating PLD in dendritic branching and cognitive functions (Oliveira, Chan et al. 2010, Zhu, Kang et al. 2012, Ammar, Humeau et al. 2013, Burkhardt, Stegner et al. 2014) and the unpublished data from our laboratory revealed that the ablation of PLD1 or PLD2 may have a differential impact in the dorsal and ventral hippocampus and in the trisynaptic circuit, our intentions include a biochemical evaluation of signaling molecules upstream and downstream of PLD such as, ras homolog gene family member A (RhoA), implicated in negatively dendritic arbor growth (Zhu, Kang et al. 2012) by PLD1, the PI3K-mTOR pathway, Wnt cascate, which PLD has been proposed as a transcriptional target (Kang, Choi et al. 2011) and APP. Furthermore, to further understand the role of PLD in synaptic plasticity, it would be interesting to evaluate the levels of metabotropic glutamate receptors (mGluR1a and mGLUR5a) at synapses of PLD1 and PLD2 knockout mice, as well as the levels of cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF), after long term potentiation (LTP)/ long term depression (LTD).

3. Evaluate the protein levels and neuronal subcellular distribution of tau using PLD inhibitors and PLD mutant animals.

PLD inhibitors such as 5-fluoro-2-indolyldes-chlorohalopemide (FIPI), blocks in vivo PA production and it is a useful tool to evaluate the many cells biological processes that have been

linked to PLD (Su, Yeku et al. 2009). Moreover, the recently published isoform specific PLD inhibitors could tease apart the specific contribution of either PLD1 or PLD2 (Lavieri, Scott et al. 2010). Finally, the usage of mouse PLD1 and PLD2 knock-out models could also contribute to understand the specific impact of either isoform in tau neuronal location (Dall'Armi, Hurtado-Lorenzo et al. 2010, Oliveira, Chan et al. 2010).

4. Evaluate the tau levels in the cytosolic and membrane fraction of the PLD1 and PLD2 knock-out mice hippocampus.

Importantly, tau is released from cells in association with exosomes, when tau is exogenously expressed or is highly phosphorylated (Saman, Kim et al. 2012, Simón, García-García et al. 2012). The tau localization is important for its propagation and a recent study from Croft et al. revealed a linked between the amount of dephosphorylated tau present at membranes and the extend of tau release (Croft, Wade et al. 2017). Thus, it would be highly relevant to understand if the PLD modulation changes the fraction of the tau that is associated with membranes and if this association with membranes could alter tau's functions.

5. Use primary neuronal culture of PLD and tau knock out mice to assess the cellular distribution and possible relocalization of tau/PLD complex.

As PLD can present itself as a tau modulator, also tau could modulate PLD. Therefore, the evaluation of the PLD/tau complex could be studied evaluating the distribution in subcellular compartments through western blotting and proximity ligation assays in PLD1/2 KO and tau KO animals.

6. Take advantage of the proximity ligation assay and co-immunoprecipitation studies to evaluate if tau is linked directly with the PLD or form a complex with other lipids/proteins such as PA or Bin1.

Among the other major risk factors for sporadic AD, there is a group of genes which are proposed to be involved in endocytosis and membrane trafficking. From these, Bin1, also known as amphiphysin 2, is the second most relevant genetic risk factor (Querfurth and LaFerla

2010). It was previously reported that Bin1 interacts with PLD1, PLD2 and tau (Lee, Kim et al. 2000, Chapuis, Hansmannel et al. 2013). We hypothesize that tau and PLD pathogenic mechanisms could be potentially shared and that Bin1 could be functioning as a hub between these two proteins.

7. The increased levels of tau observed in *pld-1*; tau animals may be explained by dissecting autophagy processes.

The impairment of protein-degradation systems might play a role in tau aggregation and tau-induced neurodegeneration (Wang and Mandelkow 2012). Additionally, it was reported that PLD1 modulates macroautophagy and that the inhibition of PLD results in increased levels of tau and p62 aggregates in organotypic brain slices (Dall'Armi, Hurtado-Lorenzo et al. 2010). Enhancing or blocking autophagy by chemical or genetic means will help us to dissect autophagic mechanisms involved in these disease process and allow us to explore with more detail the role of PLD in autophagy. Moreover, it was recently shown that blocking the endolysosomal flux, blocks autophagy and reroutes lysosomal cargo to exosomes (Miranda, Lasiecka et al. 2018). It would be relevant to assess if PLD1/2 is modulating this membrane trafficking associated pathway and if it modulates tau release in primary neuronal cultures of which could be evaluated by sensitive sandwich ELISA.

Regarding our MS-based lipidomic approach that identify potential lipid pathways associated with susceptibility to pharmacological induced-seizures, future studies should be performed:

1. To identify new therapeutical targets for excitotoxicity protection conferred by genetic mechanisms that confer resistance to pharmacological induced-seizures. To perform this we propose to use tau knockout mice and analyze its the hippocampus lipidome in the context of PTZ injections.
2. We have identified here a number of lipid modulating pathways that could be involved in seizure susceptibility/resistance in hAPP mice. The final goal would be to test if the renormalization of these lipid imbalances rescues behavioral deficits in hAPP mice.

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