Acyl-CoA Synthetase 2 Overexpression Enhances Fatty Acid Internalization and Neurite Outgrowth*

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During neurodevelopment neurons increase phospholipid synthesis to generate additional plasma membrane that makes up the growing neurites. Compared with most cell types, neurons contain a high percentage of the polyunsaturated fatty acids (PUFAs) arachidonic acid (AA) and docosahexaenoic acid (DHA). By utilizing PC12 cell lines as a model neuronal cell line, we examined the internalization rate of AA, DHA, and non-essential oleic acid (OA), as well as their effects on neurite outgrowth. When wild type cells were differentiated, the rate of AA and DHA internalization increased 50% more than the rate of OA internalization. When media were supplemented with AA or DHA, the average neurite length was increased by ~40%, but supplementation with the same amount of OA had no effect. We also increased the levels of acyl-CoA synthetase-1 (ACS1) and ACS2 proteins to determine whether they contribute to PUFA internalization or neurite outgrowth. Overexpression of ACS1 increased the rate of OA internalization by 55%, and AA and DHA uptake was increased by 25%, but there was no significant change in neurite outgrowth. In ACS2-overexpressing cells, in contrast, the rate of OA internalization increased by 90%, AA by 115%, and DHA by 70%. The average aggregate neurite length in ACS2-overexpressing cells was increased by ~40% when the media were supplemented with PUFAs, but there was no change with OA supplementation. Taken together, these results support the hypotheses that ACSs are rate-limiting for fatty acid internalization and that ACS2 enhances neurite outgrowth by promoting PUFA internalization.

Fatty acids (FAs)1 are utilized by the body as an energy source, function as signaling molecules, and are a structural component of membranes. The length of a FA and the amount and position of double bonds within that FA greatly influence its solubility and flexibility. The type of FA utilized is often linked to the primary function of the cell. For example, the primary function of skeletal and cardiac muscle is force generation, which requires enormous amounts of energy. Therefore, saturated and unsaturated FAs liberated from triglycerides (TAGs) or imported as free FAs from blood are interchangeably utilized via β-oxidation to generate ATP to power myosin movement. Adipocytes, on the other hand, are a storage depot for energy and therefore primarily function in the generation of TAGs from saturated and unsaturated free FAs that are stored in lipid droplets within the cell.

During differentiation neurons require substantial amounts of FAs for phospholipid (PL) synthesis to generate extensive neurites that are the morphological and physiological hallmarks of these cells. This process utilizes FA from two sources: de novo FA synthesis through the action of the enzyme fatty acid synthase and uptake of essential FAs, which cannot be synthesized by mammals because they lack the desaturase enzymes that introduce double bonds at the n-3 and n-6 positions (1). Despite this deficiency, the plasma membranes of neurons are enriched in PLs containing polyunsaturated fatty acids (PUFA), especially the essential FAs arachidonic acid (AA) and docosahexaenoic acid (DHA), members of the n-6 and n-3 FA series, respectively (1). Therefore, AA and DHA are internalized from dietary sources or generated by elongating shorter chain precursors within the astrocytes of the nervous system or in the liver (2, 3). Within neurons, DHA and AA are used primarily as structural components of the plasma membrane and as signaling molecules (4). A possible function for DHA and AA in the plasma membrane is to provide extra flexibility and fluidity to highly curved membranes, such as those at the neural synapses and in the disks of the photoreceptor outer segment (5). Several reports suggested that DHA may also prevent apoptosis in neurons (6). AA is believed to function directly as a signaling molecule and is a precursor of several eicosanoid molecules that also have signaling functions within the nervous system (7).

However, it remains unclear how neurons enrich for PUFAs, although other cell types, such as adipocytes, are relatively non-enriched for any specific FAs. Additionally, the mechanisms used by the cell to channel FAs toward one complex lipid pathway (for example PLs or TAGs) versus another remain unidentified. Several reports (8–10) have implicated the six members of the FA transport proteins (FATPs) family, all integral plasma membrane proteins, as the primary proteins that facilitate the transport of FAs across the lipid bilayer into the cell. Once inside the cell, FAs are activated by the addition of coenzyme A (CoA) via the action of an acyl-CoA synthetase (ACS), thus increasing the solubility of the FA and preparing it for utilization by one of many lipid metabolism pathways. Pre-
vius studies (10, 11) have shown that ACS is not only capable of activating FAs but also appears to facilitate the internalization of FAs probably by preventing the repartitioning of the FA back into the plasma membrane. Co-overexpression of ACS and FATP in 3T3-L1 or yeast cells synergistically increases the rate of FA internalization suggesting that FATP and ACS act in concert to facilitate FA internalization (12, 13). Once activated, FAs can be utilized as a signaling molecule, constructively for PL and TAG synthesis or destructively as an energy source via β-oxidation. These processes are believed to occur within specific compartments of the cell and may be influenced by the subcellular localization of specific ACS isoforms (14).

Five members of the ACS family have been identified that exhibit unique temporal and tissue expression patterns in mammals. Each enzyme activates various length FAs with different efficiencies. Biochemical studies have shown that ACS1 and ACS5 activate 16–20 carbon unsaturated FAs with equal efficiency (15, 16), whereas ACS2, ACS3, and ACS4 activate 20 and 22 carbon unsaturated FAs more efficiently (15, 17, 18). However, little is known about the specific function of these proteins within the cell. Coleman and co-workers (14, 19, 20) performed several biochemical experiments using primary liver cultures and proposed that individual ACS isoforms may channel FAs toward specific complex lipid pathways. Our analysis focused on ACS1 and ACS2 because our preliminary real time PCR studies revealed that ACS1 mRNA was significantly induced upon differentiation of 3T3-L1 cells, a model adipocyte cell line that utilizes FAs primarily for TAG synthesis, but not in differentiated PC12 cells, which use FAs primarily for phospholipid synthesis. In contrast, ACS2 mRNA was significantly induced in differentiated PC12 cells but not expressed in 3T3-L1 cells. To test the hypothesis that ACS2 functions in PFAA internalization and PL synthesis, we overexpressed it as well as ACS1 in PC12 cells. Consistent with this hypothesis, in differentiated ACS2-overexpressing cells, the rates of AA and DHA internalization were increased, and when the medium was supplemented with AA or DHA the average neurite length was significantly increased. In contrast, ACS1 overexpression had no effect on neurite length.

MATERIALS AND METHODS

Cell Culture—PC12 and 3T3-L1 cells were obtained from ATCC (Manassas, VA). PC12 cells were grown in 10% fetal bovine serum in RPMI 1640 medium on collagen-coated plates (VWR Scientific) at 37 °C in an incubator with 5% CO2. Cells were split bi-weekly at 5 × 10⁵ cells/100-mm plate. For differentiation, cells were treated for 8 min at 37 °C with 20% trypsin, triturated through a 19-gauge needle attached to a syringe, pelleted, and resuspended in 1× PBS with 0.5 μg/ml propidium iodide (to identify dead cells). The level of GFP fluorescence was determined for 10,000 cells by using a BD Biosciences FACScan. Cells were used for analysis 7–10 days after the start of infection.

Neurite Outgrowth Is Enhanced by ACS2 Overexpression

Our analysis focused on ACS1 and ACS2 because our preliminary real time PCR studies revealed that ACS1 mRNA was significantly induced on differentiation of 3T3-L1 cells, a model adipocyte cell line that utilizes FAs primarily for TAG synthesis, but not in differentiated PC12 cells, which use FAs primarily for phospholipid synthesis. In contrast, ACS2 mRNA was significantly induced in differentiated PC12 cells but not expressed in 3T3-L1 cells. To test the hypothesis that ACS2 functions in PFAA internalization and PL synthesis, we overexpressed it as well as ACS1 in PC12 cells. Consistent with this hypothesis, in differentiated ACS2-overexpressing cells, the rates of AA and DHA internalization were increased, and when the medium was supplemented with AA or DHA the average neurite length was significantly increased. In contrast, ACS1 overexpression had no effect on neurite length.

Neurite Outgrowth Analysis—Cells were plated and differentiated as described except that the differentiation media were supplemented with 7.5 μM of OA, AA, or DHA and 15 μM FA-free BSA. The cells were differentiated for 3 days before phase contrast and fluorescence images were acquired using a Spotfire software camera (Diagnostic Instruments, Inc.) mounted on a Nikon TS100 inverted microscope using Spotfire software. To determine the aggregate neurite length for each cell, the phase contrast image was converted from gray scale to CMYK in Adobe Photoshop. The black channel was then "filled" in white, and the neurites were traced in black using the pencil function in Adobe Photoshop. To determine which cells were GFP-positive, the GFP fluorescent image was "pasted" into the cyan, magenta, and yellow channels of the traced phase contrast image. To measure aggregate neurite length, the black channel, which contained the neurite tracings, was inverted, converted to gray scale, and analyzed with ImageJ software (Wayne Rasband, National Institutes of Health). The presence of GFP fluorescence within a cell was used to identify which cells were transfected. For each condition examined, significant changes in neurite length were determined using the two-tailed, Student's t test with equal variance comparing the experimental cells to the control cells.

Neurite Outgrowth Analysis—Cells were plated and differentiated as described except that the differentiation media were supplemented with the appropriate amount of free FA and 15 μM FA-free BSA. After 3 days, the cells were harvested, spun down, and resuspended in 1× SDS at a concentration of 1 × 10⁶ cells/20 μl. The cells were lysed, and the DNA was sheared by trituration through a 27-gauge needle. 40 μl of 1× loading dye was added to 0.65 ml of reagent A (Trisglyceride H kit, Wako Diagnostics), incubated for 5 min at 37 °C, followed by the addition of 0.325 ml of reagent B and incubated at 37 °C for 10 min. The absorbance at 600 nm was determined using a Shimadzu UV-1601 spectrophotometer.
FA Uptake Analysis—Cells were plated and differentiated as described. The cells were removed from the plates by trituration with a Pasteur pipette, washed in serum-free RPMI 1640, counted, and resuspended in serum-free RPMI 1640 at $5 	imes 10^5$ cells/mL. 1 ml of cells was added to a 1-ml mixture of 10 mM taurocholate, 1/18 PBS that contained $5 \mu M$ 14C-radiolabeled FA that had been sonicated for 15 min to create mixed micelles. Every 30 sec for 2 min, 450/262 l of the reaction was collected and the reaction stopped by adding the aliquot to 4 ml of ice-cold 1/18 PBS, 0.1% FA-free BSA. Once all the time points were collected, the cells were harvested onto filter paper and washed extensively with 0.1% FA-free BSA, 1/18 PBS using a Brandel multisample collector to remove non-cell-associated FAs. Cells were lysed in 4 ml of EcoScint H (National Diagnostics), and the amount of cell-associated radioactivity was determined with a scintillation counter. The counts were plotted versus time to obtain the slope, which is the rate of uptake.

Lipid Analysis—FA uptake assays were performed as described in the previous section, and the filter paper was placed in 4.8 ml of methanol/chloroform/water (2:1:0.8) and sonicated for 30 min 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, 2 ml of chloroform/water (1:1) was added, the sample was vigorously mixed for 1 min, and the phases were separated by centrifugation at 3,000 rpm for 5 min. The chloroform phase was collected, evaporated under a stream of nitrogen, resuspended in chloroform, and loaded on a silica G plate (Analtech) for separation by TLC. Complex lipid species were resolved by elution with petroleum ether/diethyl ether/acetic acid (90:10:1). The plates were then sprayed with En3hance (DuPont) and exposed to film overnight at $80^\circ$C to locate the phospholipids, diacylglycerides, free fatty acids, and triglycerides. These lipid species were scraped from the plate, and the amount of radioactivity was determined with a scintillation counter.

RESULTS

FA Internalization, Neurite Outgrowth, and TAG Accumulation upon Neuronal Differentiation of PC12 Cells—To determine whether enrichment of PUFAs within the plasma membrane of neurons could be a consequence of an increased rate of PUFAs internalization, we used radiolabeled OA, AA, and DHA to examine the change in the rate these FAs are internalized
into undifferentiated and differentiated PC12 cells, a model neuronal cell line. 3T3-L1 cells, a model adipocyte cell line, were analyzed in parallel. When PC12 cells were differentiated for 3 days, the rate of OA internalization increased 5-fold from 0.19–0.98 fmol/cell/min, the rate of AA internalization increased 7-fold from 0.24 to 1.77 fmol/cell/min, and the rate of DHA internalization also increased 7-fold from 0.21 to 1.51 fmol/cell/min (Fig. 1, A–D). The 7-fold increase in the internalization rates for AA and DHA was statistically different (p < 0.05) from the 5-fold increase in the rate of OA internalization.

In contrast, uptake of all three FAs was increased 3.3-fold upon differentiation of 3T3-L1 cells into adipocytes, with no statistically significant difference between PUFAs and OA (Fig. 1, A–D). Increased PUFA internalization into PC12 cells is consistent with the hypothesis that selective PUFA internalization contributes to their enrichment within the plasma membrane of neurons.

To confirm that the short term FA uptake assays presented in Fig. 1 measured FA internalization and not FA binding to the plasma membrane, undifferentiated and 3-day differentiated PC12 cells were incubated for 2 min with 5 μM radiolabeled OA, AA, or DHA, and the metabolic fates of these FAs were determined by TLC and scintillation counting. Consistent with internalization, only 10–15% of the radiolabeled OA was free FA, whereas the majority was metabolized to PL, DAG, and TAG (Fig. 1E). Similar results were obtained with radiolabeled AA and DHA (data not shown).

To determine whether increased availability of PUFAs in the extracellular medium alters the amount of neurite outgrowth or TAG accumulation in differentiating PC12 cells, we supplemented differentiation media that contained 0.25% horse serum in RPMI 1640 with OA, AA, or DHA bound to 15 μM of FA-free BSA, differentiated the cells for 3 days, and examined their neurite lengths and TAG levels (Fig. 2). Addition of 7.5, 15, or 30 μM OA resulted in no significant increase in neurite outgrowth compared with cells differentiated in media that were not supplemented with additional FA (Fig. 2A). However, there was a significant 95% increase in TAG levels at 30 μM OA but not at 7.5 or 15 μM (Fig. 2D). In contrast, supplementation at low concentrations of AA (7.5 or 15 μM) significantly increased aggregate neurite length by ~70% (Fig. 2B), and the same concentrations of DHA increased aggregate neurite

Fig. 2. Low concentration of PUFAs increases neurite outgrowth, whereas high FA concentration increases TAG levels. PC12 cells were differentiated for 3 days in 0.25% horse serum supplemented with the indicated amount and type of FA. For neurite outgrowth, random fields of cells were photographed, and the aggregate neurite length of each isolated cell was determined as described under “Materials and Methods.” The aggregate neurite length is the sum of the length of all of the neurites extending from a cell, and only cells with aggregate neurite lengths >25 μm were analyzed. At least 100 cells were analyzed for each condition, and the graphs represent the combined results from four independent experiments. A–C, the distribution of neurite lengths in cells supplemented with 7.5, 15, or 30 μM OA (A), AA (B), or DHA (C). Cells were grouped according to their aggregate neurite lengths and the groups plotted as a percentage of total cells. The numbers to the left of the legend on each graph are the mean aggregate neurite lengths for each condition. *, p values <0.01 were considered significant using the Student’s t test to compare each group of FA-supplemented cells to cells not supplemented with FA. Note that the 0 μM supplemented cells used in the three graphs are the same group of cells. D, for each FA condition, 3,000,000 cells were also collected to determine the level of TAGs as described under “Materials and Methods.” The relative TAG level with standard error for each condition was the average value from four experiments. *, p values <0.05 were considered significant by using the Student’s t test to compare each set of supplemented cells to unsupplemented cells. Note that the 0 μM supplemented cells presented with the three FAs conditions are the same group of cells.
length by \(-40\%\) (Fig. 2C). At 30 \(\mu M\) supplementation, aggregate neurite length was slightly reduced to 38\% with AA supplementation and slightly increased to 51\% with DHA supplementation, both still significantly different from the control (Fig. 2, B and C).

Similar to OA, supplementation with 30 \(\mu M\) of AA or DHA increased TAG accumulation by 121 and 123\%, respectively (Fig. 2D). At 15 \(\mu M\), AA supplementation significantly increased TAG accumulation by 55\%, whereas DHA supplementation increased TAG accumulation by 35\%. However, at 7.5 \(\mu M\) of AA or DHA, a level that resulted in significantly increased neurite outgrowth, no change in TAG accumulation was detected. The finding that 7.5 \(\mu M\) PUFAs increases neurite outgrowth but not TAG synthesis in PC12 cells is consistent with the hypothesis that elevated PUFA internalization in PC12 cells is preferentially coupled with increased PL synthesis resulting in increased aggregate neurite length.

Expression of ACS mRNAs in Undifferentiated and Differentiated PC12 and 3T3-L1 Cells—Because ACS proteins have been shown by several laboratories to act in concert with FATP to facilitate the internalization of FAs from the extracellular medium (12, 13), and because different ACS isoforms activate PUFAs with different efficiencies (15–18), we hypothesized that differential expression of ACS isoforms may contribute to selective PUFA internalization and PL synthesis in differentiated PC12 cells. Therefore, we performed real time PCR on undifferentiated and differentiated PC12 and 3T3-L1 cells, and identified which ACS mRNAs were present and which were induced upon differentiation of each cell type. The level of each message was normalized to cyclophilin B to minimize variation between RNA samples. To control for differences in amplification rates due to primer sequences, real time PCR was also performed with genomic DNA because each gene is present in exactly two copies and should therefore amplify equally. All five ACS mRNAs were present in undifferentiated PC12 cells with ACS1, ACS2, and ACS3 mRNAs equally expressed at about one-third and one-fifth the level of ACS4 and ACS5 mRNAs, respectively (Fig. 3A). Upon PC12 cell differentiation, ACS2, ACS4, and ACS5 mRNAs were significantly increased by 27, 63, and 110\%, respectively, whereas levels of ACS1 and ACS3 mRNAs were unchanged.

In undifferentiated 3T3-L1 cells, ACS1, ACS3, ACS4, and ACS5 mRNAs were all expressed at relatively low levels, and ACS2 mRNA was not detected (Fig. 3B). Upon 3T3-L1 differentiation, ACS1 mRNA increased \(-160\)-fold, whereas levels of ACS2, ACS3, ACS4, and ACS5 mRNAs remained unchanged. Induction of ACS1 mRNA is consistent with the hypotheses that ACS1 activates 16–20 carbon FAs non-specifically and promotes TAG synthesis. Because ACS2 is expressed in PC12 cells, but not 3T3-L1 cells, and because biochemical studies indicate that it activates PUFAs more readily than ACS1 (15), we hypothesized that ACS2 may be partly responsible for the enhanced PUFA internalization in differentiated PC12 cells and increased neurite outgrowth when differentiating cells are supplemented with PUFAs.

Generation of a PC12 Cell Line That Is Efficiently Transformed by Murine Stem Cell Virus Retrovirus—In order to perform biochemical analysis, such as FA internalization or TAG levels, of cells with overexpressed ACS proteins, we require large numbers of transformed PC12 cells. However, introduction of exogenous DNA into PC12 cells by standard methods, such as transfection or retroviral infection, normally transforms less than 5\% of the cells. Additionally, transgene-independent phenotypes often occur when stable PC12 cell clones are generated. We hypothesized that if we increased the number of retroviral receptors, we should increase transformation efficiency. To this end, we generated 12 stable PC12 cell lines that overexpressed the murine ecotropic receptor and examined the ability of each line to extend neurites and be infected by retrovirus, as compared with the parental cell line. Although all of the isolated clones exhibited a significant increase in their ability to be infected by retrovirus, only two clones extended neurites that were of similar length and arborization to those seen with the parental cell line. We decided to exploit clone Eco1 for our analysis because it extended neurites that were indistinguishable from wild type PC12 cells (Fig. 4, A and B) and because it was transformed nearly 2 orders of magnitude more efficiently than the parental cell line as determined by FACS analysis of GFP expression (Fig. 4, C and D). Additionally, the distribution of GFP expression per cell varied over a 100-fold range in a typical Poisson distribution (24). By using Eco1 cells, we generated populations of cells that overexpressed ACS1 or ACS2 protein, and we examined how neurite outgrowth, FA internalization, and TAG levels were affected.
A Method to Determine Accurately Neurite Outgrowth in Gene-altered PC12 Cells—The rate and extent of neurite outgrowth by PC12 cells are influenced by extracellular factors, such as the amount and quality of the collagen that is on the plate and the density of cells. Therefore, to determine accurately whether neurite outgrowth is altered in cells with modified levels of ACS protein, we developed a methodology that would eliminate the effects of variations due to plating density, collagen coating differences, and investigator bias between and within samples.

To examine neurite outgrowth, we infected Eco1 cells with a bicistronic retrovirus vector that expressed GFP as well as ACS1 or ACS2 (Fig. 5A), which allowed us to distinguish transformed cells from untransformed cells. Typically, we transformed between 40 and 60% of the cells, because at this infection level each plate contained an approximately equal number of control and experimental cells. After 3 days of differentiation, a phase contrast image of the live cells was collected (Fig. 4E), and then the filter and light sources were changed, and a GFP image of the same field was collected (Fig. 4F). The aggregate neurite length for each isolated differentiated cell was determined by tracing the neurites of each cell in the phase contrast images as described under “Materials and Methods” (Fig. 4G). After the neurites of all the isolated cells within an image were traced, we overlaid the GFP image to determine which cells were transformed (Fig. 4H). The aggregate neurite length for each cell was determined using ImageGauge PhosphorImaging software to quantitate the number of pixels, which was converted to neurite length. Experimental cells (GFP-positive) were compared with the uninfected control cells (GFP-negative) to determine whether there was a change in mean aggregate neurite length. It is important to note that this method reduced selection and tracing bias because each field was selected solely on its phase contrast image and neurite tracings occurred before the GFP image was added. At both of these times the transformation state of each cell was unknown to the investigator.

FA Internalization, Neurite Outgrowth, and TAG Synthesis in Cells with Increased Levels of ACS1 and ACS2—Because published biochemical experiments indicated that ACS2 preferentially activated PUFAs and because our real time PCR studies revealed that ACS2 was present in PC12 cells and not 3T3-L1 cells (Fig. 3), we examined whether ACS2 contributed to selective PUFA internalization and/or PUFA-enhanced neurite outgrowth. To this end, we generated populations of Eco1 cells that stably overexpressed ACS2, ACS1, or the empty
vector, and we examined FA internalization, neurite outgrowth, and TAG accumulation. Seven days after infection, the percentage of cells transformed was determined by FACS analysis of GFP expression (Fig. 5B). Typically, we obtained transformation rates between 60 and 75%. Real time PCR of undifferentiated cells revealed that the level of ACS2 mRNA was increased at least 90-fold, and ACS1 mRNA expression was increased 55-fold over the mRNA level in control empty vector cells and at least 100 untransformed cells that were differentiated into adipocytes (Fig. 5C). The levels of the other ACS mRNAs were not significantly changed.

Consistent with the hypothesis that ACS2 and ACS1 are rate-limiting for FA internalization, the rate of AA internalization in ACS2-overexpressing differentiated cells was increased by ~115%; OA uptake was increased by ~90% and DHA uptake by ~70% relative to control cells (Fig. 6A). In ACS1-overexpressing differentiated cells, OA internalization was increased by ~55%, whereas AA and DHA internalizations were increased by only ~25%. Compared with the increase in OA internalization, PUFA internalization was more significantly increased in ACS2-overexpressing cells than in ACS1-overexpressing cells suggesting that ACS2 contributes more to PUFA internalization than does ACS1.

To determine whether the relative increase in PUFA internalization observed in ACS2-overexpressing cells would result in increased neurite outgrowth, the aggregate neurite lengths were measured for at least 100 ACS2- or ACS1-transformed cells and at least 100 untransformed cells that were differentiated for 3 days in 0.25% horse serum supplemented with 7.5 μM OA, AA, or DHA. In ACS2-transformed cells, the mean aggregate neurite length of cells supplemented with AA was increased by 44% (Fig. 6J), compared with non-transformed cells, and was increased by 32% when supplemented with DHA (Fig. 6K). Neurite length was decreased by 10% when the media were supplemented with OA (Fig. 6I). As controls, cells were infected with the empty vector or ACS1-overexpressing constructs, and in both cases the mean aggregate neurite length of infected cells was not significantly different from uninfected cells (Fig. 6, C–H). After 3 days of differentiation in media supplemented with a 30 μM mixture of OA, AA, and DHA, TAG levels in ACS2-overexpressing cells were increased by 150% over empty vector control cells and were increased by 50% in ACS1-overexpressing cells (Fig. 6B). These results are consistent with the hypotheses that ACS2 and ACS1 contribute to FA internalization and as a by-product stimulate TAG synthesis. What is most striking is that, compared with ACS1, ACS2 overexpression preferentially promotes PUFA internalization and PUFA enhanced neurite outgrowth, presumably by increasing PL synthesis.

**DISCUSSION**

This paper makes several key points concerning the relationship of PUFA uptake, PL synthesis, and neurite outgrowth. Upon differentiation of PC12 cells, PUFA uptake increased significantly more than OA uptake suggesting that selective PUFA internalization may enrich PUFAs in neuronal cells. At low FA concentrations, neurite outgrowth was enhanced when PC12 cells were differentiated in media supplemented with PUFAs but not when supplemented with OA. However, when supplemented with high FA concentrations, both PUFAs and OA significantly increased TAG accumulation. When ACS1 or ACS2 protein was overexpressed, the cells differentiated normally, and FA uptake and TAG accumulation were both significantly increased. However, relative to OA internalization, PUFA uptake was increased significantly more in ACS2-overexpressing cells than in ACS1-overexpressing cells. Most significantly, when ACS2-overexpressing cells were differentiated in media supplemented with PUFAs, neurite length was significantly increased relative to control cells without ACS overexpression.

During the course of our analysis, we generated a PC12 cell line that overexpresses the murine ecotropic receptor which allows us to efficiently transform PC12 cells with a murine stem cell virus-based retrovirus. Finally, we developed an unbiased method for analyzing neurite outgrowth in cells that express altered levels of proteins.

**Increased PUFA Internalization in PC12 Cells Promotes Neurite Outgrowth**—Previous work established that the plasma membrane of neurons is highly enriched with PLs containing PUFAs. As a possible contributing factor, we found that PUFA internalization was increased significantly more than OA internalization when PC12 cells were differentiated but not when 3T3-L1 cells were differentiated into adipocytes. A simple explanation is that PC12 cells express proteins that preferentially interact with PUFAs, whereas 3T3-L1 cells do not.

Two models have been proposed to explain how cells internalize FAs, one is based primarily on concentration gradient-driven diffusion and the other on protein-mediated transport through the membrane. In the diffusion model, exogenous FAs readily partition into the outer leaflet of the plasma membrane due to their hydrophobicity (25). Once in the plasma membrane, FAs “flip-flop” to the inner leaflet and are extracted by proteins such as FATP, ACS, or FABP. Interaction of the FA with any of these proteins reduces the cytosolic concentration...
of free FAs, thus maintaining a concentration gradient across the plasma membrane (see Ref. 11). In the facilitated uptake model, FATPs are believed to directly interact with FA and translocate them across the plasma membrane. Once across the membrane, ACSs or FABPs interact with the FA, “freeing up” FATP to internalize the next FA. Regardless of which model is correct, enhanced PUFA internalization requires proteins that interact preferentially with PUFAs. Biochemical
The two primary mechanisms by which PUFAs are incorporated into PLs are by the replacement of a non-PUFA with a PUFAs via deacylation and reacylation or through de novo synthesis by the acylation of AA or DHA into the sn-2 position of a glycerol 3-phosphate backbone to form DAG. Deacylation/reacylation is probably the dominant mechanism in mature neurons when maintenance of the PUFA concentration in the membrane is most important. During neuronal maturation, a massive supply of additional PL is required, most likely provided by de novo PL synthesis. DAG is the common precursor for de novo synthesis of PL or TAG, and it has been reported that its level increases significantly upon PC12 differentiation (33). Biochemical studies of ACS2, ACS3, and ACS4 suggest that these enzymes possess significant ability to activate PUFAs (17, 18, 34). Because the levels of ACS2 and ACS4 mRNAs both increase significantly upon PC12 differentiation (Fig. 3), it seems reasonable that this would increase PUFA activation. Moreover, providing more PUFA-CoA for monoacylglycerol acyltransferase to synthesize DAG molecules that contain PUFAs.

An intriguing possibility is that the FA composition of a DAG molecule might influence how efficiently it is converted into a PL. An example of an enzyme that could do this is DAG kinase, which preferentially phosphorylates DAG molecules that contain AA in the sn-2 position (35). PLs containing PUFAs may be more readily delivered to the plasma membrane of the extending neurite, thereby promoting neurite extension either as a structural component of the membrane or as a signaling molecule.

ACS2 Preferentially Increases PUFA Internalization and May Be Responsible for PUFA Enhancement of Neurite Outgrowth—An important clue that ACS1 contributes to FA internalization and is rate-limiting was established when it, along with FATP1, was isolated during an expression cloning screen. Overexpression either of ACS1 or FATP1 significantly increased the internalization rate of a fluorescent FA analogue (10). In PC12 cells, overexpression of ACS1 or ACS2 also increased the rate of FA internalization, further supporting the hypothesis that normal levels of ACS proteins limit FA internalization. Indirect evidence supporting involvement of ACS2 in PUFA internalization includes biochemical experiments where ACS2 activated PUFAs more efficiently than non-PUFAs (34). This is supported also by our findings that the level of ACS2 mRNA increased significantly upon PC12 differentiation and that ACS2 was not expressed in TAG-storing 3T3-L1 adipocytes. The relative rate of PUFA internalization is significantly greater in PC12 cells overexpressing ACS2 than those overexpressing ACS1, providing direct evidence that ACS2 contributes significantly to PUFA internalization. Based on these data, it seems likely that the complement of ACS proteins within PC12 cells accounts for the preferential internalization of the PUFAs over the unsaturated FAs observed upon neuronal differentiation.

Significant changes in neurite outgrowth occurred only when ACS2 protein was overexpressed and the cells were differentiated in media supplemented with AA or DHA. This is consistent with the hypothesis that ACS2 overexpression further increased the level of PUFA-CoA, possibly by enhancing PUFA internalization into the cell. What is surprising is that overexpression of ACS1 or ACS2 significantly increased the internalization of all three FAs, yet overexpression of ACS1 did not enhanced neurite outgrowth. This suggests that the absolute amount of PUFA taken into the cell is not the critical factor in promoting neurite outgrowth; rather, it may be the relative percentage of PUFAs that are taken up. The simplest explanation of our data is that ACS1 may more efficiently remove 18:1 from FATP or, independently, from the plasma membrane, as suggested by our FA internalization assay (Fig. 6) and previous biochemical studies (15), and that ACS2 may more efficiently remove AA and DHA. Therefore, a small change in internalization and increased ability to activate AA and DHA by ACS2 would create a larger pool of PUFA-CoA which could then get incorporated into DAG, ultimately becoming a PL that contains a PUFAs.

Efficiently Transformed PC12 Cell Line, an Improved Model Neuronal Cell Line—The need for a method that efficiently introduces foreign DNA into PC12 cells without the use of an amphotropic virus led us to generate a strain of PC12 cells that is efficiently infected by a murine stem cell virus-based retrovirus that specifically recognizes the murine ecotropic receptor. This approach yields a high transformation efficiency that enables us to introduce genetic changes into this widely exploited neuronal model system, as well as to generate populations of stably transformed cells that are subject to non-transgene-mediated alterations. Another advantage of this method is that pools of cells, not clones, can be used for analysis as soon as 48 h after the start of infection, thus minimizing compensatory effects or lethality resulting from altered gene expression.

New Method To Determine Neurite Length In Gene-altered Cells Improves Accuracy—The methodology we have developed to analyze neurite outgrowth is a good measure of how FAs are utilized for PLs synthesis within the differentiating cells. Because we can reproducibly infect any desired fraction of cells by using a retrovirus vector, we always have control cells on the same plate as the experimental cells. This is critical to eliminate plate to plate variability that occurs due to different samples being plated at different densities and allows us to pool results from several plates and experiments. Additionally, PC12 cells tend to clump together, and it is often very difficult to plate different samples of cells at the same density. By plating the cells at low density and selecting fields of differentiating cells using phase contrast optics where the genotype of the cell remains hidden, we can focus on the largest cells for analysis, and only after the neurites have been traced do we learn the identity of the cell. This strategy is important because if we examined cells that were plated at high density, and if the transgene caused a significant increase in neurite outgrowth, we would not identify the change because most of these large cells would not be analyzed because their processes would touch another cell. This is critical as it is necessary to examine at least 100 each of infected and non-infected cells due to the fact that there is a 50-fold difference between the cells with smallest and largest aggregate neurite lengths.

The development of this system will allow us to systemat-
Neurite Outgrowth Is Enhanced by ACS2 Overexpression

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Acknowledgments—We thank Drs. Moonkyoung Um, Tzu-Shuen Tsao, and Saghie Ghaffari for helpful critical comments during the course of this work. We also thank current and former members of the Lodish laboratory for their contributions.
Additions and Corrections

Vol. 279 (2004) 23882–23891

Acyl-CoA synthetase 2 overexpression enhances fatty acid internalization and neurite outgrowth.

Joseph R. Marzalek, Claire Kitidis, Artya Dararutana, and Harvey F. Lodish

Page 23883, “Materials and Methods”: Under the section headed “Isolation and Construction of ACS1 and ACS2,” two errors occurred. First, the incorrect primer sequence was included for ACS1 antisense. It should read 5’-CCC GGAT CCTCAGGGCACAATCTTGTAGGTTGGAG-3’, not 5’-CCC GGATCCTTAAATCTTGATGGTTGGAGTAC-3’. Second, the GenBankTM accession number referenced for the rat ACS2 sequence used in the study should be AY625254, not D10041. These two sequences are alternatively spliced variants of rat Acs16, each containing a different variant of exon 13.

Although these minor corrections do not change the data or interpretation of the data in the paper, they are important for accuracy and reproducibility of the results by other investigators.

Vol. 280 (2005) 2361–2369

Pro-angiogenic signaling by the endothelial presence of CEACAM1.

Nerbin Kilic, Leticia Oliviera-Ferrer, Jan-Henner Wurmbach, Sonja Loges, Fariba Chalajour, Samira Neshat-Vahid, Joachim Weil, Malkanthi Fernando, and Suleyman Ergun

Page 2368: Dr. Neshat-Vahid’s last name was misspelled. The correct spelling is shown above.

Vol. 280 (2005) 3605–3612

NMR structural comparison of the cytoplasmic juxtamembrane domains of G-protein-coupled CB1 and CB2 receptors in membrane mimetic dodecylphosphocholine micelles.

Xiang-Qun Xie and Jian-Zhong Chen

Page 3612: Add new Ref. 37, Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) Biochemistry 31, 1647–1651. The reference list will now include a total of 37 references. As a result, the following reference citation should be changed:

Pg. 3608, right column, line 11 from the top: “(26, 27)” should be “(37).”

Vol. 280 (2005) 3802–3811

Activation of the phagocyte NADPH oxidase by Rac guanine nucleotide exchange factors in conjunction with ATP and nucleoside diphosphate kinase.

Ariel Mizrahi, Shahar Molshanski-Mor, Carolyn Weinbaum, Yi Zheng, Miriam Hirshberg, and Edgar Pick

Pages 3803–3811: The word “on” was omitted from the running title. The correct running title should read as follows: NADPH Oxidase Activation Dependent on GEF, ATP, and NDPK.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.