

MAPPING THE NEUROANATOMY OF DEREGULATED LIPID METABOLISM AND SIGNALING PATHWAYS IN PHARC SYNDROME

A Thesis

Submitted in partial fulfillment of the requirements

Of the degree

Doctor of Philosophy

By

SHUBHAM SINGH

20152014



INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

2021

DEDICATION

I dedicate this work to my parents

ASHUTOSH KUMAR SINGH & GEETA SINGH

CERTIFICATE

Certified that the work incorporated in the thesis entitled “Mapping the neuroanatomy of deregulated lipid metabolism and signaling pathways in PHARC Syndrome” submitted by Shubham Singh was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.


Supervisor

Date: 14th Jan, 2021

DECLARATION

I declare that this written submission represents my ideas in my own words and where others' ideas have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources, which have thus been properly cited, or from whom proper permission has not been taken when needed.

A handwritten signature in black ink, appearing to read 'Shubham Singh', with a horizontal line drawn underneath the name.

(Signature)

Shubham Singh

20152014

Date: 14th Jan, 2021

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ABSTRACT

Mapping the neuroanatomy of deregulated lipid metabolism and signaling pathways
in PHARC Syndrome. (January 2021)

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Chair of Research Advisory Committee: Dr. Siddhesh S. Kamat

My doctoral research described in this thesis largely entails mapping deregulated lipid metabolism and signaling pathways focused around the neurological disorder, PHARC, an autosomal recessive human genetic disease. It is an age dependent neurodegenerative disease presumed to be caused by defective lysophosphatidylserine (lyso-PS) signaling arising out of disrupted lyso-PS metabolism. Lyso-PS is principally metabolized by two enzymes of serine hydrolase family – ABHD16A (biosynthetic) and ABHD12 (degradative) in the mammalian central nervous system. As a part of my doctoral research, I purified and biochemically characterized lyso-PS biosynthetic enzyme i.e. ABHD16A. I mapped the spatial metabolic route of lyso-PS in the mammalian brain and identified its crucial signaling effects in neurons and microglial cells. I have also demonstrated how defective lyso-PS signaling leads to the aberrant microglial activation (in turn neuroinflammation) through Toll like receptor 2 (TLR2) and its participation in granule neuron – Purkinje neuron communication (through phosphorylation pathways) in the cerebellum. I found that the loss of ABHD12 leads to elevated phagocytosis under immune stress possibly through oxidized phosphatidylserine accumulation and signaling. I could successfully reduce the age dependent neuroinflammation in ABHD12 knockout (KO) mice by administering minocycline and also by blocking ABHD16A activity. In short, we show that TLR2 and/or ABHD16A could be a potential therapeutic target and minocycline as one of the active small molecules for treating neuroinflammation in ABHD12 KO mice. Going ahead, it remains an interesting scientific pursuit to test if sensory motor defects of ABHD12 KO mice can be rescued by blocking ABHD16A and/or TLR2 receptor.

CHAPTER I

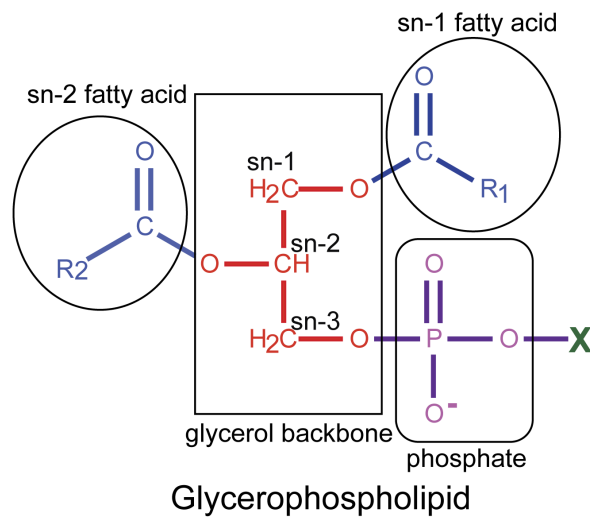
INTRODUCTION

Lipids are central to the very existence of cellular life as they drive crucial physiological processes that include structural, cellular communication, cell death and its renewal. Structurally, self-assemble into bi-layered membrane to give boundary to basic unit of living entities called cells and allow subcellular compartmentalization of eukaryotic cells. They function as signaling molecules in signal transduction pathways by directly acting as ligands to G-protein coupled receptors¹ (e.g. prostaglandins) and nuclear receptors² (e.g. steroid hormones) or indirectly as secondary messengers³ e.g. diacylglycerol. In addition to their role in cellular architecture and signaling, they modulate membrane protein (receptors, ion channels, transporters, etc.) activity, structure and functions by acting as co-factors⁴. Lipids are also appended to proteins post-translationally (e.g. palmitoylation) to modulate protein function and maintenance⁵. The advantage of lipids as a signaling molecule lies in the fact that it can act through cell surface receptors, can be transported into cells via ABC transporters⁶ or can easily diffuse through the cell membrane and act through intracellular protein-lipid interaction or through nuclear receptors. A large set of signaling lipids like phosphatidylinositol 4,5-bisphosphate (PIP2) and arachidonic acid derivatives originate from phospholipids (PLs) in the membrane through action of phospholipases.

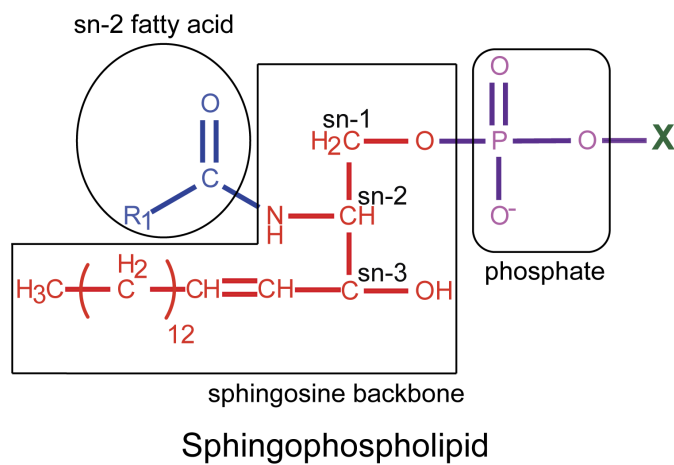
PLs can be classified into two broad classes based on their backbone – glycerophospholipids and sphingophospholipids. Glycerophospholipids have a glycerol backbone esterified with fatty acids at sn-1 and sn-2 carbon centers on glycerol (often referred as tail groups) and is phosphorylated at the sn-3 carbon (**Fig 1.1A**). On the other hand, sphingophospholipids have sphingosine backbone with fatty acid at sn-2 carbon and phosphate at the sn-1 carbon (**Fig 1.1B**). The phosphate group of phospholipids is attached to different moieties (sugars/amino acids or their derivatives) like ethanolamine, choline, serine, or inositol, which constitute the head group along with phosphate (**Fig 1.1C**).

Phospholipids are metabolized by phospholipases and acyltransferases, phospholipases catabolize phospholipids whereas acyltransferases are mostly anabolic in function. Combined activity of phospholipases and acyltransferases recycle and remodel the fatty acid

A



B



C

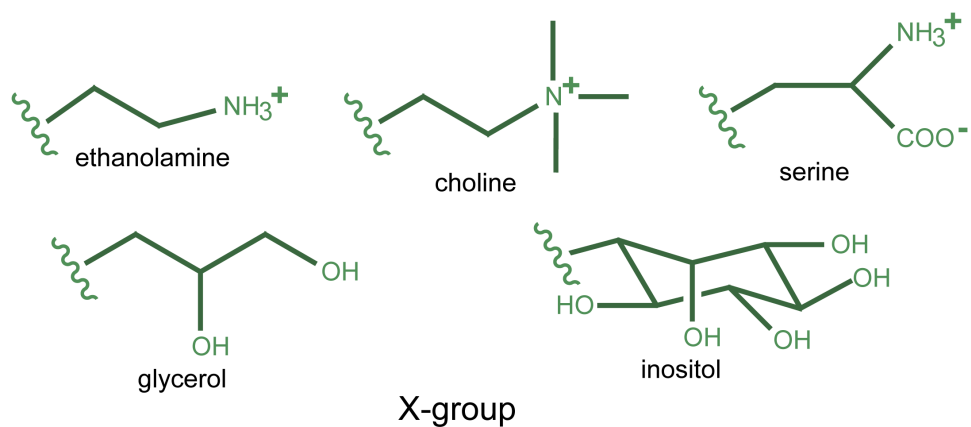


Fig.1.1: Structure of phospholipids. A- Structure of glycerophospholipids. B- Structure of sphingophospholipids. C- Structure of head groups - sugar or amino acid derivatives. Sn-1, sn-2, and sn-3 denote the carbon positions on the glycerol or sphingosine backbone.

composition of different cellular phospholipids. Phospholipases are classified into four types depending on the bond they cleave on phospholipids. Phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂) hydrolyze ester bonds at sn-1 and sn-2 positions respectively and release the free fatty acids, phospholipase C (PLC) hydrolyzes phosphodiester bond between glycerol and phosphate group at sn-3 position and releases phosphorylated head group. Phospholipase D (PLD) hydrolyzes the bond between the phosphate group and the head group moiety (choline, inositol, glycerol, ethanolamine and serine), and releases head group of the phospholipids (**Fig 1.2**).

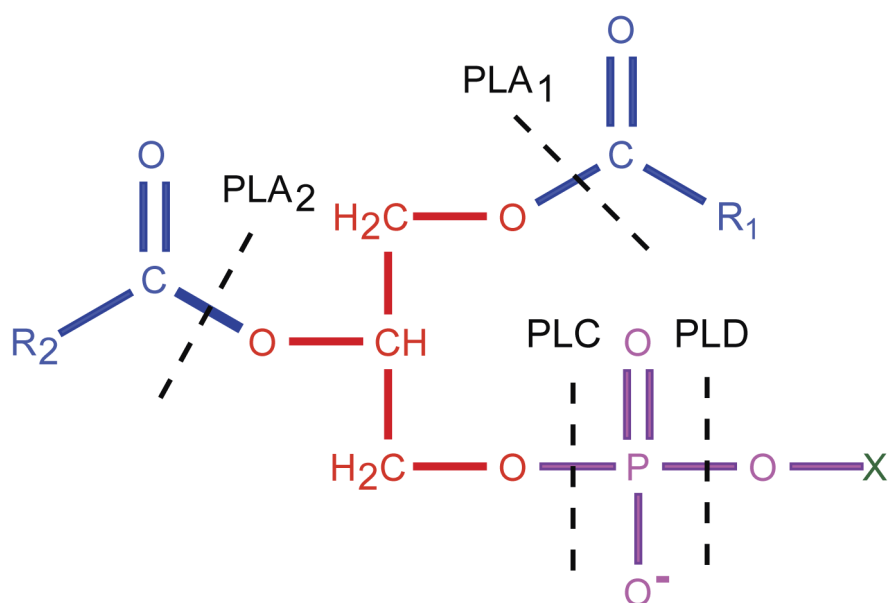


Fig.1.2: Structure of glycerophospholipids and site of action of phospholipases on glycerol backbone. PLA₁ – Phospholipase A₁, PLA₂ – Phospholipase A₂, PLC – Phospholipase C, PLD – Phospholipase D.

The action of PLA₁ and PLA₂ on phospholipids generates lysophospholipids (lyso-PLs) that have been identified as ligands to several receptors through which they mediate a long list of critical biological pathways like angiogenesis, immune modulation, neuronal axon growth, and tumor migration⁷. Lyso-PLs once synthesized, are either transported out of the cells through transporters like solute carriers or ABC transporters, where they act as extracellular ligands or they can act as intracellular ligands by interacting with the receptors on the organelle membrane apart from interacting with other intracellular components. Brain and immune cells (e.g. macrophages, T cells) exhibit high expression profile for the known lyso-

PL receptors⁷. This has sparked an interest in investigating these molecules as bioactive compounds in several neurological and/ or neuro-immunological processes.

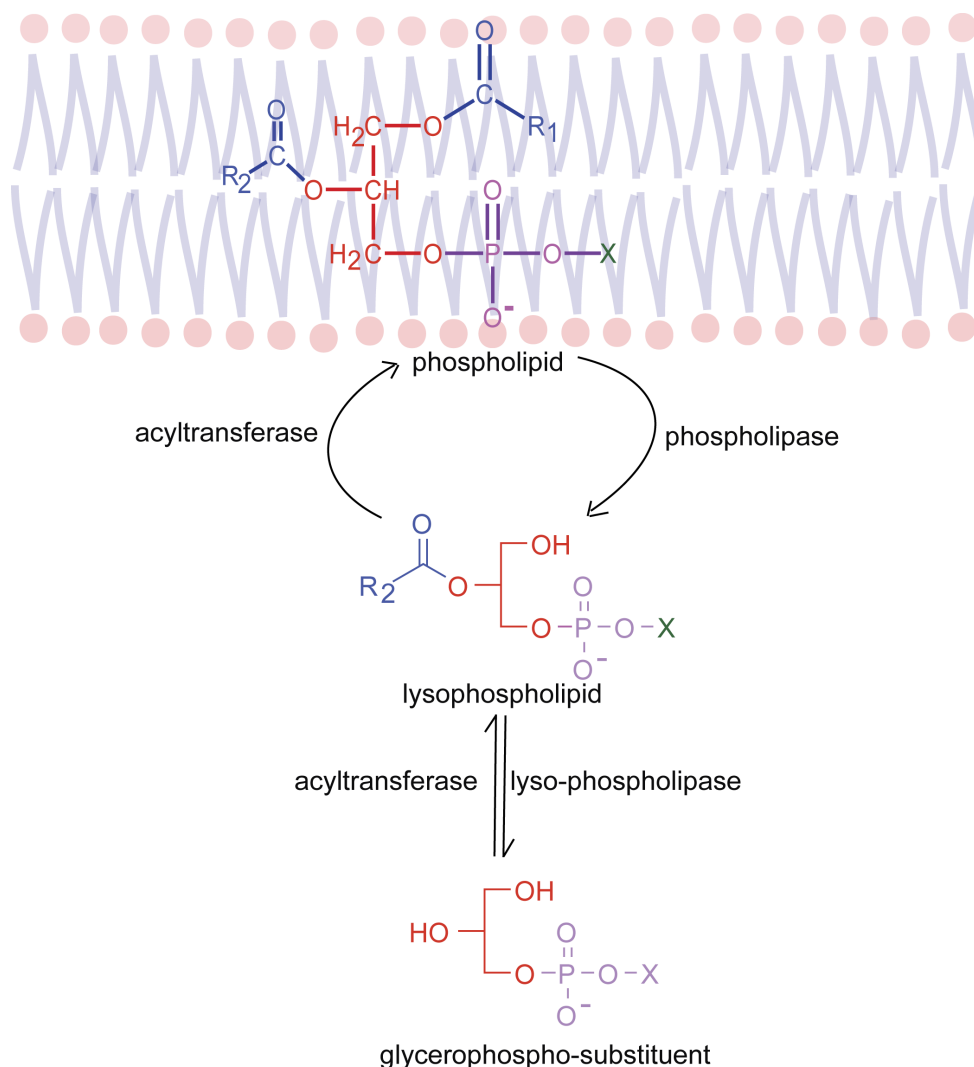


Fig.1.3: Scheme of Lands cycle as proposed by William Lands. Lands cycle proposes a mechanism for recycling of fatty acid composition of phospholipids and lysophospholipids are formed as intermediate.

History and biochemical pathways that regulating lyso-PLs.

In 1914, Delezenne found that snake venom converts dually acylated lecithin to singly acylated lecithin (lyso-PLs). Belfanti first isolated lyso-PLs in 1924 but metabolic enzymes for lyso-PL were unidentified and unknown till mid-20th century. Lands cycle proposed by

William Lands hypothesized that lyso-PLs are formed as an intermediate in process of fatty acid remodeling of PLs through enzymatic activity of phospholipases and lyso-PL acyltransferases (**Fig 1.3**). Lyso-PLs can also be generated from the ab initio phospholipid synthesis pathway (as an intermediate) or it can be produced by hydrolysis of one ester bond at sn-1 or sn-2 carbon on the PLs. Lyso-PLs can be broadly classified on the basis of backbone as glycerol-lyso-PLs (**Fig 1.4 A-B**) and sphingo-lyso-PLs (**Fig 1.4 C-D**), both these categories of lyso-PLs follow slightly different metabolic pathways. PLA₁ and PLA₂ hydrolyze the ester bonds at sn-1/sn-2 carbon on membrane glycerol-PLs and release a free fatty acid and corresponding glycerol-lyso-PLs. This results into the formation of different glycerol-lyso-PLs like lysophosphatidic acid (lyso-PA), lysophosphatidyl-serine (lyso-PS), lysophosphatidyl-choline (lyso-PC), lysophosphatidyl-glycerol (lyso-PG), and lysophosphatidyl-ethanolamine (lyso-PE) (**Fig 1.4**). Lyso-PLs are broadly classified as *sn*-1 or *sn*-2 lyso-PL depending on the fatty acid esterification site on glycerol backbone (**Fig 1.4 A-B**).

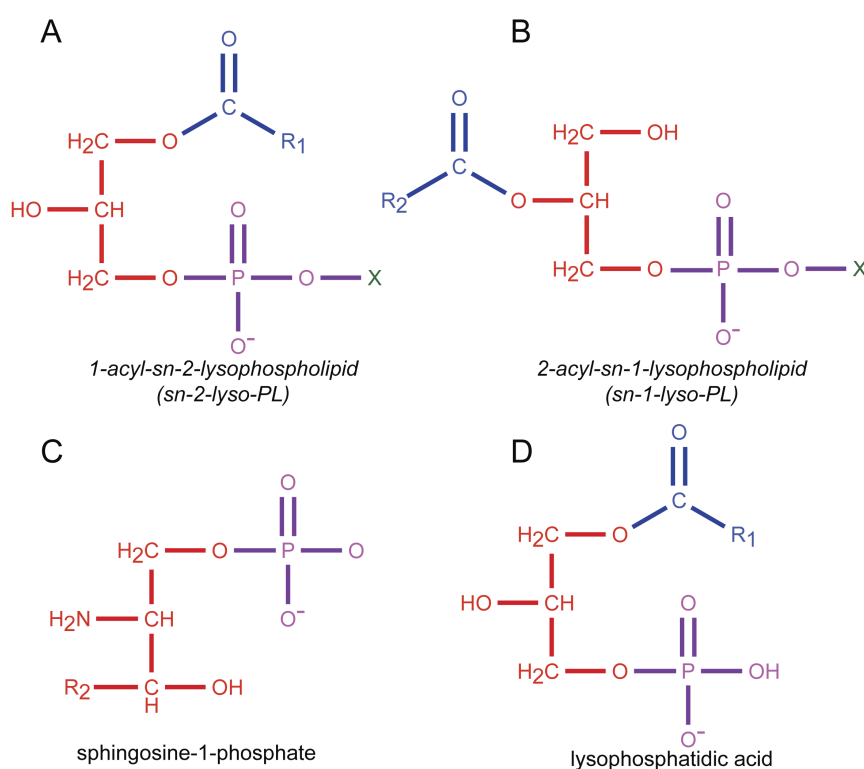


Fig.1.4: Structure and classification of lysophospholipids. A– sn-2 lysophospholipid, B – sn-1 lysophospholipid, C – lysosphingophospholipid (sphingosine-1-phosphate), D – lysophosphatidic acid. X represents the head group of lysophospholipids.

PLA₁ and PLA₂ type enzymatic activity generates 2-acyl-sn-1-lyso-PL (sn-1 lyso-PL) and 1-acyl-sn-2-lyso-PL (sn-2 lyso-PL) respectively (**Fig 1.5**). Additionally, lysophosphatidic acid (lyso-PA) is generated by lyso-PL lipase D enzymes that removes the head group substituents of lysophospholipids with head group e.g. autotaxin (ATX) removes choline from lyso-phosphatidylcholine (lyso-PC) and converts it to lyso-PA (**Fig 1.5**). Lyso-PA can also be produced through a two-step alternate pathway, where the first step involves PLD catalyzed removal of head group substituent from membrane phospholipids (formation of phosphatidic acid), followed by one fatty acid removal through membrane bound PLA₁ (**Fig 1.5**). Lyso-PLs are further metabolized to glycerophospholipids by lyso- phospholipases or converted back to phospholipids by acyltransferases as proposed by Lands' cycle (**Fig 1.3**). Lyso-PLs can be dephosphorylated by enzymes like lyso-phosphate phosphatases (LPP), which are membrane proteins with their active site lumenally oriented and act on lyso-PA to generate glycerol and phosphate. In contrast to glycerol-Lyso-PLs, sphingo-Lyso-PLs are synthesized by sphingosine kinase from cytosolic sphingosine. Sphingomyelinase converts membranous sphingophospholipid (sphingomyelin) to ceramide, which is further metabolized to cytosolic sphingosine (**Fig 1.5**). Sphingosine kinases add phosphate to the sphingosine and synthesize sphingosine-1-phosphate (S1P). S1P can be either converted back to sphingosine by S1P phosphatase (SPP) or can be degraded by S1P lyase to phosphoethanolamine and free fatty acid (**Fig 1.5**).

The functional role of lyso-PLs was first annotated when Smith et al. reported that lyso-PS is 1000 times more potent co-stimulator for histamine release from mast cells than phosphatidylserine (PS) on concanavalin co-stimulation⁸. Several other studies demonstrated involvement of lyso-PA in platelet aggregation, cell proliferation, and was reported to have several other messengers like properties when bound to serum albumin. Since then, other lyso-PLs were also found to be involved in several physiological processes, Here, I would limit my discussion to lyso-PS in cellular signaling as my thesis work describe in upcoming chapters is limited to investigation of lyso-PS metabolism and function.

Lyso-PS as a signaling molecule in the mammalian physiology.

Recently, three lyso-PLs namely lyso-PA, S1P, and lyso-PS have been implicated in several pathophysiological conditions like cancer and neuro-immunological conditions. Lyso-PS is a potent lyso-PL molecule that signals through Toll-like-receptors (TLRs)⁹ or G-Protein

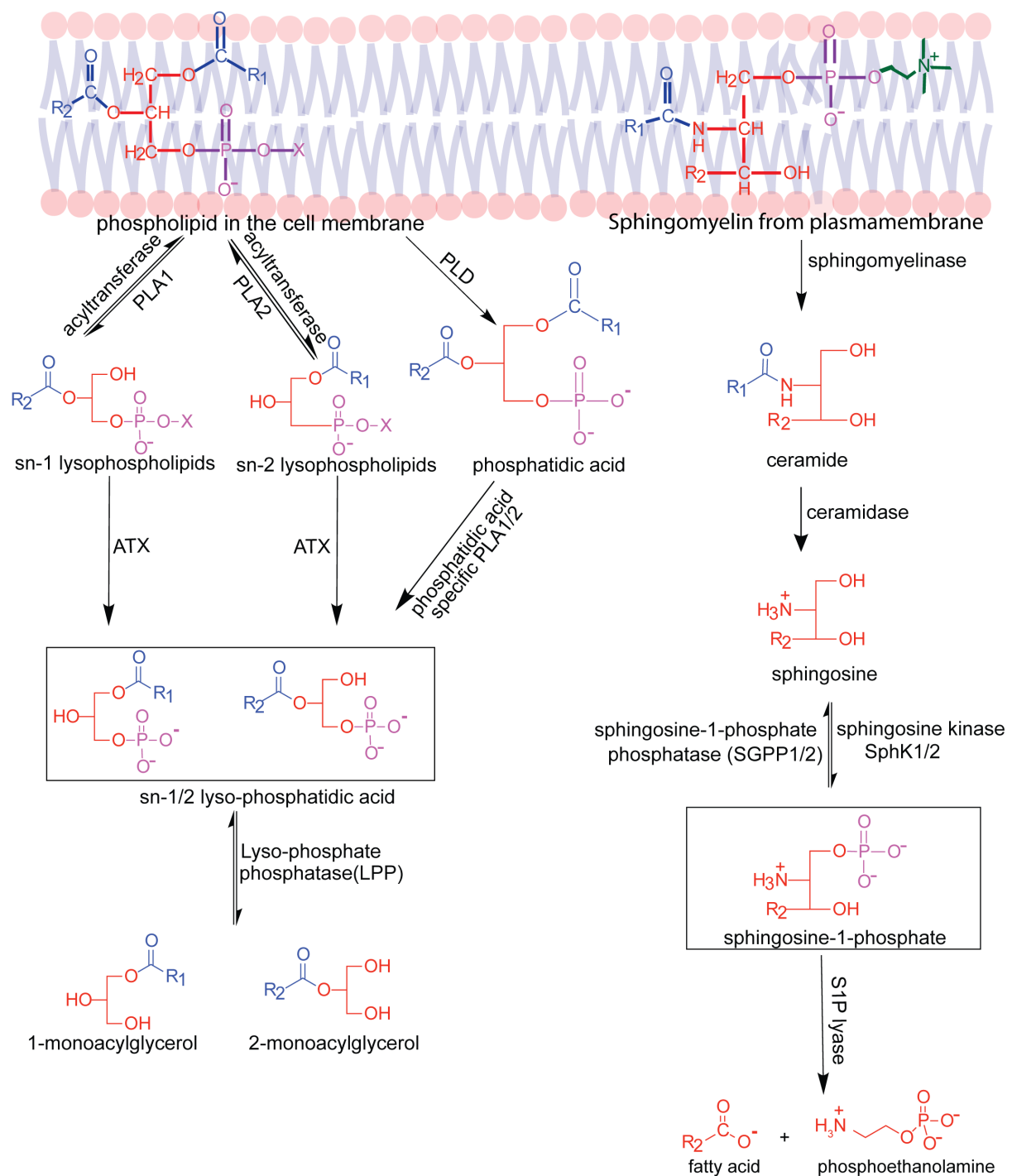
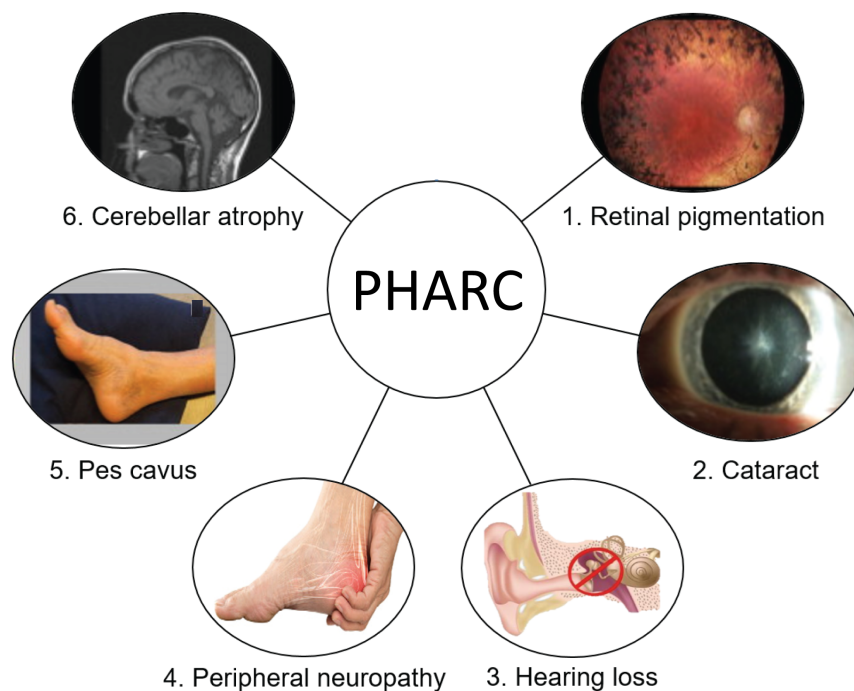


Fig.1.5: General scheme for the metabolism of glycerol-lysophospholipids and sphingo-lysophospholipids. ATX – autotaxin, S1P lyase – sphingosine-1-phosphate lyase.

Coupled Receptors (GPCRs)¹⁰ expressed on cancer and immune cells, and modulates mast cell degranulation¹¹, macrophage activation¹², stimulation of leukemic cells¹³, blood coagulation¹⁴, clearance of apoptotic cells¹⁵ and chemotactic movement of human glioma and colorectal cancer cells¹⁶. Defects in lyso-PS metabolism and signaling is proposed to be a

major underlying factor driving the pathophysiology of PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract) syndrome¹⁷. PHARC is a rare autosomal recessive genetic disorder caused by mutation in *Abhd12* gene, which encodes for (α/β)-hydrolase domain containing # 12 protein (ABHD12) – a lyso-PS lipase^{17,18}. PHARC human subjects exhibit cerebellar atrophy related sensory motor defects, peripheral neuropathy and early onset of cataract, blindness, hearing loss, and demyelination of sensorimotor neurons (**Fig 1.6**). These symptoms appear first at late childhood or early teen age, and worsen progressively with age^{19,20}, thus making PHARC an age dependent neurological disorder. The murine model of PHARC, i.e. the ABHD12 knock out (KO) mice have elevated levels of lyso-PS especially in the neural tissues and it recapitulates a few of the neurobehavioral abnormalities like hearing loss and locomotor defects seen in PHARC human subjects. ABHD12 KO mice greater than one year of age show cerebellar microgliosis marked by higher number of activated microglial cells i.e. neuroinflammation in brain (especially cerebellum), succeeding which they suffer from locomotor defects in an age dependent manner¹⁷. Pharmacological inhibition of ABHD12 in adult mice increased lyso-PS levels in their brain but it failed to cause sensory neuronal hearing loss as seen in ABHD12 KO mice, suggesting that hearing loss in ABHD12 KO mice may arise at an early developmental stages²¹.



*Fig.1.6: Symptoms of PHARC syndrome in humans. PHARC subjects bear loss of function mutation in *abhd12* gene.*

The aforementioned findings generated an active interest in finding an enzyme, a putative PS lipase, acting upstream of ABHD12, which can produce lyso-PS by hydrolyzing PS lipids (precursors of lyso-PS). Kamat *et al.* showed through target metabolite profiling and enzymology studies that ABHD16A, a poorly characterized brain serine hydrolase enzyme, is a major PS lipase in mammalian brain. They further extended the work, showing that ABHD16A and ABHD12 are the principal regulators of immunomodulatory lyso-PS in mammalian brain and immune system²². It is evident from the above findings that loss of ABHD12 activity leads to accumulation of lyso-PS synthesized by ABHD16A in the mammalian brain, which possibly leads to neuro-inflammation and locomotor defects (Fig.1.7).

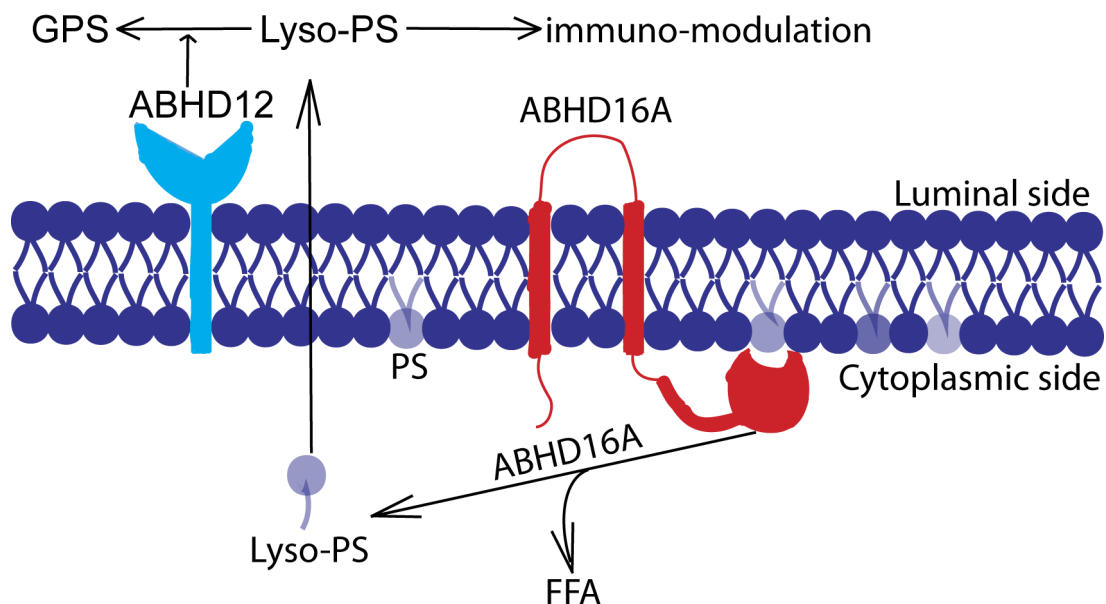


Fig.1.7: Interplay of ABHD16A and ABHD12 in regulation of lysophosphatidylserine in mammalian immune and neuronal cells.

The molecular pathways downstream of lyso-PS that contribute to neuroinflammation and other behavioral defects associated with PHARC syndrome is not known and needs thorough investigation. The annotation of ABHD16A as an enzyme upstream of ABHD12, makes it an attractive therapeutic target for PHARC and other lyso-PS induced pathophysiological conditions. However, ABHD16A remains poorly characterized both biochemically, and at a cell biological level. ABHD16A – lyso-PS – ABHD12 is an important biochemical and

signaling nodal axis and to better understand their broader role and relate it to the symptoms seen in PHARC subjects, one needs to map their localization at cellular and subcellular scale. Mapping the cellular space in which ABHD16A and ABHD12 localizes, will help further in understanding the metabolic flux and regulation of PS and lyso-PS lipids inside cells and will also provide additional mechanistic insights of the potential role of lyso-PS pathway in PHARC pathophysiology.

As a part of this thesis, I developed methods to purify ABHD16A and biochemically characterized it from bacterial and mammalian sources (Chapter II). I generated a subcellular localization map of ABHD16A and ABHD12 (Chapter III) in mammalian systems. It provides new insights into PS and lyso-PS metabolism at sub-cellular precision in mammalian cells. I also mapped the gross and cell specific localization of ABHD16A and ABHD12 in mice brain (Chapter IV). Brain has been reported to exhibit highest levels of lyso-PS among mammalian tissues but its significance in basal physiology was under-investigated. Here, along with localization studies in the mammalian brain, I show that ABHD16A – lyso-PS – ABHD12 pathway modulate the Purkinje neuron physiology in cerebellum of the mouse brain (Chapter V). ABHD12 KO mice accumulates lyso-PS in brain especially cerebellum leading to microglial activation hypothesized to be a contributory factor in behavioral defects of PHARC syndrome. I investigated the role of the lyso-PS pathway on brain macrophages (microglial cells) and show that lyso-PS activates microglial cells through Toll like receptor 2 (TLR2) (Chapter VI). I also show that loss of ABHD12 activity leads to elevated phagocytosis by microglial cells under the conditions of oxidative stress (Chapter VI). Thus lyso-PS acts as a crucial signaling molecule in the central nervous system and modulates the neuro as well as neuro-immunophysiology of the mammals, and any defects in its metabolism and signaling can lead to disorders like PHARC Syndrome (Chapter VII). Taken together, my thesis characterizes ABHD16A, maps the ABHD16A – lyso-PS – ABHD12 biochemical axis, investigates and establishes its role in the mammalian neuro-immunophysiology and provides exciting new insights into the pathophysiology of PHARC syndrome.

CHAPTER II

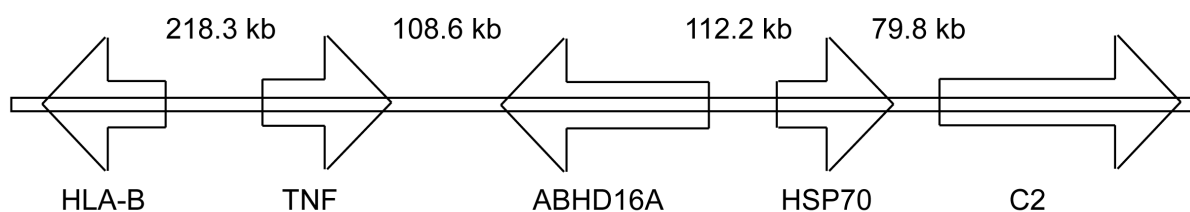
Expression, Purification and Characterization of ABHD16A

ABHD16A is a serine hydrolase family enzyme annotated as the principal phosphatidylserine (PS) specific lipase in the mammalian neuronal and immune cells but its structural or thorough biochemical characterization is lacking. Here, I assayed ABHD16A over expressing HEK293T membrane lysates for phospholipase activity and found that ABHD16A prefers phosphatidylserine as substrate compared to other phospholipids and acts as a phospholipase A₂ (PLA₂) type enzyme. I also developed protocol to purify recombinant ABHD16A from the bacterial overexpression system. Biochemical assays with purified protein too showed that ABHD16A acts as a PS specific PLA₂ type enzyme. ABHD16A hydrolyses ester bond of PS substrates and generates 1-acyl-sn-2-lysophosphatidylserine along with one free fatty acid. This is the first report of successful ABHD16A purification and its annotation as PS specific phospholipase A₂ type enzyme. My findings here are interesting to the fact that lyso-PS receptors show differential activity for sn-1 and sn-2 lyso-PS species. The purification protocols developed described here can be scaled up for structural characterization of ABHD16A.

Introduction

α/β – hydrolase domain containing protein #16A (ABHD16A) is a poorly characterized member of a large family of enzymes – serine hydrolases. Serine hydrolases constitute approximately 240 member enzymes in mammals, divided into two subfamilies i.e. serine proteases that include ~ 125 members and metabolic serine hydrolases consisting of ~ 115 members²³. Metabolic serine hydrolases have a further subdivision of enzymes characterized by α/β – hydrolase domain containing protein²⁴, ABHD16A is a member of this enzyme family. *Abhd16a* gene lies in the major histocompatibility complex III gene cluster on chromosome 6 (6p21.33) in humans and chromosome 17 (17B1|17) in mouse, hence ABHD16A is also named human lymphocyte antigen B-associated transcript 5 (BAT5) and speculated to be involved in immunological processes^{25,26} (**Fig. 2.1**).

Human - chromosome 6



Mouse - chromosome 17

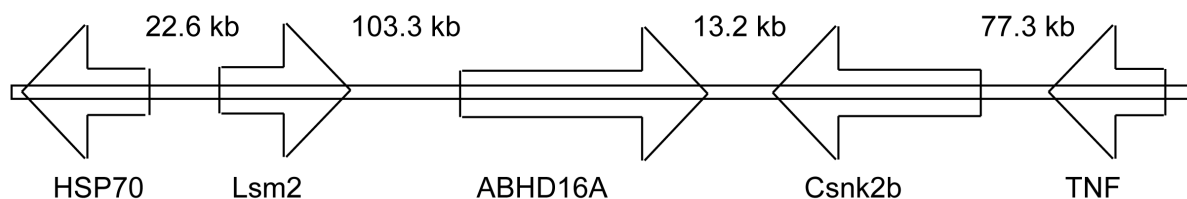


Fig.2.1: ABHD16A also called BAT5 genetic locus in major histocompatibility complex III gene cluster of chromosome 6 in the humans and chromosome 17 in the mouse.

Polymorphism in ABHD16A has been associated to a few pathological conditions including Kawasaki disease, coronary artery aneurysm and black fat metabolism suggesting its role in lipid metabolism and defects in which might lead to physiological disorders or diseases^{27,28}. It is greater than 95% conserved in mammals like mice, rat, humans, camels, and alpaca (**Fig. 2.2**), suggesting that ABHD16A is closely evolved and possibly perform a conserved function in all the mammals. It has four domains which include two predicted trans-membrane domains (TM1 and TM2), an α/β – hydrolase domain (ABHD) for hydrolysis of ester bonds and a predicted inactive acyl transfer domain (HxxxxD)²⁹. In the ABHD domain lies the catalytic triad (Serine – Histidine - Aspartate, SHD) – canonical active site residues of serine hydrolases that allow hydrolysis reaction to take place with the help of water molecules in the enzyme active site²⁹ (**Fig. 2.2**). The lipase like motif in ABHD16A – GxSxxG has one extra amino acid insertion compare to canonical lipase motif –GxSxG²⁹ (**Fig. 2.2**). ABHD domain of ABHD16A is oriented towards the cytosolic leaflet of the membrane (**Fig. 2.3**), interesting to the fact that PS is often enriched towards the cytosolic leaflet of the membrane under basal conditions²².

ABHD16A is active on dually acylated lipids and hydrolyses one ester bond of glycerophospholipids to generate glycerolysophospholipids – a singly acylated version of

```

BAT      MAKLLSCVLGPRLYKIYERDSEARASGAPETPASVTAPPPSSWDTTYQPRALEKHADSI
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Rat      MAKLLSCVLGPRLYKIYERDSDRAATSVPETPTAVPAASSSSWDSYYQPRALEKHADSI
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Alpaca   MAKLLSCVLGPRLYKIYERDSEARAPSGVPGTPTSVTTTPSSSSWDTTYQPRALEKHADSI
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Rat      NPQYRQFITILEATHRNQSAENKRQLANYNDFRSWVPDFHWEESPSSRKGSRG
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Alpaca   NPQYRQFITILEATHRNQSAENKRQLANYNDFRSWVPDFHWEESPSSRKESRG
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BAT      ALLRPEPLH
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Rat      ALLRPEPLH
Human    ALLRPEPLH
Alpaca   ALLRPEPLH
Camel    ALLRPEPLH
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Mouse    LEAG
Rat      LEAG
Human    LEAG
Alpaca   LEAG
Camel    LEAG
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Rat      EEASIYSRWEVEEDWCVSVLRSYQAEHGPDPFWSVGEDMSVDGRRQALFLARKHLHNFE
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Rat      ATHCTPLPAQHFQMPWHL
Human    ATHCTPLPAQHFQMPWHL
Alpaca   ATHCTPLPAQHFQMPWHL
Camel    ATHCTPLPAQHFQMPWHL
*****:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:.*:

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Fig.2.2: Multiple sequence alignment of ABHD16A protein primary structure in mammals like Bat (*Myotis lucifugus* - XP_006104671), Mouse (*Mus musculus*) NP_848707.1, Rat (*Rattus norvegicus*) NP_997696.1, Human (*Homo sapiens*) BAB63383.1, Alpaca (*Vicugna pacos*) XP_006215394, Camel (*Camelus ferus*) XP_006178831.

glycerophospholipids and a free fatty acid. It is shown that ABHD16A has highest specific hydrolase activity for PS with Michaelis- Menten constant (K_m) of $40 \pm 5 \mu M$ and V_{max} of $35 \pm 3 \text{ nmol mg}^{-1} \text{ min}^{-1}$ for a C18:0/C18:2 PS substrate²².

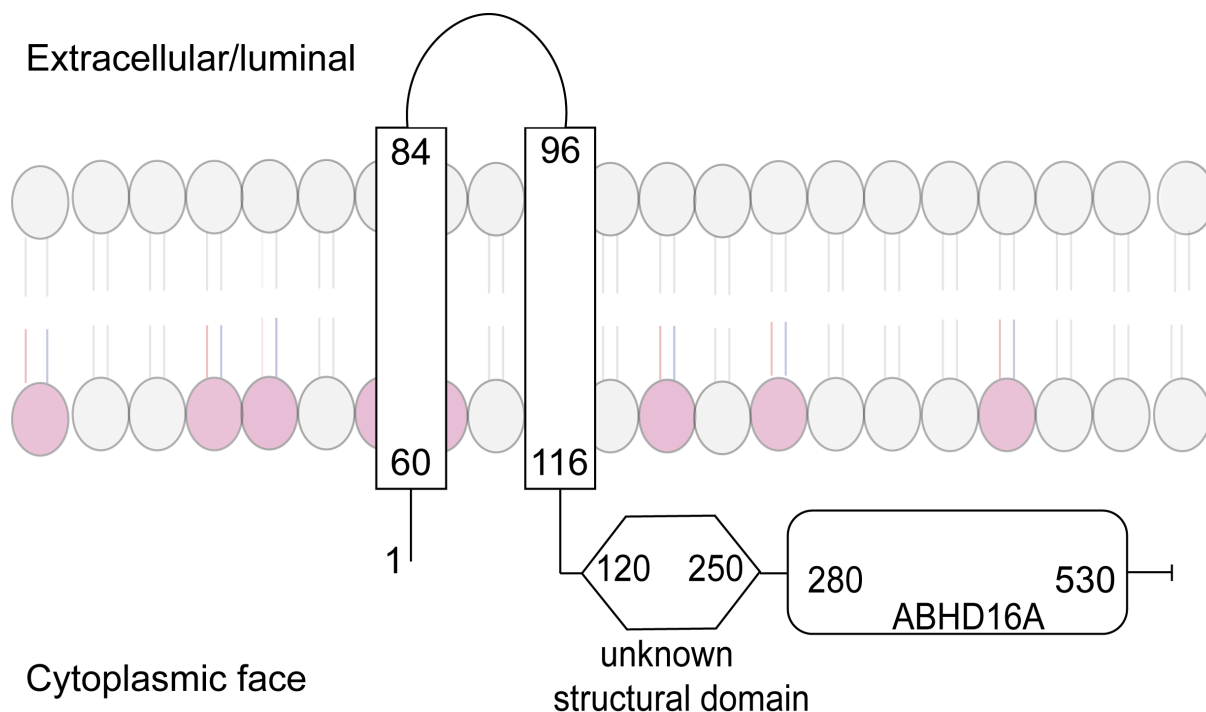


Fig.2.3: Topology, orientation and predicted structural domains of ABHD16A in mammals. Phospholipids in bilayer colored pink project the orientation of PS in cellular membrane.

It shows 3-4 fold lower hydrolysis activity on other phospholipids (PC, PE, PI, PG, and PA) and no activity on neutral lipids or lysophospholipids²². It is not known if it cleaves sn-1 or sn-2 ester bond differentially on glycerol backbone of PS i.e. if it acts as phospholipase A₁ (PLA₁) or phospholipase A₂ (PLA₂) or both. A few proteomics studies suggest that ABHD16A undergoes posttranslational modification such as palmitoylation but significance of palmitoylation on ABHD16A activity is unclear but largely. ABHD16A is inhibited by broad-spectrum serine hydrolase inhibitor – methylfluorophosphonate (FP) (**Fig. 2.4A**) and serine lipase inhibitor – tetrahydrolipstatin (THL) (**Fig. 2.4B**)^{22,30}. Based on its sensitivity to THL, β -lactone warhead (pharmacophore) based chemical library were screened and KC01 was found to be a suitably specific and active ABHD16A inhibitor. Along with KC01, KC02 was discovered as an inactive structural mimic that can be used as a structural control for KC01 (**Fig. 2.4D**). Under cellular settings, KC01 inhibits ~95% activity of ABHD16A with few off

target effects while KCO2 doesn't alter or inhibit ABHD16A activity²². This makes KC01 a suitable inhibitor for pharmacological studies of ABHD16A under in vitro or in situ experiments.

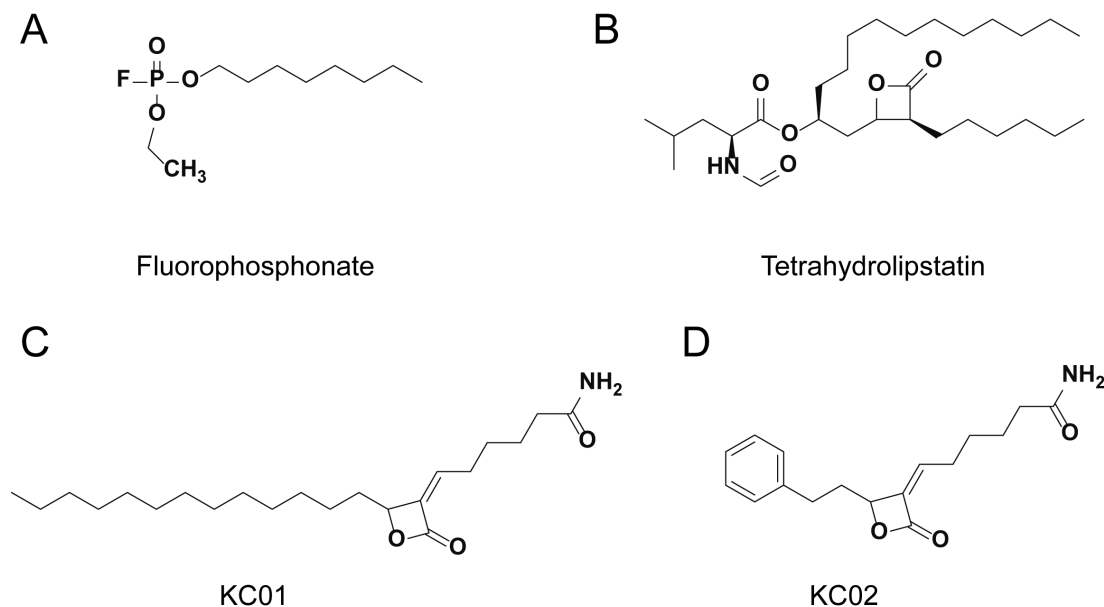


Fig.2.4: Structure of ABHD16A inhibitors. A- methylfluorophosphonate (FP), B- Orlistat or Tetrahydrolipstatin (THL), C- KC01, D- KC02. THL, KC01 and KC02 are molecules with β -lactone warhead or pharmacophore.

In this chapter, I developed protocols for expression, purification, reconstitution and biochemical assays of ABHD16A. I assayed recombinant ABHD16A for phospholipase activity and other pharmacological inhibition studies using KC01, and report that ABHD16A acts as PS specific PLA₂ type lipase and generates sn-2 lyso-PS species as a function of its activity.

Materials and Methods

Materials. Chemical probes FP-Rhodamine, WHP01 and inhibitors KC01 and KC02 were generous gift from Prof. Benjamin F. Cravatt, The Scripps Research Institute. All synthetic lipids and lipid standards used were procured from Avanti Polar Lipids. Lemo21 (DE3) competent *E. coli* cells, DNA polymerase and restriction enzymes were purchased from New England BioLabs (NEB). Plasmid isolation kits were brought from Qiagen. Tissue culture

dishes, culture media and fetal bovine serum were bought from Hi-Media. All the antibiotics were brought from MP-Biomedicals. All other chemicals and salts were purchased from Sigma-Aldrich until otherwise mentioned.

Cloning and mutagenesis. The codon optimized human ABHD16A was commercially synthesized and cloned into the pET-22b(+) vector between NdeI and HindIII restriction sites with a C-terminal 6X Histidine-tag for expression in the bacterial host (from Genscript). The plasmid for human ABHD16A for mammalian expression (pSPORT6 vector, C-terminal FLAG-tag) was a generous gift from the Prof. Benjamin F. Cravatt, The Scripps Research Institute. The active site Serine355 to Alanine355 (S355A) mutant ABHD16A for both plasmids was generated using whole plasmid polymerase chain reaction (PCR) with Phusion High-Fidelity DNA polymerase. Next, native plasmid (template DNA) was digested using DpnI restriction enzyme as per as protocol described by NEB. Post digestion, DpnI enzyme was denatured by heating the PCR mixture at 95 °C for 15 mins and whole PCR mixture was used to transform chemically competent Lemo21 (DE3) *E. coli* cells using heat shock method.

Expression of human ABHD16A into HEK293T cells and preparation of membrane lysates. HEK293T cells were cultured in DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS) and 1X Penicillin-Streptomycin maintained at 37 °C, 5% (v/v) CO₂. HEK293T cells were allowed to grow till 30% confluency in a 150 cm tissue culture plate, and were transfected with ABHD16A-FLAG-pSPORT6 plasmid using polyethyleneimine “MAX 40,000 MW (PEI, Polysciences Inc.) in 1:3 plasmid:PEI ratio. Cells were grown at 37 °C, 5% (v/v) CO₂ until 80% confluency (~ 2 days). Thereafter, cells were washed (3X) with cold sterile calcium and magnesium free Dulbecco’s phosphate-buffered saline - HIMEDIA (DPBS), pH 7.4. Cells were scraped using ice cold sterile DPBS, followed by 1000 g spin for 5 mins to pellet cells. Harvested cells were re-suspended in ice cold DPBS and lysed by probe sonication on ice with 3 sec on, 3 sec off at 60% amplitude for 30 sec total time. Lysed cells were centrifuged at the 100,000 g for 45 mins at 4 °C to pellet microsomes (enriched membrane proteome). Supernatant (soluble proteome) was discarded and pellet (microsomes) was stored at -80 °C for future substrate assays. Overexpression of ABHD16A in microsomes was confirmed by activity based protein profiling (ABPP) gel and western blot using anti-FLAG and anti-ABHD16A antibody.

Expression of codon optimized human ABHD16A in Lemo21 (DE3) E. coli cells. A sequence verified and codon optimized clone of human ABHD16A with 6X Histidine tag (pET-22b (+)-ABHD16A) was transformed into chemically competent *E. coli* Lemo21 (DE3) cells using heat shock (42 °C, 30 sec). These cells are a derivative of the standard BL21 (DE3) cells specifically engineered for the optimal expression of toxic or membrane proteins. The Lemo21 (DE3) cells keep protein expression optimal by expressing *lysY* - a variant of T7 lysozyme lacks amidase activity but inhibits T7-RNA polymerase when induced with *L*-Rhamnose. IPTG and *L*-rhamnose were titrated to get the optimal expression of ABHD16A without lysing host *E. coli* cells or its expression in the inclusion body. Temperature, IPTG and *L*-Rhamnose concentrations were screened to get optimal expression of ABHD16A in active form. Expression of ABHD16A on IPTG induction was checked by resolving membrane proteome on 10% SDS-PAGE gel using activity based protein profiling (ABPP) gel and Coomassie staining. All gels were visualized and imaged using Syngene G-Box Chemi-XRQ gel documentation system.

Detergent screen to solubilize ABHD16A from Lemo21 (DE3) E. coli cells. Lemo21 (DE3) *E. coli* cells overexpressing 6X-Histidine (6X-His) tagged ABHD16A were re-suspended in 20 mM HEPES, 150 mM NaCl, pH 7.4 and lysed by probe sonication with 1 sec on 3 sec off at 60% amplitude for 10 mins on ice. The lysed cells were centrifuged at 18,000 rpm for 45 mins to pellet the insoluble fraction enriched with membrane fraction. All detergents were screened against this insoluble fraction under various possible solubilizing conditions like varying detergent concentrations preferably at critical micelle concentration (CMC) of detergent and 10X CMC of detergent, in different buffers (like HEPES, Tris-HCl and PBS) and at different temperatures (4 °C, 25 °C, 37 °C) for differing time points (0.5, 2, 6 or 14 h). Screened detergents were of different classes including cationic, anionic, zwitterionic and non-ionic detergents. ABHD16A solubility was monitored by established gel based ABPP assays and coomassie staining using a 10% SDS-PAGE gel visualized using Syngene G-Box Chemi-XRQ gel documentation system.

Affinity chromatography (Ni-NTA) purification of ABHD16A. 6X-His tagged ABHD16A from ABHD16A overexpressing Lemo21 (DE3) *E. coli* cells was purified using a Ni-NTA column (HisTrap FF, GE Life Sciences). The detergent solubilized membrane proteome was

mixed with 5 mM imidazole and loaded onto the HisTrap 5 mL column pre-equilibrated with 5 column volumes (CV) solubilization buffer (20 mM HEPES, 150 mM NaCl, 2 mM SDS or 5 mM lauroylsarcosine sulfate (LSS), pH 7.4) supplemented with 5 mM imidazole. Post loading, the column was washed with 10 CV of the aforementioned buffer and bound protein was eluted with a step gradient of increasing imidazole concentrations in solubilization buffer with pH maintained at 7.4. Post elution from His-Trap column, imidazole was removed by dialyzing it against 20 mM HPEPS, 150 mM NaCl, 5 mM LSS, pH 6.8 for 5 h at 4 °C. All steps in this process were done in cold room maintained at 4 °C and all buffers used were ice-chilled in cold room.

Calcium hydroxyapatite (CHT) column chromatography. CHT chromatography was performed using a Calcium Hydroxyapatite column (CHT Type II, Bio-Rad). Ni-NTA eluate free of imidazole was loaded into CHT column pre-equilibrated with 10 CV of equilibration buffer comprising of 20 mM HEPES, 150 mM NaCl, 5 mM LSS, 5 mM sodium phosphate, pH 6.8). Post loading the column was washed with 10 CV of equilibration buffer and eluted with increasing step gradient of sodium phosphate in equilibration buffer, pH 6.8. Phosphate salts were removed out from eluted protein by dialyzing it against 20 mM HEPES, 150 mM NaCl, 5 mM LSS, pH 7.4. Purity of the purified ABHD16A was assessed by Coomassie staining using a 10% SDS-PAGE gel.

Refolding and reconstitution of purified ABHD16A. The detergent used for solubilization resulted in inactive ABHD16A, presumably due to denaturation and misfolding. I therefore have reconstituted detergent solubilized ABHD16A in lipid vesicles using 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) as reported for other integral membrane proteins. Briefly, POPC was dried under a stream of N₂ gas, re-suspended in 20 mM HEPES containing 150 mM NaCl at pH 7.4 and dispersed to form vesicles using a water bath sonicator for 5 mins. Purified ABHD16A with detergent and POPC was mixed to get final concentration of 1 mg/mL protein and 0.1 mM or 1.0 mM POPC in 20 mM HEPES containing 150 mM NaCl at pH 7.4. Detergent was gradually dialyzed out against 20 mM HEPES containing 150 mM NaCl at pH 7.4 and the reconstituted protein into liposomes (proteoliposomes) was assessed for activity using established gel based ABPP gels or substrate based lipase assays.

Gel based activity based protein profiling assays. Proteome or purified protein at 1 mg/mL final concentration was incubated with 2 μ M fluorophosphonate-rhodamine (FP-Rh) probe at 37 °C for 30 mins with constant shaking. Thereafter, reaction was quenched using 4X SDS loading dye and proteome was denatured by boiling the quenched reaction mixture at 95 °C for 10 mins. Denatured proteome was resolved on 10% SDS-PAGE gel and image was acquired under fluorescent channel in using Syngene G-Box Chemi-XRQ gel documentation or Typhoon scanner.

Western blots. Denatured proteome (40-50 μ g) was resolved over 10% SDS-PAGE gel and transferred to methanol activated PVDF membrane (GE Healthcare) at 60V for 12 h at 4 °C in cold room. Post-transfer, membrane was blocked with 5% (w/v) milk in DPBS (pH 7.4) containing 0.1% (w/v) Tween 20 (DPBST), and subsequently incubated with primary antibody (1:1000 dilution in 5% (w/v) milk in DPBST) overnight at 4 °C. Membrane was washed with DPBST (5X) at 25 °C and incubated with secondary antibody (1:10,000 dilution in 5% (w/v) milk in TBST) for 90 mins at 25 °C. Thereafter, membrane was washed in DPBST (5x) at 25 °C and developed using Thermo West Pico western blotting substrate (Thermofisher Scientific) and visualized using Syngene G-Box Chemi-XRQ gel documentation system. Primary antibodies used were: anti-ABHD16A (Rabbit, Abcam, ab185549), anti-FLAG (Rabbit, Sigma-Aldrich, F7425). Secondary antibodies used in the study were HRP-conjugated anti-Rabbit IgG (Goat, Thermofisher Scientific, 31460).

Lipase assays for recombinant ABHD16A. Lipase assays were performed as described previously by Cravatt and co-workers²². Briefly, 5 μ g of purified protein or 20 μ g of membrane proteome was incubated with 100 μ M lipid substrate in DPBS (100 μ L total volume) at 37°C, 30 mins with constant shaking. Reaction mixture was quenched with 250 μ L of 2:1 CHCl₃/MeOH containing 1 nmol of 17:1 free fatty acid (FFA) and 17:1 lysophosphatidylserine (lyso-PS) as internal standards. The mixture was vortexed and centrifuged at 3,000 g for 3 mins to separate aqueous (top) and organic (bottom) phase. The aqueous phase was treated with formic acid (5 μ L) and extracted again with 250 μ L of 2:1 CHCl₃/MeOH. Organic phases were pooled and dried under stream of N₂, re-solubilized in 100 μ L of 2:1 CHCl₃/MeOH for LC-MS analysis. Area under the curve was measured and normalized to the respective internal standards (IS) for relative quantification of free fatty acid and lyso-PL release as a result of lipase activity. Area normalized with IS was further

normalized to the time of reaction and amount of proteome used in the reaction to calculate hydrolysis rate. Hydrolysis rate from heat denatured lysates were subtracted from hydrolysis rate obtained for testing sets.

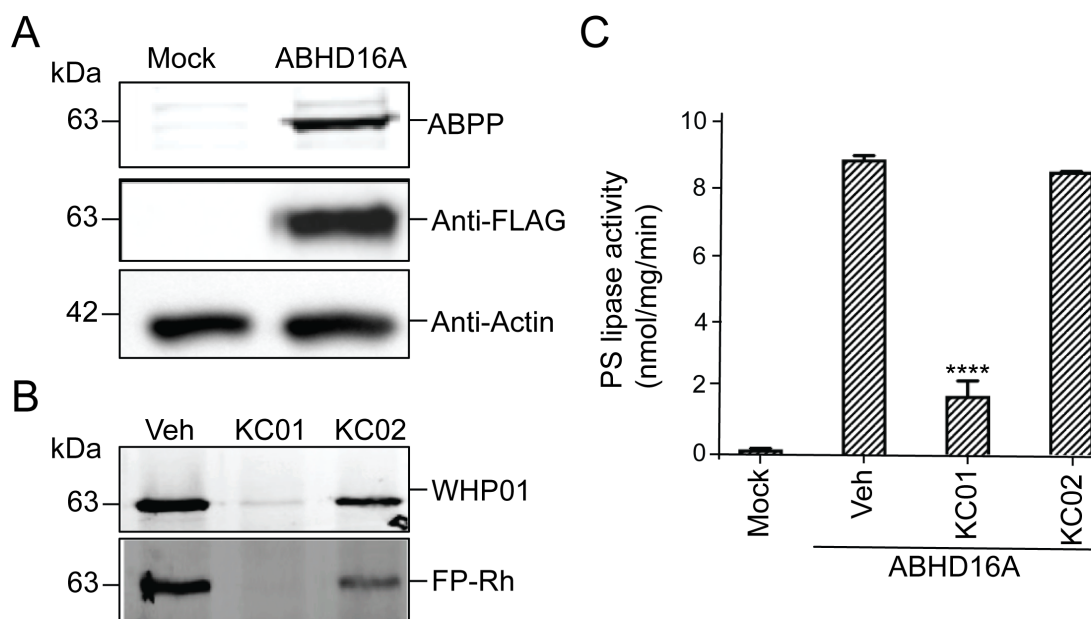
Protein Estimation. All purified protein or lysates were quantified for protein concentration using BCA reagent (Thermo Fisher) as per as manufacturer's protocol using Bovine Serum Albumin as standard.

Results

ABHD16A expression in HEK293T cells. I cloned and overexpressed human ABHD16A in HEK293T cells with a C-terminal FLAG-tag by transfection using PEI, and confirmed ABHD16A overexpression and activity in the membrane lysates of ABHD16A-transfected HEK293T cells by gel based ABPP assay and western blot respectively. Gel based ABPP using both the broad-spectrum fluorophosphonate-rhodamine (FP-Rh) probe and western blot using anti-FLAG antibody showed that ABHD16A is successfully overexpressed in BAT5-FLAG transfected HEK cells but not in mock transfected cells used as control (**Fig. 2.5A**). Next, I used competitive gel based ABPP assay, where membrane proteome was pre-treated with KC01 (β -lactone warhead based ABHD16A inhibitor) or KC02 (inactive structural mimic of KC01) and it was chased by FP-Rh or WHP01 (fluorescently tagged KC01). These competitive experiments show that overexpressed ABHD16A was successfully inhibited by KC01 but not by KC02, thus overexpressed ABHD16A was active in HEK293T cell membrane lysates and can be directly used for substrate assay (**Fig. 2.5B**).

Having confirmed successful overexpression and activity of ABHD16A using gel based ABPP and competitive assays, next I used substrate based lipase assays to validate the activity of overexpressed ABHD16A. I used 1-palmitoyl-2-linoleoyl-sn-glycero-3-phospho-L-serine (16:0-18:2 PS) as substrate against overexpressed ABHD16A HEK membrane and monitored for lyso-PS release using liquid chromatography coupled mass spectrometry (LC-MS). WT ABHD16A overexpressing membrane lysates showed $\sim 9 \text{ nmol mg}^{-1} \text{ min}^{-1}$ PS-lipase activity, which decreased to $<1 \text{ nmol mg}^{-1} \text{ min}^{-1}$ upon treatment with KC01 (ABHD16A inhibitor) but remained approximately same on treatment with KC02 i.e. $\sim 8 \text{ nmolmg}^{-1}\text{min}^{-1}$ (**Fig. 2.5C**). Given that I could express active ABHD16A from HEK293T

cells, I used ABHD16A overexpressing membrane lysates obtained from HEK293T cells for further substrate assays.



*Fig.2.5: Overexpression of ABHD16A in HEK293T cells. A – Confirmation of ABHD16A expression in transfected HEK293T cell membrane proteome by activity based protein profile gel and western blot. B – Competitive ABPP gel to confirm the activity of ABHD16A overexpressed in the membrane of HEK293T cells. C – Substrate assay confirming PS lipase activity of ABHD16A in transfected HEK293T cell membrane lysate. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value PS lipase activity (vehicle versus KC01) <0.0001 .*

ABHD16A expressed in HEK membrane lysates acts as PLA₂ type lipase. While ABHD16A is successfully annotated as a PS-lipase, its substrate preference in terms of *sn*-1 versus *sn*-2 ester bond on phospholipid is unclear. It is shown *in vitro* that besides PS, ABHD16A can accept other dually acylated phospholipids as substrate and is tolerant to different phospho-head groups. I thus assayed ABHD16A for its PLA₁ versus PLA₂ type activity by using ABHD16A transfected HEK293T membrane lysates, with HEK293T mock transfected membrane lysates as negative control, against a library of commercially available phospholipids and plasmalogens. For each substrate other than lysoplasmalogens, I could see release of 1-acyl-*sn*-2 -lyso-PL (**Fig. 2.6A**) derived from a given phospholipid substrates and

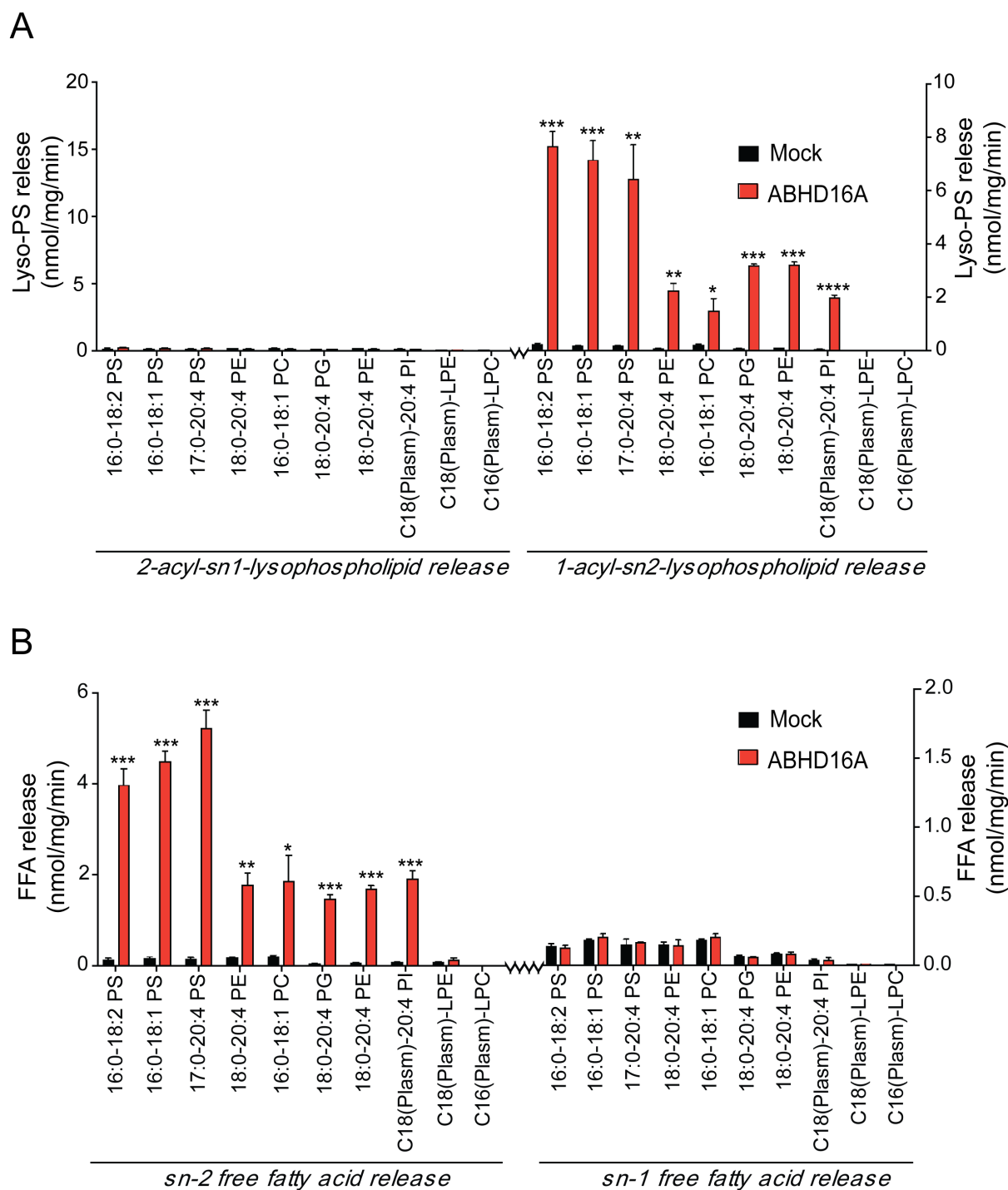


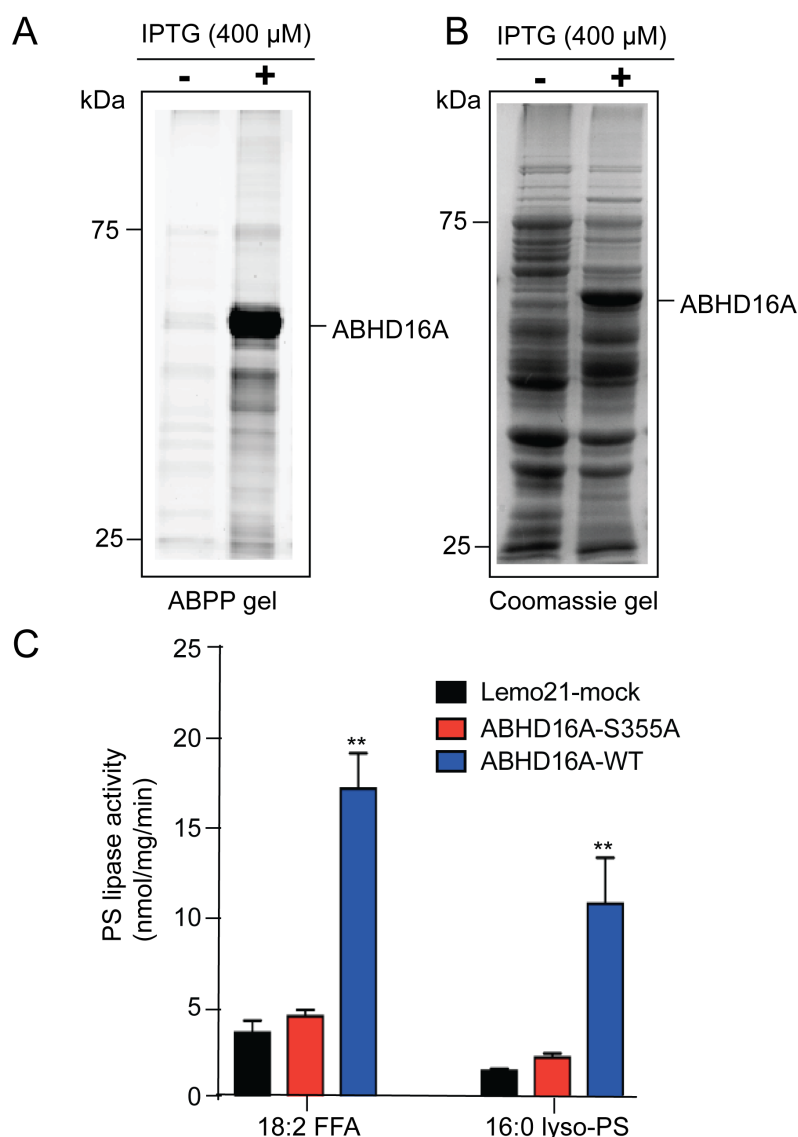
Fig.2.6: Phospholipase activity of ABHD16A. A – Lyso-PS release as a function of ABHD16A activity. B – Free fatty acid release as a function of ABHD16A activity. PS = phosphatidylserine, PE = phosphatidylethanolamine, PC – phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, Plasm = plasmalogen. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value lyso-PS release (mock versus ABHD16A) for 16:0-18:2 PS = 0.0002, 16:0-18:1 = 0.0007, 17:0-20:4 PS = 0.0083, 16:0-20:4 PE =

0.0015, 16:0-18:1 PC = 0.0139, 18:0-20:4 PG = 0.0002, 18:0-20:4 PI = 0.0003, C18(Plasm)-20:4 PC < 0.0001. *p*-value FFA release (mock versus ABHD16A) for 16:0-18:2 PS = 0.0005, 16:0-18:1 = 0.0004, 17:0-20:4 PS = 0.0002, 16:0-20:4 PE = 0.0038, 16:0-18:1 PC = 0.0229, 18:0-20:4 PG = 0.0001, 18:0-20:4 PI = 0.0003, C18(Plasm)-20:4 PC = 0.0002.

(ABHD16A inhibitor) but remained approximately same on treatment with KC02 i.e. ~8 nmolmg⁻¹min⁻¹ (**Fig. 2.5C**). Given that I could express active ABHD16A from HEK293T cells, I used ABHD16A overexpressing membrane lysates obtained from HEK293T cells for further substrate assays.

ABHD16A expression in bacterial Lemo21 (DE3) E. coli cells. Having found that ABHD16A acts as PLA₂ type enzyme, I wanted to confirm this finding by assaying purified ABHD16A. I started with the expression of human ABHD16A in bacterial system so as to get better yield of purified protein. Out of several screened host cell lines the Lemo21 (DE3) *E. coli* cells were the only one to give successful expression of human ABHD16A. The N-terminal His-tagged ABHD16A did not express (data not shown), while C-terminal His-tagged ABHD16A showed successful overexpression (**Fig. 2.7A**), suggesting that any alterations at N-terminus perturbs the expression of ABHD16A, possibly due to the disruption of signal peptide at N-terminus of ABHD16A. The Lemo21 (DE3) *E. coli* cells have *L*-rhamnose inducible pLys expression, which cleaves IPTG-induced T7 polymerase thus regulating protein expression. I titrated IPTG and *L*-rhamnose to get the optimal expression of ABHD16A in Lemo21 (DE3) cells, and observed that 400 μM IPTG in the absence of any external *L*-rhamnose gave optimal expression of ABHD16A (**Fig. 2.7A, B**), when cells were grown at 30 °C for 8-10 h post induction with IPTG. It was expected based on previous literature, as basal level *L*-rhamnose produced by Lemo-21 (DE3) *E. coli* cells are enough to keep the levels of T7-polymerase under control. The expression and activity of ABHD16A in IPTG-induced Lemo21 (DE3) cell membrane lysates was confirmed by ABPP gel (**Fig. 2.7A**) and coomassie staining (**Fig. 2.7B**) respectively. Further, I established a LC-MS based PS-lipase activity assay using ABHD16A expressing Lemo21 (DE3) *E. coli* cell membrane lysates using 16:0-18:2 PS as a substrate. I also generated the active site “catalytically dead” S355A mutant as an additional control in these assays. As expected I found that Lemo21 (DE3) cell membrane lysates expressing wild type (WT) ABHD16A had significantly 3-4 fold more lipase activity than the Lemo21 (DE3) *E. coli* cell membrane lysates expressing S355A

ABHD16A mutant or untransformed Lemo21 (DE3) *E. coli* cell membrane lysates (**Fig. 2.7C**).



*Fig.2.7: Overexpression of ABHD16A in bacterial host – Lemo21 (DE) E. coli cells. A – ABPP gel showing successful overexpression of catalytically active ABHD16A. B – Coomassie stained gel showing successful overexpression of ABHD16A in the bacterial membrane lysates. C –Substrate assay confirming PS lipase activity of ABHD16A in Lemo21 (DE) E. coli cell membrane lysate. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value PS lipase activity (WT versus S355A ABHD16A) 18:2 FFA release = 0.0015, 16:0 lyso-PS release = 0.0033.*

Solubilization and purification of ABHD16A from Lemo21 (DE) E. coli cell membrane. The next goal was to solubilize and extract ABHD16A from membrane, towards which I screened more than 20 detergents of different classes in different buffers looking for solubility of ABHD16A from WT ABHD16A overexpressing Lemo21 (DE3) *E. coli* cell membrane lysates. Detergents that could solubilize ABHD16A were SDS (2 mM) or its milder derivative LSS (5 mM) (**Fig. 2.8A**), however both of them lead to the loss of ABHD16A activity. I chose LSS over SDS given its stability at 4 °C, as this temperature is required for downstream protein purifications to maintain protein stability. The LSS solubilized ABHD16A overexpressing Lemo21 (DE3) cell membrane lysate was loaded on Ni-NTA HisTrap FF Column (GE Life Sciences) and bound protein was eluted with increasing gradient of imidazole. ABHD16A purified from the Ni-NTA column was > 80% pure (**Fig. 2.8B**). Since I needed >95% pure protein for biochemical assays, I explored other chromatography techniques like size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC), but none of them were able to improve on the purity of ABHD16A (data not shown). In case of size exclusion chromatography, injected protein eluted as the mega-Dalton complex in the void volume of column and in case of hydrophobic interaction chromatography, nothing bound to the column (data not shown). I attribute this unusual observation to the detergents used during the purification process. I thus focused on using a Ceramic Hydroxyapatite (CHT) column, reported for the purification of SDS-solubilized proteins. It gave me >95% pure ABHD16A on elution with increasing gradient of phosphate salt (buffer maintained at pH 6.8) (**Fig. 2.8C**). The purified protein was pure enough to use for biochemical or structural studies.

Reconstitution of ABHD16A purified from Lemo21 (DE) E. coli cell membrane. The WT ABHD16A protein obtained from the CHT column was not active; hence I needed to reconstitute its activity in liposome-based systems. I tried liposome based membrane protein reconstitution experiments and have successfully reconstituted the WT ABHD16A using a 16:0-18:1 PC based liposome system as checked by gel based ABPP (**Fig. 2.9A**). Additionally, I have also assayed both the WT and S355A ABHD16A reconstituted in the liposome based vesicle system by the LC-MS based PS lipase assay, and found that reconstituted WT ABHD16A has several fold more activity than the ABHD16A solubilized in detergent (**Fig. 2.9B**). There was no significant difference between ABHD16A reconstituted in 0.1 mM POPC or 1.0 mM POPC (**Fig. 2.9A and B**).

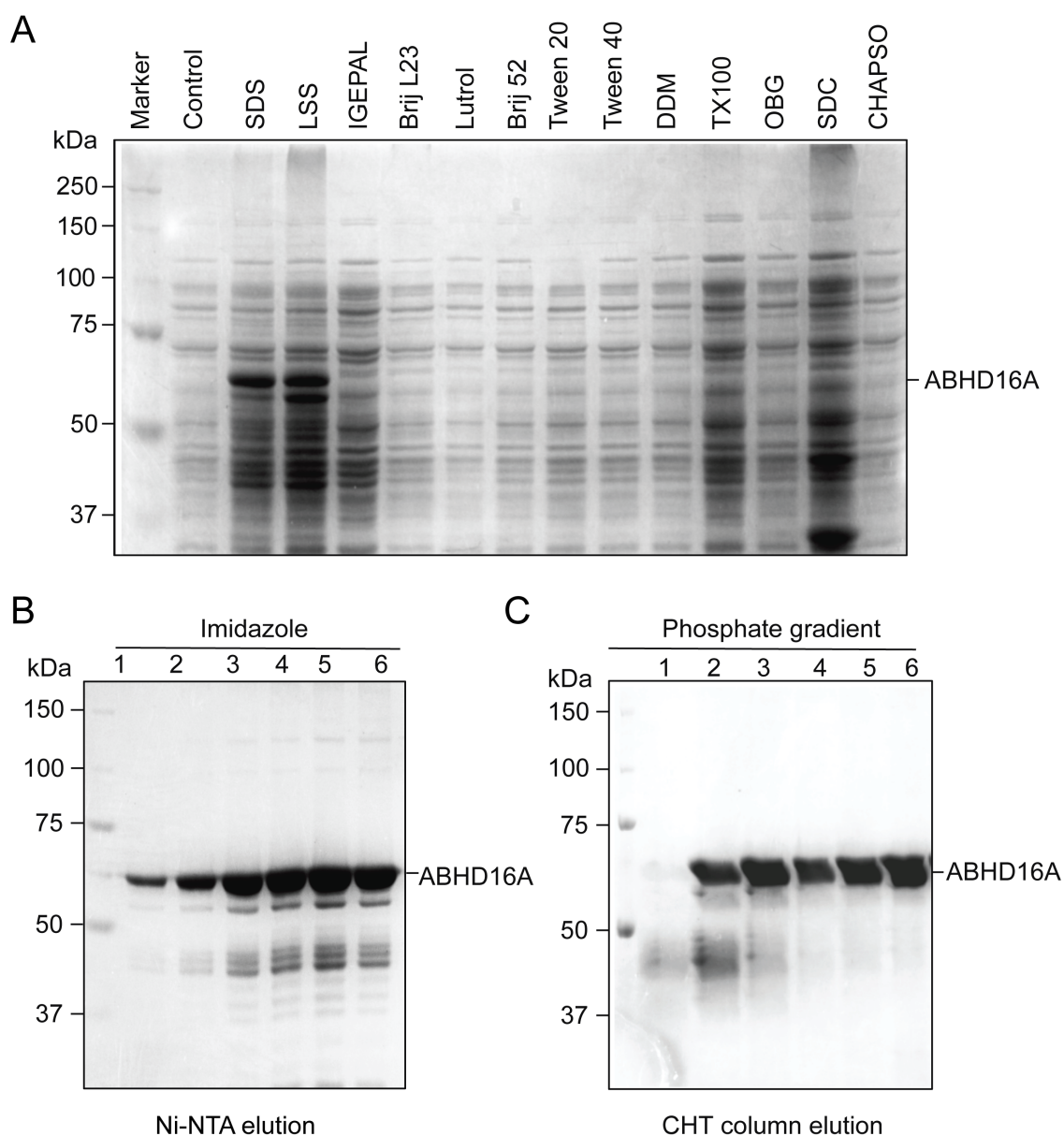
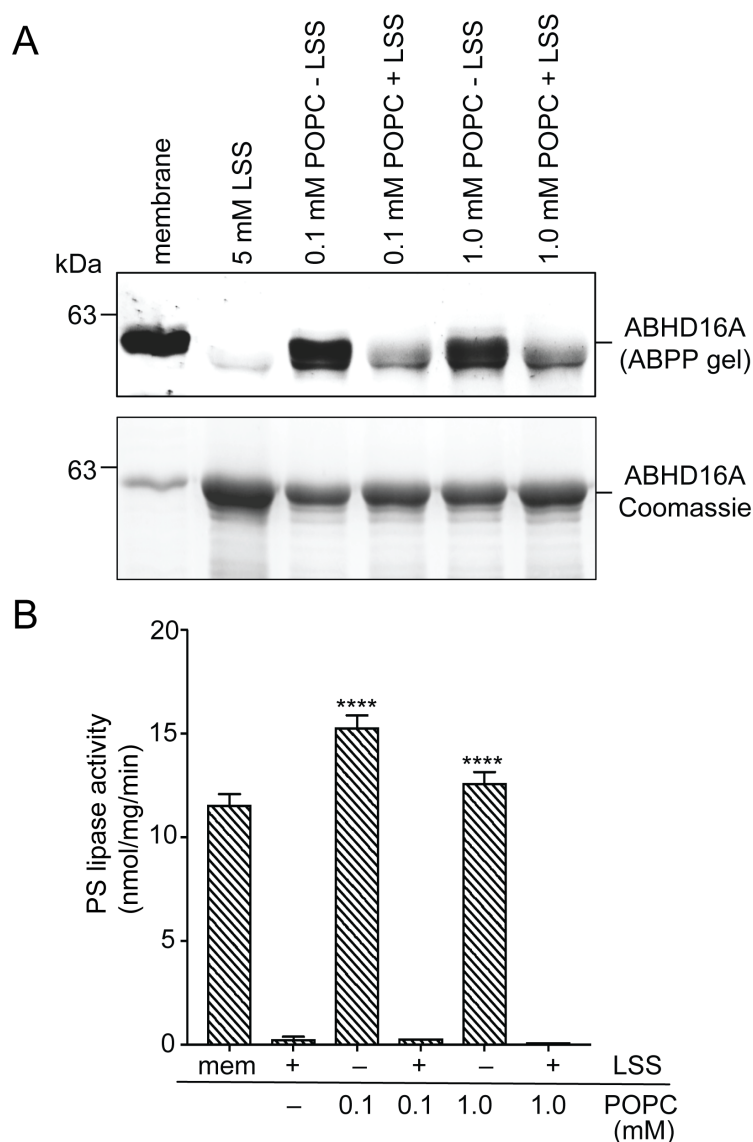


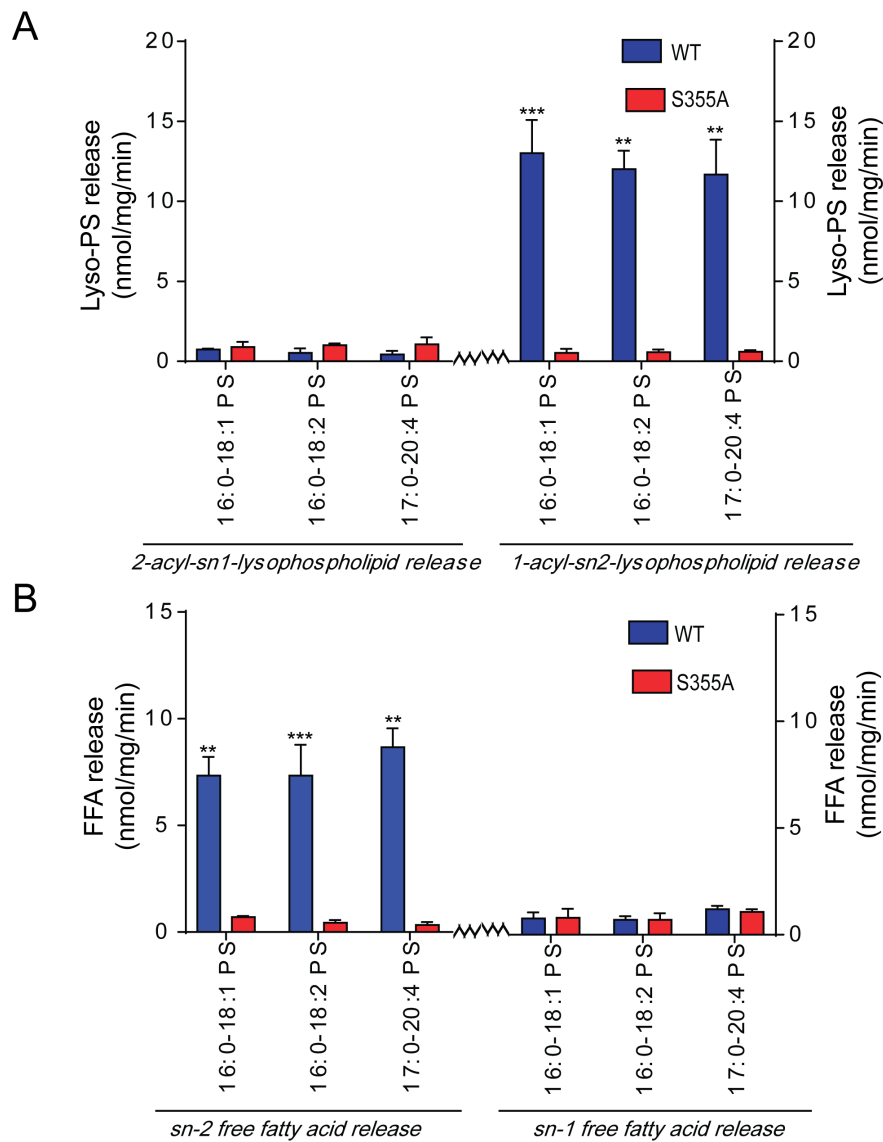
Fig.2.8: Solubilization and purification recombinant ABHD16A from bacterial Lemo21 (DE) E. coli cells. A – Representative SDS-PAGE gel showing detergent screen to solubilize ABHD16A from the membrane fraction of Lemo21 (DE) E. coli cells. 1-6 is 10-60 mM imidazole. B- Representative SDS-PAGE gel showing Ni-NTA elution profile of ABHD16A with step gradient of imidazole. C- Representative SDS-PAGE gel showing CHT column elution profile of ABHD16A with step gradient of phosphate concentration. 1-6 is 100-160 mM phosphate salt. Gels stained by Coomassie staining.



*Fig.2.9: Reconstitution of purified ABHD16A. ABPP gel showing activity of reconstituted ABHD16A. B – Lipase activity of liposome reconstituted ABHD16A compared to detergent solubilized ABHD16A. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value PS lipase activity (LSS solubilized versus liposome reconstituted ABHD16A) <0.0001 (both 0.1 mM and 1.0 mM POPC).*

Next, I assayed purified and reconstituted ABHD16A against commercially available PS. Consistent with my findings with ABHD16A overexpressing HEK membranes, I found that purified WT ABHD16A activity hydrolyses sn-2 ester bonds on glycerol backbone of PS, thus releasing 1-acyl-sn-2-lyso-PS (**Fig. 2.10A**) and corresponding FFA esterified at sn-2 position (**Fig. 2.10B**). Active site catalytic triad serine mutant i.e. S24A ABHD16A didn't

show any hydrolase activity (**Fig. 2.10B**). This confirmed that ABHD16A is a PS-PLA2 type serine hydrolase.



*Fig.2.10: PS lipase activity assay for purified ABHD16A. A- Lyso-PS release as a function of reconstituted WT and S355A ABHD16A activity. B- FFA release as a function of reconstituted WT and S355A ABHD16A activity. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value lyso-PS release (WT versus S355A ABHD16A) for 16:0-18:1 PS = 0.0001, 16:0-18:2 = 0.0005, 17:0-20:4 PS = 0.0017. *p*-value FFA release (WT versus S355A ABHD16A) for 16:0-18:1 PS = 0.0003, 16:0-18:2 = 0.0002, 17:0-20:4 PS = 0.0028.*

Discussion

ABHD16A is a transmembrane serine hydrolase enzyme known for its specific phosphatidylserine lipase activity *in vivo*²². It is often difficult to obtain pure and stable transmembrane proteins, which is pre requisite for their structural and thorough biochemical characterization. To biochemically characterize ABHD16A, I expressed it in HEK293T cells and used its membrane lysate directly to assay for PS lipase activity against commercially available lipid library. Membrane lysates of mock-transfected proteome was used as a viable control and lipids in denatured membrane lysates normalized to protein concentration were used as background signal coming from the reaction mixture. I found that ABHD16A hydrolyzes ester bond at sn-2 position on the tested phospholipids which includes PS, PI, PG, PE and PC and releases 1-acyl-sn-2-lyso-PL as a function of its activity²². Thus, it acts as PL-PLA₂ enzyme and hydrolysis rate was 2-3 folds more for PS than any other PL. Next goal I kept was to obtain purified protein for structural characterization. It is often cheap and easy to obtain recombinant proteins in abundance from prokaryotic sources. Hence, I cloned and over expressed ABHD16A in Lemo21 (DE3) *E. coli* bacterial host - best known and used for obtaining toxic or membrane protein recombinant overexpression. The ABHD16A protein expressed with conditions optimized here, allowed me to obtain active ABHD16A. It was great as often recombinant membrane proteins overexpression shunts them to inclusion body in cells and denatures them. To confirm that ABHD16A overexpressed in Lemo21 (DE3) *E. coli* membranes did not get shunted to inclusion body or were active in bacterial membrane, I used substrate based assays and confirmed that it is indeed active in bacterial membranes.

Next, I developed protocols to successfully purify ABHD16A from Lemo21 (DE3) *E. coli* membranes. I screened different detergents, buffers at different conditions of temperature and ionic strength. I could solubilize ABHD16A from bacterial membranes using SDS or LSS detergent and purified it using two step column chromatography – first with Ni-NTA affinity column and second with CHT chromatography which is a kind of ion exchange chromatography widely used for purification of detergent solubilized proteins and antibodies. Unfortunately, detergents used here (SDS or LSS) were harsh and denatured the purified ABHD16A. There are several successful reports of reconstitution of denatured proteins to regain their activity that allows assaying these tricky proteins and using them for structural studies. In my screen for suitable liposome composition to reconstitute ABHD16A, I started with mono-phospholipid mixtures at different lipid concentrations. ABHD16A successfully

regained its catalytic activity on being reconstituted with 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) liposomes – called ABHD16A proteoliposome complex. It showed PS-PLA₂ type lipase activity for PS substrate under *in vitro* conditions, thus successfully establishing that ABHD16A acts as PS specific PLA₂ type enzyme and generates 1-acyl-sn-2-lyso-PS (sn-2 lyso-PS) and free fatty acid. In addition to my studies on ABHD16A, there is another annotated enzymes PS-PLA1 which hydrolyzes ester bond at sn-1 position and releases 2-acyl-sn-1-lyso-PS (sn-1 lyso-PS) and free fatty acid. Both these enzymes are enriched in different tissues of the mammals thus possibly regulate PS and lyso-PS metabolism in discrete manner in mammalian physiology. It is also interesting to note that 1-acyl-sn-2-lyso-PS and 2-acyl-sn-1-lyso-PS both show differential activity on reported lyso-PS receptors i.e. P2Y₁₀, GPR34 and GPR174¹⁰. I would provide some insights into expression map of ABHD16A and lyso-PS flux in Chapter III and IV.

Conclusion

I report here successful protocol development for expression, reconstitution and purification of recombinant ABHD16A from bacterial host Lemo21 (DE3) competent *E. coli* cells. My studies based on substrate assays to be specific *in vitro* lipase assays using recombinant ABHD16A from mammalian or bacterial sources suggest that ABHD16A is a PS-specific PLA₂ type enzyme and generates sn-2 lyso-PS. The purification protocol reported here can easily be scaled and used to perform structural studies for ABHD16A in future.

CHAPTER III

Subcellular localization of ABHD16A and ABHD12 in the mammalian systems

ABHD16A is an intracellular PS lipase expressed widely in the mammalian tissues and acts as a major anabolic enzyme for lyso-PS metabolism in the mammalian neuronal and immune cells. Lyso-PS is catabolized by another serine hydrolase enzyme ABHD12. I studied subcellular localization of ABHD16A and ABHD12 using several parallel biochemical approaches to better understand enzymatic control of cellular PS and lyso-PS flux in the mammalian cells. I employed biochemical subcellular fractionation, immunoblots, substrate assays and stimulated emission depletion super resolution microscopy to probe for the subcellular localization of ABHD16A in the mammalian primary and secondary cell lines. I report that both ABHD16A and ABHD12 are enriched at the endoplasmic reticulum of the mammalian cells and regulate lyso-PS levels/flux from endoplasmic reticulum where bulk of PS is synthesized. Additionally, I show that overexpression of ABHD16A doesn't change PS levels instead it reduces PC and PE levels in the cells, suggesting that PS levels are tightly regulated in the mammalian cells.

Introduction

Phospholipids in the biological membrane especially plasma membrane exhaustively participate in a series of cellular signal transduction and intracellular transport either directly or by acting as a precursor for molecules with high signaling potency. One such phospholipid is phosphatidylserine (PS) constituting roughly about 3-5 mol percent or 2-10% of the total phospholipids in a cell³¹ but it plays an active role in crucial cellular physiological processes like apoptosis^{32,33}, vesicular transport^{34,35}, blood coagulation³⁶, recruitment and activation of signaling molecules like protein kinase C³⁷, synaptotagmin³⁸, etc. Two enzymes phosphatidylserine synthase 1 (PSS1) and phosphatidylserine synthase 2 (PSS2) principally synthesize bulk PS from PC and PE respectively at endoplasmic reticulum (ER) mitochondrial contact sites (also called mitochondrial associated membranes) in the mammalian cells³¹ (**Fig. 3.1**). Both these enzymes seem to be slightly redundant in their function as their individual rodent knockouts (KO) are viable and do not show substantial changes in bulk PS levels. Any aberrant PS levels can prove detrimental to cells and animals

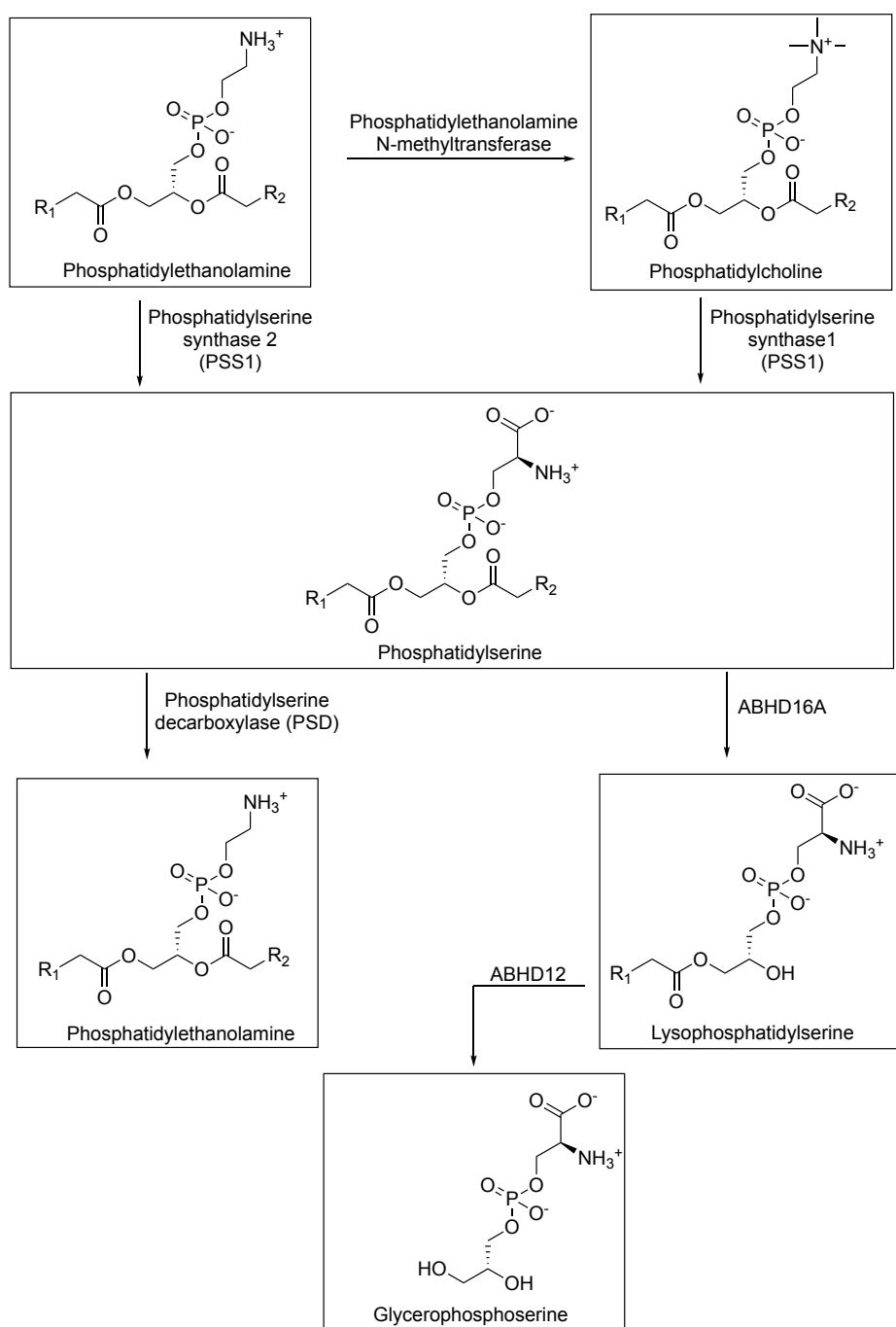


Fig.3.1: Phosphatidylserine metabolism inside the mammalian cells. PS is synthesized from PC and PE by PSS1 and PSS2 enzymes respectively. PSD converts PS to PE and ABHD16A degrades PS to make lyso-PS. Lyso-PS is further degraded by ABHD12.

as observed in PSS1 and PSS2 double knockout rodents, which are not viable underscoring the importance of PS in the mammalian physiology. The spatial and temporal localization of PS is as important as PS abundance and its physiological levels, it's asymmetrically and

excessively abundant towards the inner leaflet of the plasma membrane and this asymmetry in PS localization is maintained in an energy intensive process³⁹. Any loss or defects in this asymmetry in PS localization at plasma membrane acts as a signal for programmed cell death (or apoptotic death) of the cell, prompting its clearance through macrophages⁴⁰. In addition to the participation of PS directly in cellular signaling and physiology, PS also acts a precursor of lysophosphatidylserine (lyso-PS) – a potent signaling molecule²² in the mammalian physiology. Lyso-PS participates in immune physiology by inducing mast cell degranulation for histamine release and vasodilation^{41,42}, activation of macrophages to clear neutrophils¹⁵ and other apoptotic cells and inhibits differentiation of regulatory T cells (Treg)⁴³. In addition to its role in immune signaling, it triggers chemotactic migration of mammalian cells (like leukemic, colorectal cancer cells and human glioma cells)^{13,16,44} and glucose homeostasis⁴⁵. It induces glucose transport independent of insulin in cultured myotubes and adipocytes suggesting its active role as hormones like other lipid derivatives notably steroids⁴⁵. Two major metabolic enzymes annotated for lyso-PS production in mammalian physiology are phosphatidylserine specific phospholipase A₁ (PS-PLA₁) and ABHD16A^{22,46}.

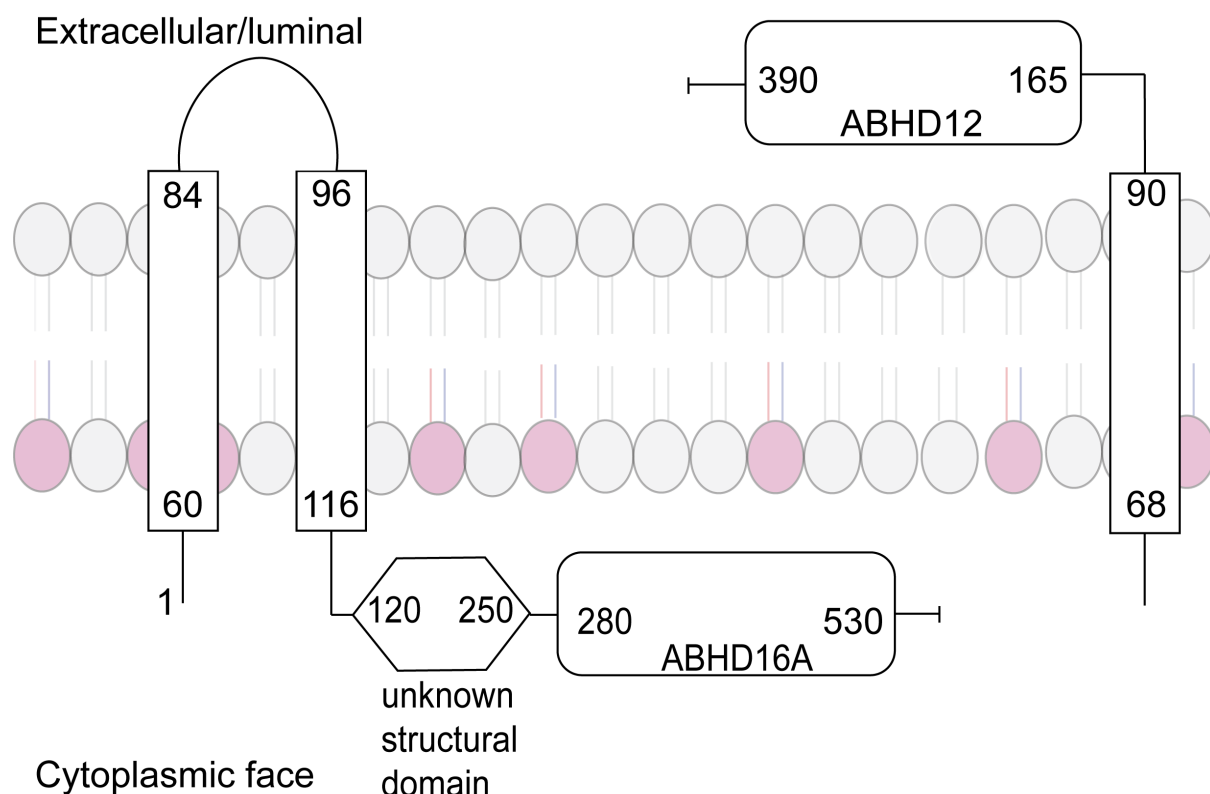


Fig.3.2: Differential topology and orientation of ABHD16A and ABHD12 at cellular membrane in the mammalian cells.

Both are PS-lipases that generate lyso-PS from PS and currently there are no de novo pathways annotated to synthesize lyso-PS. PS-PLA₁ is a secretive enzyme and has very limited access to PS with conscience of the fact that PS is enriched in the inner/cytosolic leaflet of plasma membrane or other organelle membranes. ABHD16A is the first and only intracellular PS specific lipase discovered till date and it might regulate the cellular pools of PS and lyso-PS in the mammalian cells. Lyso-PS generated as a result of ABHD16A activity is catabolized by ABHD12 enzyme^{17,22}. ABHD12 hydrolyses ester bond on lyso-PS to release free fatty acid (FFA) and glycerophosphoserine, thus ABHD12 is the nodal enzyme that degrades lyso-PS and regulates/terminates the signal triggered by lyso-PS. It is established that ABHD16A and ABHD12 together regulate both the intracellular and secretive lyso-PS in the mammals²² (**Fig. 3.1**). Protease experiments suggested that their active site is oriented differentially, ABHD domain of ABHD16A is oriented towards cytosolic side whereas that of ABHD12 is oriented towards luminal or extracellular side of the cellular membranes^{17,22} (**Fig. 3.2**). How both these enzymes fit in the subcellular space and control subcellular flux of PS/lyso-PS metabolism of the cells was not explored?

Here, I probed for subcellular localization of ABHD16A and ABHD12 in the mammalian cells and investigated its potential role in regulating cellular PS and lyso-PS levels. I found that ABHD16A and ABHD12 are preferentially enriched at the ER and controls lyso-PS flux from ER in cells. The pharmacological inhibition of ABHD16A in the mammalian cells is reported to decrease lyso-PS levels but it did not change the bulk cellular PS pools²². Here, I found that ABHD16A overexpression leads to an increase in lyso-PS levels and a decrease in PS precursors like PE and PC levels but no changes in bulk PS levels suggesting that PS levels are strictly regulated in the HEK293T cells.

Materials and Methods

Materials. Chemical probes FP-Rhodamine, WHP01 and inhibitors KC01, KC02 were generous gift from Prof. Benjamin F. Cravatt, The Scripps Research Institute. Lipid internal standards were purchased from Avanti Polar Lipids. Immortalized secondary cells Neuro-2a were procured from ATCC. MCF7 and Lipofectamine 2000 were generous gift from Dr. Mayurika Lahiri at IISER-Pune. Anti-GM130 and anti-Calnexin antibody were generous gift from Dr. Nagaraj Balasubramanian at IISER-Pune. Sec61-mCherry and mito-mCherry clones and COS-7 cells were a generous gift from Dr. Thomas Pucadyil at IISER-Pune. Plasmid

isolation kits were brought from Qiagen. All primary antibodies were brought from Abcam and secondary antibodies were brought from Thermofisher Scientific until otherwise mentioned. All tissue culture plates and tissue culture media were brought from Hi-Media. All other chemicals and salts were purchased from Sigma-Aldrich until otherwise mentioned.

Cellular fractionation studies. Subcellular fractionation from mice brain and cultured cells were performed as described before with modifications⁴⁷. Briefly, cells were harvested from 2 x 15 cm tissue culture plates or half mice brain (from mice 8-10 weeks old) was collected washed (3 X) with ice-cold sterile calcium and magnesium free Dulbecco's phosphate-buffered saline - HIMEDIA (DPBS), pH 7.4, and homogenized in 3 mL of ice-cold 250-STM homogenization buffer (containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 25 µg/mL spermine and 25 µg/mL spermidine, 1 mM PMSF) until isolated floating nuclei were seen under the phase contrast microscope. The unlysed cells were pelleted at 200 g for 3 mins at 4 °C and resulting supernatant was centrifuged at 1000 g for 15 mins at 4 °C to pellet the nuclear fraction. The supernatant from this step, was centrifuged at 10,000 g for 15 mins at 4 °C to pellet mitochondrial enriched fraction. Left over supernatant was centrifuged at 100,000 g for 60 mins at 4 °C to pellet microsomes and supernatant was collected as soluble cytoplasmic fraction. Mitochondrial enriched fraction was further purified by re-suspending it in 2M-STMDPS buffer (2 M sucrose, 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 25 µg/mL spermine and 25 µg/mL spermidine, 1 mM PMSF) and centrifuged it at 6,000 g for 15 mins at 4 °C to pellet a pure mitochondrial fraction. Each pellet fraction was re-suspended in DPBS and protein was estimated using Bradford reagent (Sigma-Aldrich). Proteome from each fraction was denatured by boiling with 4X loading dye at 95 °C for 15 mins and denatured proteome was separated over 10% SDS-PAGE gel and used for western blot.

Western blot analysis. Denatured proteome (40-50 µg) was resolved over 10% SDS-PAGE gel and transferred to methanol activated PVDF membrane (GE Healthcare) at 60 V for 12 h at 4 °C. Post-transfer, membrane was blocked with 5% (w/v) milk in DPBS (pH 7.4) containing 0.1% (w/v) Tween 20 (DPBST), and subsequently incubated with primary antibody (1:1000 dilution in 5% (w/v) milk in DPBST) overnight at 4 °C. Membrane was washed with DPBST (5X) at 25 °C and incubated with secondary antibody (1:10,000 dilution in 5% (w/v) milk in DPBST) for 90 mins at 25 °C. Thereafter, membrane was washed in DPBST (5x) at 25 °C and developed using Thermo West Pico western blotting substrate

(Thermofisher Scientific) and visualized using Syngene G-Box Chemi-XRQ gel documentation system. Primary antibodies used were: anti-ABHD16A (Rabbit, Abcam, 185549), anti-ATP5A (Mouse, Abcam, ab14748), anti-GM130 (Mouse, Abcam, ab169276), anti-Calnexin (Rabbit, Abcam, ab10286), anti-PMP70 (Mouse, Sigma-Aldrich, SAB4200181), anti-Lamin (Rabbit, Cloud Clone Corp, CAF548Hu01), anti-Tubulin (Rabbit, Cloud Clone Corp, CAB870Hu01), anti-GAPDH (Mouse, Cloud Clone Corp, CAB932Hu01), anti- β -actin (Rabbit, Cloud Clone Corp, CAB340Hu01). Secondary antibodies used in the study were HRP-conjugated anti-Rabbit IgG (Goat, Thermofisher Scientific, 31460), or HRP-linked anti-Mouse IgG (Goat, Cloud Clone Corp, SAA544Mu19).

Substrate Assays. 20 μ g proteome of different organelle fractions were incubated with 100 μ M of phosphatidylserine (Avanti Polar Lipids) in DPBS (total reaction volume = 100 μ L) at 37 °C for 30 mins with constant shaking. After 30 mins, reaction was quenched with 250 μ L of 2:1 CHCl₃: MeOH along with 1 nmol of 17:1 FFA and 17:1 lyso-PS each as internal standard. Mixture was vortexed and spun at 2,800 g for 5 mins, and organic layer was extracted. Thereafter, aqueous fraction was acidified with 5 μ L of formic acid, vortexed and extracted again with 250 μ L of 2:1 CHCl₃: MeOH. Organic layers from both the steps were pooled, dried under N₂-stream. Extracted lipids were re-solubilized in 100 μ L of 2:1 CHCl₃: MeOH and 10 μ L was injected into LC-MS X500R QTOF instrument. Lipids were separated on Gemini 5U C18-reverse phase column (Phenomenex). LC solvents were as follows: - Solvent A, 95:5 (v/v) H₂O/MeOH + 0.1% (v/v) NH₄OH, Solvent B, 60:35:5 (v/v/v) IPA/MeOH/Water + 0.1% (v/v) NH₄OH. All lipids were analyzed with established multiple reaction monitoring high resolution (MRM-HR) method on Sciex X500R QTOF instrument coupled to an Exion-series UPLC with quaternary pump. Data collected over the instrument was analyzed using SciexOS software. MS parameters were as follows: - ESI mode of ionization, curtain gas 1 = 60, curtain gas 2 = 40, ion spray voltage = -5,500 V, temperature = 550°C. All quantifications were done by calculating area under the curve and normalizing it to the area under the curve for respective internal standards and amount of proteome used.

Cellular immunofluorescence studies. Neuro-2a, COS7 and MCF7 cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) (HiMedia) with 10% (v/v) fetal bovine serum (FBS) (Thermo Fischer Scientific) and 1X penicillin-streptomycin (MP Biomedicals) at 37 °C, 5% (v/v) CO₂. Cells were counted using Trypan blue staining method on a BTC20 automated cell counter (Bio-Rad) as per as manufacturer's instructions. Cells were seeded on

a 18 mm coverslip in 35 mm tissue culture dish at 3×10^6 and cultured for 24 h, washed with sterile DPBS (3x), fixed with 4% (w/v) paraformaldehyde (PFA) in DPBS for 15 mins at room temperature (RT). Subsequently, cells were washed with sterile DPBS (4x), permeabilized with 0.5% (v/v) Triton X-100 in DPBS containing 5% (w/v) bovine serum albumin (BSA) for 30 mins at RT. Permeabilized cells were blocked with 5% (w/v) BSA in DPBS for 45 mins at RT and incubated with primary antibody (1:100) for 90 mins at 25 °C. Cells were washed in DPBS by gentle swirling (10X) and incubate with secondary antibody (1:1000) for 60 min at 25 °C. Cells were further washed in DPBS by gentle swirling (10X) and stained with 4', 6-diamidino-2-phenylindole (DAPI, nuclear marker; Sigma-Aldrich) for 3 mins. Coverslips were further washed in DPBS by gentle swirling (5X) and mounted on glass slides with FluoromountTM aqueous mounting medium (Sigma Aldrich). Slides were dried at 25 °C overnight in dark and imaged using Leica SP8 Confocal Laser Scanning Microscope fitted with 63X, 1.4 oil immersion objective. For stimulated emission depletion (STED) microscopy: the coverslip was mounted with Mowiol-DABCO mounting media, slides were dried at 25 °C in the dark. Slides were imaged on Leica TCS STED super resolution microscope with HC PL APO CS2, 100X, 1.4 oil immersion objective. Raw images were de-convoluted using Huygens Professional Software (Scientific Volume Imaging BV) and all microscopy images were analyzed using ImageJ 1.50i software (NIH, for Windows OS). The primary antibodies used in these studies were anti-ABHD16A (Rabbit, Abcam, ab1855409), anti-ABHD12 (Rabbit, Abcam, ab182011), anti-ABHD12 (Mouse, Abcam, ab68949), anti-Sec61A (Rabbit, Abcam, ab15575), anti-Sec63 (Mouse, Abcam, ab68550), anti-ATP5A (Mouse, Abcam, ab14748, mitochondrial marker), anti-PMP70 (Mouse, Sigma-Aldrich, SAB4200181). Goat anti-rabbit IgG (H&L) conjugated with DyLight 488 (Thermofisher Scientific, 35553), or goat anti-mouse IgG (H&L) conjugated with Alexa Fluor 568 (Thermofisher Scientific, A-11004) were used as secondary antibodies.

Peritoneal Macrophage Harvest. Peritoneal macrophages were elicited by 3 mL of 4% (w/v) thioglycollate injection intraperitoneally (I.P.) using standard protocols described at (<http://www.lipidmaps.org/>). At 3rd day of injection, peritoneal macrophages were harvested in chilled DPBS and red blood cells (RBCs) were removed using RBC lysis buffer. Cells were pelleted at 200 g for 3 mins at 4 °C and re-suspended in RPMI 1640 with 20% (v/v) FBS, 1 x penicillin-streptomycin. Cells were grown at 37 °C, 5% (v/v) CO₂ and allowed to adhere for 90 mins. Cells were washed with DPBS (2X) and treated with 5 µg/mL lipopolysaccharide (LPS) for 7 h in serum free RPMI 1640 media.

Cellular transfection studies. Cells were grown on coverslips till 70% confluency with 10% (v/v) serum in DMEM. Cells were serum starved for 1 h and co-transfected with ABHD16A-GFP and Sec61 β -mCherry (Addgene Plasmid No. – 49155) or mito-mCherry (Addgene Plasmid No. – 59352) for 3 h. Thereafter, culture media was supplemented with FBS and cells were grown overnight. Cells were washed with DPBS, fixed with 4% (w/v) paraformaldehyde for 10 mins at RT and mounted with FluoromountTM aqueous mounting medium (Sigma Aldrich). Slides were dried at RT overnight in dark and imaged using Leica SP8 Confocal Laser Scanning Microscope fitted with 63X, 1.4 oil immersion objectives.

Poly-D-Lysine coating of glass slides. 0.1% (w/v) solution of sterile Poly-D-lysine hydrobromide in MQ was prepared. Slides were cleaned with 0.1% HCl and 70% ethanol, dried and were incubated in the diluted Poly-D-lysine solution for 15 mins at room temperature (RT) under aseptic conditions. Thereafter, slides were dried in the incubator at 37 °C for 2 hours. Slides were stored at 4 °C until used for plating primary neurons.

Primary neuronal culture. Brain was dissected out of E18 mice embryos and meninges were stripped under the stereotaxic microscope. Thereafter, dissected brains were minced in DMEM supplemented with 10% (v/v) heat inactivated FBS and 1X antibiotic-antimycotic solution. Minced tissue was trypsinized with 0.25% (w/v) Trypsin for 10 mins over ice and incubated with DNase (0.1 mg/ml) for 5 mins over ice. Cells were pelleted down by spinning at 300 g for 3 mins and washed with Hank's Balanced Salt Solution (2X). Cells were re-suspended in DMEM supplemented with 10% (w/v) heat inactivated FBS and 1X antibiotic-antimycotic solution and plated on Poly-D-lysine coated coverslips or plates. Cells were allowed to adhere to tissue culture plate for 3 hat 37°C, 5% (v/v) CO₂. Thereafter, culture media was changed to Neurobasal Medium supplemented with 1X antibiotic-antimycotic, 1X B-27 (Thermo-Scientific), 1X Glutamine (Gibco) at 37°C with 5% (v/v) CO₂. Cells were grown for 14 days and media was changed every alternate day to achieve healthy differentiated neurons.

Results

ABHD16A and ABHD12 are localized at the ER in the mammalian tissues and cells. Trypsin digestion and deglycosylation studies have shown that ABHD16A and ABHD12 are an

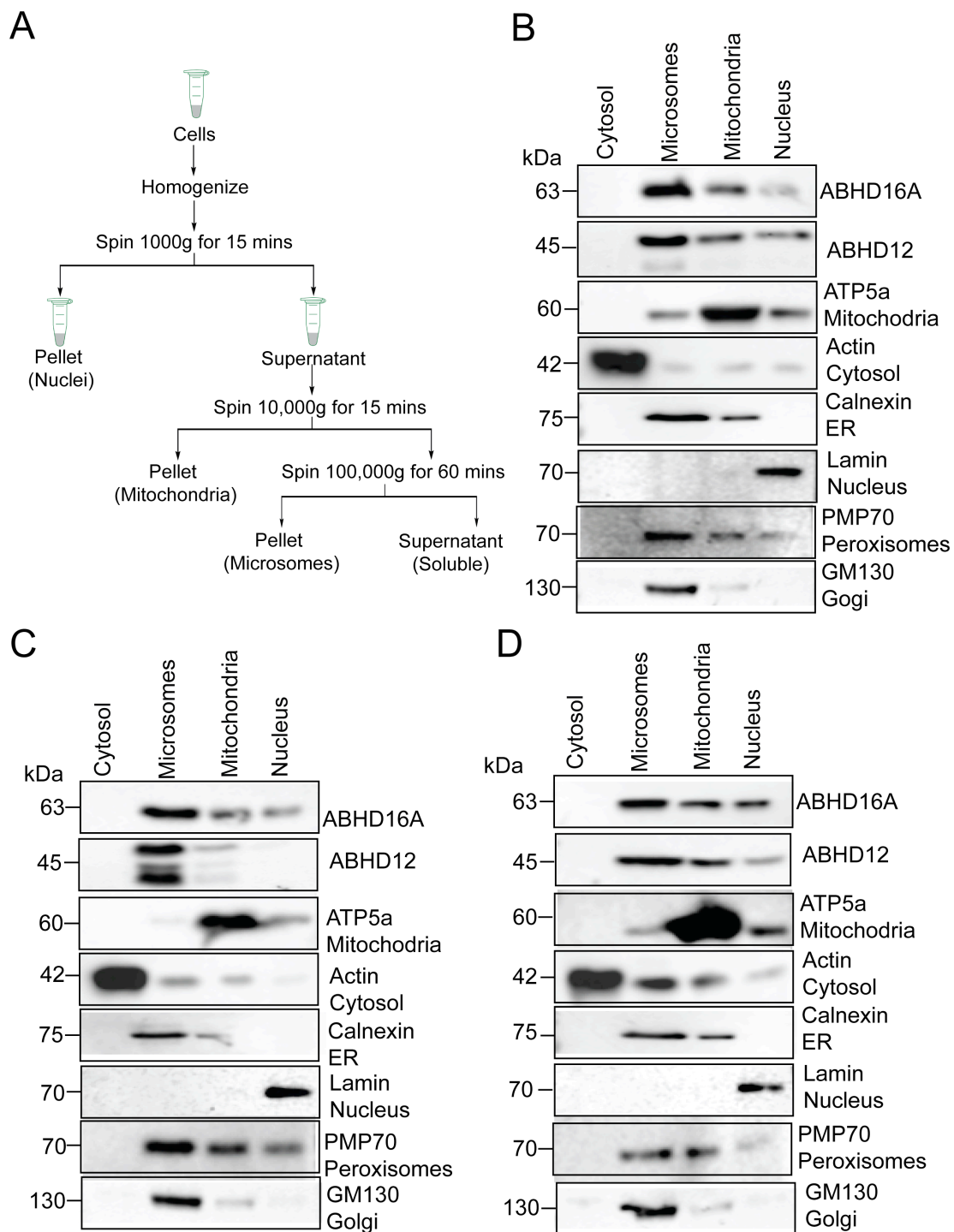


Fig.3.3: Subcellular fractionation showing ABHD16A and ABHD12 is enriched in microsomal fraction of the mammalian tissues and cells. A – Biochemical scheme to fractionate mammalian tissues and cells. B-D – Western blot probing ABHD16A, ABHD12 and organelles markers in mice brain (B), Neuro-2a cells (C) and MCF7 cells (D).

integral membrane proteins with differential orientation in cellular membranes^{17,22}. Enzymes that synthesize PS are enriched on the mitochondrial- associated membranes (MAMs), which are the contact sites between ER and mitochondrial in the cells⁴⁸. Mapping localization of ABHD16A and ABHD12 in subcellular organelles will provide an additional understanding of PS - lyso-PS flux and its metabolism inside the cell. It will help us establish if there are any cross talk between organelles or cells for PS and lyso-PS metabolism. To map the subcellular localization of ABHD16A and ABHD12 and, I took three parallel approaches: -

i) Subcellular localization of ABHD16A and ABHD12 using subcellular fractionation and western blots. Firstly, I fractionated subcellular components of the mammalian cells and probed for ABHD16A and ABHD12 in their lysates using western blots (**Fig. 3.3A**). I isolated cytoplasm, microsomes (primarily plasma membrane and endoplasmic reticulum), mitochondria and nucleus from the mice brain (**Fig. 3.3B**), and immortalized cell lines like Neuro-2a (**Fig. 3.3C**) and MCF7 cells (**Fig. 3.3D**). These fractions were approximately 90% pure as assessed by probing for organelle specific markers on western blot (anti-ATP5a for mitochondria, anti-Calnexin for ER, anti-Lamin for nucleus, anti-PMP70 for peroxisomes, anti-GM130 for Golgi, and anti-Actin for cytosol). I found that both ABHD16A and ABHD12 are enriched in the microsomes of the mice brain that primarily consist of endoplasmic reticulum (anti-Calnexin) (**Fig. 3.3B**). Next, I probed for ABHD16A in subcellular fractions of immortalized cell lines Neuro-2a and MCF7 and found that both ABHD16A and ABHD12 is enriched in their microsomal fraction (**Fig. 3.3C** and **Fig. 3.3D**), which is consistent with its localization in the brain fractions.

ii) Subcellular localization of ABHD16A and ABHD12 using substrate assays. Secondly, I measured PS-lipase activity (for ABHD16A) assay using PS 18:0-18:1 as substrate on isolated organelle fractions from the brain and cells (**Fig. 3.4A**). PS-lipase activity was maximal in the microsomal fractions of the mouse brain (**Fig. 3.4B**) and immortalized cell lines like Neuro-2a (**Fig. 3.4C**) and MCF7 cells (**Fig. 3.4D**). Treatment with KC01 – an active ABHD16A inhibitor, significantly inhibited PS-lipase activities in the microsomal fractions of mouse brain (**Fig. 3.4B**), Neuro-2a (**Fig. 3.4C**), and MCF7 cells (**Fig. 3.4D**) while it was largely unaffected on treatment with KCO2 – an inactive structural analog of KC01 (**Fig. 3.4B**, **Fig. 3.4C**, and **Fig. 3.4D**). This establishes that PS lipase activity on microsomes is largely coming from ABHD16A activity. Next, we tested these organelle fractions for lyso-PS lipase activity (for ABHD12) assay using lyso-PS 18:0 as a substrate

(**Fig. 3.4E**) and found that lyso-PS activity is also maximal in the microsomal fractions of the mouse brain (**Fig. 3.4F**) and immortalized cell lines like Neuro-2a (**Fig. 3.4G**) and MCF7 cells (**Fig. 3.4H**) and it was largely ablated on treatment with JJH350 – a specific inhibitor of ABHD12 (**Fig. 3.4F**, **Fig. 3.4G**, and **Fig. 3.4H**).

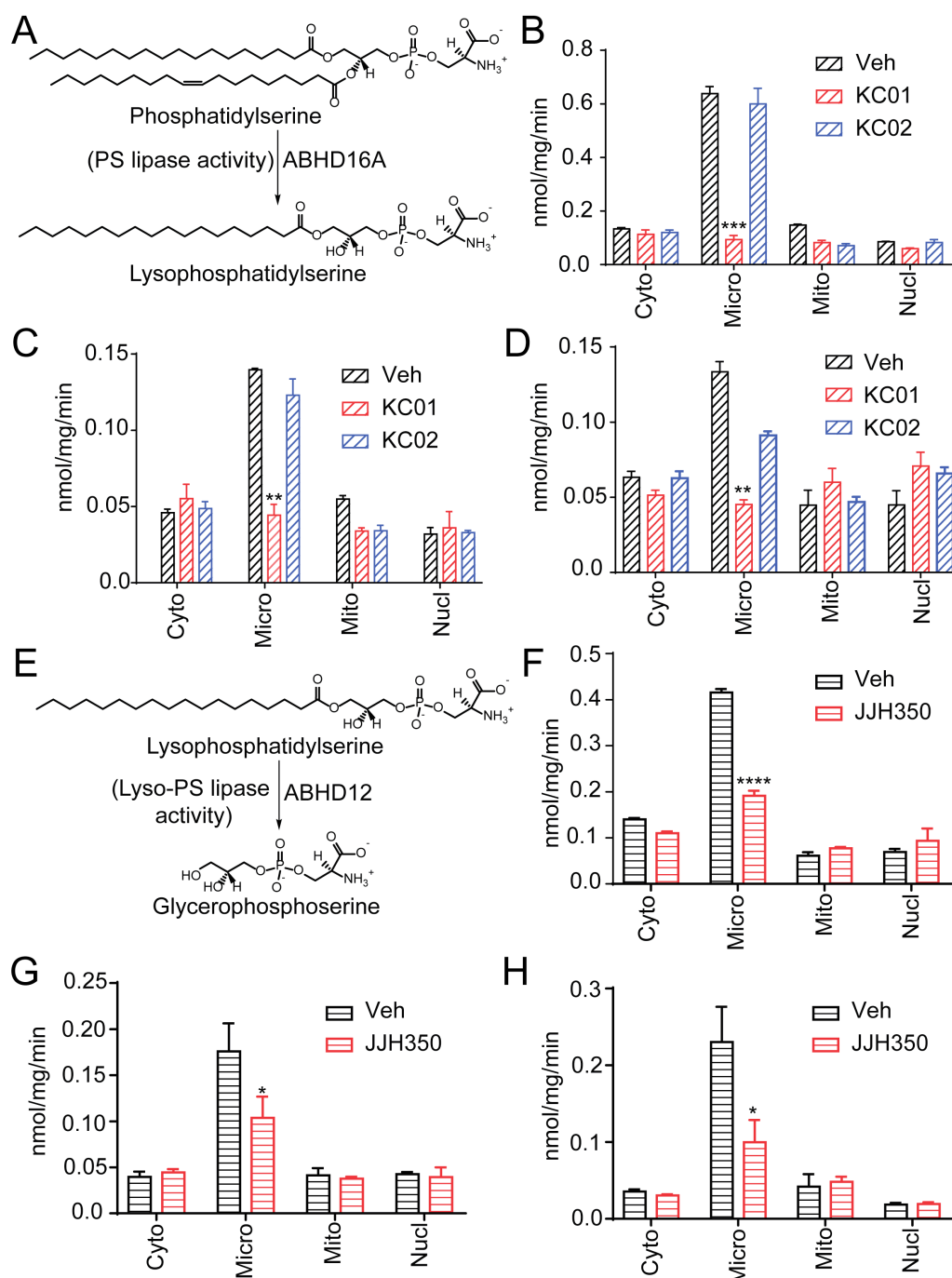


Fig.3.4: Substrate based assays on subcellular fractions show ABHD16A and ABHD12 are enriched in microsomes. A – PS lipase activity assays. PS lipase activity assays on

subcellular fractions of brain (B), Neuro-2a cells (C), MCF7 cells (D). E – Lyso-PS lipase activity assays. Lyso-PS lipase activity assays on subcellular fractions of brain (F), Neuro-2a cells (G), MCF7 cells (H). Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value PS lipase activity (vehicle versus KC01) mice brain = 0.0004, Neuro-2a = 0.0034, MCF-7 = 0.0023. Lyso-PS lipase activity (vehicle versus JJ350) mice brain <0.0001, Neuro-2a = 0.035, MCF-7 = 0.0140.

iii) *Subcellular localization of ABHD16A and ABHD12 using immunofluorescence assay and microscopy.* I confirmed ABHD16A localization at the ER using stimulated emission depletion (STED) microscopy and found that ABHD16A co-localizes well with Sec63 – an ER marker in Neuro-2a (**Fig. 3.5A-i**) and in MCF-7 cells (**Fig. 3. 5B-i**). I did not find any significant co-localization of ABHD16A with the mitochondrial marker (anti-ATP5a) or the peroxisome marker (anti-PMP70) in Neuro-2a (**Fig. 3. 5A-ii, iii**) or MCF-7 cells (**Fig. 3. 5B-ii, iii**). Next, I probed for ABHD12 and found that ABHD12 too co-localizes with Sec61a – an ER marker in Neuro-2a (**Fig. 3.5C-i**) and MCF-7 cells (**Fig. 3.5D-i**). Largely, ABHD12 did not co-localize with markers of any other cellular organelles like mitochondria (anti-ATP5a) or peroxisome (anti-PMP70) in these cells (**Fig. 3.5C-ii, iii** and **Fig. 3.5D-ii, iii**). Taken together, both ABHD16A and ABHD12 seem to localize at the ER in the secondary mammalian cell lines.

Subcellular localization of ABHD16A and ABHD12 in primary mammalian cells. With the finding that ABHD16A is enriched at the ER in the immortalized cells. I wanted to confirm this finding in the primary mammalian cells. Keeping the fact in mind that ABHD16A and ABHD12 controlled lyso-PS has major role in neuronal and immune physiology. I decided to perform these localization studies in primary neuronal and immune cells. For immune cells, I isolated primary peritoneal macrophages (PPMs) from ABHD16A WT/KO and ABHD12 WT/KO mice. I show using confocal microscopy that ABHD16A and ABHD12 are enriched at ER (anti-Sec63) in the PPMs (**Fig. 3.6A** and **Fig. 3.6B**), whereas signal for ABHD16A or ABHD12 in PPMs isolated from their individual KO mice was negligible (**Fig. 3.6A** and **Fig. 3.6B**). I also isolated primary neurons from E18 pups and probed for ABHD16A and ABHD12 and show that both enzymes co-localize with Sec63 (**Fig. 3.6C** and **Fig. 3.6D**). Knockout neurons were difficult to obtain, as it was difficult to genotype embryos and culture

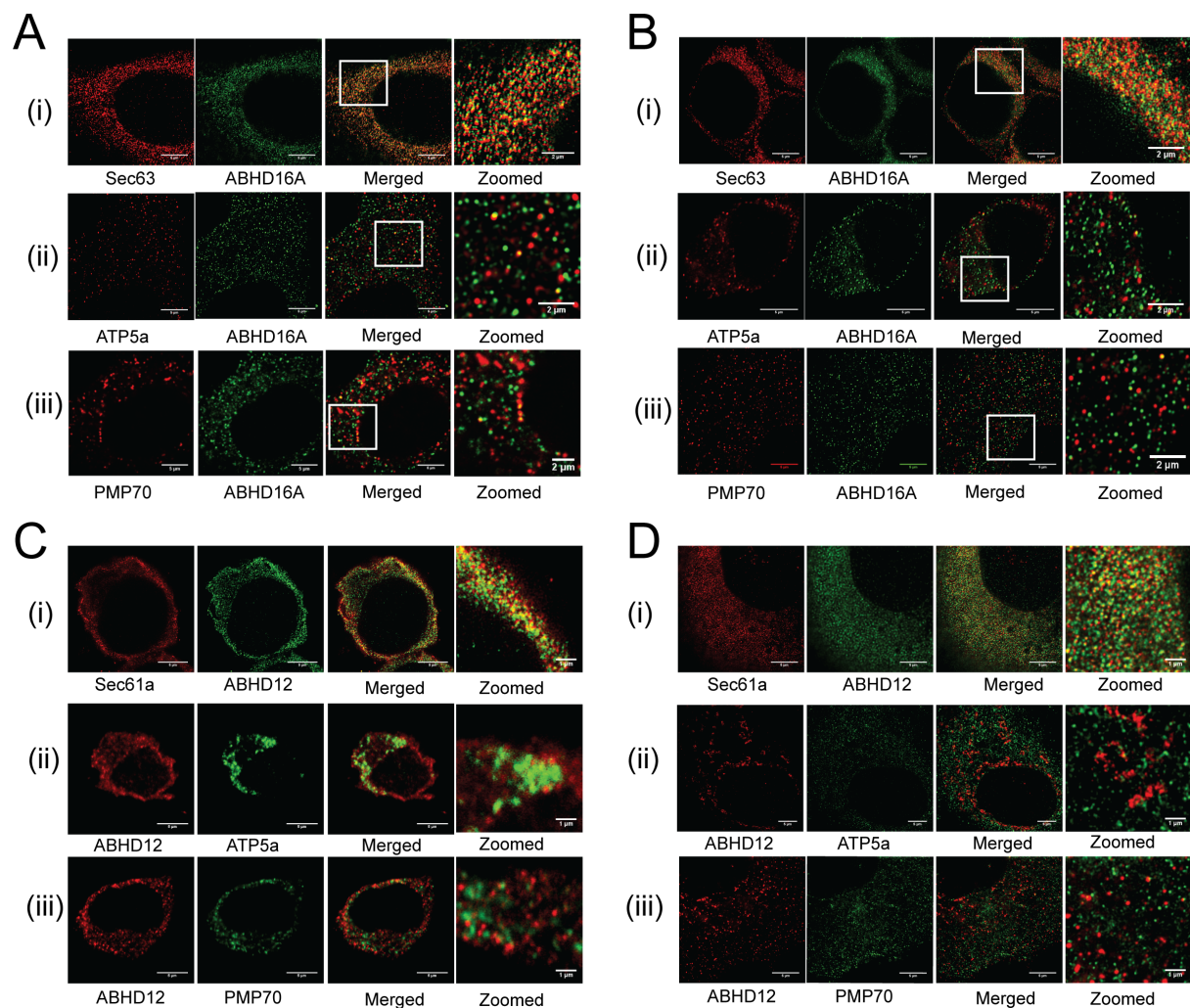


Fig.3.5: Immunofluorescence assay showing enrichment of ABHD16A and ABHD12 at ER in the mammalian cells. Co-localization studies with Sec63 or Sec61a - ER marker (i), ATP5a-mitochondrial maker (ii), PMP70- peroxisomal marker (iii). A – ABHD16A localization in Neuro-2a cells. B – ABHD16A localization in MCF7 cells. C– ABHD12 localization in Neuro-2a cells. D – ABHD12 localization in MCF7 cells. Scale bar = 5 μm . Scale bar (Zoomed image in A & B = 2 μm . Scale bar (Zoomed image in C & D = 1 μm .

primary neurons from HET x HET cross and ABHD16A KO x KO mice doesn't breed successfully under laboratory conditions. Next, I probed for ABHD16A and ABHD12 colocalization and found that both these enzymes co-localize in the Neuro-2a cells (**Fig. 3.6E**) and primary neurons (**Fig. 3.6F**). These studies confirmed that both ABHD16A and ABHD12 are enriched at the ER in primary immune and neurological cells of mammals.

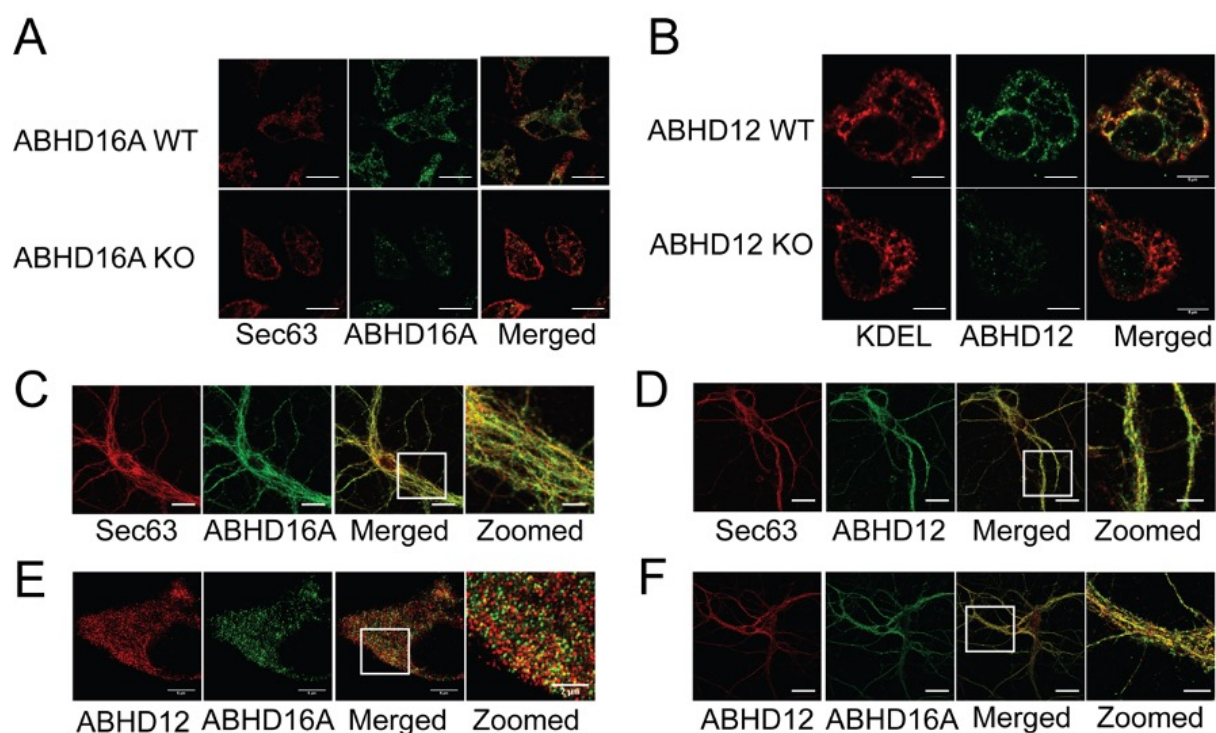


Fig.3.6: Immunofluorescence assays showing localization of ABHD16A and ABHD12 in primary mammalian cells. A– ABHD16A co-localization with Sec63- ER marker in WT PPMs with ABHD16A KO PPMs as control. B– ABHD12 co-localization with KDEL- ER marker in WT PPMs with ABHD12 KO PPMs as control. C– ABHD16A co-localization with Sec63- ER marker in primary neurons. D– ABHD12 co-localization with Sec63- ER marker in primary neurons. E– ABHD16A and ABHD12 co-localization in Neuro-2a cells. F– ABHD16A and ABHD12 co-localization in primary neurons. Scale bar (A,B,C,D,E,F) = 5 μ m. Scale bar (Zoomed image in C, D, E, F) = 2 μ m.

All parallel and complementary experimental approaches establish that ABHD16A localizes at the ER of the mammalian cells. The previously reported trypsin digestion and deglycosylation assays and our current findings together conclude that ABHD16A – lyso-PS – ABHD12 biochemical pathway is regulated from the endoplasmic reticulum with ABHD16A being active towards the cytosolic side while ABHD12 being active on the luminal side. I currently do not understand the exact reason for differential active site orientation of ABHD16A and ABHD12 but lyso-PS imaging strategies could probably shed some light on it in near future.

ABHD16A overexpression does not change ABHD16A localization or PS levels in the mammalian cells. Next, I asked if overexpression of ABHD16A could trigger its localization

to other organelles especially to plasma membrane where most of PS is enriched. Towards this end, I used green fluorescent protein tagged ABHD16A (ABHD16A-GFP) and confirmed its expression in HEK cells using activity based protein profiling gel and western blot with anti-ABHD16A/anti-GFP antibodies (**Fig. 3.7A**). I checked ABHD16A subcellular localization by transfecting GFP tagged ABHD16A and found that it co-localizes with Sec61 β -mCherry (ER marker) on Neuro-2a (**Fig. 3.7B**) and MCF7 cells (**Fig. 3.7D**). ABHD16A-GFP doesn't co-localize with mito-mCherry (mitochondrial marker) in either Neuro-2a (**Fig. 3.7C**) or MCF7 cells (**Fig. 3.7E**). Neuro-2a and MCF7 cells are slightly smaller in size and often COS7 cells are better models for subcellular studies as they have distinctly separated organelles. Hence, I performed colocalization studies in COS7 cells by co-transfecting ABHD16A-GFP and mito-mCherry or Sec61 β -mCherry, and I found that ABHD16A co-localizes with Sec61 β -mCherry (ER marker) (**Fig. 3.7F**) but not with mito-mCherry (**Fig. 3.7G**). Overall, my studies here robustly show that ABHD16A is localized at ER in the mammalian cells.

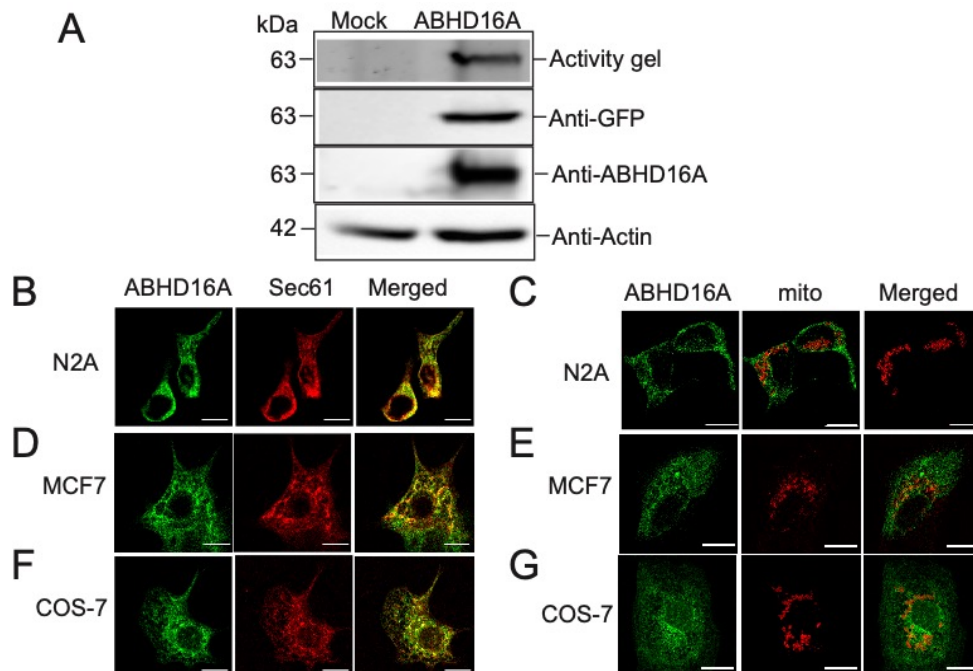


Fig.3.7: Subcellular localization of ABHD16A on overexpression. A – GFP tagged ABHD16A expression check using ABPP gel and western blot. B-E – ABHD16A-GFP co-localization with Sec61 β -mCherry and mito-mCherry in Neuro-2a (N2A) cells (B and C), in MCF7 cells (D and E) and Cos7 cells (F and G). Scale bar = 5 μ m.

ABHD16A regulates bulk lyso-PS flux in the mammalian cells but not PS. Kamat et al.²² showed that ABHD16A knockdown reduces lyso-PS drastically but have very minimal changes in PS levels. To check what effects ABHD16A overexpression might have on PS and lyso-PS levels. I overexpressed ABHD16A in HEK293T cells. HEK293T cells show maximal transfection rate hence they are apt for transient overexpression based metabolomic studies. With ABHD16A overexpressed cells, I found that lyso-PS was increased (**Fig. 3.8A**) but there were no changes in PS levels (**Fig. 3.8B**). We did find significant decrease in PE and PC levels which act as precursor to PS in cells (**Fig. 3.8C & D**). It suggests that PS levels are tightly regulated in HEK cells, possibly through phosphorylation of PSS1. It was previously reported that excessive PS levels trigger phosphorylation of PSS1 and inhibiting PS synthesis, thus providing PS triggered negative feedback for PS synthesis⁴⁹.

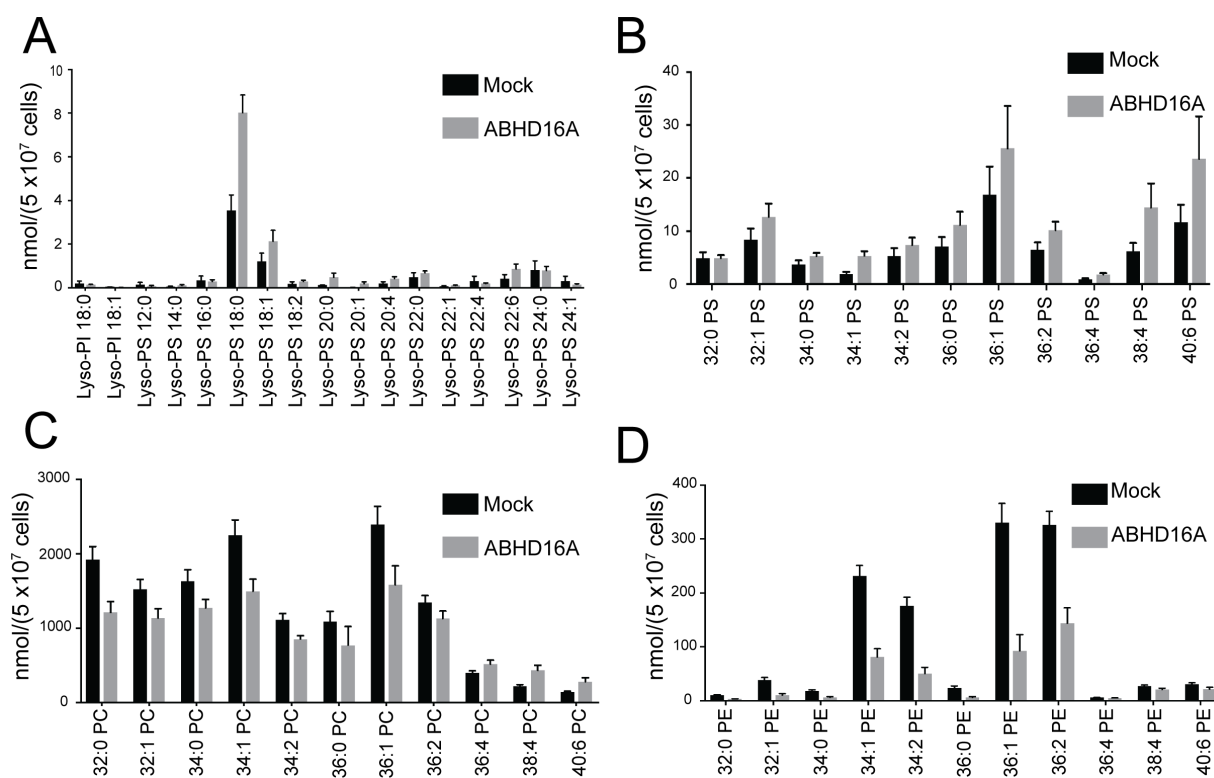
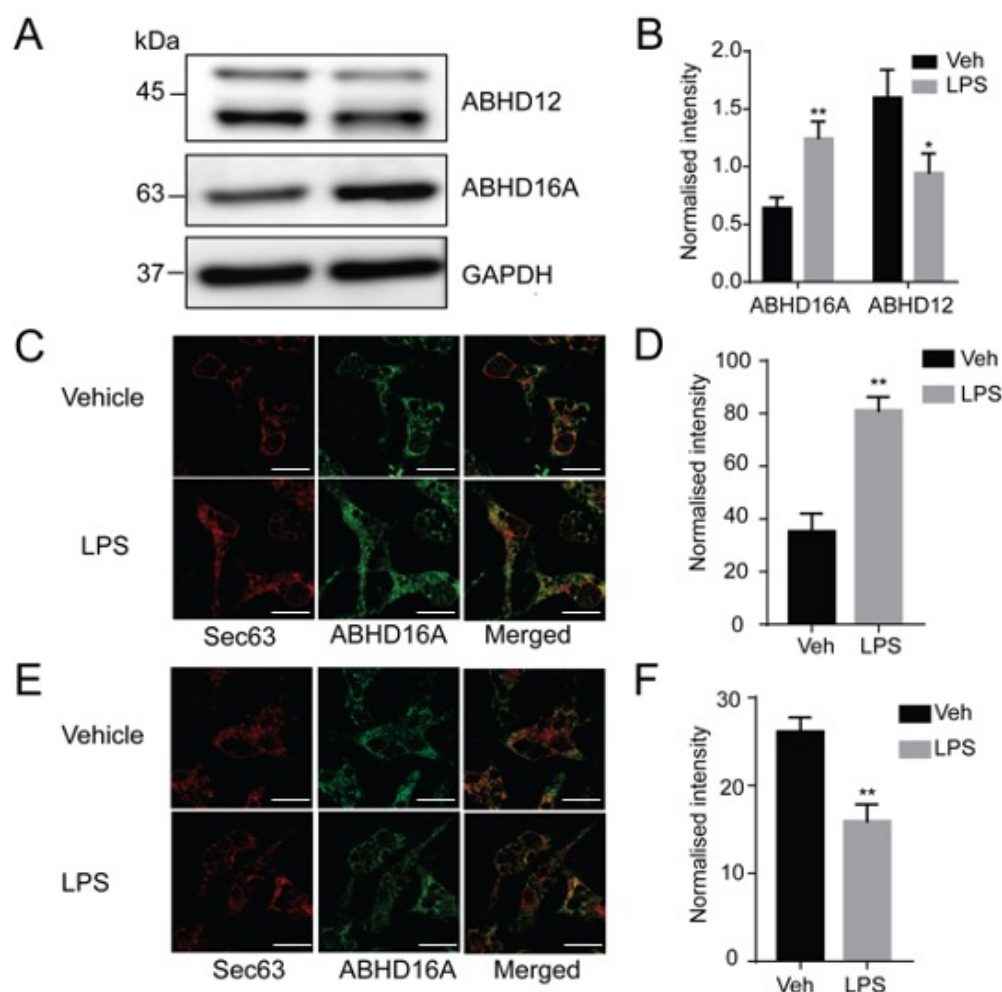


Fig.3.8: Lipidomics experiment as a function of ABHD16A overexpression in HEK293T cells. Cellular measurements of A – Lyso-PS and Lyso-PI levels, B – Phosphatidylserine, C – Phosphatidylcholine, D – Phosphatidylethanolamine on ABHD16A overexpression with mock as control. 17:1 FFA used as internal standard. Data plotted as mean $\pm$ standard deviation.

ABHD16A and ABHD12 are differentially regulated at ER on immune challenge. Kamat et.al²² and data from BioGPS database showed differential regulation of ABHD16A and ABHD12 expression in PPMs on acute immune challenge i.e. lipopolysaccharide (LPS) treatment for 7 hours. I checked ABHD16A and ABHD12 expression levels using western blot and found 2-fold increase in ABHD16A and 0.5 fold decreased in ABHD12 on LPS treated PPMs (**Fig. 3.9A & B**). To check if immune challenge can trigger changes in cellular localization of ABHD16A and ABHD12 on immune challenge, we performed immunofluorescence assay on LPS treated PPMs. We found that ABHD16A and ABHD12 both are present at endoplasmic reticulum (anti-Sec63) in PPMs (**Fig. 3.9C & E**). There was 2-fold increase in ABHD16A and half a fold decrease in ABHD12 levels on LPS treatment to PPMs but there was no change in their subcellular localization (**Fig. 3.9D & F**). It further adds to our conclusion that lyso-PS flux is regulated by ABHD16A and ABHD12 from ER in PPMs.

Discussion

PS is central to many crucial cellular processes and its levels are regulated by several metabolic enzymes which involve their synthesis, degradation or its transformation to other biomolecules. PS is synthesized at MAMs in the mammalian cells by synthase 1 (PSS1) and phosphatidylserine synthase 2 (PSS2)⁴⁸. Once PS is synthesized it is either fluxed in the mitochondria and converted to phosphatidylethanolamine (PE) by phosphatidylserine decarboxylase (PSD) or shuttled from E.R. to the plasma membrane (PM) in exchange of phosphatidylinositol-phosphate (PIP4)^{31,39}. PS from ER can also be transported through vesicular systems to Golgi apparatus³⁴ and then to PM. Until recently, PSS1, PSS2 and PSD were the major annotated enzymes that metabolically regulate PS inside the cell. None of these established enzymes were a PS specific lipase, thus it was presumed that PS is interconverted to other phospholipids and recycled then being directly degraded by any enzyme. Recently, ABHD16A was annotated as the PS specific cellular lipase and its activity generates a very promising and potent signaling molecule lyso-PS. Lyso-PS was already reported to be metabolized by ABHD12 another member enzyme of serine hydrolase family. I aimed to study the subcellular localization of ABHD16A and ABHD12 in the mammalian cells and also test how these enzymes regulated cellular PS and lyso-PS pools. I used crude and cheap method of subcellular fractionation and immunoblots to test for subcellular



*Fig.3.9: ABHD16A-ABHD12 levels in PPM on LPS treatment. A,B – Western blot and quantification probing ABHD16A and ABHD12 levels on immune challenged PPMs. C,D – ABHD16A does not change localization but is upregulated at ER on LPS treatment. E, F – ABHD12 does not change localization but is downregulated at ER on LPS treatment. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value (vehicle versus LPS) western blots ABHD16A = 0.0036, ABHD12 = 0.0181; microscopy assays ABHD16A = 0.0022, ABHD12 = 0.0018. Scale bar = 5 μ m.*

localization of ABHD16A and ABHD12 in mammalian cells. Both these enzymes turned out to be enriched in microsomes of the cells that primarily consist of ER and plasm membrane fractions. PS and lyso-PS lipase activity were also maximum in the microsomal fractions of the cells and these hydrolysis activities were sensitive to specific pharmacological inhibitors of ABHD16A and ABHD12 respectively. Immunoblots and lipase assays established that ABHD16A and ABHD12 are localized to organelles that fractionate as microsomes. Next, I

used STED and confocal microscopy based double immunofluorescence assay and report that ABHD16A and ABHD12 both co-localized with ER markers (anti-Sec63 or anti-Sec61a) in both secondary cancer cell lines (MCF7 and Neuro-2a) and primary cells (primary neurons and peritoneal macrophages). This strongly suggested that ABHD16A and ABHD12 both are localized at the ER in the mammalian cells. As per as my initial anticipation, I expected ABHD16A to be enriched at PM as PS is enriched at PM but it was localized at ER. I checked if ABHD16A overexpression can translocate ABHD16A to the PM, but GFP-tagged ABHD16A on transfection and overexpression localized at the ER in tested cell lines (Neuro-2a, MCF7 and COS7). Lyso-PS pathway involving ABHD16A and ABHD12 is triggered to produce more lyso-PS on immune challenge to PPMs, thus I probed if immune challenge that acts as a stimuli to hyper activate lyso-PS pathway trigger any changes in their localization. I found that on immune challenge ABHD16A levels increase by 2 fold while ABHD12 decreases by half a fold but subcellular localization of these enzymes remain same i.e. at the ER. From these experiments, it was established that PS synthesized at MAMs are degraded by ABHD16A at ER to generate lyso-PS and it is further degraded by an ER localized serine hydrolase - ABHD12. Additionally, lyso-PS can be also recycled back to PS as per as proposals of Lands cycle. Next goal was to check what effects ABHD16A has on PS levels in the cells, ABHD16A knock down cells have shown very subtle changes in PS levels thus, here I overexpressed ABHD16A in HEK293T cells and measured phospholipid levels and found that PS levels didn't change significantly. Instead, overexpression of ABHD16A reduced PC and PE levels; this suggests PS hydrolyzed by excessive ABHD16A is replaced by conversion of PC and PE to PS in cells. Tight control on PS is also suggested by previous reports through phosphorylation of PSS1 and it's also reported that PSS1 or PSS2 individual knockouts do not show any substantial changes in the bulk levels of PS. My findings here provide additional insights into cellular metabolism and propose a model for cellular flux of PS – lyso-PS nodal axis (**Fig. 3.10**). ABHD16A at the ER deacylates PS and forms lyso-PS that is metabolized to glycerophosphoserine and free fatty acids by ABHD12 activity at the ER (**Fig. 3.10**). The free fatty acids and glycerophosphoserine released from the activity of ABHD16A and ABHD12 are incorporated into other phospholipids and possibly recycled. A few lyso-PS species (especially C20:4) is acylated back to form PS by lysophosphatidylcholine acyltransferase 3 (LPCAT3). Currently, ABHD16A and ABHD12 are annotated as major cellular enzymes at the ER that regulate lyso-PS flux in immune and neuronal cells.

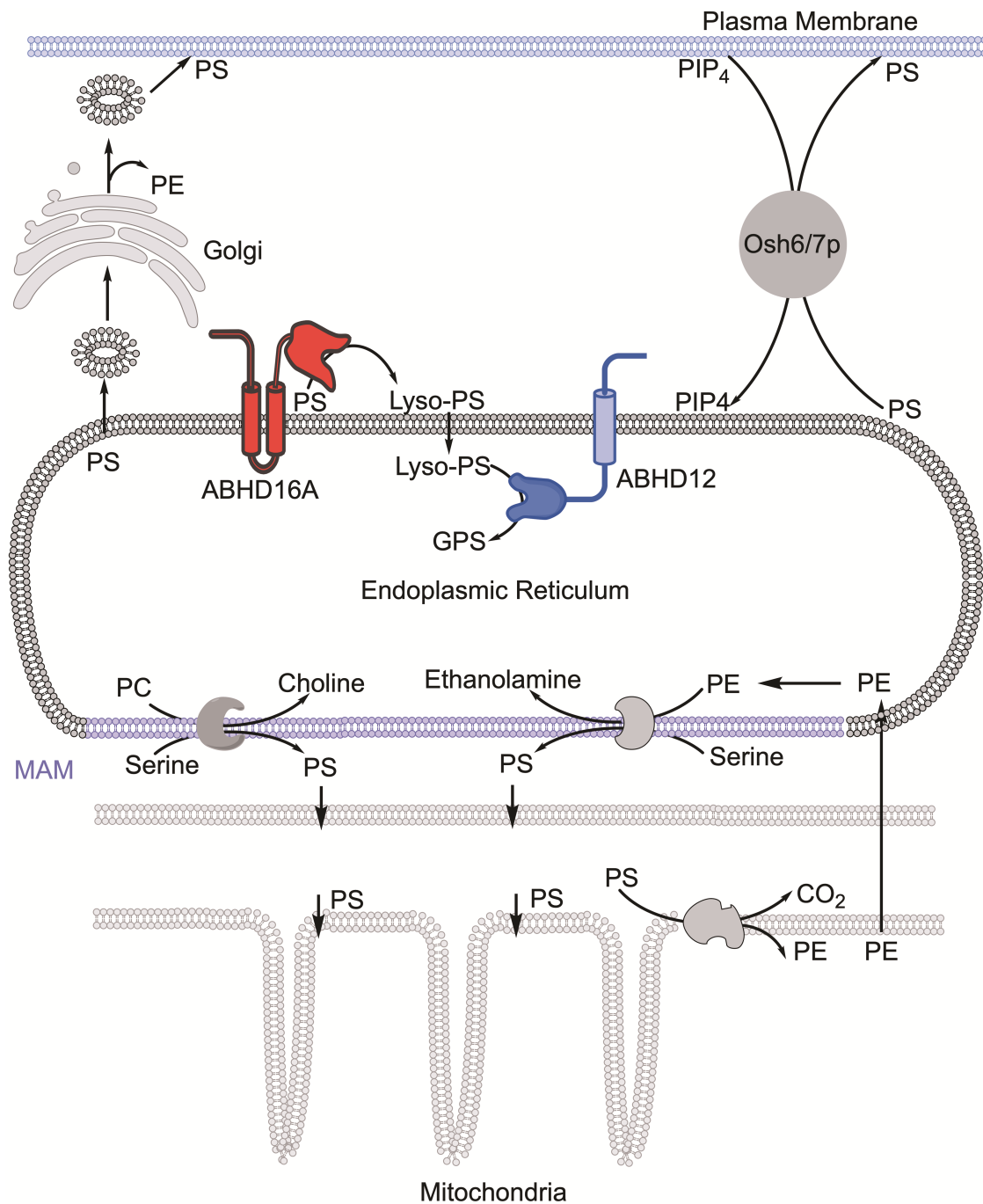


Fig.3.10: ABHD16A and ABHD12 regulate lyso-PS metabolism and flux from ER in endoplasmic reticulum. PS made at MAMs is either transported to mitochondria or plasma membrane or is metabolized to lyso-PS at ER by ABHD16A. Lyso-PS generated is further degraded by ABHD12 localized at ER in mammalian cells.

My findings provide a detailed map for PS – Lyso-PS metabolism in the mammalian cells. Further studies like vesicular transport and viral entry into cells are required to investigate the

detailed role of ABHD16A in cellular physiology as PS is central to several cellular processes.

Conclusion

Lyso-PS is regulated by ABHD16A and ABHD12 in neuro and immune cells. We robustly and confidently establish that these enzymes are localized at ER in mammalian cells and their levels are modulated in PPMs on immune challenge to make of more of lyso-PS. ABHD16A – ABHD12 – lyso-PS nodal axis doesn't seem to have a substantial effects on PS levels in basal physiology.

CHAPTER IV

Cellular expression map of ABHD16A and ABHD12 in the mammalian nervous system

Lyso-PS and its major metabolic enzymes i.e. ABHD16A and ABHD12 are most abundant in the central nervous system of the mammals. ABHD12 deletion in the mice leads to an age dependent lyso-PS accumulation in the central nervous system (CNS) followed by microglial cell activation and development of sensory motor defects similar to the phenotype seen in subjects of the PHARC syndrome. Currently, it is not known how defective lyso-PS signaling drives the pathophysiology of the PHARC syndrome. Localization studies for ABHD16A and ABHD12 in the CNS could shed some light towards understanding the lyso-PS signaling system in the mammalian CNS. Here, I mapped the cell specific localization of ABHD16A and ABHD12 using western blots, immunohistochemistry and microscopy. Additionally, I measured PS and lyso-PS in different regions of the brain using mass spectrometry to complement the localization studies. My findings provide a detailed expression atlas of ABHD16A and ABHD12 in the adult mice brain and integrate it with biochemical parameters i.e. lyso-PS and PS abundance.

Introduction

Lysophosphatidylserine (Lyso-PS) is an established signaling molecule and it's most abundant in the mammalian central nervous system (CNS) and immune tissues^{17,50}. In the mammalian CNS, α/β hydrolase domain containing protein #16A (ABHD16A) and α/β hydrolase domain containing protein #16 (ABHD12) are the major nodal enzyme that metabolize lyso-PS. ABHD16A acts as a principal phosphatidylserine (PS) lipase and synthesizes lyso-PS²² which acts as a hormone signaling molecule. Lyso-PS is further degraded by ABHD12 to glycerophosphoserine and free fatty acid¹⁷. Perturbed lyso-PS metabolism and signaling is associated with the PHARC syndrome. PHARC subjects have been found to bear loss of function mutations (more than 20 identified mutations) in *abhd12* gene¹⁸ that encodes enzyme ABHD12. PHARC rodent model i.e. ABHD12 knock out (KO) mice have elevated lyso-PS lipid levels in the CNS especially very long chain lyso-PS in an age dependent manner and it recapitulates a few of the age dependent behavioral defects observed in the PHARC human subjects like hearing loss, poor auditory brain stem response and locomotor defects (ataxia)¹⁷. ABHD12 KO mice older than 1 year of age exhibit

increased microgliosis (microglial cell activation, marked by Iba1 overexpression) in the brain compared to their wild type (WT) littermates, suggesting that elevated lyso-PS levels possibly cause or drive neuroinflammation¹⁷. Succeeding microgliosis, ABHD12 KO mice (older than 1.5 years of age) perform poorly on behavioral tests for locomotor activity like rotarod and hanging wire tests¹⁷. It is currently hypothesized that defective lyso-PS signaling triggers neuro-inflammation and it might be causative factor for PHARC pathophysiology but signaling events downstream of lyso-PS accumulation is unclear and underexplored.

Brain is a heterogeneous tissue consisting of different cell types with largely distinguished division of physiological work like neurons (that primarily conduct messages), microglia (primarily immune defense and scavenging dead cell debris⁵¹), astrocytes (metabolic support to neurons⁵² and regulates synaptic physiology⁵³), oligodendrocytes (make myelin to insulate neurons⁵⁴), etc. Additionally, brain is an anatomically one of the most evolved organ in the mammals with several anatomical distinct regions like olfactory bulb, cortex, hippocampus, thalamus, cerebellum, etc. Often, the phenotypic or behavioral output of the biochemical events in CNS is very specific to a few cell types or a specific neural circuitry in distinct brain regions, like thalamus regulating the feeding and hunger behavior of mammals. The physiological output of the signaling molecules too can be different depending on the cell types they can on. A good example for this is endocannabinoid signaling system. Endocannabinoids released from one cell act as ligand on another neuron to trigger signal transmission or they act on microglial cells to activate immune defense, thus, the same molecule can acts differently depending on the space and time of its metabolism⁵⁵⁻⁵⁷. The signaling events inside the CNS often need to be dissected out to point the different brain regions (anatomical characteristic) and cells (cellular characteristic) that work at the back end to give rise to a particular phenotype. It allows better understanding of the CNS physiology and helps us design better therapeutic strategies to treat neuro-pathological conditions. I had a few similar questions for the lyso-PS signaling system that brings me to write this chapter. Does lyso-PS act in autocrine or paracrine manner to regulate the CNS physiology? Do different cells cross talk for lyso-PS signaling and metabolism? Can it affect different cells in the CNS differently? Which regions of the CNS hosts major lyso-PS signaling? Do sensorimotor defects in ABHD12 KO mice come from defective lyso-PS signaling in cerebellum? If yes, then which cells participate in this circuitry to raise the phenotypes observed in the PHARC subjects? To answer these questions, precisely, first thing one would do is to map the localization and expression of lyso-PS metabolic enzymes, which in the

mammalian brain principally are ABHD16A and ABHD12. There are no extensive localization studies available for these two enzymes in the brain except a few proteomics studies that suggest the enrichment of ABHD16A in the astrocytes and that of ABHD12 in the microglial cells⁵⁸. A few studies also suggest that ABHD16A is enriched in the cerebellum of rodent brain which is very consistent with the fact that lyso-PS accumulates mostly in the cerebellum^{17,59}. Mapping the cell specific expression and localization pattern of ABHD16A and ABHD12 can help us better understand lyso-PS flux and its signaling effects in the CNS and, possibly it will help us identify the circuitry that gives rise to the PHARC symptoms.

In this chapter, I report the detailed cell specific expression map of ABHD16A and ABHD12 in the mouse brain. I also report the lyso-PS and PS levels as a function of ABHD16A and ABHD12 in different anatomical regions of the brain. It is interesting to note that ABHD12 and ABHD16A are localized in distinct cells of the cerebellum suggestive of lyso-PS signaling as paracrine in nature.

Materials and Methods

Materials. ABHD16A and ABHD12 transgenic (HET) breeders were provided by Prof. Benjamin F. Cravatt, The Scripps Research Institute. All primary antibodies were bought from Abcam and all secondary antibodies were brought from Thermofisher Scientific until otherwise mentioned. All lipid internal standards were purchased from Avanti Polar Lipids. DAB based bright field immunohistochemistry reagents were brought from Vector Labs. All primary antibodies were brought from Abcam and secondary antibodies were brought from Thermofisher Scientific until otherwise mentioned. All other chemicals and salts were purchased from Sigma-Aldrich until otherwise mentioned.

Animal studies. All mouse studies described in this chapter received formal approval from the Indian Institute of Science Education and Research, Pune-Institutional Animal Ethics Committee (IISER-P IAEC), under the guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All mice were maintained at National Centre for Gene Function in Health and Disease. All mice used in the study were generated by breeding either ABHD12 heterozygous (het) mice or ABHD16A het mice and had *ad libitum* access to water and food.

Timed pregnancy and genotyping of embryos. Timed pregnancies were kept as described by the Jackson Laboratory. Briefly, 8-15 week old male and non-virgin females ABHD16A het genotype with synchronized estrous cycle were kept for breeding overnight. Next morning, vaginal plugs were monitored for females and if vaginal plug was seen, the females was isolated and considered to have mated. Pregnant females were sacrificed at the days of needed embryonic stages. Dissected embryos were fixed in 4% PFA and images directly on routine Leica stereomicroscope fitted with color camera. Claws of embryos or other smaller tissues were dissected out post imaging and used for genotyping the embryos. Images and embryo samples were given numbers to map their genotype and phenotype for analysis.

Genotyping. ABHD16A mice were genotyped using the following primers: - 5'-GGCCAGCCTGAGTTCCATAG-3', 5'-CCCGGCTTTATTGTTTAACTACC-3', and 5'-TCGTGGTATCGTTATGCGCC-3'. ABHD12 mice were genotyped using the following primers: 5'-GGTGCCCAGTGAATGGCC-3', 5'-CAGTGCTGGCCTGTCAGTCG-3', 5'-TAAAGCGCATGCTCCAGACTGCC-3'. Tails (1-2 mm) were clipped from the pups at weaning stage and tail clips were digested in digestion buffer constituting 0.5 M EDTA, 5 M NaCl, 1 M Tris-HCl, pH 7.4 and 10% (w/v) SDS supplemented with 1 mg/mL of Proteinase K at 55 °C for 6 hours (h). Thereafter, digestion reaction mixture was spun down at 21000 g for 15 mins at 4 °C. Supernatant was collected and mixed with 0.7 (v/v) volumes of isopropanol to precipitate nucleic acids. The resulting mixture was spun at 21000 g for 15 mins at 4 °C and supernatant was discarded. Pellet was re-suspended for washing in 70% (v/v) ethanol and resulting mixture was spun again for at 21000 g for 30 mins at 4 °C. Supernatant was decanted and nucleic acid pellet was dried at room temperature (RT) by inverting the tubes. The dried nucleic acid pellet was re-suspended in MilliQ water and used for PCR.

Western blot analysis. Denatured proteome (40-50 µg) was resolved over 10% SDS-PAGE gel and transferred to methanol activated PVDF membrane (GE Healthcare) at 60V for 12 h at 4 °C. Post-transfer, membrane was blocked with 5% (w/v) milk in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4 containing 0.1% (w/v) Tween 20 (DPBST), and subsequently incubated with primary antibody (1:1000 dilution in 5% (w/v) milk in DPBST) overnight at 4 °C. Membrane was washed with DPBST (5X) at 25 °C and incubated with secondary antibody (1:10,000 dilution in 5% (w/v) milk in DPBST) for 90 mins at 25 °C. Thereafter,

membrane was washed in DPBST (5x) at 25 °C and developed using Thermo West Pico western blotting substrate (ThermoFisher Scientific) and visualized using Syngene G-Box Chemi-XRQ gel documentation system. Primary antibodies used were: anti-ABHD16A (Rabbit, Abcam, 185549), anti-ABHD12 (Rabbit, Abcam, 182011), and anti-Tubulin (Rabbit, Cloud-Clone Corp, CAB870Hu01). Secondary antibodies used in the study were HRP-conjugated anti-Rabbit IgG (Goat, ThermoFisher Scientific, 31460), or HRP-linked anti-Mouse IgG (Goat, Cloud Clone Corp, SAA544Mu19).

Immunohistochemical analysis. Mice were deeply anaesthetized using isoflurane and subsequently perfused with chilled phosphate buffer saline (PBS) followed by 4% (w/v) paraformaldehyde. Brains were dissected and left in 4% (w/v) paraformaldehyde overnight at 4 °C and then transferred into 30% (w/v) sucrose in PBS until brains sank to the bottom of the tubes (~ 3 days). Coronal 25 µm sections were cut on a freezing microtome maintained at -30 °C, and sections were collected in ice-cold PBS. For 3, 3'-diaminobenzidine (DAB) based bright field immunohistochemistry (IHC), endogenous peroxidases were inactivated with 3% (v/v) hydrogen peroxide in PBS for 15 min at 25 °C in dark. Thereafter the sections were washed twice with 1% (w/v) BSA in PBS and permeabilized with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA in PBS for 45 mins at 25 °C. The sections were then incubated with primary antibody in 1% (w/v) BSA overnight at 4 °C. Following overnight incubation, the sections were washed three times with 0.5% (w/v) BSA in PBS and incubated with secondary antibody, biotinylated horse-anti rabbit [Vector labs, BP-1100] or biotinylated goat-anti mouse [Vector labs, BP-9200] in 0.5% (w/v) BSA in PBS for 1 h at RT. Following this treatment, the sections were washed twice in 0.5 % (w/v) BSA in PBS, incubated with ABC Elite Vectastatin (Vector labs, PK-6100) for 1 h at RT and subsequently washed twice with excess PBS. Finally, the sections were stained with ImmPACT DAB (Vector labs, SK-4105) for 3 min in dark, transferred to excess PBS and mounted with VectaMount Mounting Medium (Vector labs, H-5000) using glass slides and coverslips. For confocal laser scanning based IHC, collected sections were directly permeabilized with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA in PBS for 45 mins at 25 °C and block with 1% (w/v) BSA in PBS for 1 h at 25 °C. Thereafter, sections were washed with 0.5% (w/v) BSA in PBS (3X) and incubated with primary antibody for 16 h at 25 °C. Sections were washed in 0.5% (w/v) BSA in PBS (3X) and incubated with secondary antibody (1:1000 dilution) for 60 mins at 25 °C. Sections were washed in excess PBS and mounted on glass slides with Fluoromount™ aqueous

mounting medium (Sigma Aldrich). Primary antibodies used in the study were anti-ABHD16A [Rabbit, abcam – ab185549, 1:100 dilution], anti-ABHD12 [Rabbit, abcam – ab182011, 1:100 dilution], anti-NeuN [Mouse, abcam – ab104224, 1:100 dilution], anti-Iba1 [Mouse, SantaCruz Biotechnology, – sc-32725, 1:250 dilution], anti-Iba1 [Rabbit, Abcam, – ab178846, 1:1000 dilution], anti-MBP [Rabbit, Abcam, – ab11223, 1:250 dilution], anti-Calbindin [Mouse, abcam – ab82812, 1:50 dilution], anti-GFAP [Mouse, abcam – ab10062, 1:1000 dilution]. Secondary antibodies used in the study were Goat anti-rabbit IgG (H&L) conjugated with DyLight 488 (Thermofisher Scientific, 35553) and goat anti-mouse IgG (H&L) conjugated with Alexa Fluor 568 (Thermofisher Scientific, A-11004).

Poly-L-Lysine coating of slides. 0.1% (w/v) solution of sterile Poly-L-lysine solution was procured from Sigma-Aldrich (P8920). Procured solution was diluted 10 fold in syringe filter (22 µm) sterilized MQ. Slides were cleaned with 0.1% HCl and 70% ethanol, dried and were incubated in the diluted Poly-L-lysine solution for 15 mins at RT. Thereafter, slides were dried in the oven at 60 °C for 2 h. Slides were stored at 4 °C until used for collecting tissue sections.

Cresyl violet staining. Cyo-sectioned 30 µm slices were collected directly on Poly-L-Lysine coated glass slides. Slides were washed (2X) in tap water to get rid of salts and polymers (4% (w/v) 2-Pyrrolidone) used for cryo-sectioning. Slides were dipped in for 3 mins (2X) in 100% absolute ethanol and sections were defatted using 100% xylene for 15 mins (3X) RT. Defatted sections were washed with 100% absolute ethanol for 10 mins (1X) and rehydrated with 95% ethanol for 3 mins (2X). Slides were washed in tap water and dipped in 0.1 % (w/v) Cresyl Violet solution in acetate buffer for 20 mins. Slides were washed in 70% (v/v) ethanol and slides were dipped once in differentiation solution (95% (v/v) ethanol with 300 µL glacial acetic acid). Slides were dipped in 100% ethanol for 3 mins (2X), cleaned in 100% xylene (2X) and sections were cover slipped with DPX Mountant. All experimental steps were done at RT.

Hematoxylin and eosin staining. Cryosectioned 30 µm slices were collected directly on Poly-L-Lysine coated glass slides. Slides were washed (2X) in tap water to get rid of salts and polymers (4% (w/v) 2-Pyrrolidone) used for cryosectioning. Sections were washed in 100% ethanol followed by 95% (v/v) ethanol followed by 70% (v/v) ethanol for 3 mins each. Slides were immersed in Mayers's haemotoxylin for 5 mins and excess stain was removed by

washing slides in running tap water. Slides were dipped 10 times in 1% (w/v) eosin solution and dipped in running tap water until eosin dye stopped streaking in the water. Slides were washed in 70% (v/v) ethanol for 5 mins, followed by wash in 95% (v/v) ethanol followed by wash in 70% (v/v) ethanol for 3 mins each. Slides were cleaned in 100% xylene (2X) and sections were cover slipped with DPX Mountant. All experimental steps were done at RT.

Luxol Fast Blue staining. Luxol Fast Blue (LFB) solution was procured from Sigma-Aldrich (L0294). It was heated to 60 °C in water bath incubator. Sections on slides were cleaned in running tap water and immersed in LFB solution for 4-6 h at 60 °C. Excessive stains were washed by rinsing the slides in running tap water and stain was differentiated by dipping the slide in 0.05 % (w/v) Lithium Carbonate for 30 seconds at RT. Slides were rinsed in tap water followed by wash in 95% (v/v) ethanol followed by wash in 70% (v/v) ethanol for 3 mins each at RT. Slides were cleaned in 100% xylene (2X) at RT and sections were cover slipped with DPX Mountant.

Quantitative lipid measurement. Mice were anesthetized with isoflurane and subsequently sacrificed with cervical dislocation. Thereafter, different regions of the mice brain were micro dissected within a minute and flash frozen in liquid nitrogen. Brain regions were weighed for their wet weight and lipids were extracted based on Folch lipid extraction method. Briefly, dissected regions were washed with chilled DPBS (3X) and homogenized in 6 mL of chilled 2:1:1 (vol/vol/vol) CHCl₃/MeOH/DPBS with 1 nmol of internal standard (37:4 PS and 17:1 lyso-PS). The mixture was centrifuged at 2,800 g for 15 mins and bottom organic layer was collected and the aqueous phase was acidified with 50 µL of formic acid. Acidified aqueous phase was vortexed, volume was adjusted to 6 mL with CHCl₃ and centrifuged at 2,800 g for 15 mins and bottom organic layer was collected. Organic extracts from both the steps were pooled and dried under inert N₂-stream gas. Dried lipid extract was re-suspended in 200 µL of 2:1 (v/v) CHCl₃/MeOH and 20 µL of it was injected for targeted LC-MS/MS analysis. Lipids were separated on Gemini 5U C18-reverse phase column (Phenomenex). LC solvents were as follows: - Solvent A, 95:5 (v/v) H₂O/MeOH + 0.1% (v/v) NH₄OH, Solvent B, 60:35:5 (v/v/v) IPA/MeOH/H₂O + 0.1% (v/v) NH₄OH. All lipids were quantified with established multiple reaction monitoring high resolution (MRM-HR) method on Sciex X500R QTOF instrument coupled to an Exion-series UPLC with quaternary pump. Data collected over the instrument was analyzed using SciexOS software. MS parameters were as follows: - ESI mode of ionization, curtain gas 1 = 60, curtain gas 2 = 40, ion spray voltage = -

5,500 V, temperature = 550°C. All quantifications were done by calculating area under the curve and normalizing it to the area under the curve for respective internal standard and weight of the wet tissues.

Results

ABHD16A mice do not show any gross histological defects in the mice brain. ABHD16A and ABHD12 are maximally enriched in the CNS of the adult mice. ABHD12 KO mice have been previously shown to have no genotypic difference in the gross anatomical organization of the brain or glial cells except microglial cell activation in mice older than 1 year¹⁷. Here, I investigated for brain anatomical organization as a function of ABHD16A. As ABHD16A KO mice suffer from partial embryonic lethality²² and only 20-25% of them are born viable, I first investigated brain morphology at embryonic stages of E15, E13, E10 and E9. ABHD16A KO embryos do not seem to have any obvious neural defects like hydrocephaly or microcephaly (**Fig. 4.1A**) except that ABHD16A KO embryos were shorter in size and looked like embryos of lower embryonic development stage than their actual developmental stage (see E10 embryos in **Fig. 4.1A**). Investigating molecular mechanisms for embryonic lethality in ABHD16A KO is beyond the scope of this chapter, hence I didn't dwell deeper into investigating it. Next, I investigated the gross anatomical changes in the mice brain of adult ABHD16A KO mice. I didn't find any obvious gross anatomical differences in adult ABHD16A KO mice compared to adult ABHD16A WT mice as accessed by cresyl violet staining for Nissl granules in hindbrain (**Fig. 4.1B**) and midbrain (**Fig. 4.1C**) or hematoxylin & eosin staining for cellular organization and morphology in hindbrain (**Fig. 4.1D**) and midbrain (**Fig. 4.1E**). Hindbrain includes cerebellum and medulla and midbrain includes cortex, hippocampus, and thalamus, hypothalamus in the slices used for the imaging. Other regions of the brain like olfactory bulb and prefrontal cortex too didn't show any gross anatomical or morphological defects (data not shown).

ABHD16A mice do not show any defects in glial cells or gross myelination across brain. I stained for glial cells to look for any defects in them as a function of ABHD16A in the adult mice brain (8-12 weeks). There were no morphological or numeric differences in microglial cells (anti-Iba1) or astrocyte reactivity (GFAP) across adult ABHD16A KO mice brain compared to the adult WT mice brain (**Fig. 4.2A and B**). Since, ABHD16A is the principal

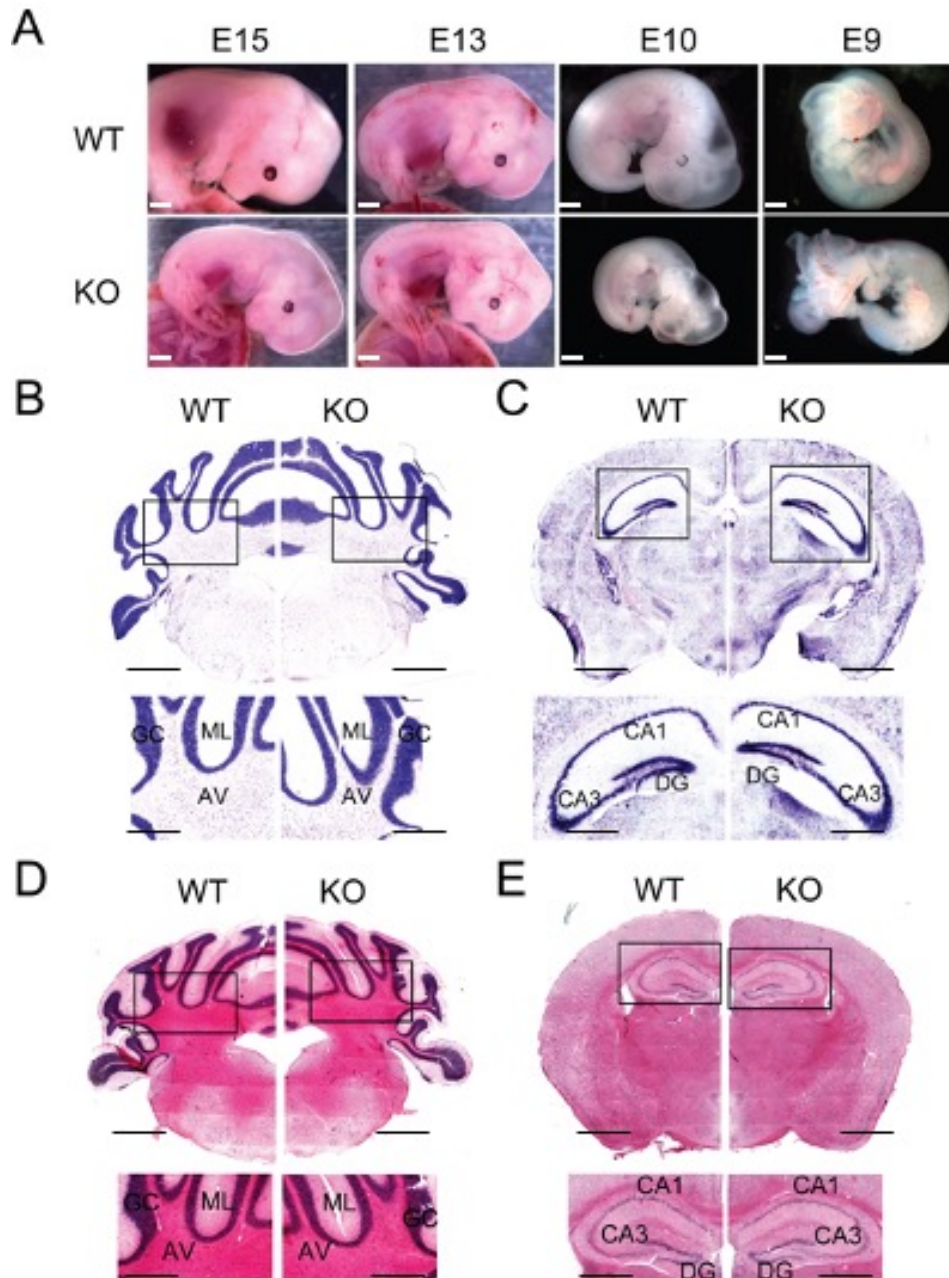


Fig.4.1: Gross characterization of ABHD16A KO mice brain. A – Representative images of ABHD16A WT and KO embryo. B, C – Representative images of Cresyl violet (Nissl Granules) stained coronal sections of ABHD16A WT and KO hindbrain (B) and midbrain (C). D, E – Representative images of Hematoxylin and Eosin (nucleus and cytoplasm of cells) stained coronal sections of ABHD16A WT and KO hindbrain (D) and midbrain (E). GC-granular layer, AV-Arbor vitae, ML-molecular layer, CA1/CA3 –pyramidal neurons in hippocampus, DG-dentate gyrus. Scale bar (A) = 1 mm, Scale bar (B,C,D,E) = 1600 μ m, Scale bar (Zoomed B,C,D,E) = 500 μ m.

PS lipase in the brain²² and PS is one of the key constituent for myelin⁶⁰, I check for the myelination status of neurons in ABHD16A KO mice brain and I probed for myelin in two ways first by staining the myelin using Luxol Fast Blue stain which is widely used for myelin histology in the neural tissues¹⁷. I didn't find any gross changes in the myelin content of ABHD16A KO mice brain compared to their WT littermates (**Fig. 4.2C**). Next, I stained for myelin basic protein to get more accurate idea of myelination in the brain but with immunohistochemistry assay as well I didn't find any significant differences in the myelin (MBP expression or gross levels of MBP) between adult ABHD16A KO and WT mice brain (**Fig. 4.2D**).

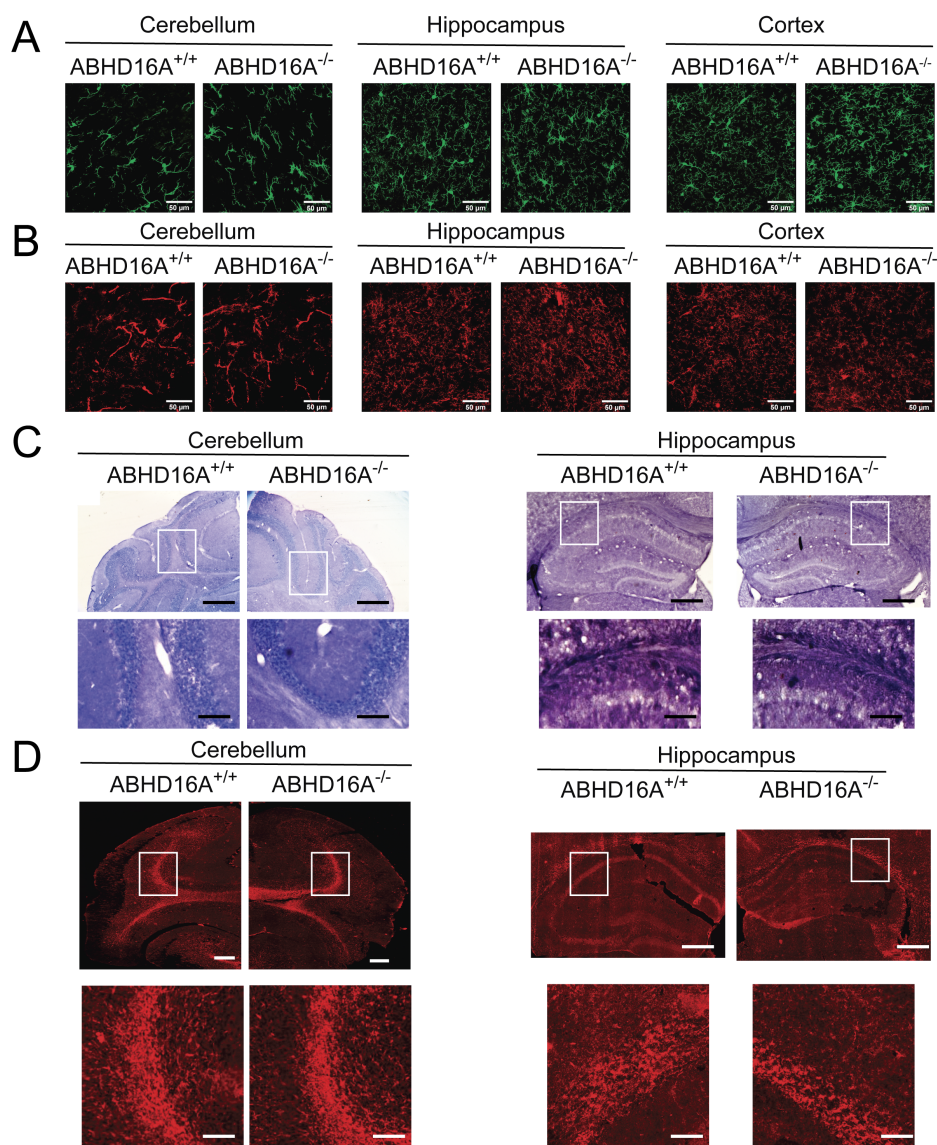


Fig.4.2: Glial and myelin characterization of ABHD16A KO mice in comparison to WT mice. A – Representative image of microglial cells (anti-Iba1) in ABHD16A KO and WT mice brain

regions. *B* – Representative image of astrocytes (anti-GFAP) in ABHD16A KO and WT mice brain regions. *C* – Luxol Fast Blue stained coronal sections of the ABHD16A KO and WT mice brain. *D* – Myelin Basic Protein (anti-MBP) stained coronal sections of the ABHD16A KO and WT mice brain. Scale bar (*A,B,C,D*) = 500 μ m. Scale bar (Zoomed *C,D*) = 100 μ m.

ABHD16A and ABHD12 expression in mice brain by western blot. First, I micro-dissected different region of the adult mice brain namely – olfactory bulb (OB), prefrontal cortex (PFC), cortex (Ctx), hippocampus (Hippo), thalamus (Thala), and cerebellum (Cereb). I probed for ABHD16A and ABHD12 in the lysates of dissected brain regions using western blot. I found that ABHD16A is approximately 3 fold more enriched in the cerebellum (**Fig. 4.3A and B**) compared to other regions of an adult brain. ABHD16A was 2 approximately 1.5 fold higher in olfactory bulb compared to other brain regions but approximately 2 fold lower than in the cerebellum. ABHD12 is ubiquitous in expression across the mice brain regions with approximately 1.5 fold higher expression in cortex and hippocampus but it was not as distinctly localized as ABHD16A. Lyso-PS is reported to be maximally accumulated in the cerebellum of ABHD12 KO mice¹⁷ and my finding that ABHD16A is maximally enriched in the cerebellum might partially explain why lyso-PS accumulates most in the cerebellum of ABHD12 KO mice.

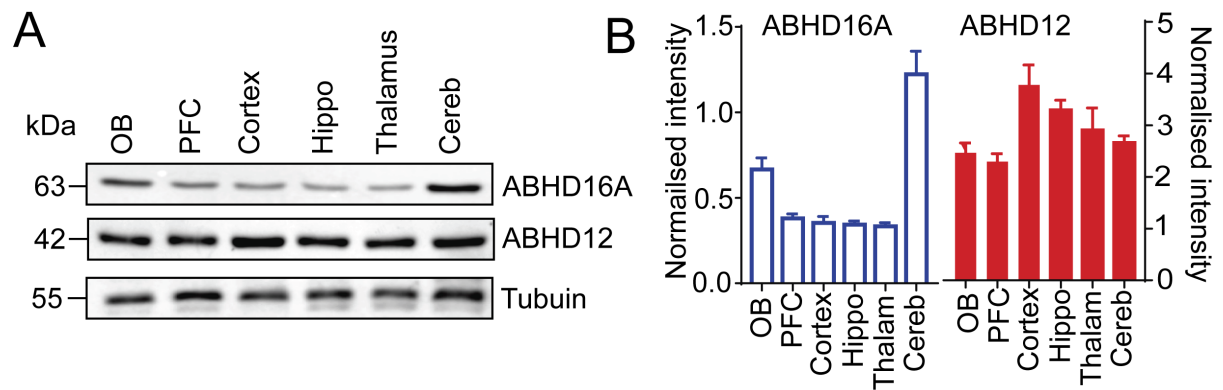


Fig.4.3: ABHD16A and ABHD12 expression in different anatomical brain regions of mice brain. A – Western blot probing ABHD16A and ABHD12 with tubulin as loading control. B – Quantification of ABHD16A and ABHD12 signal normalized to tubulin across brain regions. Data plotted as mean $\pm$ standard deviation.

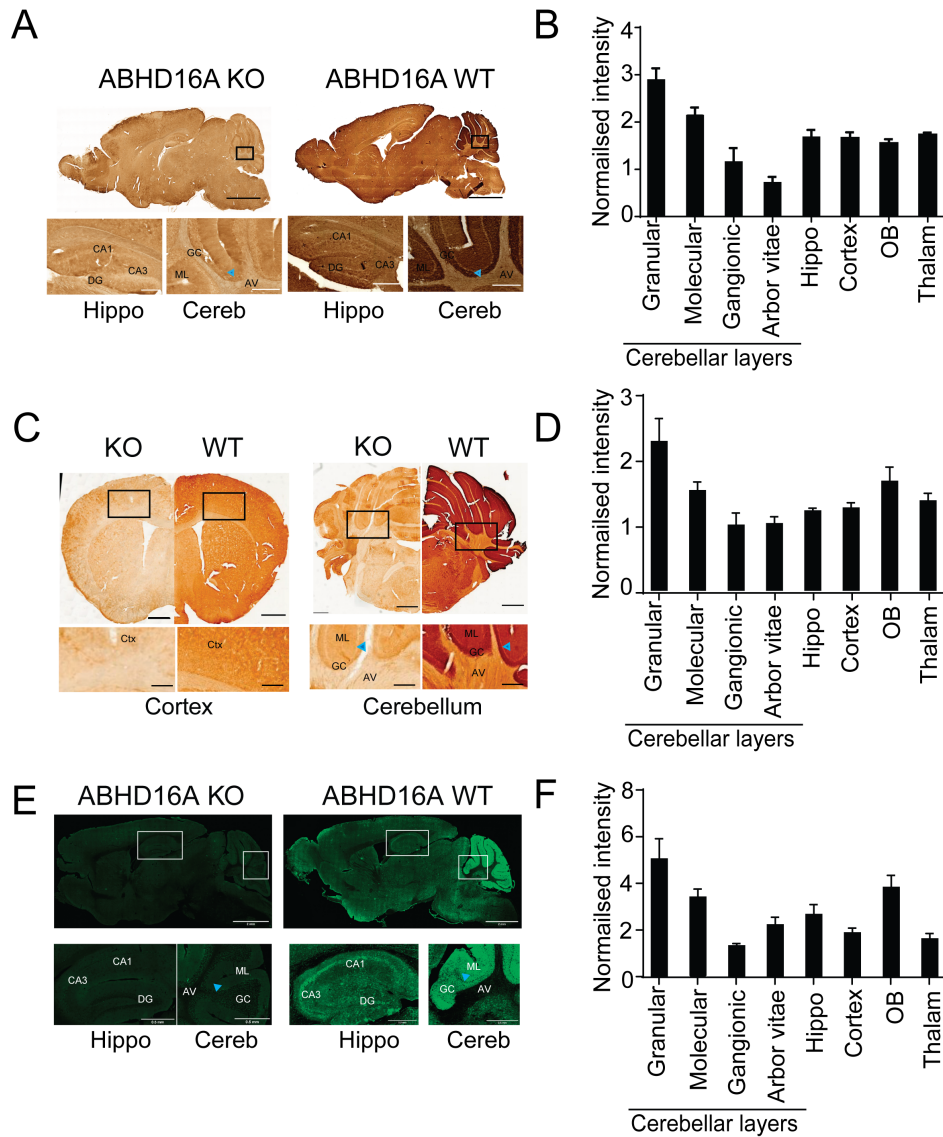


Fig.4.4: Gross localization of ABHD16A in the mammalian brain using ABHD16A KO brain as negative control. A,B,C and D – Representative images for DAB based bright field immunohistochemistry of ABHD16A in sagittal sections of the brain (A) and its relative quantification (B), and in coronal sections of the brain (C) and its relative quantification (D). Representative confocal laser scanning microscope-based immunofluorescence image of ABHD16A probed on sagittal sections of mice brain (E) and its quantification (F). GC-granular layer, AV-Arbor vitae, ML-molecular layer, CA1/CA3 –pyramidal neurons in hippocampus, DG-dentate gyrus. Blue arrows point to ganglionic layer of the cerebellum. All intensities plotted on y-axis of graphs are normalized to signal seen in KO tissues. Data plotted as mean $\pm$ standard deviation. Scale bar (A,E) = 2 mm. Scale bar (C) = 1500 μ m. Scale bar (Zoomed A,C,E) = 500 μ m.

Gross and cellular localization of ABHD16A in the adult mouse brain. Having extensively mapped the subcellular localization of ABHD16A in mammalian cells (Chapter III), I decided to map the gross and cell specific localization of ABHD16A in the adult mouse brain where it is found in abundance compared to other tissues of an adult mice. I leveraged ABHD16A KO mice brain (as negative control) and performed immunostaining studies on mice brain sections (brain slices) to map ABHD16A expression. Using, 3,3'-Diaminobenzidine (DAB) substrate based bright field immunohistochemistry, I found that ABHD16A is maximally enriched across cerebellum (granular layer >> molecular layer of the cerebellum) except cerebellar ganglionic layer of the mice brain (**Fig. 4.4A and B**). Cerebellar portion tend to roll and flip in the sagittal sections of brain while staining which might affect the staining and create artifacts (biased staining in the cerebellum). To make sure that these observations were no artifact of staining procedure, I stained for ABHD16A in coronal sections of the brain and here too; ABHD16A was found to have highest expression in the granular layer of the cerebellum (**Fig. 4.4C and D**). Additionally, I confirmed the ABHD16A enrichment in granular and molecular layer of the cerebellum using fluorescent confocal microscopy, which offers better quantification (**Fig. 4.4E and F**). These studies confirmed that ABHD16A is enriched in granular layer of cerebellum followed by olfactory bulb followed by molecular layer of the cerebellum.

To map the cell specific localization of ABHD16A in the mouse brain, we used confocal microscopy based double immunofluorescence assay with ABHD16A and cellular markers viz. anti-NeuN for neurons, anti-GFAP for astrocytes, anti-MBP for myelin, anti-Iba1 for microglial cells. I found that ABHD16A is enriched in the CA3 neurons of the hippocampus (**Fig. 4.5A**) and in the granule neurons of the cerebellum (**Fig. 4.5B**). I couldn't find any ABHD16A expression in Purkinje neurons (anti-Calbindin) whose soma is located in the ganglionic layer of the cerebellum (**Fig. 4.5C**). I did find some ABHD16A expression in the molecular layer but far lower than that in granular layer and it is likely that ABHD16A is expressed in the parallel fibers of molecular layer which originate from the granule cells in granular layer and synapse with Purkinje neurons. ABHD16A expression is absent in glial cells like astrocytes (anti-GFAP) (**Fig. 4.5D**) or microglial cells (anti-Iba1) (**Fig. 4.5E**). ABHD16A did not show any co-localization with MBP suggesting that it is absent on oligodendrocytes and it doesn't have any preferential enrichment towards myelinated parts of neurons (**Fig. 4.5F**).

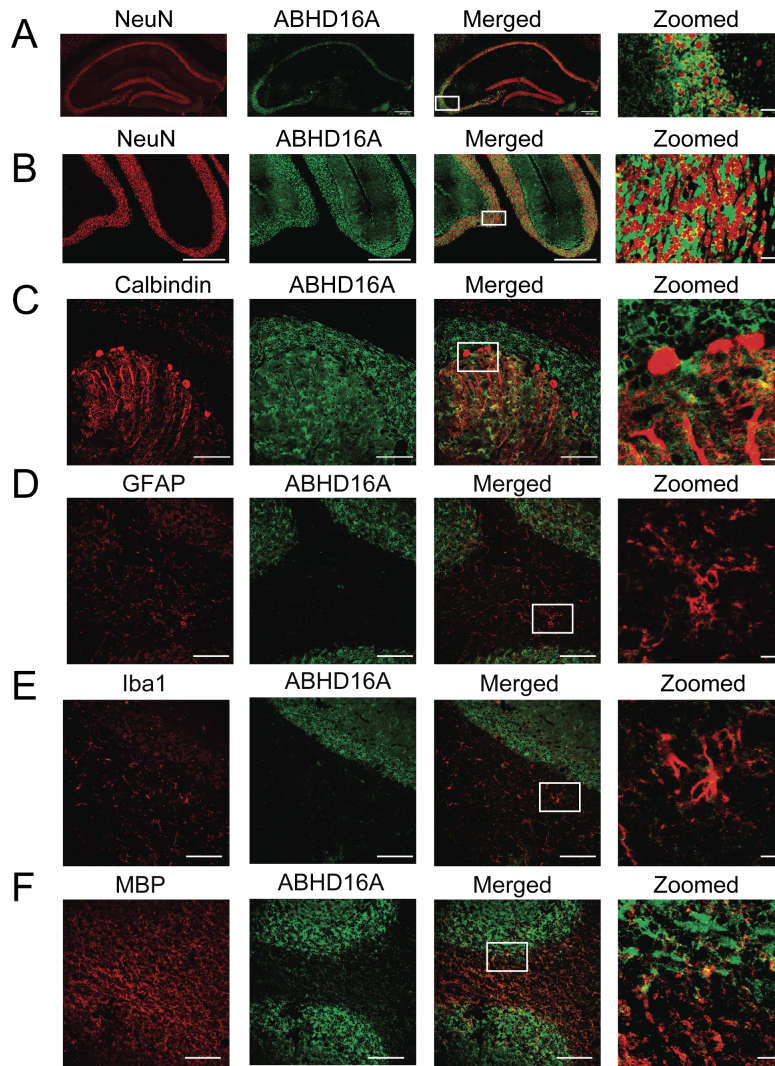


Fig.4.5: Cell specific localization of ABHD16A in the mammalian brain. A & B – ABHD16A expression in neurons stained by NeuN in hippocampus (A) and in cerebellum (B). C, D, E – ABHD16A is not expressed in Purkinje neurons of cerebellum stained by Calbindin (C), or in astrocytes stained by GFAP (D), or in microglia stained by Iba1 (E). F – ABHD16A do not show any preferential expression on MBP stained regions or cells. Scale bar (A,B) = 300 μ m. Scale bar (C,D,E,F) = 100 μ m. Scale bar (Zoomed A,B,F) = 50 μ m. Scale bar (Zoomed C,D,E) = 10 μ m.

Gross and cellular localization of ABHD12 in the adult mouse brain. I have extensively explored subcellular localization of ABHD12 in mammalian cells and have reported in Chapter III that it is enriched at the endoplasmic reticulum of the cells. Having mapped ABHD16A expression in mouse brain, next interesting protein in lyso-PS pathways is ABHD12. As discussed ABHD12 acts downstream of ABHD16A in lyso-PS metabolism and its localization studies are crucial to get an idea about lyso-PS flux in brain. I mapped the

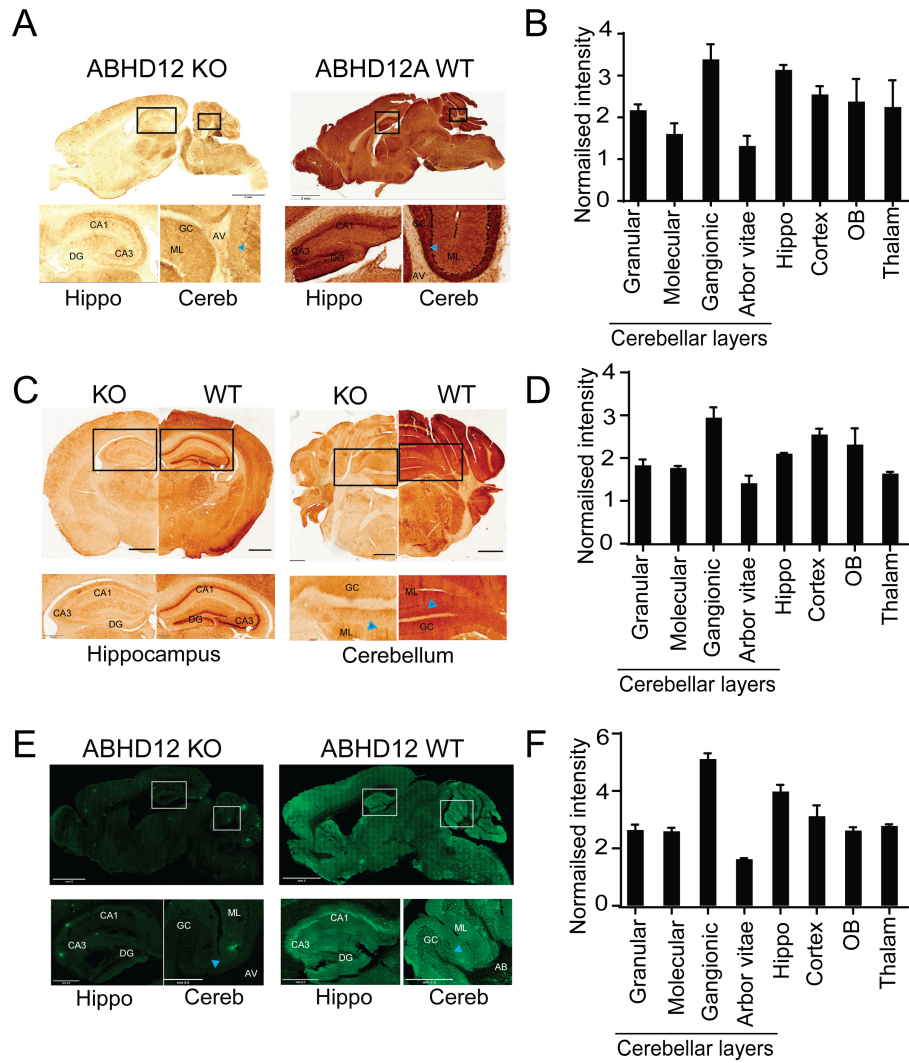


Fig.4.6: Gross localization of ABHD12 in the mammalian brain using ABHD12 KO brain as negative control. A, B, C & D – Representative images for DAB based bright field immunohistochemistry of ABHD12 in sagittal sections of the brain (A) and its relative quantification (B), and in coronal sections of the brain (C) and its relative quantification (D). Representative confocal laser scanning microscope based immunofluorescence image of ABHD16A probed on sagittal sections of mice brain (E) and its quantification (F). All intensities plotted on y-axis of graphs are normalized to signal seen in KO tissues. All intensities plotted on y-axis of graphs are normalized to signal seen in KO tissues. GC-granular layer, AV-Arbor vitae, ML-molecular layer, CA1/CA3-pyramidal neurons in hippocampus, DG-dentate gyrus. Blue arrows point to ganglionic layer of the cerebellum. All intensities plotted on y-axis of graphs are normalized to signal seen in KO tissues. Data plotted as mean $\pm$ standard deviation. Scale bar (A,E) = 2 mm. Scale bar (C) = 1500 μ m. Scale bar (Zoomed A,C,E) = 500 μ m.

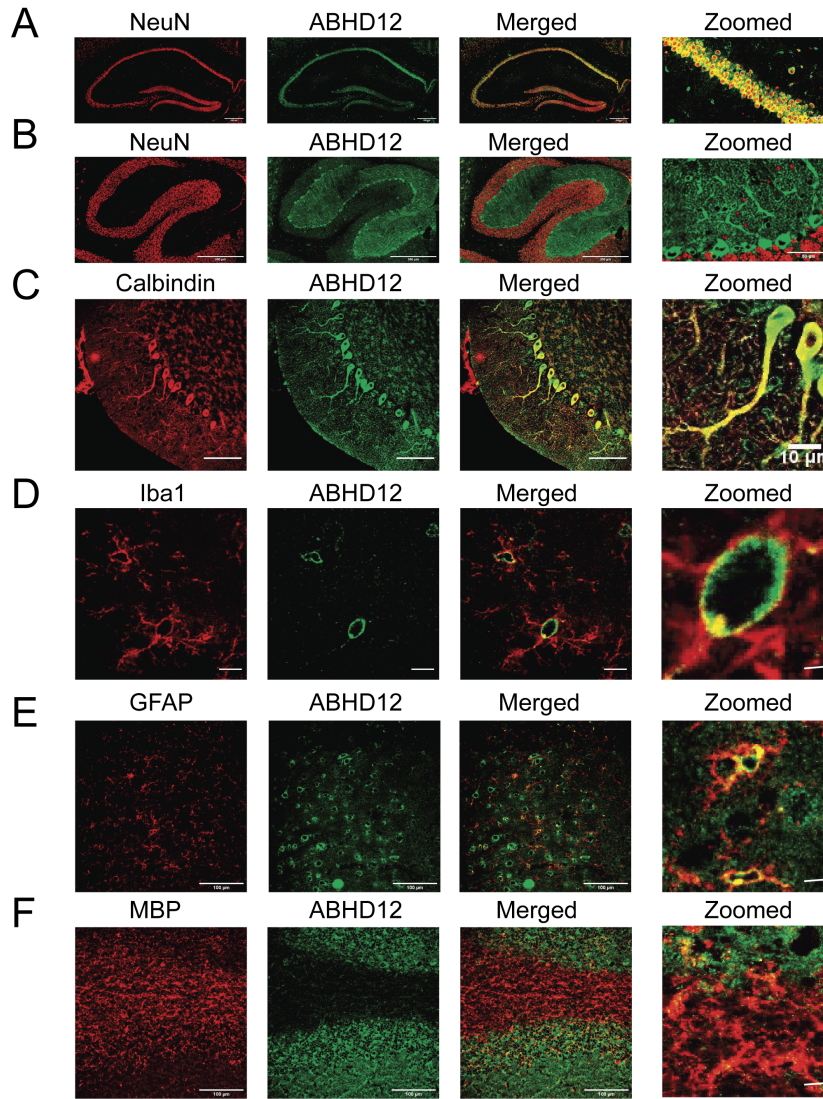


Fig.4.7: Cell specific localization of ABHD12 in the adult mice brain. A & B – ABHD12 expression in neurons stained by NeuN in the hippocampus (A) but not in neurons in the cerebellum (B). C – ABHD12 is highly expressed in the Purkinje neurons of the cerebellum stained by Calbindin. D and E – ABHD12 is also expressed in microglia labelled by Iba1 (D) and in astrocytes stained by GFAP (E). F – ABHD16A did not co-localize preferentially with MBP stained regions or cells in the brain. Scale bar (A,B) = 300 μ m. Scale bar (Zoomed A,B) = 50 μ m. Scale bar (C,E,F) = 100 μ m. Scale bar (D) = 50 μ m. Scale bar (Zoomed C, E,F) = 10 μ m. Scale bar (Zoomed D) = 5 μ m.

that ABHD12 is expressed on microglial cells (Anti-Iba1) (**Fig. 4.7D**) and on astrocytes (Anti-GFAP) (**Fig. 4.7E**) but the ABHD12 levels on astrocytes were far lower than that on microglial cells. I also check for co-localization of ABHD12 and myelin basic protein and I

couldn't find any co-localization or correlation between their expressions. Probably ABHD12 is absent from oligodendrocytes as it didn't co-localize with MBP. Contrasting ABHD16A-ABHD12 localization in the cerebellum is interesting as they show layer specific differential enrichment and PHARC rodent models i.e. ABHD12 KO mice exhibit locomotor defects and microglial activation in the cerebellum.

PS and lyso-PS levels as a function of ABHD16A and ABHD12 activity in different regions of mouse brain. To complement the ABHD16A and ABHD12 localization studies in mammalian brain, we measured their substrates i.e. PS and lyso-PS in the micro-dissected regions of the adult mouse brain (8-12 weeks old). There was a significant reduction of lyso-PS levels in ABHD16A KO mice especially in cerebellum (approximately half a fold) followed by olfactory bulb (**Fig. 4.8A**), which is in accordance with the abundance of ABHD16A in these regions of the brain. PS levels did not change significantly except for accumulation of 36:5 and 40:5 PS in ABHD16A knock out mice (**Fig. 4.8B**) suggesting that bulk PS levels are unaffected by ABHD16A activity and ABHD16A activity principally regulates lyso-PS flux at large. Lyso-PS species especially very long chain lyso-PS species were accumulated across different mice brain regions of ABHD12 KO mice (**Fig. 4.8C**). Long chain lyso-PS like lyso-PS 18:0 and lyso-PS 16:0 changed significantly only in the cerebellum while longer chain lyso-PS 22:0, 22:1, 24:0, 24:1 changed across brain regions like olfactory bulb, prefrontal cortex, cortex, hippocampus and cerebellum (**Fig. 4.8C**). Interestingly, a few levels of PS species like 38:4, 38:5 and 40:5 were elevated in the cortex, hippocampus and cerebellum of ABHD12 KO mice compared to that of their WT littermates (**Fig. 4.8D**). It was recently reported that excess lyso-PS (especially lyso-PS 24:0) is acylated by lysophosphatidylcholine acyltransferase 3 (LPCAT3) to make PS in the mammalian CNS. So, increase PS in ABHD12 KO mice is due to negative feedback for lyso-PS in the brain, which triggers LPCAT3 to convert excessive long chain lyso-PS to PS.

Discussion

Localization atlas of proteins in the tissues is crucial information to acquire. It helps in understanding the gross organization of tissues, signaling systems involving proteins in question, flux of protein products in case protein in question is an enzyme, and allows one to fit the function of the protein in bigger scheme of physiological processes at systems level. Physiological processes are often performed by a set of biochemical reactions involving

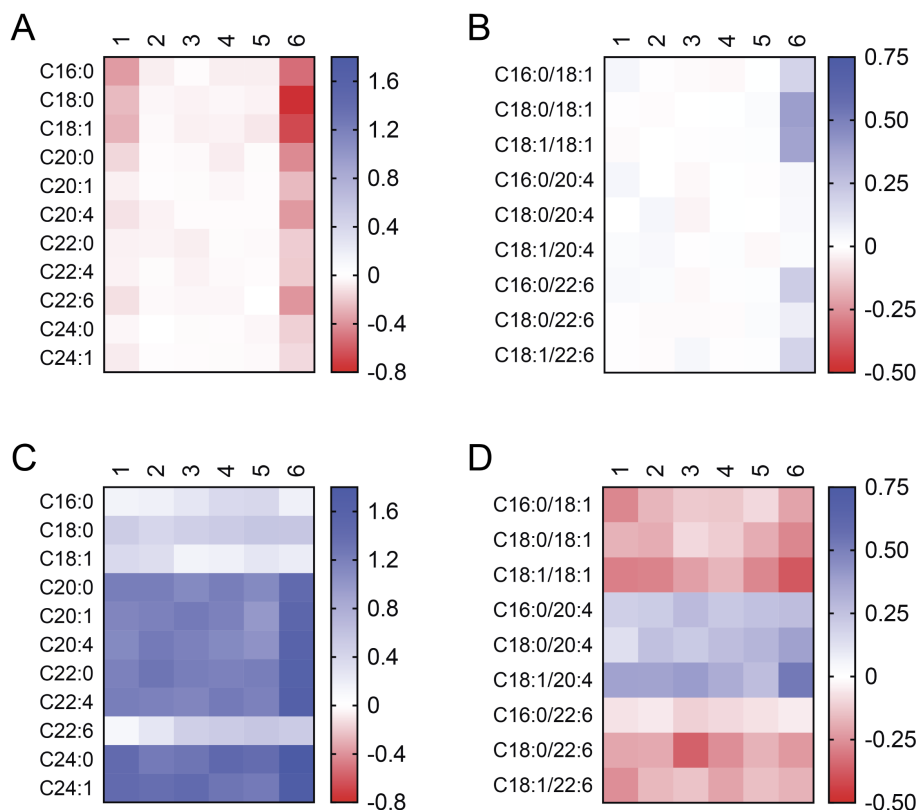


Fig.4.8: Lyso-PS and PS measurements across brain regions as a function of ABHD16A and ABHD12 in the mammalian brain. A – Lyso-PS levels in brain regions of ABHD16A KO mice compared to WT mice. B – PS levels in brain regions of ABHD16A KO mice compared to WT mice. C – Lyso-PS levels in brain regions of ABHD12 KO mice compared to WT mice. D – PS levels in brain regions of ABHD12 KO mice compared to WT mice. Values plotted are the log₁₀ values of the ratio of lipid levels in KO and WT mice. 1-Olfactory bulb, 2-Prefrontal cortex, 3-Cortex, 4-Hippocampus, 5-Thalamus, 6-Cerebellum.

synthesis and destruction of signaling molecules in the biological system. One such system is lyso-PS signaling system. Lyso-PS is principally synthesized by ABHD16A from PS and it is degraded by ABHD12 in the mammalian brain. If lyso-PS is not degraded (which could be due to loss of ABHD12 activity), it accumulates with onset of age and triggers activation of microglial cells in the brain especially in the cerebellum. ABHD12 KO mice i.e. mice lacking ABHD12 activity also develops several sensory motor defects like hearing loss and poor motor coordination which is similar to the observations made in human subjects of PHARC and interestingly they too bear loss of function mutation in *abhd12* gene. These findings established some sort of link between lyso-PS metabolism, signaling, neuro-

immunophysiology and behavior. I was interested to investigate this link from brain regions and neural circuitry point of view. So, I started to map the cell specific localization of ABHD16A and ABHD12 in different regions of the mice brain and relate it to the lipids involved in this metabolic pathway i.e. PS and lyso-PS. ABHD12 KO mice brain was anatomically investigated before to show that it doesn't show any gross organization defects¹⁷. ABHD16A KO mice was not histologically characterized but it was reported that they show partial embryonic lethality²². I isolated embryos to look for any obvious visible phenotype in ABHD16A KO embryos. I didn't find any visible change in their developing brain. Interestingly, ABHD16A KO embryos seem to have delayed development as they looked like E9 stage at E10-11 stage. It was not my goal for this chapter so I moved on but it would be interesting to investigate the role of lyso-PS signaling and metabolism in the embryonic development of mammals in the future. Next, I rigorously compared the ABHD16A KO mouse brain with that of their WT littermates using histology and immunofluorescence assay. I didn't find any gross anatomical changes in adult mice brain of ABHD16A KO mice as histologically stained by cresyl violet staining and hematoxylin and eosin staining. I didn't find any morphological or numeric defects in their glial cells i.e. astrocytes and microglial cells of ABHD16A KO mice. This established that adult ABHD16A KO mice do not have any anatomical defects in the brain. Since, ABHD16A metabolizes PS and PS is an important constituent of the myelin, I probed for myelin using Luxol Fast Blue stain and anti-myelin basic protein antibody but with both the staining approaches (dye staining and immunostaining) I couldn't find any defects in the gross myelination of the adult ABHD16A KO mice brain. Next, I probed for ABHD16A and ABHD12 gross expression in the adult mice brain using western blot and immunohistochemistry. I found that ABHD16A is enriched in the granular layer of the cerebellum and olfactory bulb while ABHD12 was ubiquitous in expression with some partial enrichment in the cortex and hippocampus. Next, I studied cell specific expression pattern of ABHD16A and ABHD12 using double immunofluorescence confocal co-localization microscopy. I found that ABHD16A was differentially enriched in the granule neurons of the cerebellum while ABHD12 expression was very high (almost exclusively) in the Purkinje neurons of the cerebellum. Interestingly, Purkinje neurons were devoid of any ABHD16A expression. Other than neurons, ABHD12 was also present on microglial cells and astrocytes but far less than on neurons. Both ABHD16A and ABHD12 didn't co-localize with myelin basic protein suggesting that these enzymes are probably not present on oligodendrocytes and do not show any preference in localization to myelinated axons. Next, after having mapped

the cellular expression atlas of ABHD16A and ABHD12 in the adult mice brain, I wanted to relate the localization results to their substrates and products. I measured PS and lyso-PS in different regions of the brain using microdissection and LC-MS. In consistent to our localization data, I found that lyso-PS was most abundant in the cerebellum followed by olfactory bulb and on ABHD16A ablation, decreased lyso-PS and increased PS levels were prominent in cerebellum followed by olfactory bulb. ABHD12 ablation in the mice brain, lead to ubiquitous increase in the very long chain lyso-PS across mice brain regions but long chain lyso-PS (like lyso-PS 18:0) was more pronounced in the cerebellar region. Overall, our lipidomic measurements related well to our localization data for ABHD16A and ABHD12 and they suggests cerebellum to be the crucial seat for lyso-PS metabolism and signaling in the mammalian brain.

Conclusion

I have provided a detailed atlas of the ABHD16A and ABHD12 and lyso-PS pathway in the mammalian brain. These studies can be extrapolated to study lyso-PS pathway at neural circuitry level in different brain regions. I report one interesting observation in terms of ABHD16A and ABHD12 i.e. both enzymes are localized on two different neurons namely granule neurons and Purkinje neurons which are reported to communicate with each other in neurophysiology. It remains interesting question to pursue what role ABHD16A – lyso-PS – ABHD12 biochemical axis plays in granule neuron – Purkinje neuron communication. I would explore this in the next chapter i.e. Chapter V.

CHAPTER V

ABHD16A – lyso-PS – ABHD12 biochemical axis modulates Purkinje neuronal physiology through paracrine cellular signaling in mammalian brain

Lyso-PS is highly abundant in the central nervous system of mammals. Its principal metabolic enzymes ABHD16A and ABHD12 are also abundant in the central nervous system of the mice. But the physiological relevance of lyso-PS in the brain (especially neurons) is under-explored and unknown. In this chapter, I investigated the role of lyso-PS in neuronal cells of the mice brain. I found that lyso-PS induces ERK1/2 phosphorylation pathways in the primary cerebellar neurons and secondary neuronal cells (Neuro-2a). Granule neurons of the cerebellum highly express ABHD16A that synthesizes bulk cerebellar lyso-PS and it acts in a paracrine manner on the cerebellar Purkinje neurons and induces ERK1/2 phosphorylation through some unannotated lyso-PS receptor. Lyso-PS signaling is terminated by ABHD12 expressed in the Purkinje neurons that breaks down lyso-PS to glycerophosphoserine and free fatty acid. I didn't find any significant morphological or structural changes in the Purkinje neurons or their synaptic densities as a result of defective lyso-PS signaling.

Introduction

Lipids participate in a gigantic number of crucial brain physiological processes in some way or other and at consequent they are one of the most widespread, robust and established signaling molecules in the central nervous system of the mammals. They indeed constitute the 50% of the brains dry weight and brain stands second to adipose tissue in terms of its lipid content⁶¹. Such higher abundance warrants for crucial functions that lipids play in regulating physiological processes of the brain. They regulate neuronal plasticity (lysophosphatidic acid⁶², 2-arachidonoylglycerol⁶³), communication⁶⁴, and response to brain injury (prostaglandins⁶⁵, thromboxanes⁶⁶, etc.). Lipids also enhance neural signaling speed, output and efficiency by insulating neurons in the form of myelin sheath^{67,68} where they constitute approximately 80% of the myelin in the brain⁶⁹. From the diverse library of mammalian lipids, the molecule at the center stage in this chapter is lysophosphatidylserine (lyso-PS). It is established to play role of a signaling molecule in peripheral tissues and cells like regulatory T cells and mast cells^{11,43} but it is most abundant in central nervous system (CNS) of the mammalian brain. In majority of cases, spatial abundance correlates well with the

function and significance of the biomolecules. The exact physiological significance of lyso-PS in the mammalian central nervous system is unclear. Since, lysophospholipids like sphingosine-1-phosphate, lysophosphatidic acid are established as potent signaling molecules in the CNS⁷⁰⁻⁷⁴ and lyso-PS in itself acts as a crucial signaling molecule in the peripheral tissues especially mast cells⁴². Thus, it's very likely that lyso-PS may be a signaling molecule in the mammalian CNS. In the mammalian CNS, ABHD16A and ABHD12 are the major metabolic enzymes of lyso-PS^{17,22}. I extensively mapped the abundance of ABHD16A, ABHD12 and lyso-PS in the mice brain. I found cerebellum to have maximum pool of lyso-PS and ABHD16A. Interestingly, ABHD16A and ABHD12 were distinctly localized on two different neurons (namely granule neurons and Purkinje neurons) of the cerebellum, which are known to communicate with each other (Chapter IV). These observations lead me to hypothesize that lyso-PS might act as some sort of signaling molecule in communication between granule neurons and Purkinje neurons. Granule neurons generate lyso-PS through ABHD16A activity and secrete it in cerebellar interstitial fluids. This secreted lyso-PS might act as the paracrine signal for Purkinje neurons and as soon as the signal is sensed through some receptor on Purkinje neurons, it is degraded by ABHD12 present on Purkinje neurons.

I tested this hypothesis using several parallel approaches described in this chapter and tried to establish the role of lyso-PS in the CNS neurons. I found that lyso-PS indeed acts as a signaling molecule for Purkinje neurons and induces phosphorylation of ERK1/2, thus activating signaling cascades in the Purkinje neurons. ERK1/2 phosphorylation indicates activation of intracellular signaling cascades in Purkinje neurons as function of lyso-PS treatment.

Materials and Methods

Materials. ABHD16A and ABHD12 transgenic (HET) breeders were provided by Prof. Benjamin F. Cravatt, The Scripps Research Institute. Small molecule inhibitors KC01, KCO2, JJH350 were generous gift from by Prof. Benjamin F. Cravatt, The Scripps Research Institute. All primary antibodies were bought from Abcam and all secondary antibodies were brought from Thermofisher Scientific until otherwise mentioned. All tissue culture reagents like culture media, serum etc. was brought from Hi-Media until otherwise mentioned. All other chemicals and salts were purchased from Sigma-Aldrich until otherwise mentioned.

Animal studies. All mouse studies described in the chapter received formal approval from the Indian Institute of Science Education and Research, Pune-Institutional Animal Ethics Committee (IISER-P IAEC), under the guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All mice were maintained at National Centre for Gene Function in Health and Disease. All mice used in the study were generated by breeding either ABHD12 heterozygous (het) mice or ABHD16A het mice and had ad libitum access to water and food.

Timed pregnancy. Timed pregnancies were kept as described by the Jackson Laboratory. Briefly, 8-15 week old male and non-virgin females of WT, or ABHD12 KO genotype with synchronized estrous cycle were kept for breeding overnight. Next morning, vaginal plugs were monitored for females and if vaginal plug was seen, the females were isolated and considered to have mated. Pregnant females were sacrificed and embryos were isolated at E18 stages. Primary neurons were isolated as mentioned in the primary neuron culture section.

Genotyping. ABHD16A mice were genotyped using the following primers: - 5'-GGCCAGCCTGAGTTCCATAG-3', 5'-CCCGGCTTTATTGTTTAACTACC-3', and 5'-TCGTGGTATCGTTATGCGCC-3'. ABHD12 mice were genotyped using the following primers: 5'-GGTGCCCAGTGAATGGCC-3', 5'-CAGTGCTGGCCTGTCAGTCG-3', 5'-TAAAGCGCATGCTCCAGACTGCC-3'. Tails (1-2 mm) were clipped from the pups at weaning stage and tail clips were digested in digestion buffer constituting 0.5 M EDTA, 5 M NaCl, 1 M Tris, pH 7.4 and 10% (w/v) SDS supplemented with 1 mg/mL of Proteinase K at 55°C for 6 hours. Thereafter, digestion reaction mixture was spun down at 21000 g for 15 mins at 4 °C. Supernatant was collected and mixed with 0.7 (v/v) volumes of isopropanol to precipitate nucleic acids. The resulting mixture was spun at 21000 g for 15 mins at 4 °C and supernatant was discarded. Pellet was re-suspended for washing in 70% (v/v) ethanol and resulting mixture was spun again for at 21000 g for 30 mins at 4°C. Supernatant was decanted and nucleic acid pellet was dried at room temperature by inverting the tubes. The dried nucleic acid pellet was re-suspended in MilliQ water and used for genotyping PCR.

Poly-D-Lysine coating of glass slides. 0.1% (w/v) solution of sterile Poly-D-lysine hydrobromide in MQ was prepared. Slides were cleaned with 0.1% HCl and 70% ethanol, dried and were incubated in the diluted Poly-D-lysine solution for 15 mins at room

temperature (RT) under aseptic conditions. Thereafter, slides were dried in the incubator at 37 °C for 2 hours. Slides were stored at 4 °C until used for plating primary neurons.

Neuro-2a cell culture. Cells were cultured in DMEM (HiMedia) with 10% (v/v) Fetal Bovine Serum (Thermo Fischer Scientific) and 1X penicillin-streptomycin (MP Biomedicals) at 37 °C, 5% (v/v) CO₂. Cells were counted using trypan blue method on a BTC20 automated cell counter (Bio-Rad) as per as manufacturer's instructions. Cells were seeded in 35 mm tissue culture dish at 3×10^6 and cultured for 24 h, washed with sterile *Dulbecco's phosphate-buffered saline (DPBS)* (3x) and treated with Lyso-PS 18:0 as described in the Lyso-PS treatment section.

Primary neuronal culture. Cerebellum was dissected out of E18 embryos and minced in *Dulbecco's Modified Eagle Media (DMEM)* supplemented with 10% (v/v) heat inactivated fetal bovine serum (HI-FBS) and 1X antibiotic-antimycotic solution. Minced tissue was trypsinized with 0.25% (w/v) Trypsin for 10 mins over ice and with DNase (0.1 mg/ml) for 5 mins over ice. Cells were pelleted down by spinning at 300 g for 3 mins and washed with Hank's Balanced Salt Solution (2X). Cells were re-suspended in DMEM media supplemented with 10% (v/v) HI-FBS and 1X anti-anti solution. Thereafter, re-suspended cells were plated on the Poly-D-lysine coated 18 mm coverslips kept in 6-well plates. Cells were cultured for 3 h to allow the cells to adhere and thereafter, media was replaced with Neurobasal™ Medium supplemented with 1X antibiotic-antimycotic, 1X B-27, 1X Glutamine at 37°C with 5% (v/v) CO₂. Cells were grown for 14 days and media was changed every alternate day.

Lyso-PS treatment to cells. Neuro-2a cells or primary cerebellar neurons were grown in Poly-D-lysine coated 6 well culture plate at 37 °C. Cells were washed with serum free media warmed at 37 °C – DMEM for Neuro-2a cells and Neurobasal for primary cerebellar neurons. Lyso-PS at 10 µM final concentration was re-suspended in respective serum free culture media. Mixture was water bath sonicated to get a homogenous suspension at 37 °C. Mixture was added at the rate of 1 mL per well and incubated at 37 °C, 5% (v/v) CO₂ for several time points. After incubation, cells were washed with DPBS supplemented with phosphatase inhibitors i.e. 1 mM of phosphatase inhibitors sodium pyrophosphate, sodium orthovanadate, sodium fluoride, β-glycerophosphate each. Thereafter, cells were either scrapped in DPBS supplemented with above mentioned phosphatase inhibitors or fixed with 4% (w/v) paraformaldehyde for 15 mins at room temperature. Scrapped cells were stored at -80 °C until

further use. Fixed cells were washed again with DPBS (3X) and processed for staining as described under cellular immunofluorescence studies.

Phosphoblot analysis. Cells were washed and scraped in DPBS supplemented with phosphatase inhibitors (1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM β -glycerophosphate). Cells were lysed on ice using lysis buffer constituting 1 mM of sodium pyrophosphate, sodium orthovanadate, sodium fluoride, β -glycerophosphate and 1% (v/v) Triton X-100. Proteome was denatured by boiling it in 4X SDS loading dye and separated over 10% SDS-PAGE gel. Thereafter, it was processed for western blot as described under western blot section.

Western blot analysis. Denatured proteome (40-50 μ g) was resolved over 10% SDS-PAGE gel and transferred to methanol activated PVDF membrane (GE Healthcare) at 60V for 12 h at 4 °C. Post-transfer, membrane was blocked with 5% (w/v) BSA in DPBS (pH 7.4) containing 0.1% (w/v) Tween 20 (DPBST), and subsequently incubated with primary antibody (1:1000 dilution in 5% (w/v) BSA in DPBST) overnight at 4 °C. Membrane was washed with DPBST (5X) at 25 °C and incubated with secondary antibody (1:10,000 dilution in 5% (w/v) BSA in DPBST) for 90 mins at 25 °C. Thereafter, membrane was washed in DPBST (5x) at 25 °C and developed using Thermo West Pico western blotting substrate (ThermoFisher Scientific) and visualized using Syngene G-Box Chemi-XRQ gel documentation system. Primary antibodies used were: anti-pERK1/2 [Rabbit, CST – 4376S, 1:400 dilution], anti-ERK1/2 [Rabbit, CST – 4695S, 1:400 dilution]. Secondary antibodies used in the study were HRP-conjugated anti-Rabbit IgG (Goat, ThermoFisher Scientific, 31460).

Immunohistochemical analysis. Mice were deeply anaesthetized using isoflurane and subsequently perfused with chilled phosphate buffer saline (PBS) supplemented with phosphatase inhibitors (1 mM each of sodium pyrophosphate, sodium orthovanadate, sodium fluoride, β -glycerophosphate), followed by another perfusion with 4% (w/v) paraformaldehyde. Brains were dissected and left in 4% (w/v) paraformaldehyde overnight at 4°C and then transferred into 30% (w/v) sucrose in PBS until brains sank to bottom of the tubes (~ 3 days). Coronal 25 μ m sections were cut on a freezing microtome maintained at -30 °C, and sections were collected in ice-cold PBS. For 3, 3'-diaminobenzidine (DAB) based brightfield IHC, endogenous peroxidases were inactivated with 3% (v/v) hydrogen peroxide in

PBS for 15 min at 25 °C in dark. Thereafter the sections were washed twice with 1% (w/v) BSA in PBS and permeabilized with 0.1% (w/v) Triton X-100 and 0.5% (w/v) BSA in PBS for 45 mins at 25 °C. The sections were then incubated with primary antibody in 1% (w/v) BSA overnight at 4 °C. Following overnight incubation, the sections were washed three times with 0.5% (w/v) BSA in PBS and incubated with secondary antibody, biotinylated goat-anti mouse [Vector labs, BP-9200] in 0.5% (w/v) BSA in PBS for 1 h at RT. Following this treatment, the sections were washed twice in 0.5 % (w/v) BSA in PBS, incubated with ABC Elite Vectastatin (Vector labs, PK-6100) for 1 h at RT and subsequently washed twice with excess PBS. Finally, the sections were stained with ImmPACT™ DAB (Vector labs, SK-4105) for 3 min in dark, transferred to excess PBS and mounted with VectaMount (Vector labs, H-5000). For confocal laser scanning based IHC, collected sections were directly permeabilized with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA in PBS for 45 mins at 25 °C and block with 1% (w/v) BSA in PBS for 1 h at 25 °C. Thereafter, sections were washed with 0.5% (w/v) BSA in PBS (3X) and incubated with primary antibody for 16 h at 25 °C. Sections were washed in 0.5% (w/v) BSA in PBS (3X) and incubated with secondary antibody (1:1000 dilution) for 60 mins at 25 °C. Sections were washed in excess PBS and mounted with Fluoromount™ aqueous mounting medium (Sigma Aldrich). Slides were imaged on Leica SP8 confocal laser scanning microscope. Primary antibodies used in the study were anti-calbindin [Mouse, abcam – ab82812, 1:50 dilution], anti-VGLUT2 [Rabbit, CST – 14487, 1:1000 dilution], anti-Gephyrin [Rabbit, Abcam – ab322206, 1:1000 dilution], anti-pERK1/2 [Rabbit, CST – 4376S, 1:400 dilution], anti-ERK1/2 [Rabbit, CST – 4695S, 1:400 dilution], anti-Sec63 (Mouse, Abcam, anti-ATP5A (Mouse, Abcam, ab14748, mitochondrial marker), anti-PMP70 (Mouse, Sigma-Aldrich, SAB4200181), anti-GM130 (Mouse, Abcam, ab169276). Secondary antibodies used in the study were Goat anti-rabbit IgG (H&L) conjugated with DyLight 488 (Thermofisher Scientific, 35553) and goat anti-mouse IgG (H&L) conjugated with Alexa Fluor 568 (Thermofisher Scientific, A-11004).

Cellular immunofluorescence studies. Fixed primary cerebellar neurons or Neuro-2a on glass cover slips were washed with sterile DPBS (4x), permeabilized with 0.5% (v/v) Triton X-100 in DPBS containing 5% bovine serum albumin (BSA) for 30 mins at RT. Permeabilized cells were blocked with 5% (w/v) BSA in DPBS for 45 mins at RT and incubated with primary antibody (1:100) for 90 mins at 25 °C. Cells were washed in DPBS by gentle swirling (10X) and incubated with secondary antibody (1:1000) for 60 min at 25 °C. Cells were further

washed in DPBS by gentle swirling (10X). Coverslips were mounted on glass slides with Fluoromount™ aqueous mounting medium (Sigma Aldrich). Slides were dried at 25 °C overnight in dark and imaged using Leica SP8 Confocal Laser Scanning Microscope. All microscopy images were analyzed using ImageJ 1.50i software (NIH, for Windows OS). Primary antibodies used were: anti-pERK1/2 [Rabbit, CST – 4376S, 1:400 dilution], anti-tERK1/2 [Rabbit, CST – 4695S, 1:400 dilution]. Goat anti-rabbit IgG (H&L) conjugated with DyLight 488 (Thermofisher Scientific, 35553), or goat anti-mouse IgG (H&L) conjugated with Alexa Fluor 568 (Thermofisher Scientific, A-11004) were used as secondary antibodies.

Results

Lyso-PS induces phosphorylation of ERK1/2 in Neuro-2a cells. To test whether lyso-PS induces activation of phosphorylation pathways in neurons, I used secondary neuronal cell line Neuro-2a as a model neuronal cell line. Lyso-PS 18:0 is the most abundant lyso-PS in the brain; hence I used lyso-PS 18:0 as a model lyso-PS to screen for phosphorylation pathways. I found that lyso-PS 18:0 treatment to Neuro-2a cell lines inducing phosphorylation of ERK1/2 (pERK1/2) within 15-mins and phosphorylation state was maintained till 30 mins (**Fig. 5.1A**), whereas corresponding fatty acid control (FFA 18:0) didn't induce any phosphorylation of ERK1/2 (**Fig. 5.1B**), suggesting that ERK1/2 phosphorylation is indeed induced by lyso-PS 18:0 not by degraded free fatty acid product of the lyso-PS. Since, lyso-PS is not a individual molecule, instead it is a library of naturally existing lipids, I screened synthetic lyso-PS library with different fatty acids appended to them and I found that long chain lyso-PS (i.e. lyso-PS 16:0, 18:0, 20:0) were most potent in inducing phosphorylation of ERK1/2 in Neuro-2a cells (**Fig. 5.1C**) compared to mid chain lyso-PS (i.e. lyso-PS 10:0, 12:0, 14:0) or very long chain lyso-PS (lyso-PS 22:0, 24:0) in similar time points tested (up to 45 mins). This observation is interesting to the fact pointed before i.e. lyso-PS 18:0 is the most abundant lyso-PS in the brain. I also looked for changes in other phosphorylation pathways like phospho-tyrosine and phospho-Akt1, etc. but lyso-PS treatments didn't bring any change in the phosphorylation of tyrosine or Akt1 in Neuro-2a cells (Data not shown). Next, I examined for any morphological changes in Neuro-2a cells on lyso-PS 18:0 treatments. There were no morphological changes in Neuro-2a cells following lyso-PS treatment for 15-30 mins (time same as used for phosphorylation reactions) (**Fig. 5.1D**). Time points to measure phosphorylation changes in the lyso-PS 18:0 might have been too small to induce any morphological changes. So, I treated Neuro-2a cells with lyso-PS 18:0 for 6-12

to look for any morphological changes, but with longer time points as well I didn't not find any significant change in cellular morphology (**Fig. 5.1E**).

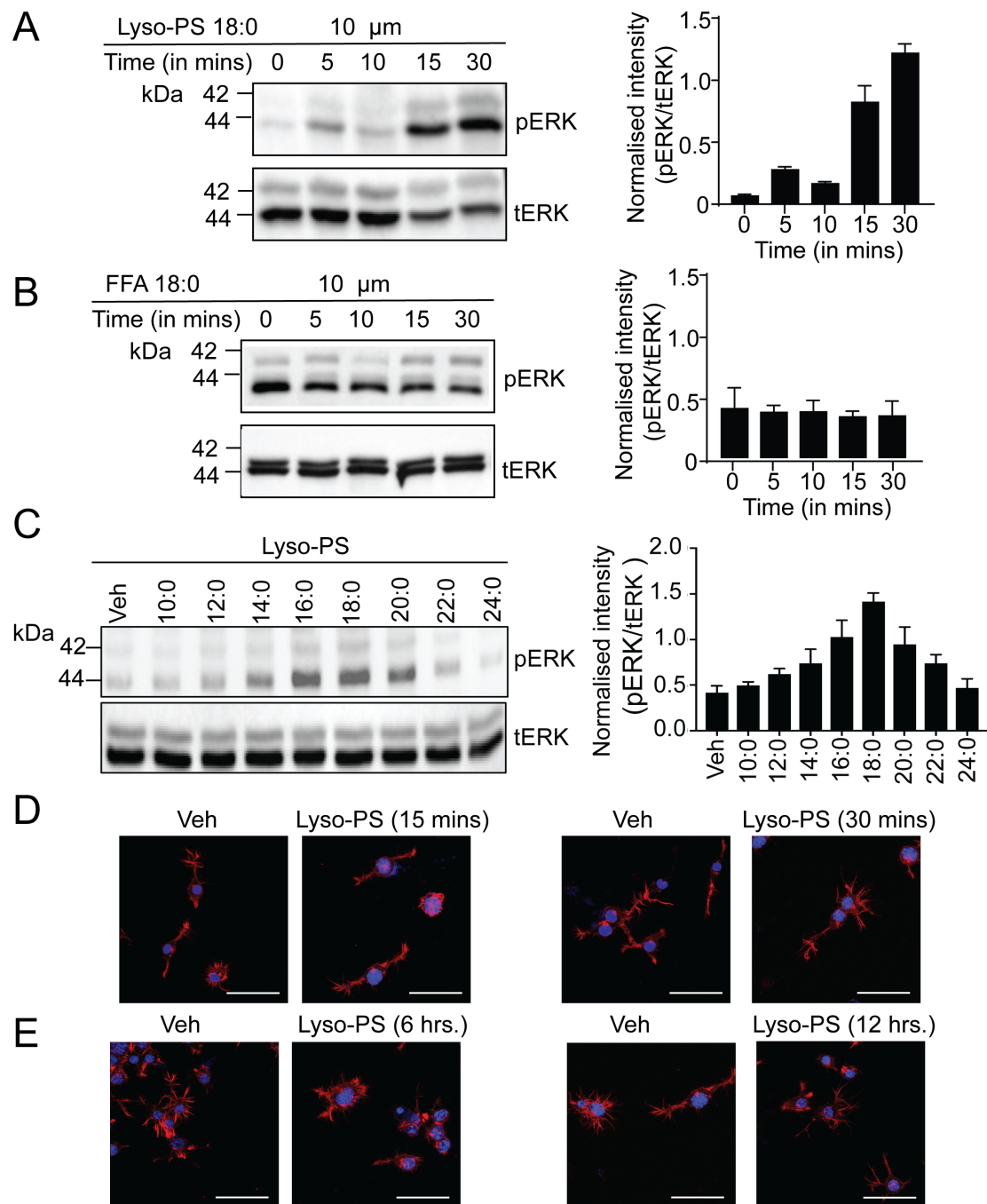


Fig. 5.1: Lyso-PS treatment induces ERK1/2 phosphorylation in Neuro-2a cells. A – Lyso-PS 18:0 induced ERK1/2 phosphorylation in Neuro-2a cells. B – Free fatty acid 18:0 did not induce any visible ERK1/2 phosphorylation in Neuro-2a cells. C – Representative blot showing long chain lyso-PS were more potent in inducing ERK1/2 phosphorylation than mid

chain (lyso-PS 10:0, 12:0, 14:0) or very long chain lyso-PS (lyso-PS 22:0, and 24:0). D and E – Lyso-PS treatment for 15-20 mins (D) or 6-12 h (E) does not bring any morphological. All plotted values on y-axis are ration of phospho-ERK (pERK1/2) to total ERK (tERK1/2). Data plotted as mean $\pm$ standard deviation. Scale bar = 50 μ m.

ABHD12 inhibition induces phosphorylation of ERK1/2 in Neuro-2a cells. Since, ABHD16A and ABHD12 are the major lyso-PS metabolic enzymes in neuronal cells. Next, I tested if inhibition of ABHD12 can induce ERK1/2 phosphorylation in Neuro-2a cells. I used small molecule inhibitor of ABHD12 (namely JJH350) to block ABHD12 activity in situ in the Neuro-2a cells. JJH350 treatment for 6 h elevated pERK1/2 levels in Neuro-2a cells. Next, I inhibited ABHD16A using KC01 inhibitor but ABHD16A inhibition did produce any effect on pERK1/2 levels (**Fig. 5.2A**). This suggests that ABHD16A-ABHD12 regulated lyso-PS levels induce ERK1/2 phosphorylation in Neuro-2a cells but there were no phenotypic or morphological changes in Neuro-2a cells on ABHD12 or ABHD16A inhibition (**Fig. 5.2B**).

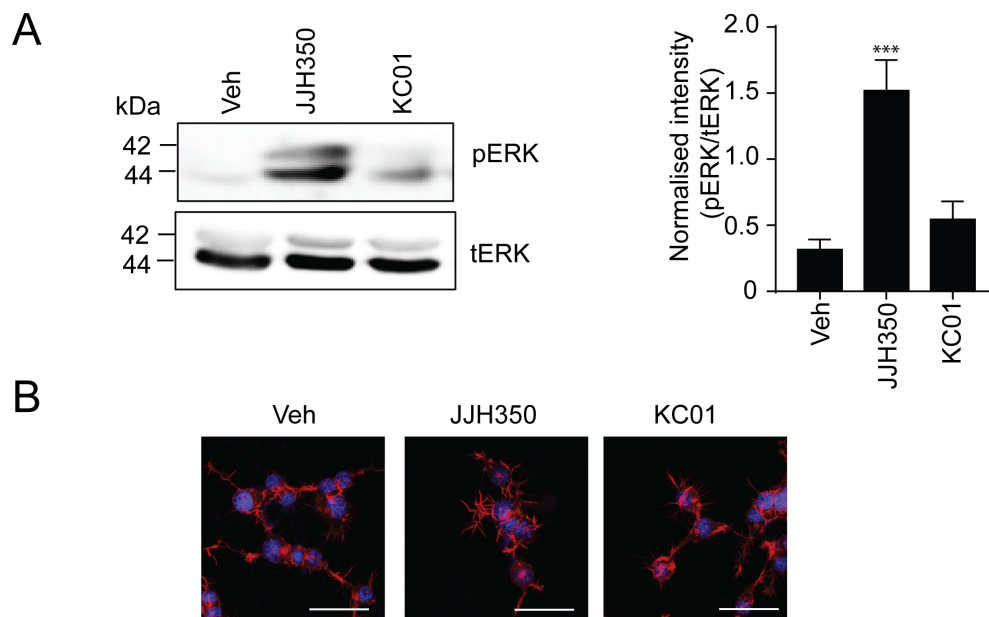


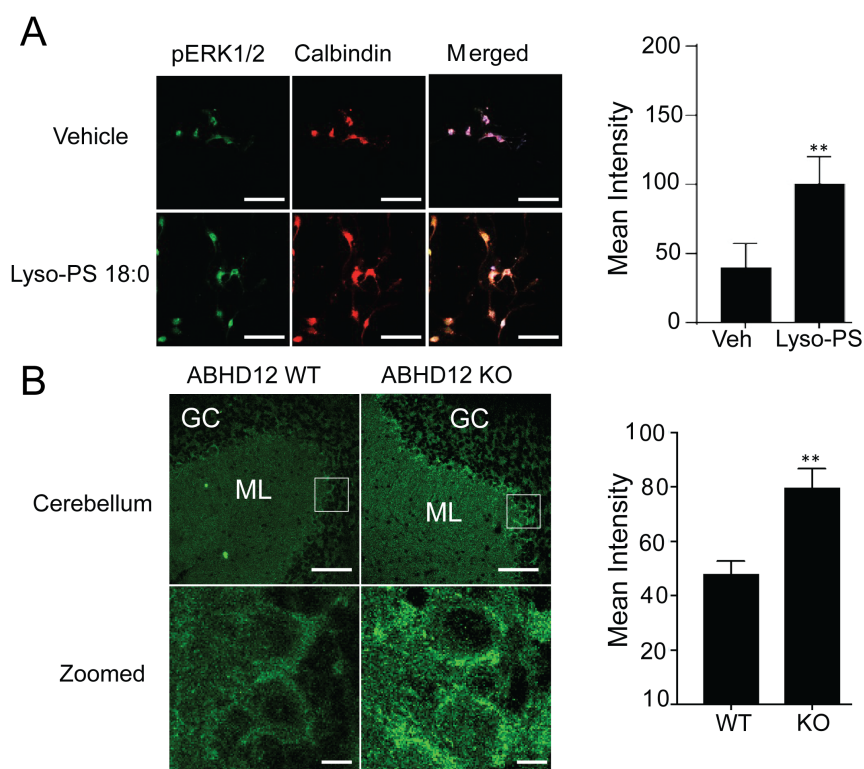
Fig.5.2: Pharmacological inhibition of ABHD16A and ABHD12 in Neuro-2a cells. A– Western blots and its quantification showing ABHD12 inhibition (using JJH350) leads to elevated phosphorylation of ERK1/2 in the Neuro-2a cells. ABHD16A inhibition does not bring any change in the phosphorylated ERK1/2 levels. B– ABHD12 or ABHD16A inhibition do not bring any change in the morphology of the Neuro-2a cells. All plotted values on y-axis

are ratio of pERK1/2 to tERK1/2. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value = 0.0001 (pERK1/2 Vehicle vs JJH350 treatment). Scale bar = 50 μ m.

Lyso-PS induced phosphorylation of ERK1/2 in Purkinje cells. Having confirmed that lyso-PS 18:0 can induce ERK1/2 phosphorylation in neuronal cells. I tested if lyso-PS can induce ERK1/2 phosphorylation in Purkinje neurons of the mammalian brain. To start with, I used primary cerebellar neurons isolated from E18 embryos and treated them with lyso-PS 18:0. Since, cerebellar primary neuronal culture include neurons other than Purkinje neurons, I couldn't pick any change in pERK1/2 levels using western blot (data not shown). I used quantitative confocal microscopy to look for phosphorylation state of ERK1/2 in the Purkinje neurons. I found that lyso-PS 18:0 increased phosphorylated ERK1/2 levels in the Purkinje neurons within 15 mins (**Fig. 5.3A**). Next, I questioned if ABHD12 KO mice (which has basal lyso-PS accumulation in the brain) show any changes in ERK1/2 phosphorylation in the Purkinje neurons. I perfused mice with saline supplemented with phosphatase inhibitors and stained the cerebellar region for pERK1/2. Purkinje neurons of ABHD12 KO mice had elevated levels of pERK1/2 compared to their WT littermates (**Fig. 5.3B**). This confirmed that granule neurons and Purkinje neurons establish some communication using lyso-PS and phosphorylation pathways.

Purkinje neurons do not show any structural defects on loss of ABHD16A or ABHD12 activity. Having confirmed that ABHD16A synthesized lyso-PS acts on Purkinje neurons and activates pERK pathways in a paracrine manner. I wanted to check if ABHD12 or ABHD16A deletion causes any structural changes in Purkinje neurons e.g. dendritic sprouting, number of Purkinje neurons or number of synaptic buttons. I probed Purkinje neurons in DAB-based fluorescent (2-D) microscopy as well as confocal based 3-D microscopy. I didn't find any structural changes in the ABHD12 KO (**Fig. 5.4A and C**) or ABHD16A KO (**Fig. 5.4B and D**) Purkinje neurons of the adult mice. This makes us strongly believe that defective lyso-PS metabolism and signaling do not cause any changes in the structure of the Purkinje neurons.

Purkinje neurons and synaptic density as the function of lyso-PS. Lipids are known to modulate the synaptic strength of the neurons. I found that lyso-PS induces the phosphorylation of ERK1/2 on Purkinje neurons. There were no structural changes in the Purkinje neurons. Next, I probed for synaptic markers in the cerebellum, so as to check if



*Fig.5.3: ERK1/2 phosphorylation in Purkinje neurons of ABHD16A and ABHD12 KO brain. A– Lyso-PS 18:0 treatment causes ERK1/2 phosphorylation in primary Purkinje neurons. B– Purkinje neurons in ABHD12 KO mice brain slice show elevated levels of pERK1/2. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value = 0.0023 (pERK1/2 Vehicle vs. Lyso-PS 18:0) & *p*-value = 0.0022 (pERK1/2 WT vs. 12 KO). Scale bar (A, Zoomed B) = 50 μ m. Scale bar (B) = 100 μ m.*

Purkinje neurons show any defect in the synaptic density as a function of lyso-PS pathway. Any changes in the synaptic density will be a direct readout of the lyso-PS modulating the synaptic communication of Purkinje neurons. I probed for Purkinje neuron synaptic density using synaptic markers anti-VGLUT2 and anti-Gephyrin in adult ABHD16A KO, ABHD12 KO and WT mice brain. I didn't find any changes in expression level of anti-VGLUT2 (**Fig. 5.5A** and **C**) or anti-Gephyrin (**Fig. 5.5B** and **D**) in ABHD16A KO or ABHD12 KO mice compared to WT mice.

Next, I probed for organelles in the Purkinje neurons in the ABHD12 and ABHD16A KO background. This was to check if defective lyso-PS signaling brings about any changes in the organelles of the Purkinje neurons. I examined peroxisomes (anti-PMP70), Golgi body (anti-

GM130), endoplasmic reticulum (anti-Sec63) and mitochondria (anti-ATP5a) but didn't find any substantial change in their morphology.

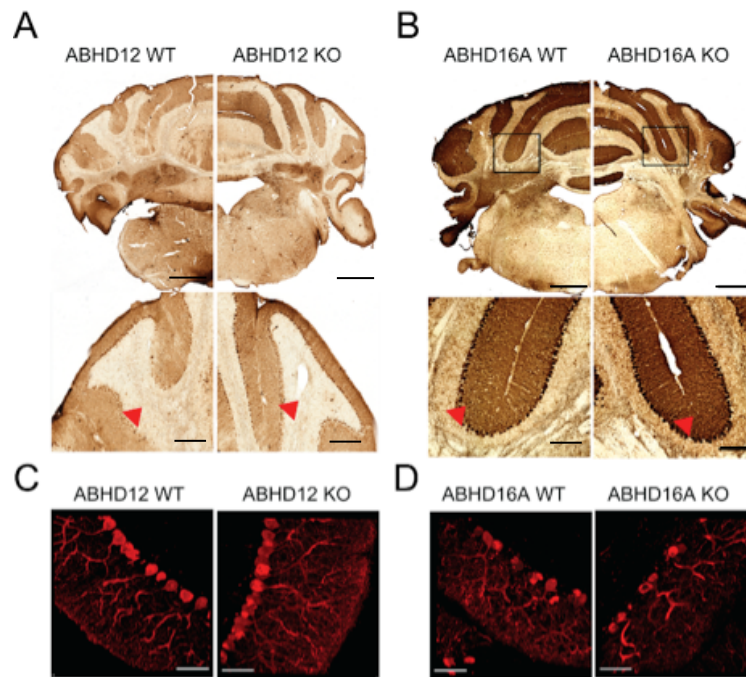


Fig.5.4: Purkinje neurons of ABHD16A and ABHD12 KO mice do not show any defects in the Purkinje neuron morphology. A and B – Representative image of DAB based IHC for Calbindin staining of Purkinje neurons in brain slices of ABHD16A KO (A) and ABHD12 KO mice (B). Representative image of fluorescent confocal microscopy based IHC for Calbindin staining of Purkinje neurons in brain slices of ABHD16A KO (C) and ABHD12 KO mice (D). Scale bar (A,B) = 1500 μ m. Scale bar (Zoomed A,B) = 500 μ m. Scale bar (C,D) = 50 μ m.

Discussion

Lipids and their derivatives actively modulate the neuron-neuron communication and glial-neuron communication in the brain. One such lipid under investigation here was lyso-PS. It is synthesized by ABHD16A and degraded by ABHD12 in the mammalian brain. My comprehensive localization studies have robustly established that ABHD16A is enriched in the granule neurons of the cerebellum, whereas, ABHD12 is enriched on the Purkinje neurons of the cerebellum (Chapter IV). It is established that granule neurons and Purkinje neurons communicate with each other directly through synapses as well as through paracrine

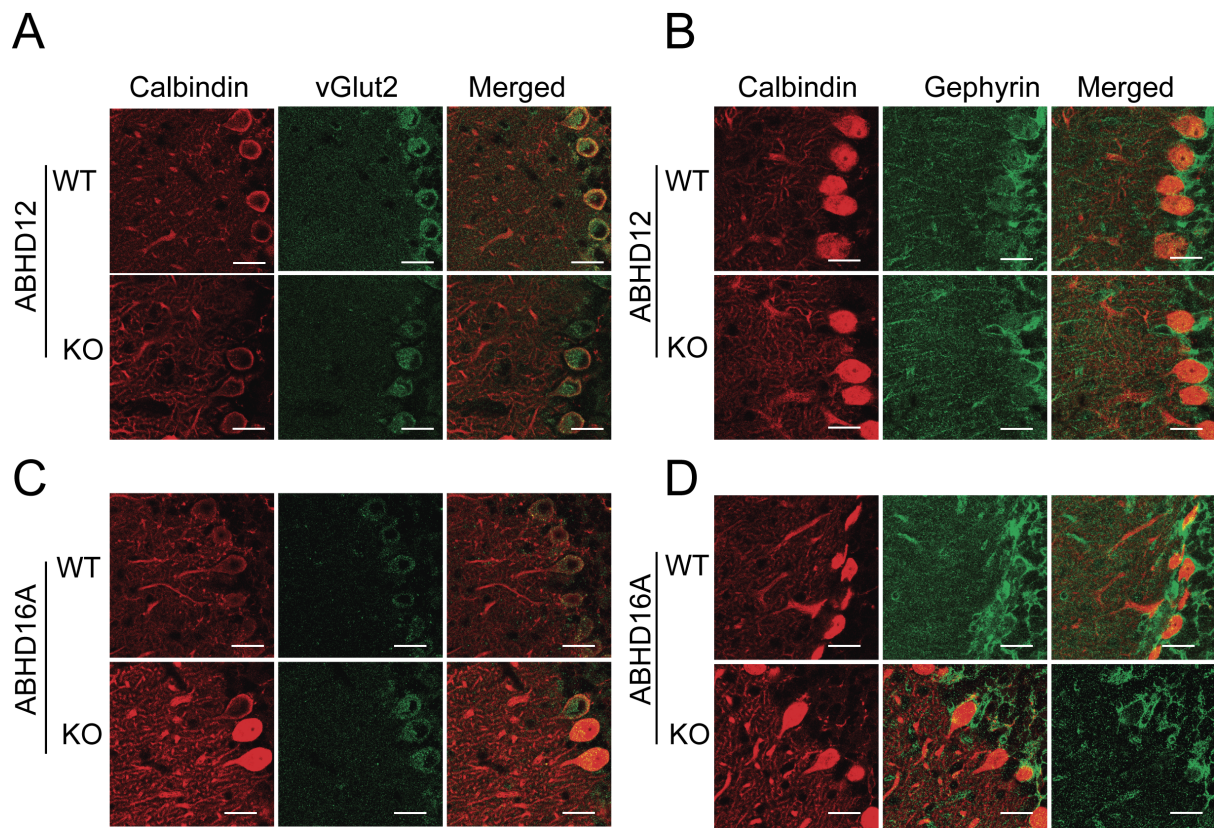


Fig.5.5: Purkinje neurons do not show any defects in synaptic density of Purkinje neurons in cerebellum. A – Synapses as stained by anti-vGlut2 antibody in ABHD12 WT and KO mice. B – Synapses as stained by anti-Gephyrin antibody in ABHD12 WT and KO mice. C – Synapses as stained by anti-vGlut2 antibody in ABHD16A WT and KO mice. D – Synapses as stained by anti-Gephyrin antibody in ABHD16A WT and KO mice. Scale bar = 50 μm.

signaling molecules⁷⁵. Lyso-PS is found to induce phosphorylation of ERK1/2 in a few cell lines⁷⁶. In order to check its effects on neurons, I treated secondary neuronal cell line Neuro-2a with lyso-PS 18:0 and I found that lyso-PS 18:0 induced phosphorylation of ERK1/2 in time dependent manner. I also found that long chain lyso-PS like lyso-PS 18:0 is more potent in inducing ERK1/2 phosphorylation compared to medium or very long chain lyso-PS. To make sure that ABHD16A-ABHD12 enzymes are the major enzymes that might call for this signaling in vitro, I used small molecule inhibitors for ABHD16A and ABHD12. ABHD16A inhibition didn't bring any visible changes in the phosphorylation of ERK1/2 whereas ABHD12 inhibition increased the phosphorylated ERK1/2 levels in the Neuro-2a cells. There were no morphological changes in the Neuro-2a cells following lyso-PS treatment. This

established that lyso-PS could act on neurons through some unannotated receptor, downstream of which phosphorylation of ERK1/2 takes place. Next, I wanted to test this in primary neurons, to make sure that these changes remain same in the brain tissue and they are not the artefacts of secondary cell lines. Since, ABHD16A and ABHD12 showed differential layer specific expression in the cerebellum, I hypothesized that granule neurons and Purkinje neurons of the cerebellum might cross talk using lyso-PS as a signaling molecule (**Fig. 5.6**).

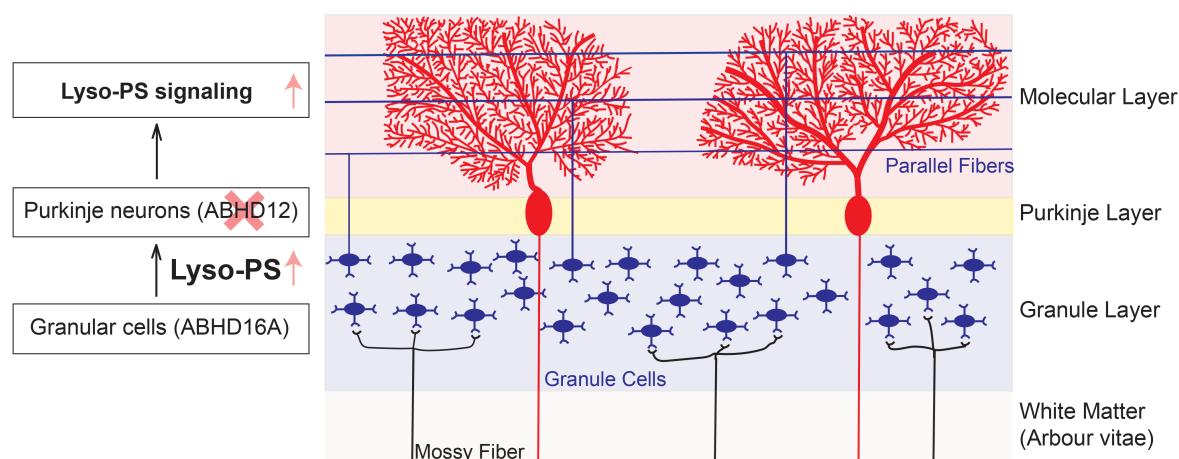


Fig.5.6: Paracrine signaling model for lyso-PS signaling in the cerebellum regulated by ABHD16A and ABHD12 enzymatic activities.

I isolated primary cerebellar neurons from the E18 embryos and treated them with lyso-PS 18:0. As speculated, lyso-PS 18:0 did increase phosphorylated ERK1/2 levels in the Purkinje neurons. Next, I checked for ERK1/2 phosphorylation in Purkinje neurons of ABHD12 KO mice where lyso-PS is basally accumulated several fold. There was an increase in ERK1/2 phosphorylation basally in Purkinje neurons of ABHD12 KO mice. Next, interesting question worth pursuing would be which receptor on Purkinje neuron is involved in lyso-PS signaling. Lyso-PS has several annotated G-protein coupled receptors like GPR34, P2Y10, GPR174, etc^{43,77,78}. It would be interesting to find their expression on Purkinje neurons and test if they act as receptors for lyso-PS in the Purkinje neurons. I also tested for the synaptic density and structure of Purkinje neurons in the adult ABHD16A KO and ABHD12 KO mice; I could not find any changes in the synaptic density or Purkinje neuron morphology. But since, it is established that lyso-PS do act as signal on the Purkinje neurons, it would be great to measure the electrophysiological properties of the Purkinje neuron as a function of lyso-PS. It would be a promising, most accurate and direct readout of what lyso-PS does to the Purkinje neuron

physiology and how it fits in the broad cerebellar physiology and behavioral output. I couldn't make it work due to lack of technical expertise and it remains an interesting aspect of lyso-PS signaling in cerebellum worth exploring.

Conclusion

My work described in this chapter provides first evidence of lyso-PS signaling on neurons in the central nervous system. Whereby, lyso-PS acts as a paracrine signaling molecule in the cerebellum and facilitates the communication between granule and Purkinje neurons of the cerebellar region of the mammalian brain. It seems from my results that lyso-PS doesn't cause any morphological changes in the neurons. Further, work is needed to establish the exact role of lyso-PS in the Purkinje neuron physiology and how it regulates the behavioral output from the cerebellum. In future studies, electrophysiology will be the gold standard tool to start with in order to establish the role of lyso-PS in the neurophysiology followed by behavioral screens.

CHAPTER VI

ABHD16A – lyso-PS – ABHD12 biochemical axis modulate neuroimmunophysiology through microglia

ABHD12 KO mice – a mammalian model organism for PHARC syndrome accumulates lyso-PS in its brain, followed by aberrant microglial cell activation (neuro-inflammation) and sensory motor defects along with onset of ageing. This suggests an important role of ABHD12 in the innate immunity of the brain. Moreover, the molecular processes that connect lyso-PS signaling, microglial activation and compromise in sensory motor behaviors are not established. Here, I investigated the role of ABHD12 and ABHD16A in innate immune physiology by leveraging their individual knockout mice. On immune challenge with lipopolysaccharide (LPS), loss of ABHD12 activity elevated microglial phagocytosis possibly through ox-PS accumulation and signaling. I also probed the role of lyso-PS in the innate immunity of the mice brain and found that very long chain lyso-PS (i.e. lyso-PS 24:0) could activate microglial cells through TLR2 receptor while long chain lyso-PS (i.e. lyso-PS 18:0) failed to activate microglial cells. My studies here robustly establish that ABHD12 is critical for innate neuro-immunity and it keeps microglial cells (and their phagocytosis) in check by regulating lyso-PS and ox-PS in the brain.

Introduction

Lipids have been central to the immunological cascades in the mammalian physiology, acting as antigens, primary signaling molecules, secondary messengers and a plethora of structural roles in the form of lipid rafts, biological membranes, phase biochemistry etc. Lipases like monoacylglycerol lipase has been annotated as the major enzyme responsible for providing substrates for immune active components like prostaglandins in the brain and several lipid receptors like cannabinoid receptor 2 is extensively demonstrated to modulate the immunity of the mammalian brain. In addition to signaling roles, defects in endo-cellular recycling and endocytic trafficking of lipids too invite immunological reactions and inflammation thus participating in the immunophysiology of the brain^{79,80}. Among several signaling lipids of neuroimmunophysiology, one is lysophosphatidylserine (lyso-PS) known for its participation and modulation of several immunological reactions. I will discuss a few of them here to emphasize on the importance and established role of lyso-PS as an immune-modulator. One

of the first annotation of lyso-PS in immune physiology dates back to early 20th century, where it was reliably established that lyso-PS can trigger release of histamine from mast cells though there is some ambiguity in the receptors through which lyso-PS brings about histamine release. Lyso-PS enhances the clearance of neutrophils (activated or dying) through efferocytosis process mediated by macrophages^{12,81}. A few reports also suggest that lyso-PS triggers the anti-oxidant activity by enhancing superoxide dismutase activity⁸². It is also reported that lyso-PS brings about immune tolerance to some proteins like Factor VIII used in enzyme replacement therapy for hemophilia⁸³. It was also found that lyso-PS inhibits the activity of Treg cells through GPR174 and also reduces the differentiation of naïve T cells to Treg cells⁴³. Deficiency in GPR174 alleviates the severity of symptoms of experimental autoimmune encephalomyelitis – a mouse model of multiple sclerosis suggesting broader roles of lyso-PS in the neuro-immunity⁴³. All these reports reliably establish lyso-PS as a potent immune modulator in mammalian physiology.

It is established in the mammalian neuro and immune cells that lyso-PS is primarily regulated by two major serine hydrolase metabolic enzymes ABHD16A and ABHD12. ABHD16A synthesizes it by hydrolyzing PS and ABHD12 degrades it to glycerophosphoserine and free fatty acid to control the optimal level of lyso-PS^{17,22}. Its physiochemical properties allow it to robustly form micelles and act as detergents thus it can be assumed that unchecked levels of lyso-PS will be toxic to the cells until buffered by other proteins⁸⁴. Immune challenge to mammals with viruses (lymphocytic choriomeningitis virus) demonstrated that excessive lyso-PS in absence of ABHD12 makes the mice more susceptible to viral infections and most of ABHD12 KO mice infected with virus succumb to the infection within 2 week²¹. Lack of ABHD12 activity also leads to the lyso-PS accumulation in the mammalian brain, followed by the elevated activation of microglial cells and neuro-behavioral deficits post a year of age¹⁷. Loss of function mutation in ABHD12 enzyme is also associated with the PHARC syndrome – a genetically recessive neuro-immunological disorder characterized by degeneration of the cerebellum as one of the major central nervous pathology in humans¹⁹. It is currently believed that defective lyso-PS signaling is one of the major driving factors towards the PHARC pathology. But, what happens downstream of lyso-PS accumulation in the brain and how it triggers microglial cell activation is unclear? There are no insights on the relevance of lyso-PS signaling in the basal immune-physiology or on chronic/immune challenge.

Here, I investigated the role of ABHD16A-ABHD12 and lyso-PS in immunophysiology of the adult mice brain, especially microglial cells (brain macrophages). I report a new function of ABHD12 i.e. an oxidized PS (ox-PS) lipase activity, in addition to its role as lyso-PS lipase. On immune challenge, lack of ABHD12 leads to the accumulation of ox-PS in the mammalian brain and increased microglial cell phagocytosis. I failed to detect ox-PS accumulation basally in the ABHD12 KO mice. I tested if lyso-PS can activate microglial cells in vivo, I found that very long chain lyso-PS activates the microglial cell and cause neuro-inflammation in Toll like receptor 2 (TLR2) receptor dependent manner.

Materials and Methods

Materials. ABHD16A and ABHD12 transgenic (HET) breeders were provided by Prof. Benjamin F. Cravatt, The Scripps Research Institute. Prof. Balaji Narayanaswamy Kithiganahalli, Indian Institute of Science, Bangalore, supplied TLR2 KO mice used in the experiments described here. Mr. Minhaj Sheikh – Department of Chemistry, IISER-Pune and Dr. Amol Mhetre – Department of Biology, IISER-Pune jointly synthesized lyso-PS 18:0 and lyso-PS 24:0. All primary antibodies were bought from Abcam and all secondary antibodies were brought from Thermo Fisher Scientific until otherwise mentioned. DAB based bright field immunohistochemistry kit was brought from Vector Labs. All other chemicals and salts were purchased from Sigma-Aldrich until otherwise mentioned.

Animal studies. All mouse studies described in the chapter received formal approval from the Indian Institute of Science Education and Research, Pune-Institutional Animal Ethics Committee (IISER-P IAEC), under the guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. All mice were maintained at National Centre for Gene Function in Health and Disease. All mice used in the study were generated by breeding either ABHD12 heterozygous (het) mice or ABHD16A het mice and had ad libitum access to water and food.

Genotyping. ABHD16A mice were genotyped using the following primers: - 5'-GGCCAGCCTGAGTTCCATAG-3', 5'-CCCGGCTTTATTGTTTTAACTACC-3', and 5'-TCGTGGTATCGTTATGCGCC-3'. ABHD12 mice were genotyped using the following primers: 5'-GGTGCCCAAGTGAATGGCC-3', 5'-CAGTGCTGGCCTGTCAGTCG-3', 5'-TAAAGCGCATGCTCCAGACTGCC-3'. Tails (1-2 mm) were clipped from the pups at

weaning stage and tail clips were digested in digestion buffer constituting 0.5 M EDTA, 5 M NaCl, 1 M Tris, pH 7.4 and 10% (w/v) SDS supplemented with 1 mg/mL of Proteinase K at 55 °C for 6 hours (h). Thereafter, digestion reaction mixture was spun down at 21000 g for 15 mins at 4 °C. Supernatant was collected and mixed with 0.7 (v/v) volumes of isopropanol to precipitate nucleic acids. The resulting mixture was spun at 21000 g for 15 mins at 4 °C and supernatant was discarded. Pellet was re-suspended for washing in 70% (v/v) ethanol and resulting mixture was spun again for at 21000 g for 30 mins at 4 °C. Supernatant was decanted and nucleic acid pellet was dried at room temperature by inverting the tubes. The dried nucleic acid pellet was re-suspended in Milli-Q water and stored at 4 °C for genotyping PCR.

Western blot analysis. Denatured proteome (40-50 µg) was resolved over 10% SDS-PAGE gel and transferred to methanol activated PVDF membrane (GE Healthcare) at 60 V for 12 h at 4 °C. Post-transfer, membrane was blocked with 5% (w/v) milk in Dulbecco's phosphate-buffered saline (DPBS), pH 7.4 containing 0.1% (v/v) Tween 20 (DPBST), and subsequently incubated with primary antibody (1:1000 dilution in 5% (w/v) milk in DPBST) overnight at 4 °C. Membrane was washed with DPBST (5X) at 25 °C and incubated with secondary antibody (1:10,000 dilution in 5% (w/v) milk in DPBST) for 90 mins at 25 °C. Thereafter, membrane was washed in DPBST (5X) at 25 °C and developed using Thermo West Pico western blotting substrate (Thermofisher Scientific) and visualized using Syngene G-Box Chemi-XRQ gel documentation system. Primary antibodies used were: anti-ABHD16A (Rabbit, Abcam, 185549), anti-ABHD12 (Rabbit, Abcam, 182011), and anti-Tubulin (Rabbit, Cloud-Clone Corp, CAB870Hu01). Secondary antibodies used in the study were HRP-conjugated anti-Rabbit IgG (Goat, Thermofisher Scientific, 31460), or HRP-linked anti-Mouse IgG (Goat, Cloud Clone Corp, SAA544Mu19).

Immunohistochemical analysis. Mice were deeply anaesthetized using isoflurane and subsequently perfused with chilled phosphate buffer saline (PBS) followed by 4% (w/v) paraformaldehyde. Brains were dissected and left in 4% (w/v) paraformaldehyde overnight at 4 °C and then transferred into 30% (w/v) sucrose in PBS until brains sank to bottom of the tubes (~ 3 days). Coronal 25 µm sections were cut on a freezing microtome maintained at -30 °C, and sections were collected in ice-cold PBS. For 3, 3'-diaminobenzidine (DAB) based brightfield IHC, endogenous peroxidases were inactivated with 3% (v/v) hydrogen peroxide in PBS for 15 min at 25 °C in dark. Thereafter the sections were washed twice with 1% (w/v)

BSA in PBS and permeabilized with 0.1% (w/v) Triton X-100 and 0.5% (w/v) BSA in PBS for 45 mins at 25 °C. The sections were then incubated with primary antibody in 1% (w/v) BSA overnight at 4 °C. Following overnight incubation, the sections were washed three times with 0.5% (w/v) BSA in PBS and incubated with secondary antibody, biotinylated horse-anti rabbit [Vector labs, BP-1100] or biotinylated goat-anti mouse [Vector labs, BP-9200] in 0.5% (w/v) BSA in PBS for 1 h at RT. Following this treatment, the sections were washed twice in 0.5 % (w/v) BSA in PBS, incubated with ABC Elite Vectastatin (Vector labs, PK-6100) for 1 h at RT and subsequently washed twice with excess PBS. Finally, the sections were stained with ImmPACT DAB (Vector labs, SK-4105) for 3 min in dark, transferred to excess PBS and mounted with VectaMount (Vector labs, H-5000). For confocal laser scanning based IHC, collected sections were directly permeabilized with 0.1% (v/v) Triton X-100 and 0.5% (w/v) BSA in PBS for 45 mins at 25 °C and block with 1% (w/v) BSA in PBS for 1 h at 25 °C. Thereafter, sections were washed with 0.5% (w/v) BSA in PBS (3X) and incubated with primary antibody for 16 h at 25 °C. Sections were washed in 0.5% (w/v) BSA in PBS (3X) and incubated with secondary antibody (1:1000 dilution) for 60 mins at 25 °C. Sections were washed in excess PBS and mounted with FluoromountTM aqueous mounting medium (Sigma Aldrich). Slides were imaged as described for cellular immunofluorescence studies. Primary antibodies used in the study were anti-ABHD12 [Rabbit, abcam – ab182011, 1:100 dilution], anti-Iba1 [Mouse, abcam – ab178846, 1:1000 dilution], anti-Iba1 [Mouse, CST – 58970S, 1:250 dilution], anti-LAMP1 [Rabbit, abcam – ab24170, 1:1000 dilution]. Secondary antibodies used in the study were Goat anti-rabbit IgG (H&L) conjugated with DyLight 488 (Thermofisher Scientific, 35553) and goat anti-mouse IgG (H&L) conjugated with Alexa Fluor 568 (Thermofisher Scientific, A-11004).

Quantitative lipid measurement. Mice were anesthetized with isoflurane and subsequently sacrificed with cervical dislocation. Thereafter, brain was micro dissected within half a minute and flash frozen in liquid nitrogen. Lipids and prostaglandins were extracted using Folch lipid extraction method. Briefly, dissected regions were washed with chilled DPBS (3X) and homogenized in 6 mL of chilled 2:1:1 (vol/vol/vol) CHCl₃/MeOH/DPBS with 1 nmol of internal standard (17:1 FFA and 17:1 lyso-PS). The mixture was centrifuged at 2,800 g for 15 mins and bottom organic layer was collected and the aqueous phase was acidified with 50 µL of formic acid. Acidified aqueous phase was vortexed, volume was adjusted to 6 mL with CHCl₃ and centrifuged at 2,800 g for 15 mins and bottom organic layer was

collected. Organic extracts from both the steps were pooled and dried under inert N₂-stream gas. Dried lipid extract was re-suspended in 200 µL of 2:1 (v/v) CHCl₃/MeOH and 20 µL of it was injected for targeted LC-MS/MS analysis. All lipids were quantified with established multiple reaction monitoring high resolution (MRM-HR) method on Sciex X500R QTOF instrument coupled to an Exion-series UPLC with quaternary pump. Data collected over the instrument was analyzed using SciexOS software. MS parameters were as follows: - ESI mode of ionization, curtain gas 1 = 60, curtain gas 2 = 40, ion spray voltage = -5,500 V or +4,500 V, temperature = 550°C. LC gradient for separation of lipids was as previously described. All quantifications were done by calculating area under the curve and normalizing it to the area under the curve for respective internal standard and weight of the tissue or amount of protein as applicable.

Results

ABHD16A–ABHD12–lyso-PS modulate microglial cell activation basally and on immune challenge. It was previously reported that loss of ABHD12 activity leads to the lyso-PS accumulation succeeding which microglial cells are activated especially in the cerebellum. My localization and lipidomics experiments (described in chapter IV) too suggest some crucial physiological role of lyso-PS pathway in the mammalian cerebellum. Here, I leveraged ABHD16A KO and ABHD12 KO mice along with their WT littermates to investigate the role of lyso-PS in the microglial cell physiology. First, I probed for the microglial cells (using anti-Iba1 antibody); I didn't find any morphological changes in microglial cells of mice <5-6 months of different genotypes (i.e. WT, ABHD12 KO, ABHD16A KO mice). Microglial cells in ABHD12 KO mice >1 year old showed significant elevation in their activation state (i.e. soma size >200 µm²), whereas ABHD16A KO mice didn't show any change in their microglial cell morphology across their life span (**Fig. 6.1A**). It suggested some role of ABHD12 and lyso-PS in the neuro-immunophysiology. To investigate any such role, I challenged mice of different genotype with acute LPS treatment paradigm (10 mg/kg body weight for 6 hours) and looked at their microglial cell morphology. On acute immune challenge, cerebellum of ABHD12KO mice had slightly more number of activated microglial cells compared to their WT controls but this increase failed to reach statistical significance (**Fig. 6.1B** and **Fig. 6.1C**). ABHD16A KO mice didn't show any differences in the number of activated microglial cells as compared to control WT littermates (**Fig. 6.1B** and **Fig. 6.1C**). On acute immune challenge, microglial cells of ABHD12 KO

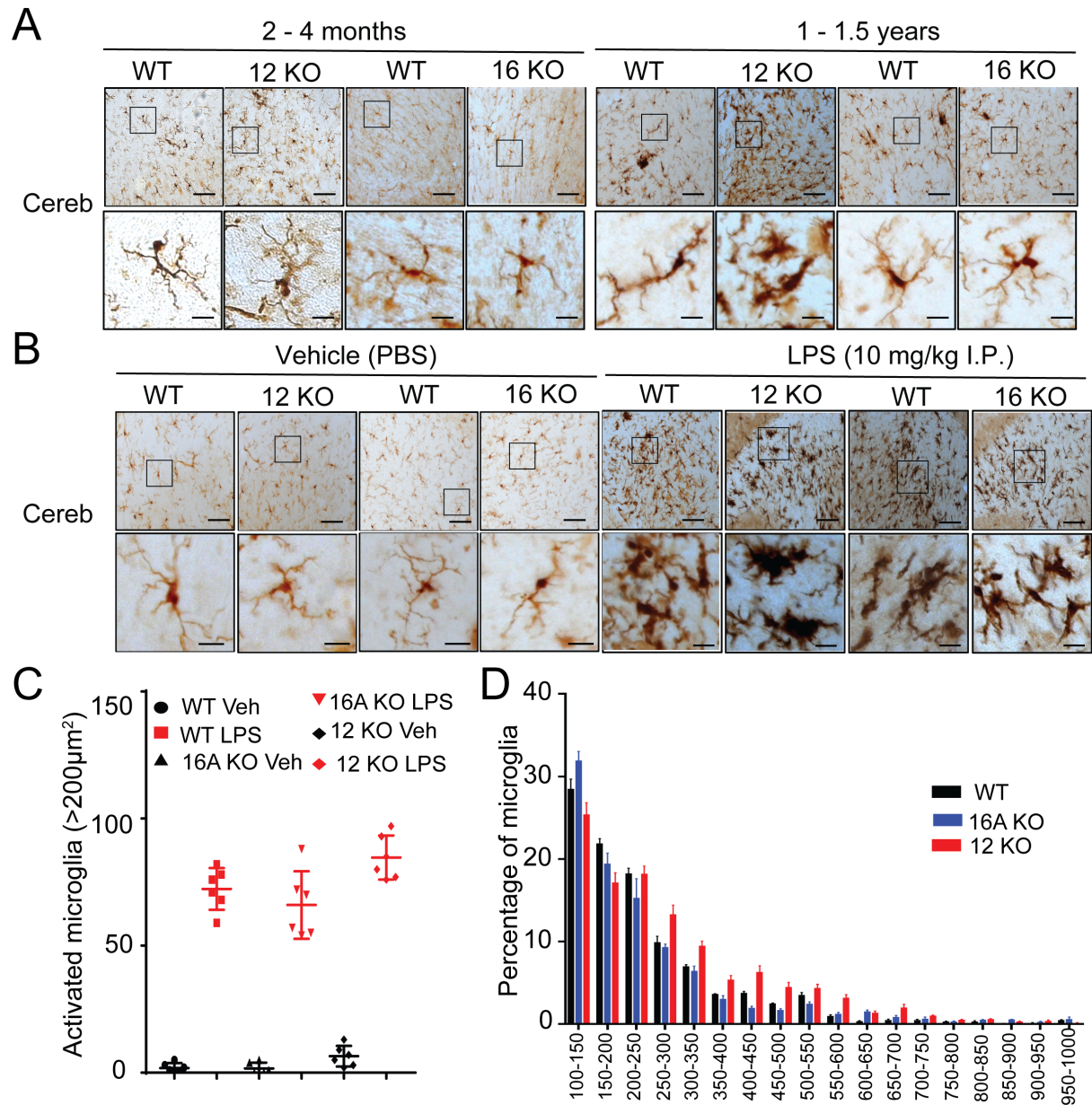


Fig. 6.1: Microglial cell morphology in ABHD16A KO and ABHD12 KO mice brain. A– Representative image of microglial cell morphology in WT, ABHD16A KO and ABHD12 KO mice cerebellum of different age group. B– LPS treatment robustly activate microglial cell across genotype and ABHD12 KO mice shows amoeboid microglial cell morphology on LPS treatment. C– Quantification of microglial cell morphology basally and on LPS treatment across genotype. D– ABHD12 KO mice shows higher soma size post LPS treatment compared to that of WT or ABHD16A KO. Data plotted as mean $\pm$ standard deviation. Scale bar = 250 μ m. Scale bar (Zoomed image) = 50 μ m.

mice showed larger soma and complete retraction of their dendrites, adopting amoeboid morphology than their WT littermates (**Fig. 6.1B** and **Fig. 6.1C**). Amoeboid morphology of the microglial cells suggests phagocytic saturation of the cell and it can no longer survey for any damage or can carry phagocytosis. Next, to get a better idea of the distribution of microglial cell morphology in immune challenged ABHD16A or ABHD12 KO mice. I plotted their size distribution and found that microglial cell in ABHD12 KO mice tend to be skewed towards larger soma size ($>250 \mu\text{m}^2$) (**Fig. 6.1D**). It is interesting as larger soma size means that microglia cells tend to be more close to amoeboid shape suggesting elevated phagocytosis.

Loss of ABHD12 elevates microglial cell phagocytosis on immune challenge. Lyso-PS is found to play a role in phagocytosis⁵⁰ hence it's likely that unchecked lyso-PS levels due to the lack of ABHD12 leads to higher degree of phagocytosis in ABHD12 KO mice. To check for the phagocytosis state of the ABHD12 KO and ABHD16A KO on immune challenge, I stained for LAMP1 – a phagocytosis (rather lysosome) marker in brain sections of LPS treated mice along with their controls. Basally, in mice 8-10 weeks of age, I didn't find any changes in expression of LAMP1 protein in the microglial cells across ABHD16A or ABHD12 genotypes (**Fig. 6.2A**). But, there was significant increase in LAMP1 expression in microglial cell across all genotypes on immune challenge with LPS (**Fig. 6.2A**). Interestingly, ABHD12 KO microglial cells accumulated more LAMP puncta on immune challenge compared to their WT and ABHD16A KO counterparts (**Fig. 6.2A**). To supplement these findings, next I checked if there were any changes in expression level of ABHD12 and ABHD16A as a function of immune challenge. I used western blot and activity based protein profiling based semi quantitative proteomics to probe for ABHD12 and ABHD16A, I found no significant changes in bulk levels of ABHD16A or ABHD12 on immune challenge to the mice except for a very minor upregulation of ABHD12 on LPS treatment (**Fig. 6.2B, C, and D**). It suggests that ABHD12 and ABHD16A largely don't change at bulk level in brain in conditions of neuro-inflammation. Next, I used immunohistochemistry to measure the ABHD12 expression on different cells (neurons, microglia, etc.) in the brain following immune challenge with LPS. Interestingly, ABHD12 expression was elevated on the microglial cells following LPS treatment when compared to vehicle treatment (**Fig. 6.2E and F**). Presumably, microglial cells overexpress ABHD12 to keep a check on phagocytosis and participate in alleviating neuro-inflammation and injury. In consistent to this thought, we see that loss of ABHD12 leads to aberrant phagocytosis in the mice brain.

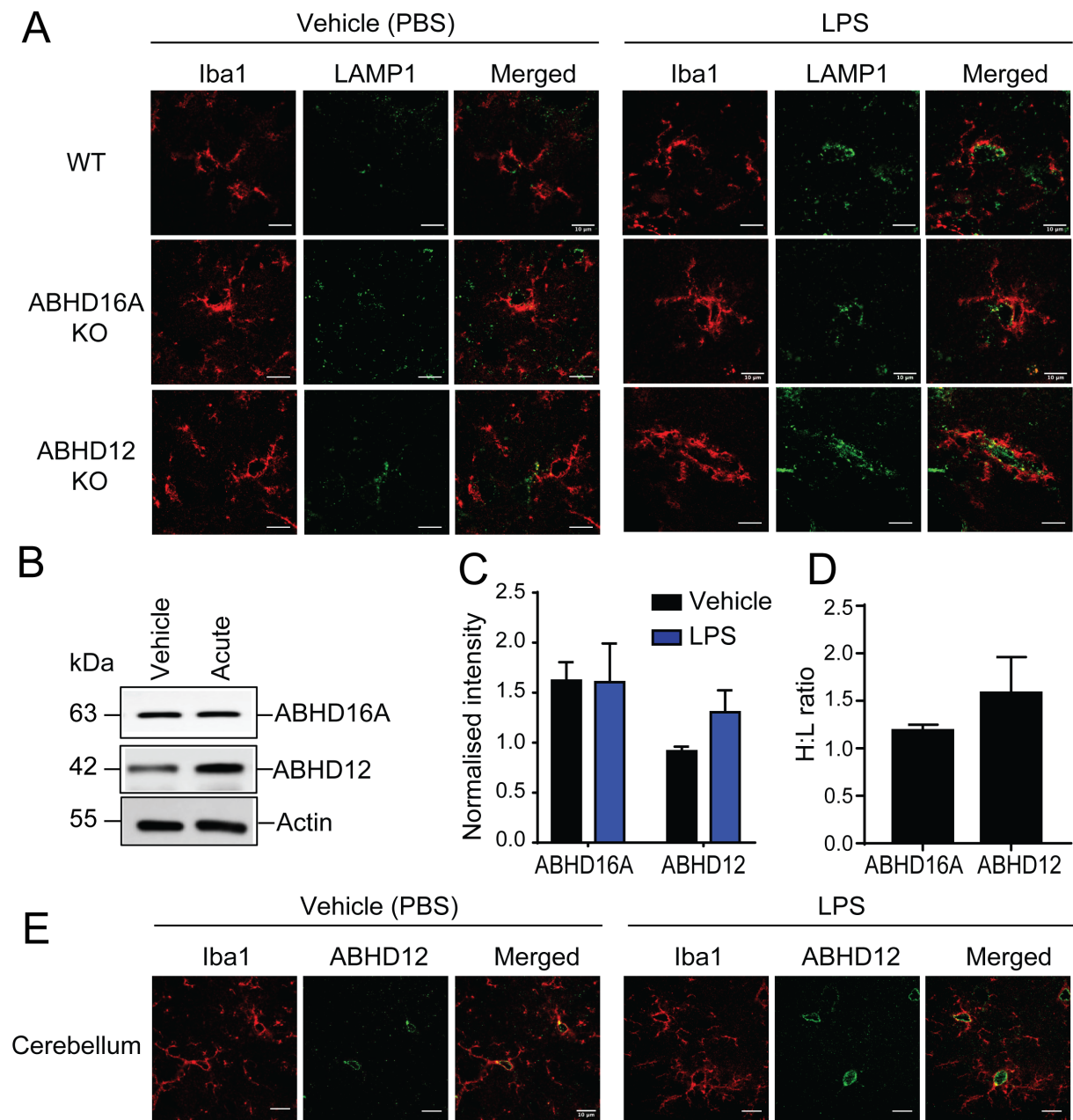


Fig. 6.2: ABHD12 KO microglial cell show elevated phagocytosis and ABHD12 is upregulated on LPS treatment. A – ABHD12 KO microglial cell show higher LAMP1 expression post LPS injection. B – Western blot probing ABHD16A and ABHD12 in the LPS treated brain. C – Quantification of western blots showing mild upregulation of ABHD12 following LPS treatment. D – ABPP based semi-quantitative estimation of ABHD16A and ABHD12 in the LPS treated brain. E – ABHD12 is upregulated in the microglial cells post LPS injection. Data plotted as mean $\pm$ standard deviation. Scale bar = 10 μ m.

Loss of ABHD12 activity leads to the accumulation of oxidized phosphatidylserine in the brain following acute immune challenge with LPS. It was interesting to ask if elevated lyso-PS brings about the aberrant (rather elevated) phagocytosis by microglial cells in the ABHD12 KO mice following immune challenge with LPS. In order to test this, I measured the lipids involved in the ABHD16A–ABHD12 metabolic axis i.e. PS and lyso-PS. In addition to PS and lyso-PS, I also measured oxidized PS (ox-PS) in the brain as they are reported to be elevated in dying cells due to the oxidative stress caused by immune challenge and inflammation. First, to benchmark the mass spectrometry studies and confirm the successful induction of inflammation, I measured prostaglandins (PGE2 and PGD2) in the brain. I found that prostaglandin levels go 3-4 fold high in the brain on LPS treatment (6 hours) (**Fig. 6.3A**). There were no differences in the prostaglandin levels of WT and ABHD12 KO mice basally as well as under immune challenge (**Fig. 6.3A**). Next, I checked if PS or lyso-PS changes on immune challenge to mice brain. I didn't find any changes in lyso-PS or PS levels in both chronic and acute immune challenge (**Fig. 6.3B and C**). To confirm that excess lyso-PS produced on inflammation was not degraded by ABHD12 in my experimental time scales, I measured their levels in immune challenged ABHD12 KO mice. I didn't find any change in lyso-PS levels as a function of inflammation in ABHD12 KO mice as well (**Fig. 6.3C**). Though, in comparison to WT mice ABHD12 KO mice accumulated 4-5 more lyso-PS but it was independent of immune challenge with LPS treatment (**Fig. 6.3C**). Next, I measured the ox-PS and LPS treated mice showed elevated ox-PS levels in their brain. I could not find any differences in the ox-PS levels basally in the brain of ABHD12 WT and ABHD12 KO mice. Interestingly, ABHD12 KO mice showed several fold higher ox-PS levels in the brain following LPS treatment compared to that of LPS treated WT mice (**Fig. 6.3D**). Further parallel work in the lab (includes chemical genetic screen, cellular and biochemical characterization) by others successfully annotated ABHD12 as an ox-PS lipase. It is interesting finding that suggests that ABHD12 can possibly regulate ox-PS in the mammalian systems with a special emphasis to its role as eater of eat me signal itself (i.e. ox-PS), thus protecting the cells from unwanted phagocytosis.

Lyso-PS activates microglial cell basally in the brain of ageing ABHD12 KO mice. Once, ABHD12 was annotated as the ox-PS lipase and ox-PS was accumulated in brain under the conditions of oxidative stress, an another interesting question that arose was whether activated microglial cells in the ABHD12 KO mice brain was due to accumulated lyso-PS or

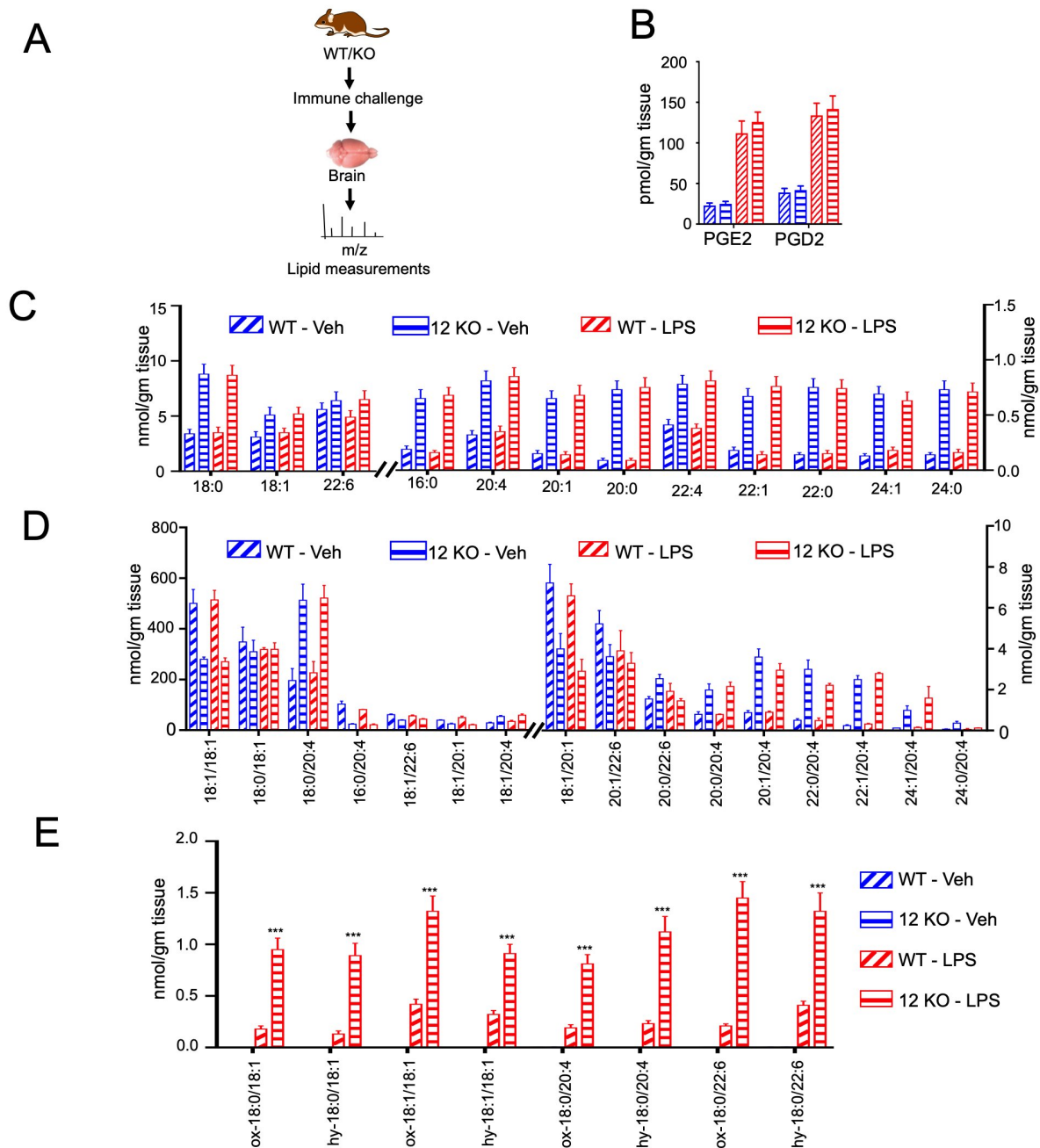
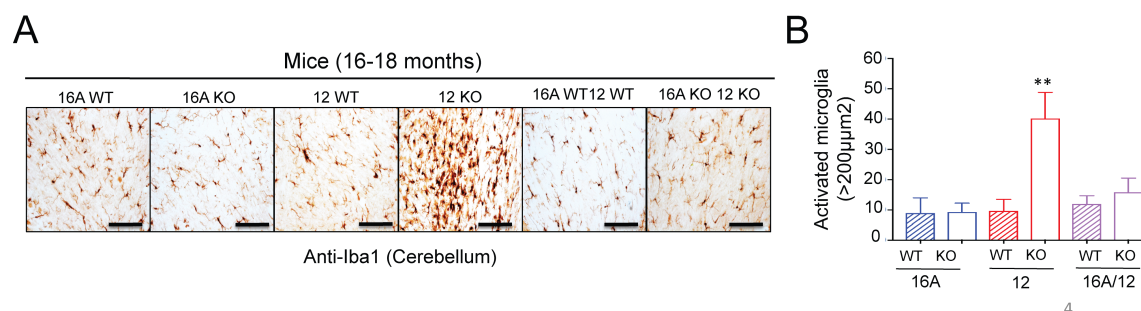


Fig. 6.3: Lipidomic measurements in brain of WT and ABHD12 KO mice administered with LPS. A – Scheme of experiment for lipidomic measurements. B – Prostaglandins increase 2-3 fold in brain post LPS treatment in both WT and ABHD12 KO mice brain. B – PS doesn't change in brain post LPS treatment in WT or ABHD12 KO mice. C – Lyso-PS doesn't change in brain post LPS treatment in WT or ABHD12 KO mice. D – ABHD12 KO mice brain accumulated 2-3 fold higher ox-PS following LPS treatment compared to WT mice. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value = 0.0001 (Acute LPS ABHD12 WT vs. Acute LPS ABHD12 KO).

ox-PS. Though, we didn't find any ox-PS accumulation in the ABHD12 KO mice brain basally, there is always a very chance of ox-PS accumulation in ABHD12 KO mice below the limit of quantification/detection of the mass spectrometer. To check on this, if we revisit lyso-PS metabolism in the mammalian brain, it is synthesized by ABHD16A. Loss of ABHD12 activity fails to degrade the lyso-PS flux coming from ABHD16A activity, thus causing lyso-PS build up. If one can ablate ABHD16A activity in ABHD12 KO mice, in principle it should substantially reduce the levels of lyso-PS in the neural tissues of ABHD12 KO mice. If microglial cells were activated due to elevated lyso-PS, ABHD16A disruption should alleviate microglial cell activation and neuro-inflammation. With this logic, I created double knockout mice i.e. ABHD16A KO ABHD12 KO mice and examined their microglial cell once they were more than 1 year old. I didn't find any morphological differences in microglial cells of ABHD16A KO mice. ABHD12 KO mice >1 year in age showed an increased number of activated microglial cells as reported previously (**Fig. 6.4 A and B**). Interestingly, in case of ABHD16A/ABHD12 double knock out mice there was no atypical activation of microglial cells in contrast to what is observed in ABHD12 KO mice (**Fig. 6.4 A and B**). It means that microglial cell activation seen on loss of ABHD12 was alleviated by ablation of lyso-PS synthetic enzyme ABHD16A (**Fig. 6.4 A and B**). It strongly suggests that microglial cell activation in ABHD12 KO mice basally was due to defective lyso-PS signaling.



*Fig. 6.4: Microglial cell activation of ABHD12 KO mice is ablated in ABHD16A KO ABHD12 KO mice. A – Representative image of microglial cell (anti-Iba1 stained) in the cerebellum of WT, ABHD16A KO, ABHD12 KO, ABHD16A KO ABHD12 KO mice. B – Quantification of activated microglial cell in mice brain of different genotype older than 1 year. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value = 0.0022 (ABHD12WT vs. ABHD12 KO). Scale bar = 250 μ m.*

Long chain lyso-PS activates microglial cell through Toll like receptor 2 (TLR2). Double knock out studies suggested that lyso-PS could be a potent signaling molecule that activates microglia. To confirm this, I tested if lyso-PS injection could trigger neuro-inflammation and activate microglial cells in the mice brain. I tested two lyso-PS species – one long chain lyso-PS 18:0 and one very long chain lyso-PS 24:0. I administered these through intravenous (I.V.) route to WT mice and monitored their response by probing their pain phenotype and microglia. At dose of 10-mg/kg-body weight, lyso-PS was lethal to the mice with immediate lung collapse (Data not shown). With reduce dose of 1 mg/kg intravenous (I.V.), long chain lyso-PS 18:0 didn't show any visible pain phenotype but lyso-PS 24:0 triggered visible immune stress and pain phenotype (orbital tightening, nose bulging, cheek bulging, reduced

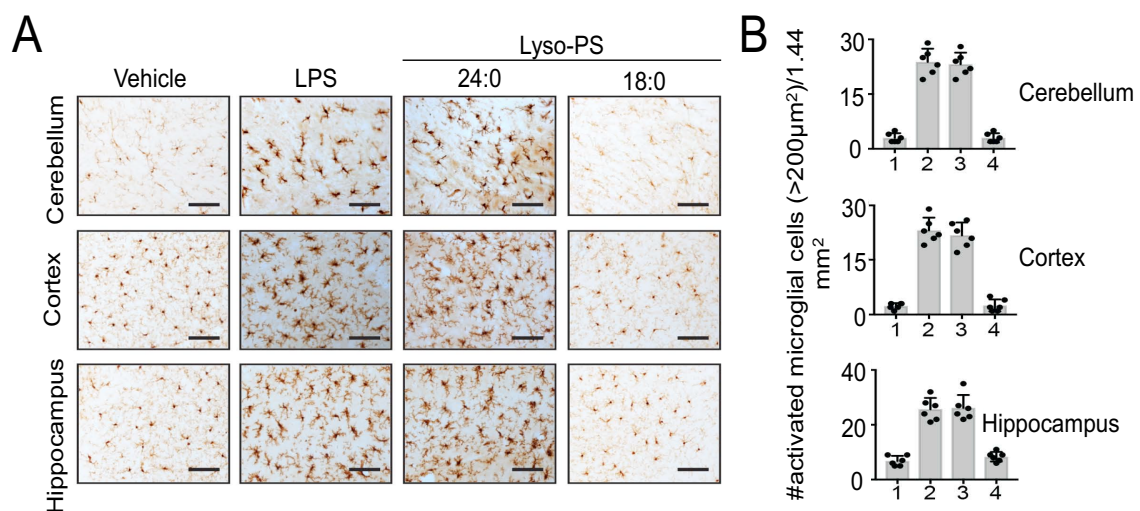
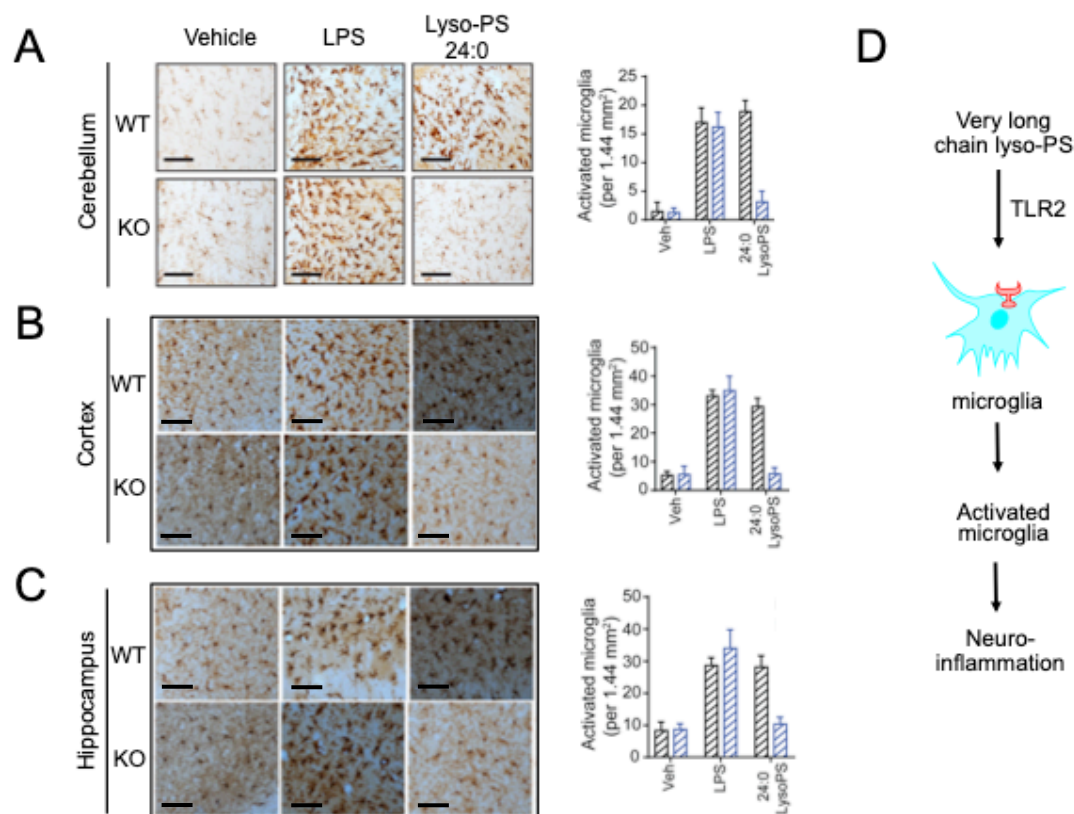


Fig. 6.5: Very long chain lyso-PS triggers microglial cell activation and cause neuro-inflammation. A – Representative image of microglial cells (anti-Iba1 stained) in cerebellum, cortex, and hippocampus of mice administered with lyso-PS and LPS. B – Quantification of activated microglial cells showing increase in number of activated microglial cells in LPS and lyso-PS treated mice. 1-Vehicle, 2-LPS, 3-LysoPS 24:0, 4-LysoPS 18:0. Data plotted as mean $\pm$ standard deviation. Scale bar = 250 μ m.

or negligible motor activity, shivering, etc.). I probed for microglial cell in these mice and found that there was increased microglial cell activation following very long chain lyso-PS 24:0 injection. The number of activated microglial cells post lyso-PS 24:0 injection was comparable to numbers seen post LPS treatment, suggesting that lyso-PS 24:0 acts almost as good antigen as LPS (**Fig. 6.5**). Long chain lyso-PS 18:0 didn't show any changes in

microglial cell activation when compared to vehicle (PBS) treated mice (**Fig. 6.5**). I found these observations to be true for all regions of brain investigated including cerebellum, cortex and hippocampus (**Fig. 6.5 A and B**). This suggests that very long chain lyso-PS can activate microglial cell to cause neuro-inflammation but long chain lyso-PS doesn't.

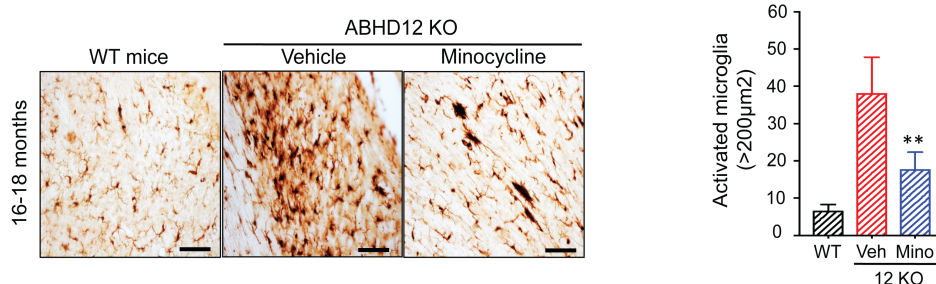


*Fig. 6.6: Very long chain lyso-PS activates microglial cells in TLR2 dependent manner. A,B,C – Representative images of microglia (anti-Iba1) in WT and TLR2 KO mice brain following LPS and Lyso-PS 24:0 administration and quantification of activated microglial cells in cerebellum (A), cortex (B), hippocampus (C). D – Scheme showing very long chain lyso-PS 24:0 activates microglial through TLR2 receptor and causes neuro-inflammation. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value <0.001 (Lyso-PS 24:0 treatment WT vs. TLR2 KO). Scale bar = 250 μ m.*

Next, I tested if TLR2 can be a receptor for very longer chain lyso-PS species to bring about neuro-inflammation in mammals. Based on screening in literature, I chose TLR2 as a possible receptor as it was highly expressed in microglial cells and was not investigated ever before in context to lyso-PS induced neuro-inflammation, and there were some reports of

TLR2 as a receptor for lyso-PS in worms. TLR2 KO mice basally didn't show any changes in the morphology or number of microglial cells. I injected TLR2KO mice with LPS and lyso-PS 24:0 LPS treated TLR2KO mice showed microglial cell activation comparable to what is seen in WT mice. Interestingly, Lyso-PS 24:0 treatments to TLR2KO didn't trigger any microglial cell activation across brain regions including cerebellum (**Fig. 6.6A**), cortex (**Fig. 6.6B**), hippocampus (**Fig. 6.6C**) in contrast to robust microglial cell activation seen in lyso-PS 24:0 injected WT mice. This strongly suggests that very long chain lyso-PS acts in TLR2 dependent manner in the mammalian physiology. A few studies by others in the lab further confirmed TLR2 as very long chain lyso-PS receptor in the peritoneal macrophages of the mice.

Minocycline can alleviate neuro-inflammation in ABHD12 KO mice. After establishing that lyso-PS causes neuro-inflammation in the ABHD12 KO mice, I started to look for small molecules that can alleviate the neuro-inflammation seen in ABHD12 KO mice. I found minocycline hydrochloride – a tetracycline antibiotic but robustly shown to deplete microglial cells and reduce cytokine secretion from macrophages. I found that minocycline administration every day for 4 weeks could successfully reduce the number of activated microglial cells in ABHD12 KO mice older than a year and it is likely that minocycline reduced neuroinflammation in ABHD12 KO mice by inhibiting cytokine secretion induced through accumulated very long chain lyso-PS signaling (**Fig. 6.7**).



*Fig. 6.7: Minocycline reduces number of activated microglia in the ABHD12 KO mice brain. A – Representative image of cerebellar microglia stained with anti-Iba1 and quantification of activated microglia. Data plotted as mean $\pm$ standard deviation. Student's *t*-test *p*-value = 0.0043 (Vehicle versus minocycline). Scale bar = 250 μ m.*

Discussion

Lyso-PS is an established immunomodulatory molecule with its active participation in the diverse immune signaling cascades. In the mammalian brain, its unregulated levels in excess trigger neuro-inflammation by activating microglial cells as observed in ABHD12 KO mice brain. This suggested some sort of role for lyso-PS in the neuro-immunophysiology. As discussed several times before in this this, lyso-PS is regulated by two major serine hydrolase family metabolic enzymes ABHD16A (anabolic) and ABHD12 (catabolic) in the mammalian CNS. My localization studies have reliably established that ABHD12 is present on the microglial cells in vivo. Here, I investigated the role of ABHD16A – ABHD12 – lyso-PS in the microglial cells and neuro-immunophysiology. First, I phenotyped the microglial cells from ABHD12 KO and ABHD16A KO mice in an age dependent manner. In consistent to previous studies¹⁷, ABHD12 KO mice older than 1 year of age show elevated microgliosis whereas ABHD16A KO mice didn't show any changes in the microglial cell morphology across their life span. Next, I tested for the role of ABHD16A – ABHD12 – lyso-PS in the immune challenged mice brain. I treated mice with LPS in order to trigger immune reactions and looked the microglial cell morphology. Mice <5 months old were chosen for these experiments in order to avoid microglial cell activation noise from the age dependent microgliosis in ABHD12 KO mice. On LPS treatment, there was enhanced microglial cell activation across all LPS treated mice groups irrespective of genotype compared to vehicle treated mice groups. Interestingly, ABHD12 KO mice showed increased microgliosis compared to their WT control treated with LPS and microglial cells in ABHD12 KO mice brain adopted amoeboid shape following LPS treatment. Amoeboid shape of the microglial cell often refers to the exaggerated phagocytosis and saturation of phagocytic capacity of a microglial cell. In order to look at the state of microglial cell phagocytosis, I probed for lysosomes and I found that LAMP1 expression was higher in LPS treated ABHD12 KO microglial cells compared to the ABHD16A KO and WT microglial cell. Next, I probed for ABHD16A and ABHD12 in the immune challenged mice brain. I found a slight (statistically non-significant) increase in the bulk ABHD12 levels and no change in the ABHD16A levels in the brain. Minor expression changes in ABHD12 at low abundant cells like microglial cell would be difficult to pick in crude brain lysates, so, I used immunohistochemistry and probed for ABHD12 in different cells of the brain. I found slight overexpression of the ABHD12 on microglial cells following LPS treatment. Loss of ABHD12 have caused aberrant elevated phagocytosis by microglial cell following LPS treatment, and increase in ABHD12

expression on the microglial cells as a function of LPS treatment suggests that ABHD12 regulates the phagocytosis process. Next, to measure if LPS triggered exacerbated microglial cell activation in the ABHD12 KO brain is caused by the lyso-PS, I measured lyso-PS, PS and ox-PS in the brain. Lyso-PS levels were several folds higher in the ABHD12 KO mice brain compared to their WT littermates. But, I didn't find any changes in lyso-PS or PS levels as a function of LPS treatment across genotypes, thus lyso-PS levels were largely unaffected by the immune challenge. Interestingly, I found elevated levels of ox-PS in the ABHD12 KO mice brain post LPS treatment, suggesting an elevated oxidative stress in the mice brain of the ABHD12 KO mice. Further studies by others in the lab established that ABHD12 can act as an ox-PS lipase. These studies report an important discovery and annotation of ABHD12 as an ox-PS metabolic enzyme. Increased ABHD12 expression in microglial cells and no measurable changes in the lyso-PS levels on LPS treatment, strongly suggests that ABHD12 increased activity could be to quench the excessive ox-PS generated under the conditions of oxidative stress. Loss of ABHD12 leads to accumulation of ox-PS on immune challenge and calls for elevated microglial cell phagocytosis as evident from my observation of amoeboid shaped microglial cell and increased LAMP1 expression on LPS treated ABHD12 KO microglial cell cells.

Is the excessive microglial cell activation observed in the ABHD12 KO mice arising from lyso-PS or ox-PS? To partially answer this, I generated ABHD16 KO ABHD12 KO mice, where elevated lyso-PS levels of ABHD12 KO mice were ablated and these mice didn't show any microgliosis contrary to what is seen in ageing ABHD12 KO mice. To further confirm that lyso-PS in the brain can cause microgliosis (neuro-inflammation), I injected mice with two different lyso-PS species, one was lyso-PS 18:0 belonging to the long chain lyso-PS lipid class and other one was lyso-PS 24:0 belonging to the very long chain lyso-PS lipid class. On injection, I found that very long chain lyso-PS (i.e. lyso-PS 24:0) triggered microglial cell activation almost equal to that of LPS, whereas long chain lyso-PS (like lyso-PS 18:0) didn't not cause any change in the microglial cell morphology. Next was to find out a receptor for the very long chain lyso-PS through which it activates microglial cell in the mammalian brain. Among the reported receptors of the lyso-PS, TLR2 is the most abundant receptor present in the microglial cells but remains uninvestigated. I injected TLR2 KO mice and their WT littermates with LPS, lyso-PS 18:0, and lyso-PS 24:0 intra-venous. LPS treated mice showed microglial cell activation and neuro-inflammation in both WT and TLR2 KO mice whereas lyso-PS 18:0 didn't show any signs of neuro-inflammation or any microglial cell

activation consistent to what was observed for WT mice. Interestingly, lyso-PS 24:0 robustly activated microglial cells in the WT mouse brain but it failed to trigger any microglial cell activation or neuro-inflammation in the TLR2 KO mice. A few in situ studies by others on primary peritoneal macrophages found that lyso-PS 24:0 treatment didn't trigger any pro-inflammatory cytokines secretion from the TLR2 KO cells. This strongly suggested that TLR2 may be a receptor for very long chain lyso-PS though a few studies with microglial cell specific TLR2 knockout mice are needed to fully establish that TLR2 is a very long chain lyso-PS receptor present on microglial cells and signaling through this receptor calls for its activation. Going further, one must behaviorally characterize ABHD16A KO - ABHD12 KO and ABHD12 KO - TLR2 KO to establish if the PHARC symptoms especially locomotor defects arise due to neuro-inflammatory activity of lyso-PS described here or due to neuronal signaling aspect of lyso-PS signaling. ABHD16A KO - ABHD12 KO and ABHD12 KO - TLR2 KO mice ideally have alleviated neuro-inflammation due thus any alleviation in the behavioral defects will suggest that neuro-inflammation was causing those defects. It remains a very interesting aspect for future investigations.

Conclusion

Overall, my studies here make two important discoveries. First, ABHD12 is an ox-PS lipase thus helps mammalian cells survive the oxidative stress without being phagocytized. Second, very long chain lyso-PS signals through the TLR2 receptor to activate microglial cells thus inviting neuro-inflammation in the mammals. My studies here and findings provide some crucial insights into the role of ABHD16A–ABHD12–ox-PS in the mammalian neuro-immuno-physiology. Further studies will shed more light on the broader physiological roles of the ox-PS and ABHD12 nodal axis. Therapeutic strategies targeting ABHD16A and TLR2 can also be tested as a therapy to treat PHARC syndrome.

CHAPTER VII

Summary and Conclusion

PHARC syndrome is an age dependent genetically recessive neurological disorder primarily marked by a set of 5 symptoms that includes peripheral neuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract. More than 20 mutations have been mapped in *abhd12* gene in PHARC human subjects. *Abhd12* gene encodes a serine hydrolase enzyme namely α/β – hydrolase containing protein #12 (ABHD12). Extensive biochemical investigations of ABHD12 lead to its annotation as a lysophosphatidylserine (lyso-PS) lipase in the mammalian brain. ABHD12 knock out (KO) mice accumulate lyso-PS in the neural tissues, followed by elevated microglial activation (neuro-inflammation) and sensory motor defects like hearing loss, ataxia and poor coordination, similar to what is observed in PHARC human subjects. This led to the proposal that defective lyso-PS signaling resulting due to uncontrolled lyso-PS metabolism may drive the pathophysiology of PHARC Syndrome. To further map the biosynthetic route of lyso-PS, Kamat et al. found ABHD16A as a major lyso-PS biosynthetic enzyme in the mammalian nervous and immune system. These studies fairly established that ABHD16A – lyso-PS – ABHD12 biochemical axis play a crucial role in neurophysiology and defects in it can cause pathological conditions like PHARC syndrome. Taking these studies further, my doctoral research described here broadly accomplishes the following broad aims: -

1. Purification and biochemical characterization of ABHD16A.
2. Mapping the cellular and neuro-anatomical expression of ABHD16A-ABHD12 in the mammalian nervous system.
3. Establishing functional role of ABHD16A and ABHD12 in the neuro-immunophysiology.

I successfully developed protocols to overexpress and purify recombinant ABHD16A from bacterial host cells and assayed its lipase activity to show that ABDH16A is a phosphatidylserine specific phospholipase A₂ enzyme and my purification protocols can be scaled to perform the structural studies and explain the substrate specificity of ABHD16A activity. Next, I moved on to in situ systems and mapped the subcellular localization of ABHD16A and ABHD12 in the mammalian cells so as to better understand cellular lyso-PS metabolism and flux. I used three parallel but complementary approaches to probe the

localization of ABHD16A and ABHD12 in the mammalian cells – i) subcellular fractionation and western blot ii) lipase activity assays in sub-cellular fractions iii) immunofluorescence assay for ABHD16A and ABHD12. With all the three parallel and complementary approaches, I showed that both ABHD16A and ABHD12 are present in the endoplasmic reticulum of the mammalian cells – namely primary neurons (mice), primary peritoneal macrophages (mice), MCF7, COS-7 and Neuro-2a cells. I also showed that under the conditions of biological stimuli (immune challenge) where levels of ABHD16A and ABHD12 expression change differentially, subcellular localization of these enzymes are retained in the endoplasmic reticulum (ER) and they regulate bulk lyso-PS levels and flux from the ER in the mammalian cells.

Having subcellular localization of ABHD16A and ABHD12 mapped, I moved on to probe for these proteins in different anatomical regions of the adult mice brain. I showed that ABHD16A was several fold higher in cerebellum of the mice brain whereas ABHD12 was pretty much ubiquitous across brain regions. Consistent with this observation, I also found that loss of ABHD16A or ABHD12 has major disturbances in PS and lyso-PS levels in the cerebellum of the adult mice brain. This observation was interesting to the fact that cerebellum is the regions which undergoes age dependent degeneration (or atrophy) contributing to ataxia and motor defects. Next, I probed for ABHD16A and ABHD12 expression in the adult mice brain using immunohistochemistry experiments and found that ABHD16A and ABHD12 are expressed on two different cerebellar layers that typically host different neuronal cells in the mice brain. ABHD16A was highly enriched on the granule neurons (granular layer) while ABHD12 was almost exclusively expressed in the Purkinje neurons (ganglionic layer) of the cerebellum. It made me hypothesize that lyso-PS could acts as a paracrine-signaling molecule between granule neurons and Purkinje neurons of the cerebellum in mammalian brain. Using several parallel approaches, I showed that lyso-PS secreted from granule neurons induce phosphorylation of ERK1/2 in the Purkinje neurons, thus strongly suggesting that granule neurons and Purkinje neurons cross talk using lyso-PS as one of the signaling molecule, though there were no phenotypic or morphological or synaptic density defects in the Purkinje neurons on lack of ABHD16A or ABHD12 activity. Further investigations using lyso-PS library lead to the observation that long chain lyso-PS species (like lyso-PS 18:0) is most potent in inducing phosphorylation of ERK1/2.

ABHD12 KO mice show lyso-PS build up and elevated microgliosis (activated microglial cells and neuro-inflammation) in an age dependent manner suggesting some role of ABHD16A – ABHD12 – lyso-PS in the neuro-immunophysiology of mammalian brain. I found that loss of ABHD12 activity leads to elevated phagocytosis by microglial cells thus causing deregulated injury to the neural tissues. Further investigations lead to the establishment that ABHD12 acts as an oxidized PS lipase, which is a signaling molecule for phagocytosis. Thus, it is likely that elevated phagocytosis by microglial cells in ABHD12 KO mice is driven by accumulated oxidized PS under the conditions of oxidative stress. Next, I checked if basal neuro-inflammation in ABHD12 KO mice contributed by lyso-PS or ox-PS. To test this, I generated ABHD16A and ABHD12 double knock out mice that had rescued levels of accumulated lyso-PS in the brain. Interestingly, double knock out mice older than 1 year didn't show any microglial activation or neuro-inflammation suggesting that lyso-PS is the metabolite that triggers neuro-inflammation in ABHD12 KO mice. To confirm this, I administered mice with lyso-PS and I found that very long chain lyso-PS like lyso-PS 24:0 activates microglia through Toll like receptor 2 (TLR2). To bring down the neuro-inflammation in the ABHD12 KO mice, I screened a set of small molecules and found that minocycline administration for a few weeks could successfully alleviate the neuro-inflammation in the ABHD12 KO mice (**Fig. 7**). Overall, I successfully demonstrate that long chain lyso-PS like lyso-PS 18:0 acts as a signaling molecule in the neurons through some unidentified receptor whereas very long chain lyso-PS like lyso-PS 24:0 signals through TLR2 to activate microglial cells leading to neuro-inflammation (**Fig. 7**). My work described in this thesis elegantly establish that ABHD16A and TLR2 can be a good therapeutic target to alleviate neuro-inflammation in the ABHD12 KO mice and minocycline can be a promising small molecule candidate to manage neuro-inflammation (**Fig. 7**).

Going ahead, it would be interesting to investigate if neuro-behavioral defects in ABHD12 KO mice arise due to lyso-PS triggered neuro-inflammation or its signaling role on Purkinje neurons in the cerebellum or may be both (**Fig. 7**). To answer this, one should screen ABHD16A KO – ABHD12 KO mice, ABHD12 KO – TLR2 KO mice for sensory motor behavior, defects in which are seen in PHARC patients or PHARC mice models. It also remains an interesting question to pursue how lyso-PS mediated granule neuron – Purkinje neuron communication drives the broader neurophysiological processes or behavioral output.

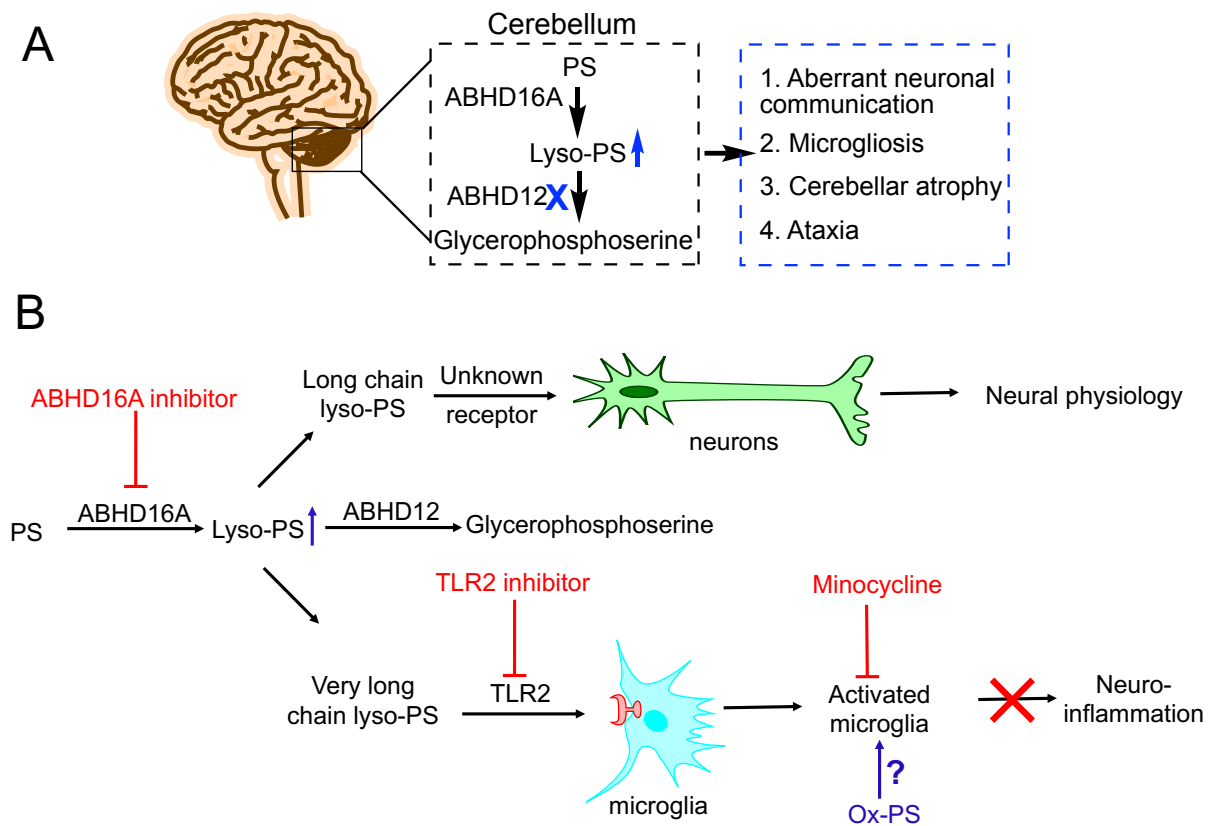


Fig. 7: Schematic showing how loss of ABHD12 might drive PHARC manifestations and possible strategies to alleviate PHARC. A – Scheme showing changes in cerebellum on loss of ABHD12. B – Possible signaling roles of long chain lyso-PS and very long chain lyso-PS in the cerebellum. Currently we don't understand how ox-PS contributes to PHARC pathophysiology. Nodes marked in red show possible strategies to alleviate PHARC symptoms. ABHD16A inhibitor can reduce PHARC manifestations by reducing levels of PHARC. TLR2 inhibitor and minocycline could also possibly reduce PHARC manifestation by alleviating neuroinflammation in the brain.

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PUBLICATIONS

1. Khandewal[#], N., Sheikh[#], M., Mhetre[#], A., **Singh[#], S.**, Sajeevan, T., Joshi, A., Balaji, N. K., Chakrapani, H., Kamat, S. S., (2020) Fatty acid chain length drives lysophosphatidylserine dependent immunological outputs. (#Equal contribution) (Accepted in [Cell Chemical Biology](#)).
2. **Singh^{*}, S.**, Joshi, A., Kamat^{*}, S. S. (2020) Mapping the neuroanatomy of ABHD16A-ABHD12 and lysophosphatidylserine provides new insights into the pathophysiology of the human neurological disorder PHARC, *Biochemistry* 2020, 59, 24, 2299-2311. (*Co-corresponding author).
3. Kelkar[#], D. S., Ravikumar[#], G., Mehendale[#], N., **Singh[#], S.**, Joshi, A., Sharma, A. K., Mhetre, A., Rajendran, A., Chakrapani, H., Kamat, S. S. (2019) A chemical genetic screen identifies ABHD12 as an oxidized phosphatidylserine lipase, [Nature Chemical Biology](#) 15, 169-178. (#Equal contribution).
4. Joshi[#], A., Shaikh[#], M., **Singh[#], S.**, Rajendran, A., Mhetre, A., Kamat, S. S. (2018) Biochemical characterization of the PHARC associated serine hydrolase ABHD12 reveals its preference for very long chain lipids, [Journal of Biological Chemistry](#) 293 (44), 16953-16963. (#Equal contribution).

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**Mapping the Neuroanatomy of ABHD16A, ABHD12, and Lysophosphatidylserines Provides New Insights into the Pathophysiology of the Human Neurological Disorder PHARC**
Author: Shubham Singh, Alaumy Joshi, Siddhesh S. Kamat
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Appendix - I

Enlisting values used in the graphs presented in the thesis.

Fig. 2.5C

Sr. No.	Sample	ABHD16A activity - nmol/mg/min		
		Replicate_1	Replicate_2	Replicate_3
1	Mock	0.178	0.122	0.137
2	ABHD16A-Veh	8.766	8.998	8.356
3	ABHD16A + KC01	2.211	1.308	1.789

Fig. 2.6A

Lyso-PS release Assay

Sr. No.	Substrate	2-acyl-sn1-lysophospholipid release (nmol/mg/min)					
		Mock			ABHD16A		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	0.127	0.268	0.252	8.702	6.852	7.390
2	16:0-18:1 PS	0.146	0.158	0.190	8.444	5.941	7.012
3	17:0-20:4 PS	0.198	0.092	0.167	5.030	8.982	5.217
4	18:0-20:4 PE	0.049	0.078	0.029	2.683	2.248	1.711
5	16:0-18:1 PC	0.108	0.209	0.231	1.082	2.400	0.928
6	18:0-20:4 PG	0.047	0.051	0.087	3.054	3.127	3.284
7	18:0-20:4 PE	0.055	0.087	0.078	2.980	3.154	3.420
8	C18(Plasm)-20:4 PI	0.044	0.035	0.040	2.084	2.057	1.759
9	C18(Plasm)-LPE	0.000	0.000	0.000	0.000	0.000	0.000
10	C16(Plasm)-LPC	0.000	0.000	0.000	0.000	0.000	0.000

Sr. No.	Substrate	1-acyl-sn2-lysophospholipid release (nmol/mg/min)					
		Mock			ABHD16A		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	0.112	0.184	0.255	0.301	0.221	0.140
2	16:0-18:1 PS	0.127	0.118	0.189	0.245	0.185	0.111
3	17:0-20:4 PS	0.194	0.125	0.156	0.167	0.239	0.132
4	18:0-20:4 PE	0.186	0.151	0.167	0.149	0.121	0.149
5	16:0-18:1 PC	0.092	0.188	0.232	0.136	0.161	0.119
6	18:0-20:4 PG	0.140	0.123	0.125	0.124	0.149	0.120
7	18:0-20:4 PE	0.186	0.151	0.167	0.149	0.121	0.149
8	C18(Plasm)-20:4 PI	0.115	0.112	0.167	0.145	0.106	0.100
9	C18(Plasm)-LPE	0.000	0.000	0.000	0.000	0.000	0.000
10	C16(Plasm)-LPC	0.000	0.000	0.000	0.000	0.000	0.000

Fig. 2.6B

FFA Release Assay							
		sn-2 FFA release (nmol/mg/min)					
		Mock			ABHD16A		
Sr. No.	Substrate	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	0.178	0.122	0.102	0.095	0.112	0.163
2	16:0-18:1 PS	0.165	0.190	0.190	0.167	0.244	0.199
3	17:0-20:4 PS	0.127	0.225	0.086	0.172	0.159	0.160
4	18:0-20:4 PE	0.165	0.103	0.171	0.222	0.103	0.103
5	16:0-18:1 PC	0.165	0.190	0.190	0.167	0.244	0.199
6	18:0-20:4 PG	0.056	0.071	0.069	0.068	0.049	0.049
7	18:0-20:4 PE	0.067	0.086	0.090	0.073	0.108	0.062
8	C18(Plasm)-20:4 PI	0.026	0.058	0.018	0.050	0.005	0.053
9	C18(Plasm)-LPE	0.000	0.000	0.000	0.000	0.000	0.000
10	C16(Plasm)-LPC	0.000	0.000	0.000	0.000	0.000	0.000

		sn-1 FFA release (nmol/mg/min)					
		Mock			ABHD16A		
Sr. No.	Substrate	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	0.113	0.161	0.178	3.256	4.320	4.363
2	16:0-18:1 PS	0.122	0.165	0.228	4.358	4.216	4.947
3	17:0-20:4 PS	0.120	0.220	0.134	6.019	4.717	4.968
4	18:0-20:4 PE	0.176	0.141	0.220	2.049	1.252	2.049
5	16:0-18:1 PC	0.113	0.234	0.238	1.145	2.995	1.469
6	18:0-20:4 PG	0.046	0.050	0.050	1.274	1.570	1.569
7	18:0-20:4 PE	0.078	0.060	0.051	1.554	1.736	1.797
8	C18(Plasm)-20:4 PI	0.094	0.083	0.074	1.791	1.666	2.278
9	C18(Plasm)-LPE	0.093	0.062	0.097	0.116	0.093	0.210
10	C16(Plasm)-LPC	0.000	0.000	0.000	0.000	0.000	0.000

Fig. 2.7C

ABHD16A Activity (nmol/mg/min)							
		18:2 FFA			16:0 Lyso-PS		
Sr. No.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Lemo21-mock	4.189	3.020	3.509	1.499	1.432	1.538
2	ABHD16A-S355A	4.226	4.794	4.570	2.369	2.032	2.169
3	ABHD16A-WT	15.307	19.126	17.743	8.301	13.298	11.898

Fig. 2.9B

ABHD16A Activity (nmol/mg/min)				
Sr. No.	Membrane	Rep-1	Rep-2	Rep-3
1	ABHD16A+LSS	0.372	0.151	0.280
2	ABHD16A + 0.1 mM POPC - LSS	15.888	14.724	15.438
3	ABHD16A + 0.1 mM POPC + LSS	0.278	0.223	0.258
4	ABHD16A + 1 mM POPC - LSS	13.150	12.073	12.579
5	ABHD16A + 1 mM POPC + LSS	0.102	0.102	0.112

Fig. 2.10A**Lyso-PS release Assay**

		2-acyl-sn1-lysophospholipid release (nmol/mg/min)					
		WT			S355A		
Sr. No.	Substrate	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	15.467	12.345	13.245	1.689	0.124	0.321
2	16:0-18:1 PS	10.423	11.572	13.185	2.213	0.221	0.134
3	17:0-20:4 PS	14	9	12	0	0	1.167
		1-acyl-sn2-lysophospholipid release (nmol/mg/min)					
		WT			S355A		
Sr. No.	Substrate	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	0.123	0.231	1.123	1.097	0.553	1.134
2	16:0-18:1 PS	0.421	0.301	1.231	0.456	0.331	0.644
3	17:0-20:4 PS	0.321	1.089	0.688	1.321	0.912	0.367

Fig. 2.10B**Lyso-PS release Assay**

		sn-2 FFA release (nmol/mg/min)					
		WT			S355A		
Sr. No.	Substrate	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	15.241	12.157	13.705	1.207	0.379	0.272
2	16:0-18:1 PS	10.812	11.573	13.515	2.185	0.382	0.619
3	17:0-20:4 PS	14.216	9.841	12.197	0.289	0.993	1.806
		sn-1 FFA release (nmol/mg/min)					
		WT			S355A		
Sr. No.	Substrate	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	16:0-18:2 PS	0.933	0.463	1.376	1.898	0.899	1.362
2	16:0-18:1 PS	0.678	0.657	1.14	0.357	0.972	0.462
3	17:0-20:4 PS	0.43	1.182	0.665	1.116	1.619	0.345

Fig. 3.4B

PS lipase assay (nmol/mg/min) - Brain													
		Cytosol			Microsomes			Mitochondria			Nucleus		
Sr.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	0.138	0.123	0.138	0.617	0.608	0.690	0.151	0.150	0.142	0.088	0.083	0.083
2	KC01	0.100	0.145	0.094	0.124	0.080	0.076	0.092	0.089	0.066	0.057	0.065	0.057
3	KC02	0.104	0.137	0.119	0.484	0.659	0.658	0.056	0.075	0.082	0.104	0.068	0.075

Fig. 3.4C

PS lipase assay (nmol/mg/min) - Neuro-2a cells													
		Cytosol			Microsomes			Mitochondria			Nucleus		
Sr.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	0.040	0.049	0.045	0.139	0.142	0.137	0.057	0.056	0.048	0.041	0.038	0.025
2	KC01	0.046	0.048	0.043	0.037	0.065	0.040	0.040	0.031	0.032	0.023	0.068	0.026
3	KC02	0.052	0.054	0.035	0.134	0.145	0.116	0.026	0.032	0.036	0.034	0.034	0.029

Fig. 3.4D

PS lipase assay (nmol/mg/min) - MCF7 cells													
		Cytosol			Microsomes			Mitochondria			Nucleus		
Sr.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	0.057	0.075	0.060	0.142	0.140	0.139	0.074	0.030	0.036	0.063	0.041	0.031
2	KC01	0.049	0.043	0.054	0.044	0.044	0.054	0.033	0.070	0.073	0.074	0.085	0.053
3	KC02	0.072	0.063	0.066	0.087	0.090	0.099	0.057	0.042	0.046	0.071	0.058	0.068

Fig. 3.4F

Lyso-PS lipase assay (nmol/mg/min) - Brain													
		Cytosol			Microsomes			Mitochondria			Nucleus		
Sr.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	0.143	0.140	0.137	0.409	0.414	0.424	0.068	0.062	0.053	0.074	0.072	0.061
2	JJH350	0.108	0.108	0.115	0.180	0.193	0.201	0.077	0.074	0.080	0.073	0.124	0.083

Fig. 3.4G

Lyso-PS lipase assay (nmol/mg/min) - Neuro-2a cells													
		Cytosol			Microsomes			Mitochondria			Nucleus		
Sr.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	0.044	0.033	0.041	0.211	0.160	0.156	0.038	0.036	0.050	0.040	0.043	0.044
2	JJH350	0.049	0.044	0.042	0.126	0.105	0.080	0.035	0.039	0.038	0.034	0.052	0.032

Fig. 3.4H

Lyso-PS lipase assay (nmol/mg/min) - MCF7 cells													
		Cytosol			Microsomes			Mitochondria			Nucleus		
Sr.	Sample	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	0.033	0.039	0.034	0.179	0.246	0.267	0.053	0.050	0.023	0.020	0.020	0.016
2	JJH350	0.030	0.029	0.032	0.070	0.102	0.128	0.054	0.050	0.041	0.017	0.021	0.021

Fig. 3.8A

LysoPI-LysoPS Levels (nmol/5 x 10⁷ cells)							
Sr. No.	Species	Mock			ABHD16A		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	LysoPI18:0	1.523	0.869	3.237	1.601	1.417	1.547
2	LysoPI18:1	0.187	0.254	0.186	0.000	0.281	0.000
3	LysoPS12:0	0.604	1.465	0.865	0.614	1.112	0.880
4	LysoPS14:0	0.715	0.281		1.006	0.699	1.575
5	LysoPS16:0	1.983	2.216	2.116	2.022	3.314	3.506
6	LysoPS18:0	30.897	43.587	32.130	88.313	80.297	72.070
7	LysoPS18:1	6.591	12.819	10.814	16.838	20.524	26.688
8	LysoPS18:2	1.051	1.574	0.808	2.279	2.952	3.527
9	LysoPS20:0	1.320	1.136	1.159	4.093	3.659	7.047
10	LysoPS20:1	0.388	0.000	0.303	1.950	1.206	2.763
11	LysoPS20:4	1.135	1.919	1.050	3.506	3.950	5.261
12	LysoPS22:0	3.781	2.554	6.885	5.731	7.155	7.567
13	LysoPS22:1	0.742	0.568	1.132	1.071	0.950	1.308
14	LysoPS22:4	1.410	2.311	1.432	2.171	1.636	2.122
15	LysoPS22:6	2.536	3.983	2.563	6.623	8.875	10.880
16	LysoPS24:0	5.556	3.449	12.494	5.935	9.663	8.315
17	LysoPS24:1	1.747	1.348	5.912	1.296	1.610	1.918

Fig. 3.8B

PS Levels (nmol/5 x 10⁷ cells)							
1	32:0PS	5.835	4.703	1.039	5.454	4.621	3.423
19	32:1PS	9.859	6.681	1.436	10.881	11.820	8.173
20	34:0PS	3.664	4.529	0.868	5.987	4.756	4.008
21	34:1PS	1.887	1.660	0.418	4.979	4.382	3.994
22	34:2PS	7.056	3.764	0.875	6.024	6.579	5.226
23	36:0PS	9.498	5.322	1.189	8.823	10.322	7.415
24	36:1PS	30.342	10.265	2.312	17.545	21.235	14.365
25	36:2PS	6.944	5.990	1.692	8.722	10.377	7.065
26	36:4PS	1.050	1.184	0.289	2.294	1.959	1.255
27	38:4PS	9.637	4.678	0.990	9.847	12.848	7.518
28	40:6PS	16.688	8.165	1.671	16.746	17.687	12.603

Fig. 3.8C

PC Levels (nmol/5 x 10 ⁷ cells)							
29	32:0PC	2247.854	1467.131	2107.100	1037.232	1045.998	1645.984
30	32:1PC	1818.861	1190.407	1712.875	986.316	989.640	1500.472
31	34:0PC	1964.014	1209.897	1828.572	1132.670	1121.408	1607.450
32	34:1PC	2704.522	1743.812	2490.338	1271.964	1315.196	1974.688
33	34:2PC	1282.792	890.600	1237.441	792.373	787.246	979.547
34	36:0PC	1374.716	694.519	1191.696	508.124	477.729	1522.708
35	36:1PC	2870.220	1746.650	2724.553	1288.293	1293.498	2342.355
36	36:2PC	1515.895	1125.839	1525.474	1005.301	1002.950	1421.524
37	36:4PC	457.506	353.246	377.941	449.146	432.370	666.774
38	38:4PC	264.626	197.618	201.828	369.805	319.249	619.892
39	40:6PC	163.594	123.979	133.529	223.052	195.374	433.507

Fig. 3.8D

PE Levels (nmol/5 x 10 ⁷ cells)							
40	32:0PE	11.411	7.518	10.757	2.793	2.722	4.527
41	32:1PE	49.821	27.429	43.059	7.765	7.541	17.618
42	34:0PE	21.539	12.500	19.160	5.592	4.971	10.030
43	34:1PE	266.279	176.345	250.836	65.420	63.248	127.628
44	34:2PE	214.984	137.535	201.943	38.789	40.518	84.225
45	36:0PE	31.877	14.065	27.403	4.111	4.112	11.308
46	36:1PE	394.575	226.346	389.840	61.848	65.891	182.419
47	36:2PE	373.632	259.788	363.333	111.139	113.832	229.702
48	36:4PE	5.746	6.056	5.140	5.180	5.355	5.188
49	38:4PE	32.652	25.369	26.388	18.867	17.201	27.923
50	40:6PE	36.851	26.091	30.357	18.461	16.942	30.670

Fig. 3.9B

Sr. No.	Protein	Vehicle			LPS		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	ABHD16A	0.650	0.550	0.730	1.100	1.250	1.390
2	ABHD12	1.520	1.410	1.870	1.130	0.910	0.800

Fig. 3.9D

ABHD16A- IFA Assay							
Sr. No.	Treatment	Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Vehicle	35.5	26.252	43.691	40.123	37.73	27.283
2	LPS	88.19	74.018	78.015	78.599	86.493	80.226

Fig. 3.9F

ABHD12- IFA Assay							
Sr. No.	Treatment	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Vehicle	27.375	23.764	26.405	24.956	25.684	28.388
2	LPS	13.509	14.698	15.645	15.768	16.308	19.368

Fig. 4.3B

Western Blot							
Sr. No.	Brain Region	ABHD12			ABHD16A		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	Olfactory bulb	2.68	2.31	2.41	0.74	0.65	0.61
2	Prefrontal Cortex	2.14	2.45	2.3	0.39	0.4	0.35
3	Cortex	3.35	3.89	4.1	0.37	0.38	0.31
4	Hippocampus	3.27	3.13	3.5	0.35	0.36	0.32
5	Thalamus	3.34	2.9	2.5	0.35	0.31	0.34
6	Cerebellum	2.66	2.8	2.54	1.07	1.32	1.28

Fig. 4.4B

DAB-IHC-ABHD16A-Sagittal							
Sr. No.	Brain Region	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Granule layer	3.057	2.809	3.159	3.085	2.599	2.569
2	Molecular layer	1.813	1.702	1.868	2.443	2.759	1.731
3	Purkinje neurons	1.229	1.279	1.038	0.587	1.447	1.293
4	Arbour vitae	0.700	0.608	0.747	0.820	0.522	0.864
5	Hippocampus	1.545	1.494	1.759	1.943	1.616	1.668
6	Cortex	1.452	1.584	1.600	1.718	1.836	1.703
7	Olfactory bulb	1.428	1.553	1.627	1.425	1.542	1.660
8	Thalamus	1.809	1.634	1.738	1.717	1.710	1.713

Fig. 4.4D

DAB-IHC-ABHD16A-Coronal							
Sr. No.	Brain Region	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Granule layer	1.876	2.76	2.047	1.941	2.561	2.169
2	Molecular layer	1.643	1.757	1.697	1.382	1.607	1.513
3	Purkinje neurons	0.792	1.125	0.992	1.316	0.881	1.217
4	Arbour vitae	1.055	1.155	1.082	0.929	1.192	0.933
5	Hippocampus	1.494	1.314	1.332	1.405	1.379	1.329
6	Cortex	1.287	1.351	1.491	1.449	1.539	1.425
7	Olfactory bulb	1.963	1.823	1.959	1.845	1.901	1.5004
8	Thalamus	1.507	1.41	1.66	1.59	1.585	1.397

Fig. 4.4F

DAB-IHC-ABHD16A-Fluorescence							
Sr. No.	Brain Region	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Granule layer	5.732	6.171	4.357	5.411	5.486	5.041
2	Molecular layer	3.697	3.186	3.347	3.758	3.751	3.554
3	Purkinje neurons	1.188	1.281	1.183	1.146	1.2	1.318
4	Arbour vitae	2.102	2.17	2.464	2.696	1.982	2.026
5	Hippocampus	2.833	2.746	2.583	2.151	2.979	2.953
6	Cortex	2.187	1.932	2.204	2.209	1.953	2.052
7	Olfactory bulb	4.185	3.503	3.711	4.318	4.244	3.837
8	Thalamus	2.016	1.819	1.707	1.754	1.724	2.083

Fig. 4.6B

DAB-IHC-ABHD12-Sagital							
Sr. No.	Brain Region	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Granule layer	2.467	2.121	2.109	2.084	1.977	2.094
2	Molecular layer	1.187	1.697	1.464	1.469	2.019	1.622
3	Purkinje neurons	3.158	3.694	3.989	3.046	3.166	3.112
4	Arbour vitae	1.033	1.272	1.753	1.403	1.247	1.015
5	Hippocampus	2.978	2.981	3.328	3.018	3.167	3.192
6	Cortex	2.880	2.414	2.510	2.573	2.549	2.195
7	Olfactory bulb	3.368	2.388	2.244	2.263	2.233	1.609
8	Thalamus	2.757	2.850	1.672	1.163	2.546	2.329

Fig. 4.6D

DAB-IHC-ABHD12-Coronal							
Sr. No.	Brain Region	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Granule layer	1.974	1.753	1.915	1.55	1.692	1.926
2	Molecular layer	1.618	1.657	1.711	1.771	1.799	1.822
3	Purkinje neurons	2.639	2.87	3.295	3.033	2.749	2.968
4	Arbour vitae	1.388	1.482	1.506	1.383	1.576	1.152
5	Hippocampus	2.059	2.089	2.024	1.912	2.105	2.059
6	Cortex	2.8	2.775	2.702	2.744	2.509	2.752
7	Olfactory bulb	2.958	2.614	2.603	2.307	2.354	2.167
8	Thalamus	1.895	1.726	1.75	1.8	1.778	1.742

Fig. 4.6F

DAB-IHC-ABHD12-Fluorescence							
Sr. No.	Brain Region	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	Granule layer	3.177	2.522	2.849	2.388	2.499	2.678
2	Molecular layer	2.736	2.816	2.271	2.846	2.548	2.479
3	Purkinje neurons	5.435	5.712	5.683	5.87	5.307	5.343
4	Arbour vitae	1.38	1.311	1.505	1.151	1.45	1.292
5	Hippocampus	4.304	4.292	3.79	3.941	3.888	4.107
6	Cortex	3.406	3.589	2.798	3.425	3.643	3.677
7	Olfactory bulb	2.996	2.871	3.061	2.915	3.02	2.842
8	Thalamus	3.013	3.097	2.976	3.077	3.085	3.097

Fig. 4.8A

Lyso-PS heat map ABHD16A (log10 values of AHD16A KO/ABHD16A WT)							
Sr. No.	Species	Olfactory bulb	Prefrontal cortex	Cortex	Hippocampus	Thalamus	Cerebellum
1	C16:0	-0.357	-0.071	-0.022	-0.071	-0.071	-0.509
2	C18:0	-0.252	-0.041	-0.056	-0.041	-0.056	-0.721
3	C18:1	-0.276	-0.032	-0.066	-0.056	-0.092	-0.638
4	C20:0	-0.149	-0.022	-0.032	-0.076	-0.027	-0.420
5	C20:1	-0.066	-0.018	-0.027	-0.041	-0.022	-0.252
6	C20:4	-0.108	-0.056	-0.022	-0.022	-0.022	-0.367
7	C22:0	-0.060	-0.051	-0.071	-0.027	-0.032	-0.187
8	C22:4	-0.056	-0.027	-0.056	-0.032	-0.022	-0.194
9	C22:6	-0.119	-0.032	-0.041	-0.041	-0.009	-0.377
10	C24:0	-0.041	-0.009	-0.027	-0.022	-0.041	-0.174
11	C24:1	-0.081	-0.018	-0.022	-0.027	-0.032	-0.143

Fig. 4.8C

Lyso-PS heat map ABHD12 (log10 values of AHD12 KO/ABHD12 WT)							
Sr. No.	Species	Olfactory bulb	Prefrontal cortex	Cortex	Hippocampus	Thalamus	Cerebellum
1	C16:0	0.146	0.176	0.255	0.362	0.380	0.176
2	C18:0	0.491	0.398	0.462	0.505	0.556	0.544
3	C18:1	0.362	0.322	0.146	0.176	0.255	0.204
4	C20:0	1.176	1.176	1.079	1.176	1.079	1.380
5	C20:1	1.114	1.146	1.204	1.146	0.954	1.447
6	C20:4	1.079	1.204	1.146	1.079	1.000	1.491
7	C22:0	1.146	1.255	1.176	1.146	1.176	1.544
8	C22:4	1.176	1.146	1.114	1.204	1.146	1.580
9	C22:6	0.114	0.255	0.462	0.505	0.544	0.505
10	C24:0	1.362	1.204	1.255	1.398	1.342	1.716
11	C24:1	1.362	1.322	1.380	1.255	1.204	1.653

Fig. 4.8B

PS heat map ABHD16A (log10 values of AHD16A KO/ABHD16A WT)							
Sr. No.	Species	Olfactory bulb	Prefrontal cortex	Cortex	Hippocampus	Thalamus	Cerebellum
1	C16:0/18:1	0.049	-0.009	-0.018	-0.022	0.009	0.176
2	C18:0/18:1	-0.009	-0.013	-0.004	0.009	0.021	0.380
3	C18:1/18:1	-0.018	-0.004	-0.009	0.013	0.017	0.362
4	C16:0/20:4	0.049	0.000	-0.022	0.009	-0.009	0.041
5	C18:0/20:4	-0.963	0.049	-0.032	0.009	-0.004	0.037
6	C18:1/20:4	0.021	0.037	-0.009	0.017	-0.022	0.021
7	C16:0/22:6	0.033	0.025	-0.022	0.013	0.017	0.204
8	C18:0/22:6	-0.009	-0.018	-0.018	-0.013	0.025	0.079
9	C18:1/22:6	-0.009	-0.013	0.045	-0.009	0.013	0.176

Fig. 4.8D

PS heat map ABHD12 (log10 values of AHD12 KO/ABHD12 WT)							
Sr. No.	Species	Olfactory bulb	Prefrontal cortex	Cortex	Hippocampus	Thalamus	Cerebellum
1	C16:0/18:1	-0.268	-0.167	-0.125	-0.131	-0.092	-0.208
2	C18:0/18:1	-0.174	-0.187	-0.092	-0.119	-0.187	-0.268
3	C18:1/18:1	-0.284	-0.276	-0.215	-0.167	-0.268	-0.367
4	C16:0/20:4	0.193	0.207	0.272	0.220	0.243	0.258
5	C18:0/20:4	0.130	0.250	0.217	0.258	0.290	0.371
6	C18:1/20:4	0.367	0.364	0.384	0.326	0.258	0.507
7	C16:0/22:6	-0.068	-0.056	-0.108	-0.092	-0.071	-0.051
8	C18:0/22:6	-0.201	-0.194	-0.347	-0.252	-0.174	-0.229
9	C18:1/22:6	-0.252	-0.161	-0.137	-0.208	-0.149	-0.174

Fig. 5.1A

Neuro-2a Lyso-PS 18:0 treatment pERK				
Sr. No.	Time	Rep-1	Rep-2	Rep-3
1	0 min	0.07	0.05	0.08
2	5 min	0.282529	0.3	0.25
3	10 min	0.146942	0.17	0.18
4	15 min	0.963723	0.8	0.7
5	30 min	1.151615	1.3	1.2

Fig. 5.1B

Neuro-2a FFA 18:0 treatment pERK				
Sr. No.	Time	Rep-1	Rep-2	Rep-3
1	0 min	0.799	0.582	0.318
2	5 min	0.547	0.592	0.436
3	10 min	0.535	0.660	0.398
4	15 min	0.549	0.440	0.430
5	30 min	0.442	0.672	0.337

Fig. 5.1C

Neuro-2a Lyso-PS screen pERK				
Sr. No.	Treatment	Rep-1	Rep-2	Rep-3
1	veh	0.49489002	0.34143826	0.41816414
2	10:00	0.5369026	0.45684607	0.49687434
3	12:00	0.68278573	0.56567176	0.62422875
4	14:00	0.89411875	0.58640642	0.74026258
5	16:00	1.51080715	0.84376695	1.17728705
6	18:00	1.50897492	0.822722	1.16584846
7	20:00	1.23827689	0.76125904	0.99976797
8	22:00	0.8357461	0.63826493	0.73700551
9	24:00:00	0.37370016	0.57000245	0.4718513

Fig. 5.2A

Neuro-2a ABHD12 inhibition pERK				
Sr. No.	Treatment	Rep-1	Rep-2	Rep-3
1	Veh	0.25	0.4	0.3
2	JJH350	1.75997	1.3	1.5
3	KCO1	0.68995	0.521	0.42

Fig. 5.3A

Isolated Purkinje neurons pERK						
Sr. No.	Treatment	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5
1	Veh	67.311	61.678	2.758	0.064	65.28
2	Lyso-PS	133.888	132.97	76.5	143.03	114.553

Fig. 5.3B

WT- ABHD12 KO Purkinje neurons pERK							
Sr. No.	Genotype	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	WT	49.615	51.433	49.102	48.520	50.629	38.506
2	KO	74.730	83.917	72.615	73.764	84.085	89.406

Fig. 6.1C

No. of activated microglia in cerebellum							
Sr. No.	Strain	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	WT-Veh	0	0	3	5	2	1
2	WT-LPS	76	71	68	82	78	59
3	16A KO-Veh	0	4	0	5	0	1
4	16A KO-LPS	70	54	88	55	72	57
5	12 KO-Veh	13	5	7	9	2	3
6	12 KO -LPS	76	80	93	77	85	97

Fig. 6.1D

Size distribution of activated microglia in cerebellum							
WT							
Sr. No.	Microglia soma size (µm)	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	100-150	28.1908	33.5714	30.147	27.27	25.32	26.154
2	150-200	22.2857	21.0256	22.235	24.55	20.64	20.253
3	200-250	19.084	17.1429	20.059	19.28	15.29	18.293
4	250-300	13.3495	9.48905	8.209	9.756	7.975	10.256
5	300-350	7.52427	5.92157	6.5217	7.463	7.317	6.8493
6	350-400	3.81679	3.57143	3.2967	3.226	3.797	3.7037
7	400-450	3.39806	2.97826	4.4776	3.659	3.521	4.2735
8	450-500	2.29008	2.74725	2.5641	2.456	2.439	2.0548
9	500-550	3.5045	2.45631	3.9197	2.239	3.659	4.8405
10	550-600	0.76336	1.0989	1.8471	0.602	0.629	0.5236
11	600-650	0	0	0.5495	0.743	0	0.2548
12	650-700	0.76336	0	0	0.602	0	1.0471
13	700-750	0.76336	0.54945	0	0.602	0	0.6173
14	750-800	0	0	0.5495	0.743	0	0
15	800-850	0	0.74257	0.7353	0	0	0
16	850-900	0	0	0	0.248	0	0
17	900-950	0	0	0	0.495	0	0
18	950-1000	0.76336	0	0.5495	0.495	0	0.5236
16A KO							
Sr. No.	Microglia soma size (µm)	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	100-150	30.5677	33.8017	35.58	33.67	28.46	29.215
2	150-200	16.2162	18.75	23.243	15.73	19.33	23.039
3	200-250	9.90991	10.7639	10.811	16.98	18.16	24.755
4	250-300	10.8108	8.33333	9.1892	10.16	8.011	9.0098
5	300-350	5.36036	7.84722	7.027	4.667	5.028	8.3333
6	350-400	2.85586	2.33333	4.7297	3.81	2.028	2.2059
7	400-450	1.42857	2.64835	2.6178	1.258	1.538	1.8519
8	450-500	2.07254	2.08333	1.9481	0.635	1.235	1.6961
9	500-550	2.29008	1.47059	3.6289	2.424	1.935	2.5181
10	550-600	0.97087	1.96078	1.4599	0.317	0.838	1.4706
11	600-650	1.8018	1.73611	2.1622	1.27	0.838	0.9804
12	650-700	0.72816	1.96078	0	0.635	0.559	0.7353
13	700-750	0	0	1.4599	0.746	1.227	0
14	750-800	0.48544	0	0	0	0	0.8547
15	800-850	0.9009	0.38889	0.6216	0.317	0.117	0.2451
16	850-900	0.35135	0.41667	0.6216	0.317	0.559	0.7353
17	900-950	0.8018	0.04167	0.0811	0.317	0	0
18	950-1000	0.45045	2.08333	0.5405	0	0	0

12 KO							
Sr. No.	Microglia soma s	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	100-150	20.3922	24.1781	25.543	30.6	28.22	23.077
2	150-200	22.549	15.6863	14.043	15.69	18.41	16.239
3	200-250	14.6275	16.6667	21.925	18.29	18.27	19.068
4	250-300	12.2137	11.8812	18.824	10.99	13.84	11.613
5	300-350	9.92366	9.40594	8.9005	11.83	7.273	9.2308
6	350-400	2.6699	5.83942	5.9783	6.838	5.479	4.9825
7	400-450	9.45946	6.94444	5.9459	3.81	6.676	4.7157
8	450-500	5.40541	3.26087	3.4925	2.454	5.983	5.9459
9	500-550	5.73973	4.16667	4.8649	5.317	3.235	2.451
10	550-600	4.95495	2.77778	3.7838	2.985	2.454	1.7094
11	600-650	0.48544	0.98039	1.4599	1.493	2.439	0.8547
12	650-700	3.6036	2.08333	2.7027	1.46	1.087	0.6849
13	700-750	1.35135	1.04167	1.0811	0.952	0.559	0.7353
14	750-800	0.7027	0.43056	0.8108	0.317	0.279	0.2451
15	800-850	0.24272	0.98039	0	0.46	0.63	0.7463
16	850-900	0.72816	0	0	0	0.543	0
17	900-950	0.72816	0	0	0.73	0.543	0
18	950-1000	0.48544	0	0	0	0	0

Fig. 6.2C

ABHD16A-ABHD12 levels in Brain - Western Blot							
Sr. No.	Protein	Vehicle			Acute LPS treatment		
		Rep-1	Rep-2	Rep-3	Rep-1	Rep-2	Rep-3
1	ABHD16A	1.97276	1.53579	1.3538	2.232	1.685	0.8975
2	ABHD12	0.83165	0.91786	0.9918	1.359	1.653	1.1024

Fig. 6.2D

ABHD16A-ABHD12 levels in Brain - ABPP (LPS/Vehicle)				
Sr. No.	Protein	Rep-1	Rep-2	
1	Abhd16a	1.23032	1.13805	
2	Abhd12	1.84902	1.31096	

Fig. 6.3B

Prostaglandin measurements													
Sr. No.	Species	Acute Veh ABHD12 WT			Acute Veh ABHD12 KO			Acute LPS ABHD12 WT			Acute LPS ABHD12 KO		
		Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N
1	PGE2	23	3	4	25	3	4	112	15	4	126	12	4
2	PGD2	39	5	4	42	5	4	134	15	4	142	16	4

Fig. 6.3C

Lyso-PS measurements													
Sr. No.	Species	Acute Veh ABHD12 WT			Acute Veh ABHD12 KO			Acute LPS ABHD12 WT			Acute LPS ABHD12 KO		
		Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N
1	18:0	3.4	0.4	4	8.8	0.9	4	3.5	0.5	4	8.7	0.9	4
2	18:1	3.1	0.5	4	5.1	0.7	4	3.5	0.4	4	5.2	0.6	4
3	22:6	5.6	0.6	4	6.4	0.8	4	4.9	0.6	4	6.5	0.8	4
4	16:0	0.19	0.03	4	0.65	0.08	4	0.16	0.02	4	0.68	0.07	4
5	20:4	0.32	0.04	4	0.81	0.09	4	0.35	0.05	4	0.85	0.08	4
6	20:1	0.15	0.03	4	0.65	0.07	4	0.14	0.03	4	0.68	0.09	4
7	20:0	0.09	0.02	4	0.73	0.08	4	0.09	0.02	4	0.75	0.09	4
8	22:4	0.41	0.05	4	0.78	0.08	4	0.38	0.04	4	0.81	0.09	4
9	22:1	0.18	0.03	4	0.67	0.07	4	0.14	0.03	4	0.76	0.09	4
10	22:0	0.14	0.02	4	0.75	0.08	4	0.15	0.03	4	0.74	0.08	4
11	24:1	0.13	0.02	4	0.69	0.07	4	0.18	0.03	4	0.63	0.08	4
12	24:0	0.14	0.02	4	0.73	0.08	4	0.16	0.03	4	0.71	0.08	4

Fig. 6.3D

PS measurements													
Sr. No.	Species	Acute Veh ABHD12 WT			Acute Veh ABHD12 KO			Acute LPS ABHD12 WT			Acute LPS ABHD12 KO		
		Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N
1	18:1/18:1	500.4	55.21	4	280.2	8.214	4	513.8	38.321	4	270	15.21	4
2	18:0/18:1	348.2	58.21	4	309.5	45.21	4	318.5	7.214	4	319	25.21	4
3	18:0/20:4	195.8	47.22	4	512.4	64.21	4	226.6	44.214	4	521	50.42	4
4	16:0/18:1	103.2	11.22	4	24.21	2.142	4	81.52	0.7214	4	21.5	3.241	4
5	18:1/22:6	61.28	2.985	4	39.45	1.589	4	56.55	3.4145	4	43.8	2.124	4
6	18:0/20:1	39.58	1.325	4	24.82	2.5	4	49.49	5.782	4	21.1	2.214	4
7	18:1/20:4	28.55	3.215	4	54.5	3.214	4	35.16	4	4	59.5	4.125	4
8	18:1/20:1	7.215	0.912	4	3.985	0.756	4	6.587	0.587	4	2.9	0.58	4
9	20:1/22:6	5.214	0.654	4	3.608	0.589	4	3.895	0.9875	4	3.29	0.523	4
10	20:0/22:6	1.54	0.125	4	2.53	0.214	4	1.92	0.384	4	1.45	0.114	4
11	20:0/20:4	0.789	0.125	4	1.987	0.295	4	0.785	0.0247	4	2.15	0.211	4
12	20:1/20:4	0.875	0.098	4	3.589	0.399	4	0.895	0.058	4	2.95	0.321	4
13	22:0/20:4	0.5	0.079	4	2.987	0.458	4	0.485	0.1258	4	2.22	0.093	4
14	22:1/20:4	0.242	0.046	4	2.489	0.198	4	0.321	0.0255	4	2.79	0.046	4
15	24:1/20:4	0.124	0.002	4	0.987	0.215	4	0.15	0.0245	4	1.59	0.57	4
16	24:0/20:4	0.065	0.013	4	0.358	0.099	4	0.059	0.0421	4	0.13	0.003	4

Fig. 6.3E

ox-PS measurements													
Sr. No.	Species	Acute Veh ABHD12 WT			Acute Veh ABHD12 KO			Acute LPS ABHD12 WT			Acute LPS ABHD12 KO		
		Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.	N
1	ox-18:0/18	0	0	4	0	0	4	0.18	0.03	4	0.95	0.11	4
2	hy-18:0/18	0	0	4	0	0	4	0.13	0.03	4	0.89	0.12	4
3	ox-18:1/18	0	0	4	0	0	4	0.42	0.05	4	1.32	0.15	4
4	hy-18:1/18	0	0	4	0	0	4	0.32	0.04	4	0.91	0.09	4
5	ox-18:0/20	0.002	0.001	4	0.003	0.002	4	0.19	0.03	4	0.81	0.09	4
6	hy-18:0/20	0	0	4	0	0	4	0.23	0.03	4	1.12	0.15	4
7	ox-18:0/22	0.001	0	4	0.002	0	4	0.21	0.02	4	1.45	0.16	4
8	hy-18:0/22	0.003	0.001	4	0.004	0.002	4	0.41	0.04	4	1.32	0.18	4

Fig. 6.4B

ABHD16A -ABHD12 Double knockout microglial activation study																			
Sr. No.	Strain	ABHD16A						ABHD12						Double KO-WT					
1	WT	4	3	11	8	16	12	5	6	13	8	12	14	11	9	17	12	11	12
2	KO	9	8	13	9	12	5	30	37	48	31	50	45	22	20	12	17	14	10

Fig. 6.5B

No. of activated microglia (Cerebellum)				
Replicate	Vehicle	LPS	Lyso-PS 24:0	Lyso-PS 18:0
Rep-1	4	23	21	3
Rep-2	5	29	28	4
Rep-3	2	21	25	2
Rep-4	3	19	22	2
Rep-5	2	25	24	2
Rep-6	2	26	19	5
No. of activated microglia (Cortex)				
Replicate	Vehicle	LPS	Lyso-PS 24:0	Lyso-PS 18:0
Rep-1	2	21	25	4
Rep-2	1	23	26	5
Rep-3	3	25	21	2
Rep-4	3	22	23	2
Rep-5	3	19	17	1
Rep-6	2	29	19	1

No. of activated microglia (Hippocampus)				
Replicate	Vehicle	LPS	Lyso-PS 24:0	Lyso-PS 18:0
Rep-1	6	32	23	11
Rep-2	5	24	35	8
Rep-3	9	21	24	7
Rep-4	9	22	26	6
Rep-5	7	28	27	9
Rep-6	5	27	22	9

Fig. 6.6

No. of activated microglia									
		Veh							
	Genotype	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	Rep-7	Rep-8
Cerebellum	WT	5.25	0.75	0.5	1.25	0.5	1.75	1	0.75
	TLR2 KO	0.25	0.5	1.25	1	0.75	2.25	2.25	2
Cortex	WT	5	6	5	8	6	4	4	4
	TLR2 KO	2	4	3	8	5	5	11	6
Hippocampus	WT	5	7	7	6	13	11	9	9
	TLR2 KO	8	9	10	12	8	9	6	8
LPS									
		Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	Rep-7	Rep-8
Cerebellum	WT	18	16	17	14.5	22	13.5	17.5	17.5
	TLR2 KO	16.25	19.25	17.25	16	18.5	15.5	10.75	16
Cortex	WT	32	34	32	37	30	33	32	34
	TLR2 KO	34	30	43	40	35	27	35	35
Hippocampus	WT	28	25	33	31	29	28	27	28
	TLR2 KO	28	26	34	41	42	36	30	35
LysoPS 24:0									
		Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	Rep-7	Rep-8
Cerebellum	WT	20.75	15.75	17.5	19.5	20.75	20.5	17	19.5
	TLR2 KO	1.75	2	2.75	7.5	2.5	3.75	1.75	3
Cortex	WT	31	30	31	31	33	27	24	28
	TLR2 KO	7	10	3	5	6	6	4	5
Hippocampus	WT	27	28	28	34	25	24	33	26
	TLR2 KO	13	10	11	14	9	9	9	8

Fig. 6.7

No. of activated microglia							
Sr. No.	Genotype	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6
1	WT	5	6	5	9	7	8
2	12 KO - Vehicle	49	32	31	45	26	46
3	12 KO - Minocycline	15	19	26	17	17	13