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# α-Synuclein Gene Deletion Decreases Brain Palmitate Uptake and Alters the Palmitate Metabolism in the Absence of α-Synuclein Palmitate Binding†

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ABSTRACT: α-Synuclein is an abundant protein in the central nervous system that is associated with a number of neurodegenerative disorders, including Parkinson's disease. Its physiological function is poorly understood, although recently it was proposed to function as a fatty acid binding protein. To better define a role for α-synuclein in brain fatty acid uptake and metabolism, we infused awake, wild-type, or α-synuclein gene-ablated mice with [1-$^{14}$C]palmitic acid (16:0) and assessed fatty acid uptake and turnover kinetics in brain phospholipids. α-Synuclein deficiency decreased brain 16:0 uptake 35% and reduced its targeting to the organic fraction. The incorporation coefficient for 16:0 entering the brain acyl-CoA pool was significantly decreased 36% in α-synuclein gene-ablated mice. Because incorporation coefficients alone are not predictive of fatty acid turnover in individual phospholipid classes, we calculated kinetic values for 16:0 entering brain phospholipid pools. α-Synuclein deficiency decreased the incorporation rate and fractional turnover of 16:0 in a number of phospholipid classes, but also increased the incorporation rate and fractional turnover of 16:0 in the choline glycerophospholipids. No differences in incorporation rate or turnover were observed in liver phospholipids, confirming that these changes in lipid metabolism were brain specific. Using titration microcalorimetry, we observed no binding of 16:0 or oleic acid to α-synuclein in vitro. Thus, α-synuclein has effects on 16:0 uptake and metabolism similar to those of an FABP, but unlike FABP, it does not directly bind 16:0; hence, the mechanism underlying these effects is different from that of a classical FABP.

α-Synuclein is a soluble protein that contains 140 amino acids (*1*, *2*). The protein is primarily localized in presynaptic terminals of neurons (*1*, *3*−*5*) and oligodendroglial cell bodies (*6*). α-Synuclein overexpression and mutations in the α-synuclein gene are associated with familial Parkinson's disease (*7*−*10*). Aggregates containing α-synuclein are also hallmarks of a number of neurological disorders, including Alzheimer's disease (*11*, *12*), Lewy body disease (*13*, *14*), Down's syndrome (*15*), and multiple-system atrophy (*16*). Despite the association of α-synuclein with neurodegenerative diseases, its physiological function is poorly understood.

Several lines of evidence suggest that α-synuclein may have a role in brain lipid metabolism. For instance, in yeast and HEK-293 cells, overexpression of α-synuclein leads to enhanced lipid droplet formation (*17*, *18*), suggesting altered lipid trafficking and metabolism. In addition, yeast phospholipase D (PLD)[1] activity is inhibited by this overexpression (*17*), which is also observed in vitro (*19*, *20*). The inhibition of PLD is hypothesized to be biologically important because vesicular transport processes are modulated via PLD activity (*19*, *20*). α-Synuclein's capacity for binding small phospholipid vesicles (*21*) and brain vesicles (*22*), as well as its capacity for regulating distal pool of synaptic vesicles (*23*, *24*), is in agreement with this hypothesis. In addition, α-synuclein also interacts with phospholipase C $\beta_2$ and increases the capacity for coupling of the G$\beta\gamma$ subunit to this phospholipase C, thereby increasing enzymic activity (*25*), which may account for the observed increase in the extent of inositol phosphate formation in α-synuclein-expressing PC-12 cells upon receptor stimulation (*25*). Thus,

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[1] Abbreviations: FABP, fatty acid binding protein(s); CerPCho, sphingomyelin; ChoGpl, choline glycerophospholipids; EtnGpl, ethanolamine glycerophospholipids; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; 16:0, palmitic acid; PL, phospholipids; PLD, phospholipases D; WT, wild-type; KO, α-synuclein gene-ablated; $k_i^*$, coefficient for incorporation of 16:0 from plasma into a given pool; $J_{FA,i}$, net rate of incorporation from 16:0-CoA into an individual phospholipid; $C_{br,i}$, mass of 16:0 in an individual brain phospholipid; $C_{liv,i}$, mass of 16:0 in an individual liver phospholipid; $F_{FA,i}$, fractional turnover rate of 16:0 in an individual phospholipid; $T_{1/2,i}$, half-life of 16:0 in an individual phospholipid.

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α-synuclein may have a critical function in lipid-mediated signal transduction; however, its role in brain lipid metabolism is poorly understood.

α-Synuclein has been hypothesized to function as a fatty acid binding protein (FABP) in the central nervous system (*26*). More direct evidence for this hypothesis is provided by a recent study in α-synuclein deficient astrocytes where 16:0 uptake is slowed and targeting to cellular lipid pools is altered (*27*). Like FABP, α-synuclein is a small cytosolic protein (17 kDa) (*3*, *26*) that has similar amino acid sequences and binds fatty acids with high affinity in vitro (*26*). Although FABP can facilitate fatty acid uptake and trafficking (*28*−*32*) and can enhance phospholipid (PL) biosynthesis (*32*−*34*), it is not known if α-synuclein can affect these processes in the brain. While the brain contains a number of FABP (*35*), very little is known with regard to their impact on fatty acid uptake and trafficking. However, heart FABP (H-FABP) differentially increases brain fatty acid uptake, improving 20:4 n-6 but not 16:0 uptake (*36*). In addition, H-FABP expression increases the total brain phospholipid mass and that of select individual phospholipids, as well as altering n-6 family fatty acid content of phospholipids, demonstrating that this protein has a profound affect on brain lipid metabolism (*36*). Thus, it is very intriguing that α-synuclein may function as a FABP in the brain.

To address this issue, we examined the influence of α-synuclein on 16:0 uptake and turnover kinetics in brain phospholipid pools in vivo. Further, we examined the ability of α-synuclein to directly bind 16:0 using titration microcalorimetry. As α-synuclein was not found in liver, this tissue was used as a negative control. Wild-type (WT) and α-synuclein gene-ablated (KO) mice were infused with radiolabled [1-$^{14}$C]16:0 in vivo, and kinetics were calculated using a steady-state radiotracer model (*37*−*39*). These data show, for the first time, that α-synuclein deficiency decreased brain 16:0 uptake and also decreased the incorporation rate and fractional turnover of 16:0 in a number of brain phospholipids, but increased these parameters in the choline glycerophospholipids (ChoGpl). In the absence of direct binding of 16:0 by α-synuclein, but in the presence of marked changes in 16:0 uptake and metabolism in α-synuclein deficient mice, we conclude that although α-synuclein does not behave as a classical FABP, it alters these parameters by a mechanism that affects brain 16:0 metabolism.

## EXPERIMENTAL PROCEDURES

*Mice.* This study was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 80-23) and under an animal protocol approved by the IACUC at the University of North Dakota (Protocol 0110-1). α-Synuclein gene-ablated mice were generated from the 129/SvEv strain by gene-targeted deletion (*24*). Male mice (25−30 g) were maintained on standard laboratory chow diet and water ad libitum. The ages for the mice used in this study were between 9 and 11 months in both groups.

*Western Blot Analysis of* α*-Synuclein.* Brains and livers were frozen in liquid nitrogen and pulverized under liquid nitrogen temperatures to a fine, homogeneous powder. The tissue powders were suspended in 250 μL of ice-cold homogenization buffer [10 mM Hepes (pH 7.4)] containing protease inhibitors (Sigma, St. Louis, MO). The samples were kept on ice, resuspended, and then sonicated for 10 s. The nuclei and cellular debris were pelleted by centrifugation at 12000*g* for 10 min. Solubilized material was removed and placed into a new 1.5 mL tube, and the protein concentration was measured using a dye binding assay with bovine serum albumin as a standard (*40*). For Western blot analysis, 50 μg of liver protein and 10 μg of brain protein from wild-type and gene-ablated tissues and lysate from stably transfected HEK cells expressing α-synuclein (*18*) were denatured in sample application buffer containing SDS for 5 min at 95 °C. Samples were then loaded onto 15% polyacrylamide gels and were run at a constant voltage (160 V) for 65 min.

Proteins were then transferred onto PVDF blotting membranes (Pierce, Rockford, IL), at 300 mA for 40 min, boiled for 10 min, blocked overnight with 3% BSA in TBS containing 0.1% Tween 20, and immunoblotted for 2 h with monoclonal anti-synuclein antibody 4D6 (1:1000) (Signet, Dedham, MA) or rabbit anti-Erk-2 antibody (1:20000) (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Santa Cruz Biotechnology) were used to detect bound α-synuclein or Erk-2 antibody, respectively, and visualization was carried out using SuperSignal West Pico Chemiluminescent substrate (Pierce).

The positive control was the lysate from stably transfected HEK cells expressing α-synuclein, and Erk-2 was used as a loading control.

*Tracer Preparation.* [1-$^{14}$C]16:0 was synthesized by Moravek Biochemical (Brea, CA). The ethanol from the tracer aliquot was evaporated under a constant stream of $N_2$ at 40 °C. The tracer was solubilized in 5 mM HEPES (pH 7.4) buffer containing "essentially fatty acid free" bovine serum albumin (50 mg/mL; Sigma Chemical Co.). Solubilization was facilitated by sonication in a bath sonicator for 60 min at 40 °C. The appropriate amount of radiotracer was prepared for each mouse using the mouse's weight based upon the infusion parameters of 170 μCi/kg (*41*).

*Mouse Surgery and Infusion.* The mouse surgery and tracer infusion were performed as previously described (*32*). Briefly, fasted WT and KO male mice were anesthetized with halothane (1−3%), and PE-10 catheters were inserted into the femoral artery and vein. Using an infusion pump (BS-8000, Braintree Scientific, Inc., Braintree, MA), awake (3−4 post-operative hours) male mice were infused with 170 μCi/kg of [1-$^{14}$C]16:0 via the femoral vein over 10 min at a constant rate of 30 μL/min to achieve steady-state plasma radioactivity. Prior to and during the infusion, arterial blood samples (∼20 μL) were taken to determine plasma radioactivity. Following infusion, each mouse was killed using pentobarbital (100 mg/kg, intravenous) and immediately subjected to focused microwave irradiation (2.8 kW, 1.35 s; Cober Electronics, Inc., Norwalk, CT) to heat denature enzymes in situ. The whole brain and liver were removed, frozen in liquid nitrogen, and pulverized at liquid nitrogen temperatures to a fine, homogeneous powder.

*Lipid Extraction.* Lipids from the tissue powder, plasma, and blood samples were extracted using a two-phase extraction procedure (*42*). The radioactivity in the aqueous and organic fractions was determined by liquid scintillation counting. The extracts were concentrated under a stream of

$N_2$ at 40 °C and dissolved in *n*-hexane, 2-propanol, and water (56.7:37.8:5.5 by volume).

*Thin-Layer Chromatography.* Tissue phospholipids (PL) were separated by TLC on heat-activated Whatman silica gel-60 plates (20 cm × 20 cm, 250 μm) and developed in chloroform, methanol, acetic acid, and water (55:37.5:3:2) (*43*). Brain, liver, and plasma neutral lipids (NL) were separated by TLC on heat-activated Whatman silica gel-60 plates (20 cm × 20 cm, 250 μm) and developed in petroleum ether, diethyl ether, and acetic acid (75:25:1.3 by volume) (*44*). Individual PL and NL from TLC were used to quantify individual PL and NL class fatty acid mass by GLC and to quantify individual PL and NL radioactivity by liquid scintillation counting, respectively.

*Gas−Liquid Chromatography.* The 16:0 mass in phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), ChoGpl, and ethanolamine glycerophospholipids (EtnGpl) was determined following base-catalyzed transesterification to form the fatty acid methyl esters (*45*). As acid-catalyzed methylation has a higher yield for sphingomyelin (CerPCho), individual CerPCho fatty acid mass, as well as free fatty acid mass in plasma, brain, and liver, were measured by GLC after acid-catalyzed transesterification (*46*). The gas−liquid chromatograph (Trace GC, ThermoElectron, Austin, TX) was equipped with a capillary column [SP 2330; 30 m × 0.32 mm (inside diameter); Supelco, Bellefonte, PA] and a flame ionization detector. Fatty acids were quantified using a standard curve from commercially purchased standards (NuChek Prep, Elysian, MN), and 17:0 was the internal standard.

*Acyl-CoA Extraction, Separation, and Quantitation.* Acyl-CoA from brain and liver was extracted and purified using a solid-phase extraction procedure and separated by HPLC on a C-18(2) column (Luna, Phenomenex, Torrance, CA) (*47*). The system was controlled with a Beckman (Fullerton, CA) 127 solvent module. The eluent was monitored at 260 nm using a Beckman 166 UV−vis detector. 16:0-CoA mass was determined using a standard curve from commercially purchased standards (Sigma Chemical Co.), and 17:0-CoA was the internal standard. 16:0-CoA radioactivity was determined by liquid scintillation counting.

*Liquid Scintillation Counting.* Samples were placed into 20 mL liquid scintillation vials, and 0.5 mL of $H_2O$ was added, followed by 10 mL of Scintiverse BD (Fisher). After mixing had been carried out, the samples were quantified by liquid scintillation counting using a Beckman LS5000 CE liquid scintillation counter (Beckman Instruments) at least 1 h after the addition of the liquid scintillation mixture.

*Kinetic Analysis.* Our study was performed under steady-state conditions, and calculations were carried out as previously described (*37*−*39*). Radioactivity of a given brain or liver compartment *i*, $C^*_{br,i}(T)$ or $C^*_{liv,i}(T)$ (in nanocuries per gram), was calculated by correcting its radioactivity for its residual intravascular radioactivity (*48*, *49*). Blood samples taken at the time of death, $T = 10$ after starting tracer infusion, were extracted and analyzed to make this correction. A curve of plasma radioactivity was constructed for each mouse and the area under the curve calculated. This area is the input function. Unidirectional incorporation coefficients, $k_i^*$ (in inverse seconds), of [1-$^{14}$C]16:0 from plasma into a given brain or liver compartment *i*, were calculated as follows

$$k_i^* = \frac{C^*_{br,i}(T)}{\int_0^T C^*_{pl}\,dt} \text{ or } k_i^* = \frac{C^*_{liv,i}(T)}{\int_0^T C^*_{pl}\,dt}$$

where $t$ is the time after the beginning of infusion and $c^*_{pl}$ (in nanocuries per milliliter) is the plasma concentration of radiolabeled 16:0 during infusion. The rate of incorporation of nonesterified 16:0 from brain or liver palmitoyl-CoA into brain or liver phospholipid *i*, $J_{FA,i}$, was calculated as follows

$$J_{FA,i} = \frac{C^*_{br,i}(T)}{\left(\frac{\lambda}{C_{pl}}\right)\int_0^T C^*_{pl}\,dt} = k_i^*\frac{C_{pl}}{\lambda} \text{ or}$$

$$J_{FA,i} = \frac{C^*_{liv,i}(T)}{\left(\frac{\lambda}{C_{pl}}\right)\int_0^T C^*_{pl}\,dt} = k_i^*\frac{C_{pl}}{\lambda}$$

where $c_{pl}$ (in nanomoles per milliliter) is the concentration of unlabeled nonesterified 16:0 in plasma. The dilution coefficient, $\lambda$, represents the steady-state specific activity of palmitoyl-CoA relative to that of plasma

$$\lambda = \frac{C^*_{palmitoyl-CoA}/C_{palmitoyl-CoA}}{C^*_{pl,16:0}/C_{pl,16:0}}$$

where the numerator is the specific activity of brain or liver palmitoyl-CoA and the denominator is the specific activity of plasma 16:0. The fractional turnover rate of 16:0 within compartment *i*, $F_{FA,i}$ (in percent per hour), is defined as

$$F_{FA,i} = \frac{J_{FA,i}}{C_{br,i}} \times 100\%$$

The half-life of the FA in *i* is defined as

$$\text{half-life} = 0.693/F_{FA,i}$$

Calculations were carried out individually for each animal, which allows calculation of normalized individual parameters. We then calculated averages of individual parameters to compare kinetics between groups.

*Titration Microcalorimetry.* Titration microcalorimetry was carried out using a Microcal Omega Isothermal Titration Microcalorimeter (Microcal, Northhampton, MA) and followed established procedures for assessing fatty acid binding to fatty acid binding proteins (*50*). Recombinant α-synuclein was purified as previously described (*51*). α-Synuclein was dissolved in 20 mM potassium phosphate containing 50 mM KCl (pH 7.2), and the final concentration was 40−60 μM, which was titrated with either palmitate or oleate at 27 °C. The fatty acid-containing solution was prepared just before use by weighing the fatty acid into a 50 mL Falcon tube and dissolved in a small volume of $H_2O$ containing 1.2 equiv of potassium hydroxide. This solution was adjusted to a final concentration of 0.8 mM for palmitate or 1.25 mM for oleate by the addition 20 mM potassium phosphate containing 50 mM KCl (pH 7.2). In a typical experiment, α-synuclein was placed in the calorimeter cell and was stirred at 400 rpm. The sample was titrated with 25−30 aliquots of the fatty acid-containing solution (4 μL) added at 3 min intervals. The reference cell was filled with 0.02% sodium

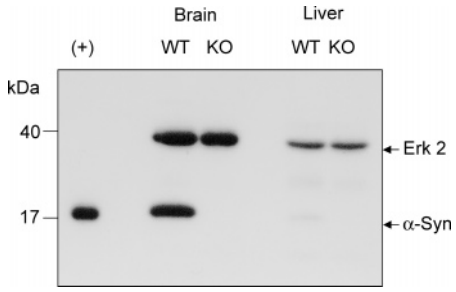


FIGURE 1: Western blot analysis of α-synuclein in brain and liver tissues from WT and KO mice: (+) 0.3 μg of total protein from α-synuclein-expressing HEK-293 cells, (WT-B) 10 μg of total protein from wild-type brain, (KO-B) 10 μg of total protein from gene-ablated brain, (WT-L) 50 μg of total protein from wild-type liver, and (KO-L) 50 μg of total protein from gene-ablated liver.

azide in water, and the instrument was calibrated by standard electrical pulses. Raw data were integrated using the supplied ORIGIN software (MicroCal, Inc.), and isotherms were analyzed as described previously (*52*, *53*). All experiments were repeated three times.

*Statistics.* Statistical analysis was done using Instat2 from GraphPad (San Diego, CA). Statistical significance was assessed using an unpaired, two-way, Student's *t*-test, with a $p$ of $<0.05$ considered to be statistically significant.

## RESULTS

*Western Blotting Demonstrates the Absence of α-Synuclein in Liver.* α-Synuclein presence in brain and liver of WT and KO mice was tested with Western blot analysis (Figure 1). The Erk-2 indicated approximately equal loading of the WT and KO brain protein, and of the WT and KO liver protein. Note that the Erk-2 mass in liver is lower than in brain (*54*). Brains from WT mice contained α-synuclein (Figure 1, lane 2), while it was absent in brains from KO animals (Figure 1, lane 3). Liver homogenates from WT and KO mice did not contain α-synuclein (Figure 1, lanes 4 and 5), and heart also did not demonstrate detectable α-synuclein (data not shown). Hence, liver tissue was used as a negative control to determine if α-synuclein gene ablation had a nonspecific effect on tissue fatty acid metabolism.

*Plasma Curves for [1-$^{14}$C]16:0 Infusion.* Plasma curve radioactivity indicates that the study was performed under steady-state conditions. The mean integrated area under the curve was 1155 ± 426 and 1260 ± 467 nCi mL$^{-1}$ min$^{-1}$ ($n = 9$) for WT and KO mice, respectively. The plasma 16:0 concentration (nanomoles per milliliter) was 68.7 ± 11.9 for WT and 76.1 ± 14.5 ($n = 8$) for KO mice. There were no differences in the mean plasma curve area or free 16:0 plasma concentration between groups.

*α-Synuclein Ablation Decreased Brain 16:0 Uptake.* The effect of α-synuclein on brain 16:0 uptake and incorporation into the organic and aqueous fractions was determined. The total and organic phase radioactivity of the brain extract from KO mice was decreased 34 and 48%, respectively, as compared to those of WT (Figure 2, top panel). Similarly, the coefficient of incorporation, $k_i^*$, into these compartments was also decreased 35 and 48%, respectively (Figure 2, bottom panel). At the same time, the free 16:0 mass in brain was decreased 20% in KO brains as compared to WT, and the values (nanomoles per gram wet weight) were

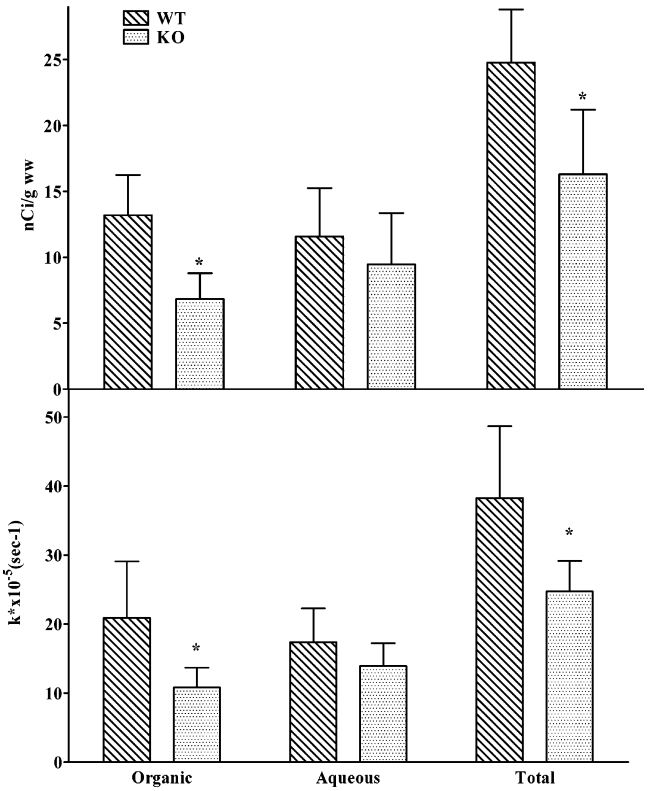


FIGURE 2: α-Synuclein expression influenced targeting of [1-$^{14}$C]16:0 to different brain metabolic pools. Uptake of [1-$^{14}$C]16:0 into brain tissue from wild-type and α-synuclein gene-ablated mice expressed as the total uptake or uptake into the aqueous and organic brain fractions. Values are expressed as nanocuries per gram wet weight (top panel) or $k^* \times 10^{-5}$ (inverse seconds) (bottom panel) and were corrected for radioactivity associated with the residual blood left in the brain tissue. Values represent the mean ± the standard deviation ($n = 7-9$). The asterisk indicates statistical significance from control mice ($p < 0.05$).

120 ± 16 ($n = 7$) for WT mice and 101 ± 15 ($n = 8$) for KO mice. There was no significant difference in tracer entering the brain aqueous compartment, which represents products of β-oxidation (*32*, *55*−*57*). Liver tissue did not show differences between groups in 16:0 uptake and incorporation into the organic and aqueous fractions (Figure 3), indicating that the observed differences were brain specific.

*α-Synuclein Affected Incorporation of [1-$^{14}$C]16:0 into the Brain Acyl-CoA Pool.* To determine the effect of α-synuclein gene ablation on incorporation of fatty acids into individual lipids, incorporation coefficients ($k_i^*$) for individual lipid classes and the acyl-CoA pool were determined. There were no significant differences between groups in $k_i^*$ for 16:0 entering brain individual neutral lipids (data not shown) or individual PL (Table 1). However, $k_i^*$ for 16:0 entering the brain acyl-CoA pool was significantly decreased 36% in KO mice; these values ($k_i^* \times 10^{-5}$ s$^{-1}$) were 0.101 ± 0.021 ($n = 7$) for WT mice and 0.065 ± 0.019 ($n = 7$) for KO mice. For liver, no differences were observed between groups in $k_i^*$ values for individual lipid classes (Table 2) or in $k_i^*$ values for 16:0-CoA; these values were 404 ± 140 ($n = 5$) for WT mice and 542 ± 215 ($n = 7$) for KO mice.

*α-Synuclein Affected the Rate of 16:0 Incorporation and Turnover Kinetics in Brain Phospholipids.* Because incorporation coefficients alone are not predictive of fatty acid

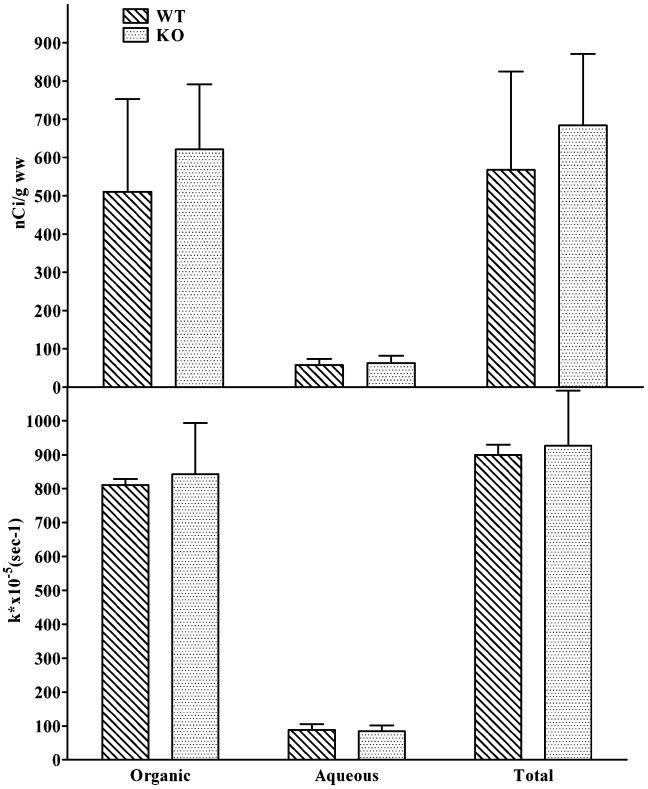


FIGURE 3: α-Synuclein expression did not influence targeting of [1-$^{14}$C]16:0 to different liver metabolic pools. Uptake of [1-$^{14}$C]16:0 into liver tissue from wild-type and α-synuclein gene-ablated mice expressed as the total uptake or uptake into the aqueous and organic brain fractions. Values are expressed as nanocuries per gram wet weight (top panel) or $k^* \times 10^{-5}$ (inverse seconds) (bottom panel) and were corrected for radioactivity associated with the residual blood left in the liver tissue. Values represent the mean ± the standard deviation ($n = 5-7$).

turnover in individual PL classes, we calculated kinetic values for 16:0 incorporation and turnover in WT and KO mouse brain phospholipids (Table 1) and liver phospholipids (Table 2). In brain tissue, the rate of incorporation ($J_{FA,i}$) of 16:0-CoA into PtdIns, PtdSer, and CerPCho was decreased 44, 55, and 51%, respectively, but was increased for incorporation into ChoGpl 1.5-fold in KO mice compared to WT mice. The fractional turnover ($F_{FA,i}$) of 16:0 in brain PtdSer and CerPCho was decreased 68 and 62%, respectively, but was increased 1.7-fold in ChoGpl in KO mice compared to WT mice. The half-life ($T_{1/2}$) for 16:0 in brain PtdSer and CerPCho was increased 2.9- and 2.7-fold, respectively, but was decreased 41% in ChoGpl in KO mice compared to WT mice. There were no significant differences in liver individual PL kinetics between groups (Table 2). Note that brain 16:0 mass ($C_{br,i}$) was significantly increased 1.2-fold in PtdSer and was decreased 30% in PtdIns in KO mice compared to WT mice (Table 1). The dilution coefficient $\lambda$ in brain was 0.054 ± 0.015 ($n = 7$) for WT mice and 0.058 ± 0.016 ($n = 7$) for KO mice. For liver tissue, there were no differences in liver individual phospholipid class 16:0 mass (Table 2) or in the dilution coefficient $\lambda$, which was 0.12 ± 0.08 ($n = 6$) for WT mice and 0.19 ± 0.07 ($n = 6$) for KO mice.

*α-Synuclein Does Not Bind Palmitic and Oleic Acid.* Because α-synuclein may bind fatty acids with a $K_d$ similar to that of FABP (*26*), we used titration microcalorimetry to determine if α-synuclein bound fatty acids with a $K_d$ similar to that of FABP. This technique has been widely used to study the ligand binding properties of a number of lipid binding proteins, including *N*-myristoyltransferase (*58*), acyl-CoA binding protein (*53*), and FABP (*50*, *59*, *60*). Using this sensitive technique, we were not able to detect any binding of palmitate (Figure 4) or oleate to α-synuclein (results not shown).

## DISCUSSION

Recently, α-synuclein has been proposed to act as a FABP in the central nervous system (*26*, *61*), but studies examining the potential function of α-synuclein in brain lipid metabolism are lacking. Because binding studies in vitro do not necessarily indicate biological function in vivo (*29*, *31*), we examined the role of α-synuclein in brain 16:0 uptake and trafficking using α-synuclein deficient mice. In addition, we re-examined the interaction of α-synuclein with fatty acids (16:0 and 18:1 n-9) using a more sensitive technique, titration microcalorimetry, to determine if α-synuclein bound fatty acids in vitro with a binding constant similar to that observed for FABP.

Despite observations by others that α-synuclein deficient mouse brain cytosol had a reduction in 22:6 n-3 mass (*61*) and that polyunsaturated fatty acids induced multimerization of α-synuclein (*62*, *63*), we examined the effect of α-synuclein deficiency on 16:0 uptake and metabolism because we have observed changes in the uptake of this fatty acid in α-synuclein deficient astrocytes cultured from these same mice (*27*). In addition, we also have observed a marked effect of α-synuclein on uptake of 16:0 into HEK-293 cells stably expressing α-synuclein (data not shown). Thus, in this study we chose to define the effect of α-synuclein on 16:0 uptake using the gene-ablated mouse as a model system.

We have shown that WT brains, but not livers, contain α-synuclein (Figure 1), confirming literature reports that α-synuclein expression is limited to a few tissues, including the central nervous system (*1*, *3*−*6*, *64*). Hence, liver tissue was used as a negative control to define if α-synuclein gene ablation had a nonspecific effect on fatty acid metabolism. Because no differences were found in 16:0 metabolism in WT compared to KO livers (Figure 3 and Table 2), we assume that observed changes in 16:0 metabolism in WT versus KO brains are not the result of a nonspecific effect of α-synuclein gene ablation, but rather distinct for the brain.

Using titration microcalorimetry, we could not demonstrate binding of 16:0 to α-synuclein in vitro. This is in contrast to the report of α-synuclein binding oleic acid ($K_d$ = 12.5 $\mu$M) using the Lipidex binding assay (*26*). However, it is important to note that this binding curve demonstrates binding similar to the observed nonspecific binding (*65*), rather than specific binding in a lipophilic binding pocket as reported elsewhere (*66*). Further, other evidence that α-synuclein is a FABP is less convincing and is based upon a similar number of amino acids and a molecular mass for α-synuclein similar to that of the majority of FABP (*26*) and because α-synuclein is a protein that associates with membranes (*21*, *67*). To confirm that α-synuclein did not bind oleic acid, we also determined the binding of this fatty acid to α-synuclein using titration microcalorimetry, but could not demonstrate any binding of the fatty acid to the

Table 1: Individual Brain Phospholipid Kinetics under α-Synuclein Deficient Conditions[a]

| | $k_i^*$ ($\times 10^{-5}$ s$^{-1}$) | | $J_{FA,i}$ (nmol/h) | | $F_{FA,i}$ (%/h) | | $T_{1/2,i}$ (h) | | $C_{br,i}$ (nmol/g wet weight) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | WT | KO | WT | KO | WT | KO | WT | KO | WT | KO |
| EtnGpl | 1.1 ± 0.4 | 0.9 ± 0.5 | 50 ± 13 | 45 ± 12 | 8.4 ± 3.2 | 6.3 ± 3.0 | 9.4 ± 3.6 | 13.5 ± 6.6 | 697 ± 170 | 758 ± 152 |
| PtdIns | 0.4 ± 0.2 | 0.5 ± 0.4 | 25 ± 9 | 14 ± 2* | 16.6 ± 5.2 | 14.1 ± 3.0 | 4.6 ± 1.6 | 5.2 ± 1.2 | 139 ± 28 | 97 ± 10* |
| PtdSer | 0.4 ± 0.3 | 0.4 ± 0.3 | 22 ± 6 | 10 ± 3* | 14.5 ± 5.8 | 4.6 ± 1.2* | 5.5 ± 2.3 | 15.7 ± 3.7* | 180 ± 38 | 223 ± 20* |
| ChoGpl | 4.7 ± 2.3 | 4.0 ± 1.3 | 175 ± 58 | 259 ± 50* | 1.6 ± 0.5 | 2.7 ± 0.6* | 46.0 ± 13.9 | 26.8 ± 5.4* | 9110 ± 1529 | 10089 ± 858 |
| CerPCho | 0.7 ± 0.4 | 0.8 ± 0.4 | 45 ± 8 | 22 ± 5* | 17.3 ± 3.1 | 6.8 ± 1.7* | 4.0 ± 0.7 | 10.7 ± 2.7* | 194 ± 48 | 199 ± 51 |

[a] Values represent the mean ± the standard deviation ($n = 6-8$). The asterisks indicate statistical significance from control ($p < 0.05$).

Table 2: Individual Liver Phospholipid Kinetics under α-Synuclein Deficient Conditions[a]

| | $k_i^*$ ($\times 10^{-5}$ s$^{-1}$) | | $J_{FA,i}$ (nmol/h) | | $F_{FA,i}$ (%/h) | | $T_{1/2,i}$ (h) | | $C_{liv,i}$ (nmol/g wet weight) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | WT | KO | WT | KO | WT | KO | WT | KO | WT | KO |
| EtnGpl | 19.5 ± 10.8 | 18.4 ± 6.2 | 172 ± 52 | 287 ± 78 | 0.2 ± 0.1 | 0.2 ± 0.1 | 2.4 ± 1.2 | 3.0 ± 0.9 | 1075 ± 199 | 1154 ± 239 |
| PtdIns | 4.2 ± 1.5 | 4.0 ± 0.9 | 65 ± 35 | 58 ± 19 | 0.5 ± 0.3 | 0.4 ± 0.1 | 1.7 ± 0.7 | 2.0 ± 0.6 | 151 ± 34 | 152 ± 39 |
| PtdSer | 3.6 ± 0.2 | 3.2 ± 1.0 | 55 ± 19 | 43 ± 12 | 0.4 ± 0.2 | 0.4 ± 0.1 | 2.1 ± 0.9 | 2.0 ± 0.6 | 147 ± 26 | 120 ± 22 |
| ChoGpl | 129.9 ± 34.8 | 149.2 ± 31.3 | 2283 ± 968 | 2224 ± 445 | 0.4 ± 0.2 | 0.4 ± 0.1 | 1.8 ± 0.6 | 1.8 ± 0.5 | 5496 ± 280 | 5418 ± 964 |
| CerPCho | 20.4 ± 5.0 | 21.4 ± 4.5 | 339 ± 88 | 306 ± 66 | 1.0 ± 0.4 | 1.1 ± 0.2 | 0.8 ± 0.2 | 0.7 ± 0.1 | 334 ± 61 | 283 ± 36 |

[a] Values represent the mean ± the standard deviation ($n = 4-6$).

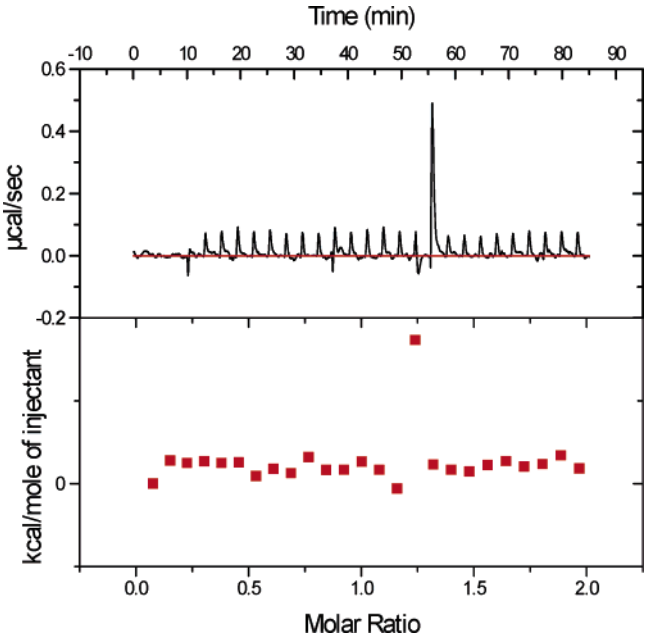


FIGURE 4: α-Synuclein does not bind palmitic acid. Titration microcalorimetry was carried out as described in Experimental Procedures. Experiments were repeated in triplicate, and no binding was demonstrated.

protein. A number of studies have demonstrated the successful use of titration microcalorimetry to determine constants for binding of fatty acids such as oleate to mammalian FABP (*50*, *60*) and to cellular retinoic acid binding proteins II (*59*). Thus, we conclude that α-synuclein does not bind 16:0 (Figure 4) or 18:1 n-9, indicating that the observed reduction in the rate of uptake could not be ascribed to a reduction in binding capacity, but rather suggests that the reduction in the rate of uptake in the absence of α-synuclein is the result of slowed 16:0 metabolism in the brain.

This is supported by our observation that α-synuclein deficiency significantly decreased the rate of brain 16:0 uptake in vivo, which was concurrent with decreased 16:0 mass in the brain free fatty acid fraction and in some brain phospholipid fractions. Although the magnitude of this decreased rate of uptake is consistent with FABP-facilitated fatty acid uptake observed in cultured cells (*29*−*31*, *68*−*70*) and in vivo (*32*), the lack of demonstrated binding of 16:0 by α-synuclein precludes the conclusion that α-synuclein functions as a FABP in vivo. Unlike FABP (*35*), α-synuclein is abundant in the mammalian brain and distributed in multiple cell types. It is localized in presynaptic terminals of neurons (*1*, *4*, *5*), in oligodendroglial cell bodies and primary processes (*6*), and in astrocytes (*6*). Thus, unlike FABP, which have specific cellular distributions, α-synuclein may have a more widespread impact on brain lipid metabolism because of its ubiquitous distribution in brain.

α-Synuclein deficiency depressed fatty acid targeting to the brain organic fraction, but did not alter targeting to the aqueous fraction (Figure 2), indicating that α-synuclein influenced targeting of 16:0 to the lipid compartment of the brain. Despite the decrease in the total $k^*$ into the organic fraction, no significant reduction in the incorporation coefficients was observed in individual phospholipids. Under these circumstances, it is difficult to estimate an effect of α-synuclein on targeting of 16:0 to individual lipid compartments in the brain.

The incorporation of 16:0 into the 16:0-CoA pool was inhibited by α-synuclein deficiency, further supporting the supposition that α-synuclein influences brain 16:0 metabolism in the absence of demonstrated 16:0 binding in vitro. The incorporation of 16:0 into the acyl-CoA pool may be altered by acyl-CoA synthase activity in the α-synuclein deficient mice. Although the brain 16:0-CoA concentration was not altered in α-synuclein deficiency (7.34 ± 0.93 nmol/g wet weight for WT mice and 6.38 ± 1.31 nmol/g wet weight for KO mice), there was a marked reduction in the rate of incorporation of 16:0 into the CoA pool, supporting this hypothesis. Whatever the underlying cause, the results indicate that α-synuclein affected the incorporation of 16:0 into the CoA pool, likely accounting for the observed decrease in the rate of 16:0 incorporation from the CoA pool into individual PLs.

Because α-synuclein did not affect the incorporation of the tracer into the aqueous phase, it is likely that α-synuclein affected specific 16:0-CoA pools. Acyl-CoA pools are spatially arranged in organelles, with pools ranging from the

mitochondrial pool to those in the endoplasmic reticulum. The lack of differences in the amount of tracer subjected to $\beta$-oxidation suggests a weaker effect on the mitochondrial pool than on the endoplasmic reticulum pool. Collectively, these results indicate that α-synuclein deficiency reduced the rate of 16:0 uptake and altered targeting into brain lipid compartments, similar to the effect of FABP in cell cultures (*29*, *30*, *69*, *71*, *72*) and in tissues (*32*), but unlike FABP, it did not affect the targeting of 16:0 for $\beta$-oxidation (*32*). Further, these effects are in the absence of demonstrated binding of 16:0 by α-synuclein, suggesting that these effects are the result of altered brain 16:0 metabolism in the absence of α-synuclein.

A number of kinetic properties of 16:0 metabolism were affected by α-synuclein gene ablation. Because $\lambda$ was not changed by α-synuclein gene ablation, the rate of 16:0 recycling between the total brain PL pool and the acyl-CoA was not altered (Table 1). However, the net rate of incorporation ($J_{FA,i}$) of 16:0-CoA into PtdIns, PtdSer, and CerPCho was decreased by α-synuclein gene ablation, while the rate of incorporation into ChoGpl was increased. As a result, the fractional turnover ($F_{FA,i}$) of 16:0 in PtdSer and CerPCho was decreased and that in ChoGpl increased in α-synuclein deficiency. This is indicative of α-synuclein facilitating brain phospholipid fatty acid turnover. FABP also bind acyl-CoA (*73*, *74*) and stimulate (~10−20-fold) an increase in the rate of PtdOH biosynthesis in vitro (*43*, *75*, *76*), by putatively delivering acyl-CoA to endoplasmic reticulum-localized lipid biosynthetic enzymes. Our results suggest that α-synuclein may have a similar role in incorporation of 16:0-CoA into PL pools by influencing endoplasmic reticulum-localized lipid biosynthetic enzymes.

However, our results also revealed that α-synuclein may limit ChoGpl fatty acid turnover in vivo. This finding is important because α-synuclein inhibits PLD, which is selective for ChoGpl (*19*, *20*) and because inhibition of PLD activity appears to be required for synaptic vesicle fusion with the target membrane (*19*, *20*, *77*). Further, PLD activity and the subsequent production of phosphatidic acid may regulate synaptic vesicle recycling (*20*, *77*), indicating the importance of PLD activity in synaptic vesicle release and uptake. This is consistent with the 50% reduction in the size of the undocked synaptic vesicle pool and the synaptic vesicle depletion after high-frequency stimulation observed in these α-synuclein gene-ablated mice (*24*). Our observed limitation of ChoGpl metabolism is consistent with the observed regulation of PLD activity by α-synuclein (*20*).

Although α-synuclein deficiency decreased the rate of fatty acid uptake in a manner consistent with a FABP, there are a number of key points in which our study suggests that α-synuclein does not function as an FABP but rather has an effect on brain lipid metabolism independent of functioning as an FABP. First, this hypothesis is consistent with the overexpression of α-synuclein in *Drosophila*, where the expression of a number of genes associated with lipid metabolism was downregulated (*78*), suggesting an effect on lipid metabolism by this protein. Second, we could not demonstrate binding of either 16:0 or 18:1 n-9 to α-synuclein, inconsistent with a FABP as FABP are known to bind fatty acids with a $K_d$ in the 5−600 nM range, dependent upon the fatty acid (*79*, *80*). Third, we have not observed any robust changes in total phospholipid mass or individual phospholipid mass (data not shown), while H-FABP expression increases brain total phospholipid mass and increases the mass of choline-containing phospholipids (*36*). Fourth, FABP expression concurrently affects fatty acid uptake and its mass in tissue phospholipids (*32*, *36*), and this effect was not seen herein or in α-synuclein deficient astrocytes (*27*). Thus, while there is evidence suggesting that α-synuclein has an impact on brain lipid metabolism, this effect is not consistent with that observed for FABP, with respect to metabolism or fatty acid binding.

In summary, α-synuclein affected brain lipid metabolism in the absence of 16:0 binding in vitro, while liver 16:0 metabolism was unaffected. This demonstrates the brain specificity of the α-synuclein deficiency on lipid metabolism. In the absence of α-synuclein, the rate of 16:0 uptake was depressed and incorporation into the 16:0-CoA pool was slowed, subsequently reducing the rates of incorporation into PtdIns, PtdSer, and CerPCho. Because there were no alterations in targeting of 16:0 for $\beta$-oxidation, we postulate that α-synuclein deficiency affected a specific metabolic 16:0-CoA pool that is destined for use in phospholipids. More importantly, α-synuclein deficiency increased the rate of 16:0 turnover in ChoGpl, possibly due to the lack of α-synuclein inhibition of PLD in these mice. These results are important because they are the first demonstration that α-synuclein impacts brain lipid metabolism in the intact animal in a manner that is consistent with an effect on ER-based lipid synthetic enzymes rather than an influence on fatty acid uptake via the direct binding of 16:0 by α-synuclein.

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