Long-chain Acyl CoA Synthetase 6 Preferentially Promotes DHA Metabolism

Joseph R. Marszalek¹, Claire Kitidis¹, Concetta C. DiRusso³,⁴ and Harvey F. Lodish¹,²,⁵

¹Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142; ²Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, 02142, ³Ordway Research Institute, Inc & The Albany Medical College, Albany, NY 12208

⁵To whom correspondence should be addressed at Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142. Telephone: (617) 258-5216 Fax: (617) 258-6768. E-mail: lodish@wi.mit.edu

Running title: DHA metabolism and AcsL6.

Abbreviations: PUFA, polyunsaturated fatty acid; FA, fatty acid; OA, oleic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; PL, phospholipids; DAG, diacylglyceride; TAG, triacylglyceride; AcsL, acyl-CoA synthetase long-chain family member; FATP, fatty acid transport protein; GFP, green fluorescent protein; FBS, fetal bovine serum; NGF, nerve growth factor; TLC, thin-layer chromatography; GC/MS, gas chromatography/mass spectrometry; MAM, mitochondrial associated membranes; CoA, coenzyme A
Abstract:
Previously we demonstrated that supplementation with the polyunsaturated fatty acids (PUFA) arachidonic acid (AA) or docosahexaenoic acid (DHA) increased neurite outgrowth of PC12 cells during differentiation, and that over-expression of rat acyl-CoA synthetase long-chain family member 6 (Acsl6 – formerly ACS2) further increased PUFA-enhanced neurite outgrowth. However, whether Acsl6 over-expression enhanced the amount of PUFA accumulated in the cells or altered the partitioning of any FA into phospholipids (PLs) or triglycerides (TAGs) was unknown. Here we show that Acsl6 over-expression specifically promotes DHA internalization, activation to DHA-CoA, and accumulation in differentiating PC12 cells. In contrast, OA and AA internalization and activation to OA-CoA and AA-CoA were increased only marginally by Acsl6 over-expression. Additionally, the level of total cellular PLs was increased in Acsl6 over-expressing cells when the medium was supplemented with AA and DHA, but not with OA. Acsl6 over-expression increased the incorporation of [14C]-labeled OA, AA or DHA into PLs and TAGs. These results do not support a role for Acsl6 in the specific targeting of FAs into PLs or TAGs. Rather, our data support the hypothesis that Acsl6 functions primarily in DHA metabolism, and that its over-expression increases DHA and AA internalization primarily during the first 24 hours of neuronal differentiation to stimulate PL synthesis and enhance neurite outgrowth.
Introduction:

Compared to other tissues, the nervous system is highly enriched in polyunsaturated fatty acids (PUFAs) that fall into two categories based on the presence of a double bond at the n-6 or n-3 positions of the FA. Arachidonic acid (AA, 20:4 n-6) and docosahexaenoic acid (DHA, 22:6 n-3) are the most abundant PUFAs, but cannot be synthesized de novo by mammals because the desaturase enzymes required to introduce double bonds at the n-3 and n-6 positions of FAs are not present (1). Therefore, AA and DHA, or their precursors, must be ingested from dietary sources and transported to the brain. During late gestation and early postnatal development neurodevelopment is especially rapid and AA and DHA are critical to ensure proper brain and retina growth. AA and DHA are normally transferred by the placenta to the fetus during the last trimester of pregnancy and in breast milk to infants (2). However, if the postnatal diet is not properly supplemented with these essential FAs, FA deficiency may manifest itself in the form of neurological and visual deficits. Premature infants are at even greater risk because they are deprived of the essential FA loading that normally occurs during the last trimester of gestational development (3).

Despite their obvious importance, the functions PUFAs perform during this critical period of development have yet to be defined. Several reports establish that supplementation of neuronal cells with AA or DHA can affect neurite outgrowth indicating that PUFAs may be critical for proper neuronal differentiation (4-11). However, little is known about the mechanism or proteins that are responsible for PUFA internalization and their subsequent metabolism.
While it remains controversial how FA cross the plasma membrane, considerable evidence implicate diffusion through the PL bilayer as well as several integral and associated plasma membrane proteins as facilitators of free FA transport across the lipid bilayer (12-14). After internalization, FAs are activated primarily by acyl CoA synthetase long-chain family members (Acsls, formerly ACSs), which utilize ATP to catalyze the formation of FA-CoA by esterifying the FA to coenzyme A (CoA). Once activated, the FA can function as a signaling molecule, be incorporated into phospholipids (PLs) or triacylglycerides (TAGs), or undergo β-oxidization in mitochondria for energy generation.

Since Acsl proteins catalyze an essential step in the metabolism of FA, it seemed likely that multiple isoforms exist with different preferences for FA species. This was first confirmed by the biochemical isolation of an arachidonoyl-specific acyl CoA synthetase in human platelets that was different from the original acyl CoA synthetase enzyme that had broad FA specificity (15). Subsequently, five mammalian Acsl proteins in all have been identified and biochemical studies using bacterially expressed Acsl proteins have shown that each protein activates different FAs with unique efficiencies. Acsl1 and Acsl5 activate most unsaturated FAs similarly (16,17), while Acsl3, Acsl4 and Acsl6 (formerly ACS2) preferentially activate PUFAs (17-19). In fact, Acsl6 activates AA and DHA two and eight-fold more efficiently, respectively, than does Acsl1. Compared to OA, the initial rate AA and DHA uptake was increased more in PC12 cells over-expressing Acsl6 than in cells over-expressing Acsl1, providing in vivo evidence that Acsl6 preferentially metabolizes PUFAs (7).

A series of biochemical and cell fractionation experiments established that different Acsl proteins have unique intracellular localizations, suggesting that each protein may partition FAs toward different metabolic fates. Acsl1 protein has been proposed to function in FA
internalization and TAG synthesis because it localizes to the plasma membrane in 3T3-L1 cells (20,21) and to ER and mitochondrial associated membranes (MAM) in primary rat hepatocytes (22). Consistent with these proposals, FA internalization in 3T3-L1 and PC12 cells (7,23) and TAG levels in PC12 cells (7,23) are increased in cells that over-express Acsl1 protein. Acsl4 protein localizes to MAM and peroxisomes in primary rat hepatocytes suggesting it functions in TAG synthesis and FA oxidation (22). Acsl5 is the only Acsl localized to mitochondria in primary rat liver hepatocytes where it likely activates FAs for β-oxidation (22). The intracellular localizations of Acsl6 and Acsl3 have yet to be determined. However, Acsl6 over-expression in PC12 cells increases FA internalization, neurite outgrowth and TAG accumulation, which are consistent with localization to the plasma membrane, ER and MAM. Taken together, these data suggest that differential expression of Acsl proteins may contribute to PUFA enrichment in the PLs of neurons.

To test the hypothesis that Acsl6 enhances neurite outgrowth by preferential PUFA accumulation and partitioning into PLs, we over-expressed Acsl6 in differentiating PC12 cells and examined the metabolic fate of internalized FAs. We show that Acsl6 over-expression selectively enhances DHA internalization, activation to DHA-CoA, and accumulation, but does not increase FA targeting to PLs relative to TAGs.
Materials and Methods:

Generation of Stable Acsl6 Over-Expressing Cells

To generate stable populations of control and Acsl6 over-expressing PC12 cells, retroviral supernatants containing control (pMSCVpuroIRES2-EGFP) or rat Acsl6 (pMSCVpuro-ratACS2-IRES2-EGFP) retrovirus were used to infect PC12 cells as described previously (7). Two days after infection, transformed cells were selected by incubation with 5 µg/ml puromycin for 48 hours. The level of GFP fluorescence was determined for 10,000 cells using a BD Biosciences FACScan to ensure that greater than 95% of the cells were transformed. The cells were then expanded for 2 weeks, the transformation rate confirmed again by FACS analysis, and aliquots of the cells were frozen until needed. The cells used for analysis were in culture for 2-8 weeks after infection.

Western Analysis of Acsl6 Protein

A peptide corresponding to the first 19 amino acids (MQTQEILRILRLPELSDLG) at the amino terminus of rat Acsl6 protein was synthesized and coupled to KLH. Antibodies to the peptide were raised in rabbits. Undifferentiated control and Acsl6 over-expressing cells were harvested, washed with 1X PBS and resuspended in 1X sample buffer (2% SDS, 150 mM TRIS pH 6.8, 10% glycerol, 700 mM 2-mercaptoethanol) at a concentration of 10^6 cells per ml. Cells were lysed and DNA sheared by trituration through a Hamilton syringe and heated at 80°C for 10 min. Equal amounts of protein extract from control and Acsl6 over-expressing cells were separated on a 7% NuPAGE Tris-Acetate gel (Invitrogen), transferred to nitrocellulose, and probed with anti-Acsl6 antibody. The membrane was then probed with HRP-conjugated sheep anti-mouse secondary antibody and Acsl6 protein levels were visualized using an ECL Western blotting detection kit (Amersham Bioscience) followed by exposure to film X-Omat Blue (Kodak).
Quantitative-PCR

Aliquots of undifferentiated control and Acs16 over-expressing cells were collected at the same time that these cells were plated for differentiation experiments. Total RNA was extracted and the amount of Acs1-6 mRNAs determined as described previously (7).

Acyl-CoA Synthetase Activity Assay

Undifferentiated control and Acs16 over-expressing cells were treated for 5 min. at 37°C with 0.25% trypsin and then washed three times with 1X PBS. Cells were pelleted, resuspended in STE (10 mM Tris base, 10 mM NaCl, 1 mM EDTA) at 10^7 cells/ml and then sonicated for two min in a cup sonicator. The extract was then centrifuged for 10 min. at 1500 rpm to remove insoluble material. The lysate was then diluted 1:4 (v/v) with STE. One hundred microliters of the extract was added to a reaction mix containing 250 µl of 2X reaction mix (0.1 M Tris base pH 8.0, 20 mM MgCl₂, 0.02% Triton X-100, 0.6 mM DTT), 50 µl of 100 mM ATP, 50 µl of 0.5 mM [14C]-OA, [14C]-AA or [14C]-DHA (500 ng of cold 5 mM OA/ 7 ng of [1- 14C]-FA (American Radiolabeled Chemicals – ARC-297 (OA), ARC-290 (AA) and ARC-380 (DHA) in 1 ml of 10 mg/ml α-cyclodextrin). The reaction was started by adding 50 µl of 2 mM CoA, incubated for 5-10’ min. at 37°C, and stopped by adding 2.5 ml isopropanol:n-heptane:1M H₂SO₄ (40:10:1 by volume). Newly synthesized [14C]-FA-CoA was separated from unesterified [14C]-FA by first adding 0.5 ml of water and then extracting three times with 2.5 ml n-heptane. The amount of [14C]-FA-CoA synthesized was determined by measuring the amount of radioactivity in 500 µl of the aqueous phase using a scintillation counter.

Differentiation of Cells for Lipid Analysis

Control and Acs16 over-expressing PC12 cells were maintained in 10% FBS in RPMI 1640 medium on collagen coated plates (VWR- Biocoat) at 37°C in an incubator with 5% CO₂.
were split bi-weekly at 5 x 10^6 cells/100 mm plate. For each analysis, cells were treated for 8 min. at 37°C with 0.25% trypsin, triturated through a 19-guage needle attached to a syringe, counted, and plated in growth media at the specified concentrations. Normal differentiation medium contains 4 mole % OA, which rises to 30.7% when supplemented with 5 µM OA. Normal differentiation medium contains no AA or DHA, and the mole % rises to 19.5% and 22.0%, respectively when supplemented with 5 µM of AA or DHA (Table I). For the 15 min. [14C]-FA labeling experiments, cells were plated at 10^6 cells per well of 6-well plates. For each [14C]-FA condition, twenty-four hours after plating, the growth media was exchanged for differentiation media (0.25% horse serum/ RPMI1640/ 75 ng/ml NGF (Alexis)/ 15 µM FA free-BSA) supplemented with 2.5 µM [14C]-FA and 2.5 µM unlabeled FA. [1-14C]-OA, [1-14C]-AA and [1-14C]-DHA were purchased from American Radiolabeled Chemicals – ARC-297 (OA), ARC-290 (AA) and ARC-380 (DHA). After 15 minutes of incubation, each well was rinsed with 1X PBS/0.1% FA-free BSA, the cells were removed and transferred to a glass tube, pelleted and the supernatant discarded. Cell pellets were capped under a stream of nitrogen and stored at -80°C until analysis. For 72 hour incubation experiments, control and Acsl6 over-expressing cells were each plated onto 100 mm plates at 7.5 x 10^5 cells/ plate. For total cellular PL measurements and GC/MS analysis, after 24-hours growth media was exchanged for differentiation media that contained 5 µM OA, AA or DHA. For 72 hour [14C]-FA labeling, 0.5 µM of the 5 µM unlabeled FA was replaced with an equivalent amount of [14C]-OA, [14C]-AA or [14C]-DHA. For each experiment, the cells were collected after 72 hours of differentiation as described for the 15 minute [14C]-FA labeling experiment.

To determine the rate of [14C]-FA removal from the media, 100 µl of media from the [14C]-FA supplemented differentiating cells was collected every 24 hours and compared to the
amount of $[^{14}C]$-FA at the start of differentiation. Detached and dead cells were removed by centrifugation at full speed in a microfuge for 5 min. and the amount of radioactivity in the media determined by adding the media to 4 ml of EcoScint H (National Diagnostics) followed by scintillation counting.

**Analysis of $[^{14}C]$-FA Incorporation into Complex Lipids**

Cell pellets were resuspended in 0.8 ml of water and vortexed vigorously to disrupt the cell pellet. A 15 µl aliquot of the cell suspension was removed and added to 45 µl of counting solution (24), and the concentration of cells in the suspension determined using a hemacytometer. Two milliliters of methanol and 1 ml of chloroform with 50 µg/ml butylhydroxytoluene (BHT) were added to the remainder of the cell suspension, the sample was mixed vigorously and then sonicated for 30 min. at room temperature in a bath sonicator. Non-solubilized material was removed by centrifugation at 3,000 rpm for 5 min. in a table top centrifuge. The supernatant was transferred to a new tube, 1 ml of chloroform (with 50 µg/ml BHT) and 1 ml of water was added, the sample was vigorously mixed for 1 min., and the phases were clarified by centrifugation at 3,000 rpm for 5 min. The chloroform phase was collected, evaporated under a stream of nitrogen and resuspended in chloroform at a concentration equivalent to $10^7$ cells/ml. Ten microliters of the $[^{14}C]$-labeled samples were added to 4 ml of EcoScint H and the amount of radioactivity determined by scintillation counting. Twenty-five microliters of each $[^{14}C]$-labeled sample was spotted on to a silica G plate (Analtech) for separation by thin-layer chromatography (TLC). Complex lipid species were resolved by elution with petroleum ether/diethyl ether/acetic acid (90:10:1). PL species were resolved by elution with chloroform/triethylamine/ethanol/water (30:30:34:8). The plates were allowed to dry overnight and then exposed for 6 hours to a phosphorimaging screen (FugiFilm). The plates
were read with a Fujifilm BAS-2500 scanner and the amount of $^{14}$C]-FA incorporated into PL and TAG species were determined using Multiguage software (FugiFilm).

**Determination of PL Levels**

Twenty-five microliters of lipid extract from three day differentiated cells that were supplemented with 5 µM unlabeled FA were evaporated to dryness in glass tubes under a stream of nitrogen. Fifty microliters of perchloric acid was added to each tube, then the tube was capped and placed in a 180°C heating block overnight to liberate phosphate from the PL. Ten microliters of each sample was added to wells of a 96 well plate. One-hundred microliters of assay solution (0.29% brilliant green, 1 M ammonium molybdate, 1.2 N hydrochloric acid and 0.05% Tween-20) was added for 15 minutes at room temperature. The absorbance at 660 nm was measured using a Molecular Dynamics Versamax plate reader to determine the amount of free phosphate which is a measure of PL abundance. PL levels were determined by comparing the unknown samples to a standard of known amounts of PC (Sigma) that had been treated identically.

**Gas chromatography/ Mass Spectrometry (GC/MS) of FAs**

Ten microliters of 1 mg/ml 17:0 FA (internal standard) and 50 µls of lipid extract from three day differentiated cells that were supplemented with 5 µM unlabeled FA were evaporated to dryness in a glass tube under a stream of nitrogen. Once evaporated, 0.25 ml of toluene and 0.5 ml of 1% Sulfuric Acid in Methanol were added, the tube were capped under nitrogen, and incubated at 50°C overnight in a heat block. The next day, 1.25 ml of 5% NaCl was added and the suspension was mixed vigorously. FA-methyl esters (FAMEs) were extracted twice with 1 ml of hexane followed by centrifugation at 3,000 rpm for 5 min. in a table top centrifuge. The hexane phase was washed with 1 ml of 2% potassium bicarbonate and collected again before
evaporation under a stream of nitrogen. The sample was resuspended in 25 µl of methyl acetate with 50 µg/ml BHT. The fatty acid methyl esters (FAMES) were analyzed on an Agilent 6890 series gas chromatograph equipped with a 5873 mass selective detector. Identity of the fatty acids was made by comparison with the National Institute of Standards and Technology (NIST) database and confirmed by comparison with standards purchased from commercial sources (Sigma and Avanti). Quantification was made by comparison with the added 17:0 internal standard.
Results:

Previously we reported that supplementation of differentiating PC12 cells with PUFAs enhances neurite outgrowth and that over-expression of Acs16 further increases PUFA-mediated neurite outgrowth (7). For the metabolic tests to determine whether Acs16 over-expression selectively enhanced PUFA accumulation or targeted PUFAs into PL relative to TAG, large numbers of control and Acs16 over-expressing cells were required. Therefore, Eco1 PC12 cells (PC12 cells over-expressing the mouse ecotropic retrovirus receptor) were infected with control or Acs16 over-expressing retroviruses that also encoded GFP and a puromycin resistance gene. After 48 hours, the cells were selected for two days with 5 µg/ml puromycin to obtain highly enriched populations of transformed cells. As identified by FACS analysis of GFP expression, over 95% of the cells contained an integrated retrovirus and synthesized additional Acs16 protein (Figure 1A-C). To obtain sufficient numbers of cells to perform kinetic and biochemical analysis of the metabolic fate of exogenous OA, AA and DHA during PC12 cell differentiation, these populations of transformed cells were expanded for 2-8 weeks before differentiation.

Quantitative-PCR (Q-PCR) was used to measure the levels of Acs1 mRNAs and determine whether long-term over-expression of Acs16 affected the expression of other Acs1 mRNAs (Figure 1D). Compared to the level of Acs16 mRNA in control cells, the level of Acs16 mRNA in Acs16 over-expressing cells was increased ~100-fold. The levels of the other Acs1 mRNAs were not significantly altered in Acs16 over-expressing cells, demonstrating that the other Acs1 mRNA are not down-regulated to compensate for increased Acs16 mRNA expression.

The level of Acs16 protein in control and Acs16 over-expressing cells was measured to determine whether the 100-fold increase in Acs16 mRNA translated to a similar increase in Acs16 protein. In Acs16 over-expressing cells, the level of Acs16 protein increased by ~15-fold
compared to control cells (Figure 1C), confirming that the increase in the level of Acsl6 mRNA led to a highly significant increase in Acsl6 protein accumulation.

To determine whether Acsl6 over-expression selectively increased total OA-CoA, AA-CoA or DHA-CoA synthetase activity, the conversion of each $[^{14}\text{C}]$-FA to $[^{14}\text{C}]$-FA-CoA was measured using total cellular extracts from undifferentiated control and Acsl6 over-expressing cells (Figure 2). In control cells, the total OA-CoA synthetase activity was only 1/7th and 1/3rd the level of total AA-CoA and total DHA-CoA synthetase activities, respectively (Figure 2A). Acsl6 over-expression increased DHA-CoA synthetase activity 5-fold compared to control cells. In contrast, OA-CoA and AA-CoA synthetase activities were increased only 1.7-fold and 0.4-fold in Acsl6 over-expressing cells compared to control cells. These findings provide direct evidence that Acsl6 preferentially converts DHA to DHA-CoA.

**Acsl6 Over-expression Selectively Increases DHA Metabolism.**

To determine whether Acsl6 over-expression specifically increased AA or DHA metabolism over the course of differentiation, control and Acsl6 over-expressing cells were supplemented with 5 µM $[^{14}\text{C}]$-OA, $[^{14}\text{C}]$-AA or $[^{14}\text{C}]$-DHA and the amount of $[^{14}\text{C}]$-FA remaining in the media was determined every 24 hours during the 3 day differentiation. After 24 hours, 200% more $[^{14}\text{C}]$-DHA, 40% more $[^{14}\text{C}]$-AA and 30% more $[^{14}\text{C}]$-OA were extracted from the media by Acsl6 over-expressing cells compared to control cells (Figure 3C and 3A, respectively; compare dotted to solid curves). After 72 hours of differentiation, each $[^{14}\text{C}]$-FA was depleted similarly from the media by control and Acsl6 over-expressing cells, except for $[^{14}\text{C}]$-DHA which was depleted 26% more from the media by Acsl6 over-expressing cells than by control cells.
To confirm that the selective $[^{14}\text{C}]-\text{DHA}$ removal from the media by Acsl6 over-expressing cells presented in Figure 3 resulted in an increase in $[^{14}\text{C}]-\text{DHA}$ accumulation (and not $\beta$-oxidation), control and Acsl6 over-expressing cells were supplemented with 5 $\mu$M $[^{14}\text{C}]-\text{FA}$ and the amounts of $[^{14}\text{C}]-\text{OA}$, $[^{14}\text{C}]-\text{AA}$ and $[^{14}\text{C}]-\text{DHA}$ present in the lipid extracts were measured 15 minutes (Figure 4A) and 72 hours (Figure 4B) after the start of differentiation. Consistent with enhanced extraction of $[^{14}\text{C}]-\text{DHA}$ from the media by Acsl6 over-expression (Figure 3C), after 15 minutes of incorporation the level of $[^{14}\text{C}]-\text{DHA}$ was increased by 90% in Acsl6 over-expressing cells compared to control cells (compare bars 5 and 6, Figure 4A). This was in contrast to the accumulation of $[^{14}\text{C}]-\text{OA}$ and $[^{14}\text{C}]-\text{AA}$ which were increased by 30% and 50% respectively, in Acsl6 over-expressing cells (compare lanes 2 to 1, and 4 to 3). Similarly, after 72 hours of incorporation the level of $[^{14}\text{C}]-\text{DHA}$ was increased in Acsl6 over-expressing cells relative to control cells by a modest 28% (Figure 4B, lanes 6 and 5), but the levels of $[^{14}\text{C}]-\text{OA}$ and $[^{14}\text{C}]-\text{AA}$ in Acsl6 over-expressing cells (lanes 1-4) were similar to those of control cells. Increased DHA extraction from the media and increased accumulation of $[^{14}\text{C}]-\text{DHA}$ in Acsl6 over-expressing cells are consistent with the notion that Acsl6 preferentially functions in DHA metabolism for lipid synthesis, and not $\beta$-oxidation.

Taken together, these results are consistent with previously reported short-term uptake studies (7) and biochemical studies (17) that suggest Acsl6 preferentially promotes AA and DHA metabolism. The highly significant increase in $[^{14}\text{C}]-\text{DHA}$ removal from the media by Acsl6 over-expressing cells during the entire 3-day period of differentiation strongly support the hypothesis that DHA-enhanced neurite outgrowth is a consequence of increased DHA availability for PL synthesis. In contrast, the modest increase in $[^{14}\text{C}]-\text{AA}$ extraction from the media only during the first 24-48 hours of differentiation suggest that AA-enhanced neurite
length in Acsl6 over-expressing cells must be a consequence of other mechanisms that could include enhanced AA incorporation into PLs, preferential delivery of AA containing PLs to the plasma membrane, or increased AA signaling that leads to increased endogenous FA synthesis.

**Acsl6 over-expression does not preferentially increase FA targeting to PLs.**

The existence of five Acsl proteins with unique subcellular localization patterns is consistent with the hypothesis that different Acsl proteins target FAs to different complex lipid pools. To test whether the PUFA-enhanced neurite outgrowth that occurs with Acsl6 over-expression is a consequence of Acsl6 preferentially targeting DHA or AA to PLs, the lipid extracts of control and Acsl6 over-expressing cells supplemented with 5 µM [14C]-OA, [14C]-AA or [14C]-DHA were analyzed by TLC 15 minutes and 72 hours after the start of differentiation. The relative incorporation of each [14C]-FA into PLs versus TAGs was determined by phosphorimaging analysis (Figure 5A-D). Inconsistent with Acsl6 primarily targeting FAs to the PL pool, after 15 min. of incorporation the relative amounts of [14C]-PLs (Figure 5A) and [14C]-TAGs (Figure 5B) in Acsl6 over-expressing cells supplemented with [14C]-OA, [14C]-AA or [14C]-DHA were similarly increased over that of control cells and paralleled the total increase in [14C]-FAs accumulation (Figure 4A). For example, in Acsl6 over-expressing cells supplemented with [14C]-DHA, the amount of [14C]-PLs was increased by 70% over that of control cells (Figure 5A, lane 6 relative to lane 5) and the amount of [14C]-TAGs (Figure 5B, lane 6 relative to lane 5) was increased by ~250% compared to control cells. In other words, of the additional [14C]-DHA incorporated by Acsl6 over-expressing cells relative to control cell, only 66% was incorporated into PLs and 33% was incorporated into TAGs. This was in contrast to control cells, where 90% of the [14C]-DHA was incorporated into PLs and
10% of the [14C]-DHA was incorporated into TAGs. These results are inconsistent with Acsl6 over-expression increasing the targeting of FAs for PL synthesis rather than TAG synthesis.

**Distribution of FAs Among PL Classes Is Not Altered by Acsl6 Over-expression.**

Earlier studies showed that in PC12 cells AA accumulates primarily in phosphatidylethanolamine (PE) and phosphatidylinositol (PI), DHA accumulates primarily in PE, and OA accumulates primarily in phosphatidylcholine (PC) (25,26). We hypothesized that DHA-enhanced neurite outgrowth in Acsl6 over-expressing cells was a consequence of Acsl6 preferentially targeting of AA and DHA to their preferred PL species. To test this hypothesis, the relative distributions of [14C]- OA, [14C]- AA and [14C]- DHA among PC, PS, PI and PE were determined in control and Acsl6 over-expressing cells using the lipid extracts described in the previous section. The PL species were separated by TLC and the amounts of [14C]-PC, [14C]-PS, [14C]-PI and [14C]-PE were measured by phosphorimager-based densitometry. Both after 15 minutes and 72 hours of incubation, the relative distribution of each [14C]- FA among PC, PS, PI and PE species in Acsl6 over-expressing cells was not significantly different from control cells (Figure 6A and 6B). These findings strongly suggest that Acsl6 does not target FAs to specific PL species.

Especially after 72 hours of incubation, there were several significant differences in the distributions of [14C]-OA, [14C]- AA and [14C]- DHA among PL species that were independent of Acsl6 over-expression. The most striking difference was that the bulk of internalized [14C]-AA and [14C]- DHA were incorporated into PE, while the majority of [14C]-OA was incorporated into PC. There were also highly significant differences in the relative distributions of [14C]- FAs into PS and PI. Compared to [14C]-OA, nearly 50% more [14C]- AA and 80% more [14C]- DHA were incorporated into PS. Likewise, the relative amount of [14C]- DHA incorporated into PS
was increased by 80% compared to $[^{14}\text{C}]-\text{OA}$. The differential incorporation of OA, AA and DHA into different PL species may play a role in PUFA-enhanced neurite outgrowth.

**Total PL Levels are Increased in Acsl6 Over-expressing Cells Supplemented With PUFAs.**

Because the amounts of $[^{14}\text{C}]-\text{AA}$ and $[^{14}\text{C}]-\text{DHA}$ incorporated into PLs by Acsl6 over-expressing cells were not substantially increased after a 3-day differentiation, it was unclear whether PUFA-enhanced neurite outgrowth was a consequence of increased levels of total cellular PLs. Therefore, the levels of total cellular PL of control and Acsl6 over-expressing cells differentiated for three day in media supplemented with 5 µM OA, AA or DHA were measured (Figure 7). Consistent with increased PL synthesis, the level of total cellular PL in Acsl6 over-expressing cells supplemented with AA and DHA were significantly increased by 11% and 14%, respectively, compared to the control cells (lane 4 relative to lane 3 and lane 5 relative to lane 6). In contrast, the level of total PL in Acsl6 over-expressing cells supplemented with OA was not different from control cells (compare lane 2 to lane 1).

Importantly, the levels of total PLs in control and Acsl6 over-expressing cells supplemented with AA (lanes 3 and 4) or DHA (lanes 5 and 6) were increased by 13-38% compared to equivalent cells supplemented with OA (lanes 1 and 2). These small but significant changes in total PL levels that occur with PUFA supplementation and Acsl6 over-expression support the hypothesis that increased levels of intracellular AA and DHA stimulate PL synthesis, and that the resulting increase in total cellular PLs stimulates neurite outgrowth.

**PUFA Supplementation Increases Levels of Both Saturated and Polyunsaturated FAs.**

We have shown that Acsl6 over-expression primarily increased DHA and to a lesser extent AA internalization (Figure 3 and 4A), but did not preferentially target $[^{14}\text{C}]-\text{FAs}$ into PLs (Figure 5 and 6). To determine whether Acsl6 over-expression increased the total amount of
cellular AA and DHA and other FA species, control and Acs16 over-expressing cells were differentiated for 3 days with 0.25% horse serum supplemented with 5 µM of OA, AA or DHA. After extraction, the total lipid extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) to measure the levels of several saturated, monounsaturated and PUFA species. Compared to control cells supplemented with DHA, the level of DHA in DHA-supplemented Acs16 over-expressing cells was increased by a highly significant 23% (Figure 8C; 22:6 (n-3)). This finding supports the hypothesis that the majority of internalized DHA was incorporated into PLs and TAGs and was not metabolized to other FA species. Additionally, compared to control cells, the level of stearic acid (18:0) in Acs16 over-expressing cells supplemented with DHA was increased by a significant 38%, and the PUFA eicosapentanoic acid (20:5 (n-3)) was increased by a highly significant 77% (Figure 8Ci).

Supplementation of Acs16 over-expressing cells with AA selectively and significantly increased the levels of palmitic (16:0) and stearic acid (18:0) compared to control cells (Figure 8B). Consistent with no change in [14C]- AA accumulation after 72 hours of incubation (Figure 4B, lanes 4-6), the level of AA was unchanged in Acs16 over-expressing cells supplemented with AA compared to control cells (Figure 8B; 20:4 (n-6)). However, it is interesting that the level of DHA was significantly increased by 63% (Figure 8Bi) in Acs16 over-expressing cells supplemented with AA compared to control cells. This finding is consistent with the hypothesis that Acs16 preferentially promotes DHA metabolism. Supplementation of Acs16 over-expressing cells with OA resulted in no significant change in the level of any FA compared to control cells (Figure 8A), consistent with no change in total PL levels (Figure 7).

Independent of Acs16 over-expression, supplementation with AA instead of OA increased the amount of AA by 3.5-fold (compare figure 8A to 8B; 20:4 (n-6)) and supplementation with
DHA in place of OA increased the amount of DHA by 9-13-fold (compare figure 8A to 8C; 22:6 (n-3)). Additionally, AA or DHA supplementation in place of OA primarily increased the level of stearic acid (18:0) by 40-70% (compare figure 8B and 8C to 8A; 18:0) and not surprisingly reduced the level of oleic acid by 30-40% (compare figure 8B and 8C to 8A; 18:1). This is consistent with published reports that PUFAs reduce the activity of stearoyl-CoA desaturase (SCD), which converts stearic acid (18:0) to oleic acid (18:1) (27). Taken together, these findings are consistent with the hypothesis that increased levels of PUFAs lead to increases in PL levels and neurite length. It seems likely that PUFA-enhanced neurite outgrowth in Acsl6 over-expressing cells is simply an exacerbation of this process due to increased AA and DHA internalization during the first 24-hours of differentiation.
Discussion:

This paper makes several key points concerning the relationship of PUFA internalization and metabolism to PL synthesis and neurite outgrowth. Upon differentiation of PC12 cells, and independent of Acs16 over-expression, internalized PUFAs were preferentially metabolized into PLs, relative to TAG, and their distribution among the major PL species differed significantly from internalized OA. After 3 days of differentiation, the respective levels of AA or DHA, as well as the level of total cellular PLs, were significantly increased in cells that were supplemented with AA or DHA relative to cells that were supplemented with OA. Additionally, compared to control cells, the levels of PLs, SFAs, and PUFAs were increased in Acs16 over-expressing cells that were supplemented with AA or DHA. The most striking finding was that, relative to OA and AA metabolism, DHA internalization, activation to DHA-CoA and incorporation into lipids was significantly increased in Acs16 over-expressing cells compared to control cells. Finally, Acs16 over-expression did not increase the partitioning of internalized FAs exclusively into PLs or TAGs nor did it alter the distribution of FAs among the major PL species. Thus Acs16 preferentially promotes DHA uptake and metabolism, presumably by preferentially activating DHA to DHA-CoA. AA uptake was only slightly enhanced by Acs16 over-expression, yet supplementation with AA also increased total PL accumulation, presumably by stimulating FA and/or PL synthesis.

PUFA Metabolism, PL synthesis, and Neurite Outgrowth

Several reports established that supplementation of neuronal cells with AA or DHA can enhance neurite outgrowth (4-11), but the mechanisms by which these FAs exert their modifying effects remain unclear. Previously, we showed that supplementation with PUFAs, not OA increased neurite outgrowth. Over-expression of Acs16, but not over-expression of Acs11
stimulated neurite outgrowth further, but again only when the medium was supplemented with AA or DHA, not OA. Consistent with the hypothesis that selective PUFA internalization contributes to PUFA-enhanced neurite outgrowth, upon PC12 differentiation, the initial rate of PUFA internalization was increased more than the initial rate of OA internalization (7). Additionally, compared to over-expression of Acsl1, over-expression of Acsl6 caused the initial rate of PUFA internalization to be increased relatively more than that of OA (7).

Based on these findings, it seemed likely that the PUFA-enhanced neurite outgrowth might simply be a consequence of PUFAs being internalized more efficiently than OA, with Acsl6 over-expression further increasing PUFA internalization. Higher levels of PUFAs would provide more FA substrate for PL synthesis that would be inserted into the plasma membrane to increase neurite length. Consistent with this hypothesis, we found that after 72 hours of incubation, the levels of total cellular PLs were significantly increased in cells supplemented with AA and DHA, compared to cells supplemented with OA. Acsl6 over-expression increased the level of PLs further when the medium was supplemented with PUFAs, not OA. However, after 72 hours of incubation, nearly equal amounts of OA, AA and DHA were taken up from the medium and incorporated into lipids of control cells, except for Acsl6 over-expressing cells where DHA internalization and incorporation were increased further. This finding suggests that PUFA-enhanced neurite outgrowth is not simply due to an increase in AA and DHA accumulation, relative to OA accumulation.

Rather, these results suggest several possible alternative mechanisms as to how PUFAs stimulate neurite outgrowth. These include preferential incorporation of PUFAs into PLs; PUFAs functioning as signaling molecules to stimulate an increase in the level of other FAs (i.e. saturated FAs) by either increasing their uptake into the cells or their synthesis within the cell; or
preferential targeting of PLs containing PUFAs to the plasma membrane relative to PLs not containing PUFAs,

Consistent with preferential partitioning of PUFAs into PLs, we found that compared to 
$[^{14}\text{C}]-\text{OA}$ a higher percentage of $[^{14}\text{C}]-\text{AA}$ and $[^{14}\text{C}]-\text{DHA}$ were partitioned into PLs relative to TAG after 15 minutes of incubation. This phenomena is biologically relevant because when rats were infused with $[^{14}\text{C}]-\text{palmitic acid (PA)}$, $[^{14}\text{C}]-\text{AA}$, or $[^{14}\text{C}]-\text{DHA}$ for 15 minutes a higher percentage of $[^{14}\text{C}]-\text{PUFAs}$, relative to $[^{14}\text{C}]-\text{PA}$ were incorporated into brain PLs even though equal amounts of label were found in the lipid fraction (28,29). However, our finding that after 72 hours, the levels of $[^{14}\text{C}]-\text{PLs}$ were similar regardless of the $[^{14}\text{C}]-\text{FA}$ used suggest that preferential internalization and incorporation of PUFAs into PLs does not provide sufficient amounts of FAs to increase the level of PLs over the entire differentiation period.

DAG is the common precursor for de novo PL and TAG synthesis and its level increases significantly upon PC12 differentiation (30). During the synthesis of PLs, saturated FAs (i.e., palmitic acid, (16:0) or stearic acid, (18:0)), are typically acylated into the sn-1 position of glycerol-3-phosphate and PUFAs (i.e., 20:4 or 22:6) are acylated into the sn-2 position to generate phosphatidic acid, which is the precursor for PI. However, most of the phosphatidic acid is dephosphorylated to generate DAG, which is further metabolized to PC, PE or TAG. There is some evidence that DAG molecules that contain PUFAs in the sn-2 position are preferentially converted to PLs. For example, DAG species that contain DHA in the sn-2 position are the preferred substrate of ethanolaminephosphotransferase, which converts DAG to PE (31), and DAG molecules that contain AA in the sn-2 position are phosphorylated by DAG kinase ε (32,33). Consistent with this hypothesis, the distributions of OA, AA and DHA among PC, PS, PI and PE are significantly different. The bulk of DHA is incorporated in PE and to a
lesser extent in PS. Likewise, the majority of AA is incorporated into PI and PE, while the bulk of OA is incorporated into PC (26,34). Consistent with this result, we found that after 72 hours of incubation, a higher percentage of $[^{14}\text{C}]-\text{DHA}$ and $[^{14}\text{C}]-\text{AA}$ were incorporated into PS, PI and PE, while the bulk of $[^{14}\text{C}]-\text{OA}$ was incorporated into PC.

Not surprisingly, when we examined the FA composition of the cells after 72 hours of differentiation, the greatest difference between the PUFA supplemented cells and the OA supplemented cells was a massive increase in the amount of each respective PUFA in the cells. Notably, however, there also were increases in the levels of several other FA species that were highly significant, most prominently in the levels of the saturated FAs stearic acid (18:0) and palmitic acid (16:0). This finding is consistent with studies that have shown that high concentrations of PUFAs repress stearoyl-CoA-$\Delta_9$-desaturase expression and activity, which in turn reduces the conversion of stearic acid (18:0) to OA (18:1)(27). This may be important to ensure that saturated FAs, whether newly synthesized or taken up from the external environment, are available for insertion into the $sn$-1 position of PLs as they are synthesized. Maintaining the proper PUFA/non-PUFA ratio in the PLs of the plasma membrane is critical for normal membrane fluidity and possibly for optimal neurite outgrowth.

**AcsL6 over-expression does not preferentially target FAs to PLs**

Several reports suggest that some AcsL or FATP proteins can influence the partitioning of FAs into either TAG or PLs (20,22,35). Therefore we had hypothesized that the increase in total cellular PL levels that occurs with AcsL6 over-expression may in part be due to an increase in the partitioning of PUFAs into PLs relative to TAG. However, after 15 minutes of incubation, the relative incorporation of $[^{14}\text{C}]-\text{OA}$, $[^{14}\text{C}]-\text{AA}$ and $[^{14}\text{C}]-\text{DHA}$ incorporated into the PL and
TAG pools of Acsl6-over-expressing cells were similar and mimicked the increases in FA accumulation.

While Acsl6 over-expression did not increase FA partitioning preferentially into PL or TAG, we hypothesized that Acsl6 might alter the partitioning of PUFAs among PL species and that this might contribute to increased neurite outgrowth. However, the distribution of $[^{14}\text{C}]-\text{OA}, [^{14}\text{C}]-\text{AA}$ and $[^{14}\text{C}]-\text{DHA}$ among PL classes of control and Acsl6 over-expressing cells were nearly identical after 15 min. of incubation. In fact, after 15 minutes of incubation, the majority of $[^{14}\text{C}]-\text{OA}, [^{14}\text{C}]-\text{AA}$ and $[^{14}\text{C}]-\text{DHA}$ were incorporated similarly into PC and PI, regardless of whether Acsl6 was over-expressed by the cells. Taken together, these data suggest that Acsl6 does not preferentially or exclusively partition FAs into TAGs, PLs or specific PL species. However, it should be noted that the subcellular distribution of Acsl6 in Acsl6 over-expressing cells may differ from its normal distribution, thus altering its normal function in FA partitioning.

**Acsl6 and DHA Metabolism**

As stated earlier, we previously reported that, compared to over-expression of Acsl1, over-expression of Acsl6 preferentially increased the initial rates of AA and DHA internalization relative to the rate of OA internalization (7). Other evidence is consistent with the notion that Acsl6 contributes to DHA enrichment in neurons. In PC12 (7) and reaggregated rat brain cell cultures (36), the level of Acsl6 mRNA is increased upon differentiation, but the level of Acsl1 remains unchanged. Additionally, biochemical studies using bacterially expressed proteins established that Acsl6 activates AA 2-fold and DHA 8-fold more efficiently than Acsl1 does (17). Here we found that, relative to control cells, $[^{14}\text{C}]-\text{DHA}$ internalization, activation to DHA-CoA and metabolism into complex lipids was significantly and selectively enhanced in Acsl6 over-expressing cells especially during the first 24 hours of differentiation (Figures 3 and
4). This is the first report to provide in vivo evidence for preferential DHA metabolism by any Acsl protein.

The precise mechanism of how FAs cross the plasma membrane is controversial, but it most likely involves integral and plasma membrane-associated proteins that facilitate FA internalization (13,14,23,37). In the first step, unesterified FAs partition into the outer leaflet of the plasma membrane due to their hydrophobicity or by their interaction with putative FA transporters such as FATP. Once in the plasma membrane, “flip-flop” of the FA from the outer leaflet to the inner leaflet occurs spontaneously or may be enhanced by FA transporters. FAs are then extracted from the inner leaflet of the plasma membrane by membrane associated FATPs or Acsls or by cytosolic FABPs. However, if the FA is not extracted, the process can be reversed and the FA may repartition back to the extracellular environment. Unesterified FAs extracted from the plasma membrane by FABPs must be transported to subcellular locations where the FAs are esterified to CoA by Acsls or possibly FATPs since there is now evidence that some FATPs possess acyl-CoA synthetase activity (38,39). Equally likely, Acsls or possibly FATPs may couple the extraction of the FA from the membrane with its activation to FA-CoA (21,40). Once activated, FAs can not repartition back into the plasma membrane due to their decrease in hydrophobicity. FA activation also decreases the concentration of unesterified FA in the cell, thus maintaining a concentration gradient that is favorable for the entry of more unesterified FAs into the cell (13,23).

There are several possible steps in FA internalization and metabolism where Acsl6 may function to promote DHA accumulation. An increase in the initial rate of FA uptake (7,23) and an increase in the accumulation of FAs in Acsl6 over-expressing over a 15 min time frame (Figure 4A) are consistent with a fraction of Acsl6 localizing to the plasma membrane where it
may activate FAs, especially DHA, as it extracts them from the plasma membrane or accepts them from another protein such as a FATP. Either way, esterification of the FA to CoA will maintain the outside > inside FA concentration gradient in the cell, thus promoting the influx of more FA into the cell. Alternatively, FABPs may extract FA from the membrane and transport the FA to subcellular compartments where the Acs1 proteins are located. The FA may would be transferred from the FABP to the Acs1, where it would be activated, thus “freeing up” the FABP to bind an additional FA. Regardless of the specific intracellular localization, increased Acs1 expression increases the rate of FA activation and metabolism, thereby promoting DHA internalization.

It is clear that AA and DHA are critical for proper brain and retina growth. Our study suggests that PUFAs may function to stimulate neuronal differentiation by being preferentially metabolized into PLs and stimulating PL synthesis. Our finding that Acs1 preferentially promotes DHA metabolism suggests that it and perhaps other proteins may function to internalize DHA efficiently.
Figure Legends:

Table I. Fatty acid composition of growth and differentiation media. The molar percentages of FA in growth medium containing fetal bovine serum (FBS) and differentiation media containing 0.25% horse serum supplemented with 5 µM OA, AA or DHA were determined by GC/MS. The supplemented FAs are in bold. Note that there was no detectable AA or DHA in unsupplemented differentiation media.

Figure 1. Stable over-expression of Acsl6 does not alter the levels of other Acsl mRNAs. Eco1 PC12 cells were infected with control or Acsl6 over-expressing retrovirus that also expressed GFP and the puromycin resistance gene. Transformed cells were selected by incubation with 5 µg/ml puromycin for 48 hours. Cells were expanded for 4-8 weeks before analysis. (A) Histogram of FACS analysis for GFP expression in drug-selected empty vector control cells. (B) Histogram of FACS analysis for GFP expression in drug selected cells transformed to over-express rat Acsl6. (C) Western blot of Acsl6 protein expression. Total extract from 180,000 undifferentiated control and Acsl6 over-expressing cells was separated on a SDS-PAGE gel and probed with an anti Acsl6 antibody as described in Materials and Methods. * indicates the position of Acsl6 protein. Note the equal signal from the cross-reacting protein at 55 kD. The numbers at the right of the blot are sizes in kD. (D) Undifferentiated control and Acsl6 over-expressing cells were harvested, total RNA extracted and analyzed Q-PCR to determine the level of each Acsl mRNA. All mRNA levels are relative to the amount of Acsl1 mRNA in control cells, which was defined as 1. As previously described (7), to correct for differences in amplification rates among the Acsl genes due to primer sequence variation, the amplification rate
for each primer pair was determined for genomic DNA where each gene is present in exactly two copies. Cyclophilin B mRNA levels were used to normalize expression between samples

Figure 2. Over-expression of Acsll6 preferentially increases DHA-CoA synthesis. Cellular extracts from undifferentiated control and Acsll6 over-expressing cells were prepared as described in Materials and Methods. The conversions of $[^{14}C]$-OA to $[^{14}C]$-OA-CoA, $[^{14}C]$-AA to $[^{14}C]$-AA-CoA and $[^{14}C]$-DHA to $[^{14}C]$-DHA-CoA were measured to determine acyl-CoA synthetase activities in control and Acsll6 over-expressing cells as described in Materials and Methods. The relative amounts of $[^{14}C]$-FA-CoA synthesized in total cellular extracts from control and Acsll6 over-expressing cells. Equal amounts of each $[^{14}C]$-FA were used in the reactions. The graph represents the average of three independent experiments performed in duplicate. *p-value < 0.05 and considered significantly increased from control cells in by Student’s t-Test.

Figure 3. DHA is rapidly removed from the differentiation media by Acsll6 over-expressing cells. Control or Acsll6 over-expressing cells were differentiated for three days in 0.25% horse serum supplemented with 5 µM of $[^{14}C]$-OA, $[^{14}C]$-AA or $[^{14}C]$-DHA, 0.1% FA-free BSA and 50 ng/ml NGF. Each day 200 µl of media was collected and the amount of $[^{14}C]$-FA remaining in the media was determined by scintillation counting. In each graph, the percent of $[^{14}C]$-FA remaining in the media after 1, 2 or 3 days was plotted with day 0 equal to 100%. (A) Supplementation with $[^{14}C]$-OA. (B) Supplementation with $[^{14}C]$-AA. (C) Supplementation with $[^{14}C]$-DHA. The average and error bars were determined from 4 or 5 independent experiments are plotted. *p-value < 0.05 and considered significant from control cells by Student’s t-Test.
Figure 4. Preferential DHA accumulation in Acsl6 over-expressing cells. Cells were differentiated in 0.25% horse serum supplemented with 5 µM of [14C]-OA, [14C]-AA or [14C]-DHA, 0.1% FA-free BSA and 50 ng/ml NGF as described in Materials and Methods. The amount of [14C]-OA (lanes 1-2), [14C]-AA (lanes 3-4) and [14C]-DHA (lanes 5-6) in the lipid extract were measured in control and Acsl6 over-expressing cells (A) 15 minutes and (B) 72 hours after the start of differentiation. Counts per minute (x10^3) per 10^5 cells are plotted. (A) is the average from three independent experiments and (B) is the average from 4 independent experiments. *p-value < 0.05 and considered significant from control cells by Student’s t-Test.

OA = [14C]- oleic acid, AA = [14C]- arachidonic acid, DHA = [14C]- docosahexaenoic acid.

Figure 5. Acsl6 increases FA incorporation into PLs and TAGs. The relative levels of [14C]-PLs (A, C) and [14C]-TAGs (B, D) in the same extracts used in Figure 4 were determined by phosphorimager analysis of the TLC plates as described in Materials and Methods. The relative levels of [14C]-PL and [14C]-TAG levels (A, B) 15 minutes or (C, D) 72 hours after the start of differentiation were plotted in arbitrary units. The same scale was used within each time point to accurately reflect relative levels of [14C]-PL and [14C]-TAG. *p-value < 0.05 and considered significant from control cells by Student’s t-Test. [14C]-OA (lanes 1-2), [14C]-AA (lanes 3-4) or [14C]-DHA (lanes 5-6).

Figure 6. The distribution of [14C]-AA and [14C]-DHA among PL species differs from the distribution of OA and is not altered by Acsl6 over-expression. The incorporation of [14C]-OA, [14C]-AA and [14C]-DHA into PC, PS, PI and PE were determined from the same extracts as...
described in Figure 4 and 5. The PL species were separated by TLC as described in Materials and Methods. The relative distribution of \(^{14}\text{C}\)-OA, \(^{14}\text{C}\)-AA and \(^{14}\text{C}\)-DHA into PC, PS, PI and PE (A) 15 minutes and (B) 72 hours after the start of differentiation. *p-value < 0.05 and considered significantly different from control cells by Student’s t-test. # p-value < 0.05 and considered significantly different from \(^{14}\text{C}\)-OA cells by Student’s t-test. O = \(^{14}\text{C}\)-oleic acid, A = \(^{14}\text{C}\)-arachidonic acid, D = \(^{14}\text{C}\)-docosahexaenoic acid, PC = phosphatidylcholine, PS = phosphatidylserine, PI = phosphatidylinositol, PE = phosphatidylethanolamine.

Figure 7. Total levels of cellular phospholipids are increased with AA and DHA supplementation and further increased by Acsl6 over-expression. Control or Acsl6-over-expressing cells were differentiated for three days in 0.25% horse serum/RPMI 1640 supplemented with 5 µM OA, AA or DHA. Lipids were extracted and phospholipid levels were determined for 5 x 10^4 cells using a molybdate/malachite green assay to determine phosphate levels as described in Materials and Methods. PL levels were determined by comparing experimental samples to a standard series of known amounts of PL. The graph represents the average with standard error from five independent experiments. Note that these samples were differentiated in parallel with the cells that were supplemented with \(^{14}\text{C}\)-FAs in Figures 4-6. *p-value < 0.05 and considered significantly different from control cells by the paired Student’s t-test. ** p-value < 0.05 and considered significantly different from OA supplemented cells by the paired Student’s t-test. OA = oleic acid, AA = arachidonic acid, DHA = docosahexaenoic acid.
Figure 8. PUFA supplementation alters the FA composition of differentiated PC12 cells.

Control and Acsl6 over-expressing cells were differentiated for 3 days in 0.25% horse serum/RPMI 1640 supplemented with 5 µM OA, AA or DHA. Lipids were extracted and FA-methyl esters (FAMEs) were generated from the total extract of 2.5 x 10^5 cells as described in Material and Methods. The FAMEs were analyzed by gas chromatography/ mass spectrometry to determine the level of each FA species. Note that these FA profiles are from the same lipid extracts that were used in Figure 7. The number under each group of bars represents the FA species. The level of each FA species in control and Acsl6 over-expressing cells (A) supplemented with oleic acid; (B) supplemented with arachidonic acid; (C) supplemented with DHA. (i) The amounts of 22:6 in cells supplemented with AA are replotted to show the increase in Acsl6 over-expressing cells. (ii) The amounts of 20:5 in cells supplemented with AA are replotted to show the increased Acsl6 over-expressing cells.*p-value < 0.05 and considered significantly different from control cells by Student’s t-test. #p-value < 0.05 and considered significantly different from OA supplemented cells by Student’s t-test.
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Table 1. Fatty acid composition of media (mole %)

| Growth Medium | 0.25% FBS | 0.25% HS | 0.25% HS/5 μM OA | 0.25% HS/5 μM AA | 0.25% HS/5 μM DHA |
|---------------|-----------|----------|------------------|------------------|------------------|
| 14.0          | 2.46      | 0.00     | 0.54             | 0.00             | 0.77             |
| 16.0          | 11.84     | 13.38    | 10.37            | 11.41            | 10.71            |
| 18.0          | 10.56     | 15.34    | 12.43            | 13.04            | 13.10            |
| 18.1 (n-9)    | 6.66      | 4.03     | 30.74            | 4.76             | 5.89             |
| 18.2 (n-6)    | 1.38      | 21.41    | 21.51            | 21.02            | 22.34            |
| 20.0          | 0.77      | 0.00     | 0.22             | 0.00             | 0.24             |
| 20.1          | 1.77      | 0.00     | 0.39             | 0.00             | 0.41             |
| 20.4 (n-6)    | 2.35      | 0.00     | 0.00             | 19.53            | 0.20             |
| 22.0          | 0.75      | 0.00     | 0.00             | 0.00             | 0.19             |
| 22.1          | 60.75     | 45.84    | 23.81            | 30.23            | 23.99            |
| 22.6 (n-3)    | 0.00      | 0.00     | 0.00             | 0.00             | 22.01            |
| 24.0          | 0.72      | 0.00     | 0.00             | 0.00             | 0.16             |
Marszalek et al. Figure 1

- **A**: Graph showing the relative mRNA levels of Acsl1. The x-axis represents the GFP intensity (log), and the y-axis represents the counts. The graph indicates a 96% decrease in expression.

- **B**: Graph showing the relative mRNA levels of another gene, possibly Acsl6, with a similar 96% decrease in expression.

- **C**: Image of a western blot showing bands at 71 and 55 kDa, marked with an asterisk.

- **D**: Bar graph comparing the relative mRNA levels of Acsl1, Acsl6, Acsl3, Acsl4, and Acsl5. The control cells are represented by light gray bars, while Acsl6 overexpressing cells are shown in dark gray. The y-axis represents the relative mRNA levels with control Acsl1 mRNA level set to 1. The bars show varying levels of expression for each gene.
Marszalek et al. Figure 2

Control

AcsL6 Overexpressing

Relative Amounts of $[^{14}C]$-Acyl-CoA

(cpm x 10$^3$/min/10$^6$ cells)

OA

AA

DHA

*
Marszalek et al. Figure 3
A

Control

Acs16 Overexpressing

15 minutes

B

120

96

72 hours

72

48

OA

AA

DHA

1
2

3
4

5
6

Marszalek et al. Figure 4
Marszalek et al. Figure 5
Marszalek et al. Figure 6

A  15 MINUTES

B  72 HOURS

Percent of Total Phospholipid

Control

Acs6 Overexpressing

PC  PS  PI  PE

O  A  D  O  A  D  O  A  D  O  A  D

*  #

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Marszalek et al. Figure 7
Marszalek et al., Figure 8
Long-chain Acyl CoA synthetase 6 preferentially promotes DHA metabolism
Joseph R. Marszalek, Claire Kitidis, Concetta C. DiRusso and Harvey F. Lodish

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