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# Acylglycerophosphate acyltransferase 4 (AGPAT4) is a mitochondrial lysophosphatidic acid acyltransferase that regulates brain phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol levels

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ABSTRACT

The acylglycerophosphate acyltransferase/lysophosphatidic acid acyltransferase (AGPAT/LPAAT) family is a group of homologous acyl-CoA-dependent lysophospholipid acyltransferases. We performed studies to better understand the subcellular localization, activity, and *in vivo* function of AGPAT4/LPAATδ, which we found is expressed in multiple mouse brain regions. Endogenous brain AGPAT4 and AGPAT4 overexpressed in HEK293 or Sf9 insect cells localizes to mitochondria and is resident on the outer mitochondrial membrane. Further fractionation showed that AGPAT4 is present specifically in the mitochondria and not in the mitochondria-associated endoplasmic reticulum membrane (*i.e.* MAM). Lysates from Sf9 cells infected with baculoviral *Agpat4* were tested with eight lysophospholipid species but showed an increased activity only with lysophosphatidic acid as an acyl acceptor. Analysis of Sf9 phospholipid species, however, indicated a significant 72% increase in phosphatidylinositol (PI) content. We examined the content of major phospholipid species in brains of *Agpat4*$^{-/-}$ mice and found also a >50% decrease in total levels of PI relative to *wildtype* mice, as well as significant decreases in phosphatidylcholine (PC) and phosphatidylethanolamine (PE), but no significant differences in phosphatidylserine, phosphatidylglycerol, cardiolipin, or phosphatidic acid (PA). A compensatory upregulation of *Agpats 1*, *2*, *3*, *5*, and *9* may help to explain the lack of difference in PA. Our findings indicate that AGPAT4 is a mitochondrial AGPAT/LPAAT that specifically supports synthesis of brain PI, PC, and PE. This understanding may help to explain apparent redundancies in the AGPAT/LPAAT family.

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## 1. Introduction

The acylglycerophosphate acyltransferase/lysophosphatidic acid acyltransferase (AGPAT/LPAAT) family is a group of proteins that have been identified based on sequence homology, where AGPAT1 was the first isoform to be characterized [1–3]. This enzyme catalyzes the formation of phosphatidic acid (PA) through the fatty acyl-CoA-dependent esterification of lysophosphatidic acid (LPA) in the synthesis of PA in the second committed step of the *de novo* Kennedy pathway for phospholipid and triacylglycerol biosynthesis [4], as reviewed in Kitson *et al.* [5]. Thus far, at least eleven AGPAT family members have been identified in mice and humans [5]. However, subsequent functional characterization of these enzymes has led to some reclassifications. For example, AGPAT7 is now known as lysophosphatidylethanolamine acyltransferase 2 (LPEAT2) [6], AGPAT8 is now acyl-CoA:lysocardiolipin acyltransferase 1 (ALCAT1) [7], and AGPAT11 is now lysophosphatidylcholine acyltransferase 2 (LPCAT2) [8].

*Abbreviations:* AGPAT, acylglycerophosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; LPA, lysophosphatidic acid; ALCAT, acyl-CoA: lysocardiolipin acyltransferase; LPEAT, lysophosphatidylethanolamine acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; DHA, docosahexanoic acid; GFP, green fluorescent protein; CL, cardiolipin; CDP-DAG, cytidine diphosphate-diacylglycerol; TLC, thin layer chromatography; HA, hemagglutinin; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HBSS, Hank's Buffered Saline Solution; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; MLCL, monolysocardiolipin; DLCL, dilysocardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol; MAM, mitochondria-associated ER membrane

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In our efforts to functionally characterize novel enzymes of importance in lipid metabolism, we studied a member of the AGPAT family of homologous proteins known as AGPAT4 (LPAAT4/LPAATδ), which is most highly expressed in brain [2,9]. In our initial investigations on AGPAT4, we found that both endogenous and overexpressed AGPAT4 chiefly localizes to the mitochondria. These findings led us to broadly test the lysophospholipid substrate specificity of this enzyme, with the idea that it may be directly involved in the synthesis of mitochondria-associated phospholipids, and therefore may be a candidate for functional reclassification. In the present work however, we found that AGPAT4 only uses LPA when assayed *in vitro*, and does not utilize lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), lysophosphatidylglycerol (LPG), or lysophosphatidylinositol (LPI) as acyl-acceptors, in agreement with previous reports [2,9], and also does not utilize monolysocardiolipin (MLCL), or dilysocardiolipin (DLCL) which we test for the first time in the present study. This precludes a direct role for AGPAT4 in the synthesis of other phospholipids, and indicates that this enzyme should remain classified as an AGPAT/LPAAT. However, further analyses *in vivo* have uncovered a novel role for AGPAT4 in the indirect regulation of other phospholipids. Our studies on AGPAT4 overexpression in Sf9 cells, and *Agpat4* gene ablation in mice, have identified this enzyme as a regulator of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine levels. Here, we report the results of our investigations on the subcellular localization, activity, and role of AGPAT4 in brain phospholipid composition using insect and mammalian cell culture models as well as *Agpat4* gene knockout mice (*Agpat4*$^{-/-}$).

## 2. Materials and methods

### 2.1. Materials

Lysophospholipids, phospholipid standards, and non-radiolabeled fatty acyl-CoAs were from Avanti Polar Lipids (Alabaster, AL). Superscript® II Reverse Transcriptase, Lipofectamine 2000, TRIzol®, cell culture medium and additives were from Life Technologies (Carlsbad, CA). Thin layer chromatography (TLC) plates were from Analtech (Newark, DE). All radiochemicals were from Moravek Biochemicals and Radiochemicals (Brea, CA). Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

### 2.2. Animals

All animal procedures were approved by the University of Waterloo Animal Care Committee. Mice were housed in a temperature and humidity controlled environment, on a 12:12 h reversed light/dark cycle, and standard rodent chow and water were provided *ad libitum*. Female C57BL/6J mice age 3–4 months were used for studies on endogenous AGPAT4 levels and localization in brain mitochondria. Heterozygous *Agpat4*$^{-/+}$ mouse embryos were cryorevived at the Mutant Mouse Regional Resource Centre, from a strain produced by Lexicon Genetics/Genentech (strain B6;129S5-*Agpat4*$^{tm1Lex}$/Mmucd). The production of these mice as part of a large phenotyping project has been reported previously, but phenotypic information on this strain in particular has not yet been published [10]. *Agpat4* gene ablation was achieved by targeted replacement of exons 4, 5, and 6 in mouse 129S5 embryonic stem cells with a LacZ/neomycin selection cassette, and positive clones were identified by Southern blotting [11]. Progeny from two clones (1A9 and 1F9) achieved germline transmission after crossing with C57BL/6J mice, and cryorevived heterozygous offspring were sent from the University of California at Davis to the University of Waterloo, where female and male heterozygous littermates were crossed to produce *wildtype* and total null (*Agpat4*$^{-/-}$) animals. Adult male *Agpat4*$^{-/-}$ mice, aged 11–17 weeks were used in studies, and in all experiments age-matched *wildtype* littermates were used as controls. DNA was isolated from ear punches and genotyping was performed using primers specific to the *wildtype* and *targeted* alleles (*wildtype*, forward: 5′-TTA GCA TAG TGG GCG AAG TTC-3′, reverse: 5′-GGT AGT GGC CAA GTT AAT AGT CCT-3′; *knockout*, forward: 5′-GCA GCG CAT CGC CTT CTA TC-3′, reverse: 5′-CTC CCA TTT CTA GGA AGG AAG CAG-3′). Loss of exons 4–6 was confirmed by RT-PCR in multiple tissues from different mice using a forward primer in the deleted fourth exon (5′-GAG TCT TCT GGG AAG ACC CCT GTC-3′) and a reverse primer in the sixth exon (5′-ATC ACG CTG ACT GCT ACG TTC GGA-3′).

### 2.3. Cloning of full-length AGPAT4 cDNA, AGPAT4-GFP, and generation of AGPAT4 baculovirus and protein in Sf9 cells

Full length murine AGPAT4 (NM_026644.2) was amplified by PCR from mouse whole brain cDNA with the addition of BglII and SalI restriction sites to the N-terminal and C-terminal ends, respectively, using the following primers: forward 5′-AGA TCT ACC ATG GAC CTC ATC GGG CTG-3′ and reverse 5′-CGT CGA CTT GTC CGT TTG TTT CCG TTT G-3′. The resulting amplicon was subcloned into pGEM-T-Easy resulting in production of pGEM-AGPAT4 that was verified by direct sequencing, and was then cut with BglII/SalI restriction enzymes for directional subcloning into pEGFP-N1 in frame with green fluorescent protein (GFP). Baculovirus was generated using the Bac-to-Bac® Baculovirus Expression System. A full length AGPAT4 amplicon with a C-terminal 6 x His tag and BglII and SalI restriction sites at the N-terminal and C-terminal ends was produced by PCR using 50 ng of pGEM-AGPAT4 as template and the following primers: forward 5′-AGA TCT ACC ATG GAC CTC ATC GGG CTG-3′; reverse 5′-GTC GAC GTG GTG GTG GTG GTG GTC CGT TTG TTT CCG TTT GTT GTC G-3′. The resulting amplicon was subcloned into pGEM-T-Easy for direct sequencing, forming pGEM-AGPAT4-6His. This vector was subsequently digested using NotI/SalI to release full-length AGPAT4 that was subcloned into NotI/SalI restriction sites in pCMV-3Tag-3A, which was then digested with KpnI/NotI releasing full-length AGPAT4 that was subcloned in frame with the 3× HA tag into pFastBacI. pFastBacI-AGPAT4 was transformed into DH10Bac chemically competent cells and plated on triple selection (Kanamycin, Gentamicin, Tetracycline) LB plates treated with Xgal and IPTG. DNA was prepared from positive white colonies, verified by direct sequencing, and transfected into Sf9 cells grown in Sf-900 III media, and incubated for 3–4 days to generate baculovirus. Control baculovirus was produced by the same method but using pFastBacI without DNA insertion into the multiple cloning site. Amplified baculovirus was titred by the end point dilution method in cultures of Sf9 cells grown in 6-well plates, and harvested at 70–80% viability as indicated by staining a subset of cells with trypan blue. Infected Sf9 cells were harvested, and lysed in Lysis Buffer 1 (100 mM Tris–HCl, pH 7.4, 5 mM NaCl, 3 mM $MgCl_2$) by sonication in an ice-slurry bath (65% output, 3 × 6 s), which efficiently disrupts mitochondria and other organelles. Unbroken cells and organelles were cleared by centrifugation (10,000 × *g* for 10 min). This process will pellet any remaining unbroken mitochondria, but not sub-mitochondrial particles generated by sonication. Protein in supernatants was quantified using Bradford assay solution and diluted to 1 μg/μL for use in radiochemical enzymatic activity assays or Western blotting.

### 2.4. RNA Extraction, Reverse Transcription (RT) PCR, and RT qPCR

Whole-embryos and mouse organs were collected and flash frozen in liquid nitrogen at the Central Animal Facility at the University of Waterloo. Total RNA was isolated from tissues using TRIzol® Reagent (1 mL/≤100 mg tissue), essentially as described by the manufacturer, and a Polytron homogenizer (VWR, Radnor PA) set at the highest speed. Isolated RNA was dissolved in DEPC water and stored long-term at −80 °C. RNA samples were quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham MA), and cDNA was synthesized from 2 μg of RNA by oligo(dT) priming using SuperScript II Reverse Transcriptase according to the manufacturer's protocol.

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