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FABP3 Protein Promotes α-Synuclein Oligomerization Associated with 1-Methyl-1,2,3,6-tetrahydropiridine-induced Neurotoxicity*

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Background: αSyn toxicity is triggered by oligomerization of αSyn, and its formation is partly regulated by PUFAs.
Results: MPTP-induced neurotoxicity and αSyn oligomerization are attenuated in *Fabp3*$^{-/-}$ mice.
Conclusion: FABP3 is implicated in arachidonic acid-induced αSyn oligomerization and promotes dopaminergic cell death.
Significance: FABP3 aggravates MPTP-induced neuronal toxicity and αSyn accumulation.

α-Synuclein (αSyn) accumulation in dopaminergic (DA) neurons is partly regulated by long-chain polyunsaturated fatty acids. We found that fatty acid-binding protein 3 (FABP3, H-FABP), a factor critical for arachidonic acid (AA) transport and metabolism in brain, is highly expressed in DA neurons. *Fabp3* knock-out (*Fabp3*$^{-/-}$) mice were resistant to 1-methyl-1,2,3,6-tetrahydropiridine-induced DA neurodegeneration in the substantia nigra pars compacta and showed improved motor function. Interestingly, FABP3 interacted with αSyn in the substantia nigra pars compacta, and αSyn accumulation following 1-methyl-1,2,3,6-tetrahydropiridine treatment was attenuated in *Fabp3*$^{-/-}$ compared with wild-type mice. We confirmed that FABP3 overexpression aggravates AA-induced αSyn oligomerization and promotes cell death in PC12 cells, whereas overexpression of a mutant form of FABP3 lacking fatty-acid binding capacity did not. Taken together, αSyn oligomerization in DA neurons is likely aggravated by AA through FABP3 in Parkinson disease pathology.

Parkinson disease (PD)[2] is a common motor disorder affecting >1% of the population over 65 years of age worldwide (1). Histopathologic features of PD are the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the presence of cytoplasmic protein aggregates, known as Lewy bodies (LBs) (2). α-Synuclein (αSyn), a 140-amino acid protein, is associated with synaptic vesicles in presynaptic nerve terminals (3), and β-sheet fibrillar aggregates, including αSyn, are major components of LBs. αSyn accumulation is associated with progressive loss of DA neurons, implicating that activity in PD pathogenesis (4). In addition, duplication/triplication (5–7) and missense mutations (A53T, A30P, E46K, H50Q, and G51D) (8–12) in the αSyn gene *SNCA* are linked to familial early onset PD, suggesting that the mutations accelerate αSyn aggregation and disease progression.

αSyn toxicity is triggered by oligomerization of αSyn *in vitro* (13) and *in vivo* (14), indicating that oligomerization underlies cytotoxic events in PD. However, mechanisms underlying αSyn oligomerization in DA neurons are unclear. Previous reports suggested that αSyn binds fatty acids, particularly long-chain polyunsaturated fatty acids (PUFAs) (15, 16), and that αSyn oligomerization and the appearance of LB-like inclusions in cultured mesencephalic neuronal cells are enhanced by exposure to PUFAs (17–19). In addition, abnormally high PUFA levels are observed in αSyn-transfected mesencephalic neuronal cells and in PD brains, whereas lower levels are seen in mice lacking αSyn (17, 18), suggesting that PUFA binding to αSyn is a key event in generating pathogenic αSyn oligomers.

Because PUFAs are insoluble in an aqueous cellular environment, fatty acid-binding proteins (FABPs) acting as cellular shuttles are essential to transport them to appropriate intracellular compartments (20). Among the FABPs, FABP3, which is expressed in neurons (21), shows a preference for binding to *n*-6 fatty acids (22). Indeed, *Fabp3* knock-out (*Fabp3*$^{-/-}$) mice exhibit a 24% reduction in incorporation of arachidonic acid (AA) into brain membranes and reduced levels of total *n*-6 fatty acids in major phospholipid classes in membranes (23), suggesting that FABP3 is critical for neuronal AA uptake and metabolism. We report herein that FABP3 is highly expressed in DA neurons and accelerates αSyn oligomerization, thereby aggravating AA-induced αSyn oligomerization and its toxicity.

EXPERIMENTAL PROCEDURES

Animals—Generation of *Fabp3*$^{-/-}$ mice was described previously (24). Adult 12-week-old mice were used in all experiments. Mice were housed under climate-controlled conditions with a 12-h light/dark cycle and provided standard food and

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[2] The abbreviations used are: PD, Parkinson disease; AA, arachidonic acid; αSyn, α-synuclein; DA, dopaminergic; DSP, dithiobis(succinimidylpropionate); FABP, fatty acid-binding protein; *Fabp3*$^{-/-}$, *Fabp3* knockout; LB, Lewy body; MPP$^+$, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-1,2,3,6-tetrahydropiridine; PUFA, polyunsaturated fatty acids; PGE_2, prostaglandin E2; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

water *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee at Tohoku University.

MPTP-treated PD Model—Mice were treated once a day for 5 days with 1-methyl-1,2,3,6-tetrahydropiridine (MPTP, Sigma; 25 mg/kg, intraperitoneally) and then subjected to behavioral (at 1–4 weeks), immunohistochemical (at 4 weeks), and biochemical (at 4 weeks) analyses.

Behavioral Tests—In training sessions for the rotarod task, mice were placed on a drum (ENV-576M; Med Associates, St. Albans, VT) rotating at 20 rpm until the latency to fall from the drum exceeded 200 s. For test sessions, mice were placed on the rotating rod and latency to fall was recorded for up to 5 min. The beam-walking task was performed as described previously (25).

Immunohistochemistry and Cell Counting—Immunohistochemistry was performed as described previously (26). Primary antibodies included the following: mouse monoclonal anti-FABP3 (1:50, Hycult Biotechnology, Uden, Netherlands), anti-ubiquitin (1:1000, Millipore, Bedford, MA), and anti-tyrosine hydroxylase (TH) (1:1000, Immunostar, Hudson, WI); rabbit polyclonal anti-FABP3 (1:500, ProteinTech, Chicago), anti-αSyn (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-TH (1:1000, Millipore). Visualization of TH immunoreactivity following diaminobenzidine (DAB) staining was performed using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). For immunofluorescence, sections were incubated with secondary antibodies, including Alexa 594 anti-mouse IgG and Alexa 448 anti-rabbit IgG (1:500, Invitrogen). FABP3 immunoreactivity was visualized using a TSA-Direct kit (PerkinElmer Life Sciences). Immunofluorescent images were analyzed using a confocal laser scanning microscope (LSM700, Zeiss, Thornwood, NY). TH- or αSyn-positive cells were counted in substantia nigra pars compacta (SNpc) on both sides of the substantia nigra region (eight sections per mouse, five to six mice per condition).

Immunoprecipitation and Immunoblotting Analysis—Immunoprecipitation and immunoblotting analysis was performed as described previously (26). Striatal tissues or substantia nigra tissues were homogenized in buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 4 mM EDTA, 4 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM DTT, and protease inhibitors (trypsin inhibitor, pepstatin A, and leupeptin) and treated with SDS buffer with (denatured samples) or without (nondenatured samples) boiling. Antibodies used included the following: rabbit polyclonal anti-FABP3 (1:500, ProteinTech), anti-αSyn (1:100, Santa Cruz Biotechnology) and anti-TH (1:1000, Millipore); mouse monoclonal anti-β-tubulin (1:5000, Sigma).

Plasmid Constructs—Human αSyn plasmid was purchased from Abgent (San Diego). FABP3 plasmid was prepared as described previously (26). Mutant FABP3(F16S) lacking fatty-acid binding capacity (27) was generated using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Cell Culture and Viability Assay—PC12 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS), and penicillin/streptomycin (100 units/100 μg/ml) at 37 °C under 5% CO_2. Cells were transfected using Lipofectamine 2000 (Invitrogen) as described previously (26). Conditioning living PC12 cells with AA was carried out as described previously (18). Briefly, at 32 h post-transfection in serum-free DMEM, fatty acid-free bovine serum albumin (BSA, Sigma)-AA (Sigma) complexes were added to the medium. These complexes were prepared by mixing BSA with AA (at a 1:5 molar ratio) in binding buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl at 37 °C for 30 min. After treatment of cells with AA for 16 h, a final concentration of 500 μM 1-methyl-4-phenylpyridinium (MPP^+, Sigma) was added for an additional 24 h. Survival experiments were performed as described previously (28). The appearance of condensed nuclear staining with DAPI (Vector Laboratories) served as an indicator of cell death. Triplicate cultures were used for each condition, and each experiment was performed at least three times.

In Vitro αSyn Oligimerization—Recombinant human αSyn (Enzo Life Sciences, Farmingdale, NY) was incubated with AA and recombinant human His-tagged FABP3 (Cayman Chemical, Ann Arbor, MI) at the indicated concentrations in binding buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl at 37 °C for 30 min. For detection by immunoblotting, samples were mixed with SDS buffer without boiling.

Chemical Cross-linking Reactions—We performed chemical cross-linking reactions to identify αSyn oligomerization (29). For *in vitro* cross-linking of recombinant proteins, dithiobis-(succinimidylpropionate) (DSP) (Pierce) was added to the incubation mixture with a final concentration of 30 μM, and the recombinant proteins were incubated at 37 °C for 30 min in PBS containing protease inhibitors. The cross-linking reactions were terminated by incubation with Tris-HCl (pH 7.5) at 50 mM final concentration for 15 min at room temperature. Samples were mixed with SDS buffer without boiling. For *in situ* cross-linking in PC12 cells, transfected cells in 60-mm dishes were washed with PBS and incubated with DSP (1 mM) at 37 °C for 30 min. The cross-linking reactions were terminated in the dishes by incubation with Tris-HCl (pH 7.5) at 50 mM final concentration for 15 min at room temperature. After chemical cross-linking, cells were collected by scraping and homogenized in buffer containing PBS with 1% Triton X-100 and protease inhibitors. Samples were mixed with SDS buffer without boiling. We also detected αSyn oligomerization in mouse brain samples using the native method without chemical cross-linking.

Statistical Evaluation—All values were expressed as means ± S.E. Comparison between two experimental groups was made using the unpaired Student's *t* test for immunoblot and immunohistochemical analyses. Behavioral tests were analyzed using two-way analysis of variance, followed by one-way analysis of variance for each group and Dunnett's tests. $p < 0.05$ was considered significant.

RESULTS

$Fabp3^{-/-}$ Mice Are Resistant to MPTP-induced DA Neurodegeneration in the SNpc—15-kDa cytoplasmic FABPs occur as 13 different isoforms that are widely distributed in various tissues. Among FABPs, FABP3, FABP5, and FABP7 are expressed in brain (30). FABP5 is predominantly expressed in immature neurons and glial cells and FABP7 is in glial cells, whereas

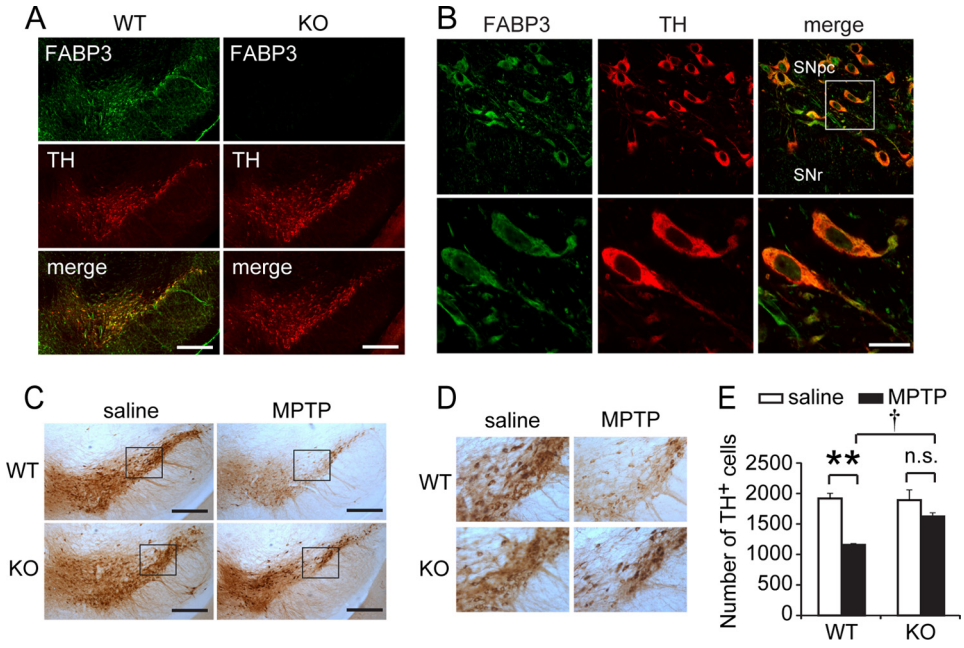


FIGURE 1. **Genetic ablation of *Fabp3* rescues DA neurons in MPTP-treated PD model.** *A,* confocal images showing FABP3 (*green*) and TH (*red*) colocalization in the substantia nigra. *B,* high magnification images of substantia nigra of wild-type mice. *Bottom,* enlarged images of *boxed area* in top merge. *C* and *D,* representative photomicrographs showing TH immunoreactivity in the substantia nigra. Enlarged images in *D* correspond to respective *boxed areas*. *E,* quantitative analysis of the number of TH-positive neurons in the SNpc. **, $p < 0.01$ in saline-treated WT *versus* MPTP-treated WT. †, $p < 0.05$ in MPTP-treated WT *versus* MPTP-treated KO. *n.s.*, not significant; *WT*, wild-type mice; *KO*, *Fabp3*$^{-/-}$ mice. *Scale bars, A* and *C*, 250 μm, and *B*, 20 μm.

FABP3 is highly expressed in mature neurons (21). In the substantia nigra, strong FABP3 immunoreactivity was observed in the SNpc but not in the substantia nigra reticular. That immunoreactivity was totally abolished in *Fabp3*$^{-/-}$ mice (Fig. 1*A*) (26). In addition, analysis of TH immunoreactivity indicated that most FABP3-positive neurons were dopaminergic (Fig. 1*B*).

To address the role of FABP3 in the pathogenesis of PD, mice were treated with MPTP (Fig. 1, *C–E*). We observed no significant difference in the number of TH-positive neurons between saline-treated wild-type (WT) and *Fabp3*$^{-/-}$ mice. In WT mice, 4 weeks after the last MPTP injection, the number of TH-positive neurons in SNpc was markedly reduced compared with numbers seen in saline-treated WT mice ($t = 5.39$, $p < 0.01$, $n = 6$ each). By contrast, the number of TH-positive neurons was unchanged by MPTP treatment in *Fabp3*$^{-/-}$ mice ($t = 0.84$, $p = 0.231$, $n = 6$ each). The number of TH-positive neurons was significantly rescued in MPTP-treated *Fabp3*$^{-/-}$ mice compared with MPTP-treated WT mice ($t = 4.38$, $p = 0.011$). These results suggest that endogenous FABP3 aggravates DA neurotoxin-induced cell death.

MPTP-induced Neurotoxicity Is Attenuated in the Striatum of Fabp3$^{-/-}$ *Mouse Brain*—Next we confirmed that DA terminals in the striatum are less damaged by MPTP in an *Fabp3*$^{-/-}$ background. TH immunoreactivity in the striatum was reduced in WT mice following MPTP treatment but was unchanged in MPTP-treated *Fabp3*$^{-/-}$ mice (Fig. 2*A*). To quantify TH immunoreactivity in striatal regions, we performed immunoblotting analysis and found that TH protein levels in the striatum of MPTP-treated animals were significantly higher in *Fabp3*$^{-/-}$ (66.6 ± 7.4%) compared with WT (46.3 ± 5.0%) mice (Fig. 2*B*) ($t = 2.27$, $p = 0.018$, $n = 8–12$).

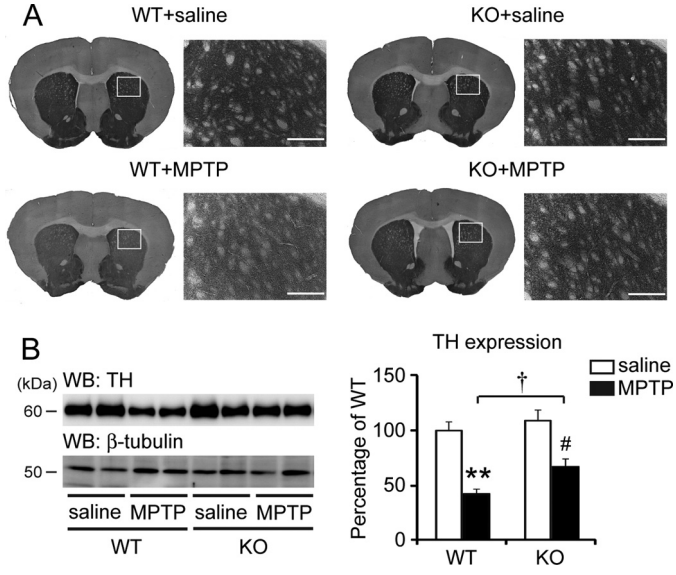


FIGURE 2. **MPTP-induced neurotoxicity is attenuated in the striatum of *Fabp3*$^{-/-}$ mouse brain.** *A,* representative photomicrographs showing TH immunoreactivity in the striatum. *B,* shown are representative immunoblots of striatal total lysates probed with various antibodies (*left*) and quantitative densitometry analysis (*right*). **, $p < 0.01$ in saline-treated WT *versus* MPTP-treated WT; #, $p < 0.05$ in saline-treated KO *versus* MPTP-treated KO; †, $p < 0.05$ in MPTP-treated WT *versus* MPTP-treated KO. *WT,* wild-type mice; *KO*, *Fabp3*$^{-/-}$ mice; *WB*, Western blot. *Scale bar,* 300 μm.

FABP3 Deficiency Attenuates Motor Deficits Induced by MPTP—We next confirmed that MPTP-induced motor deficits were attenuated in *Fabp3*$^{-/-}$ mice by assessing animals 1–4 weeks after saline or MPTP treatment using beam-walking (Fig. 3*A*) and rotarod (Fig. 3*B*) tasks. Saline-treated WT and *Fabp3*$^{-/-}$ mice showed no significant differences in performance on either motor coordination task. However, MPTP-

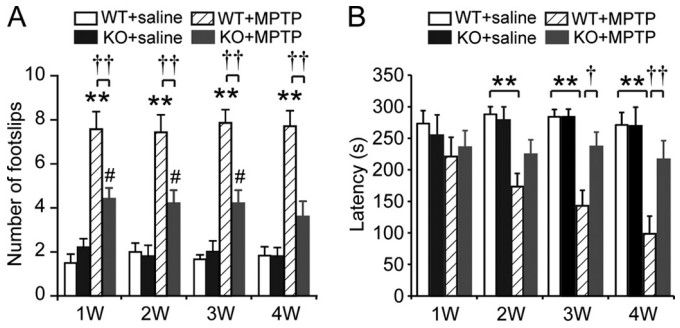


FIGURE 3. **Genetic ablation of *Fabp3* attenuates motor deficits induced by MPTP.** Quantitative analyses of motor coordination using the beam-walking (*A*) and rotarod (*B*) tasks. *A*, "footslips" are defined as errors in a beam-walking task. **, $p < 0.01$ in saline-treated WT *versus* MPTP-treated WT; #, $p < 0.05$ in saline-treated KO *versus* MPTP-treated KO; †, $p < 0.05$; ††, $p < 0.01$ in MPTP-treated WT *versus* MPTP-treated KO. *WT,* wild-type mice; *KO*, *Fabp3*$^{-/-}$ mice.

treated WT mice showed profoundly impaired motor performance on both beam-walking and rotarod tasks, although MPTP-treated *Fabp3*$^{-/-}$ mice showed a much improved performance relative to WT mice, especially in the rotarod test (beam-walking ($F_{(3,71)} = 13.5$, $p < 0.01$) and rotarod ($F_{(3,87)} = 18.2$, $p < 0.001$)).

FABP3 Deficiency Attenuates MPTP-induced αSyn Accumulation in the SNpc—We next asked whether the resistance to MPTP-induced DA neurodegeneration in *Fabp3*$^{-/-}$ mice is associated with reduced αSyn oligomerization. Immunoblot analysis using an αSyn-specific antibody showed that although the levels of αSyn 15-kDa monomer and oligomers were unchanged in both denatured and nondenatured extracts of substantia nigra from saline-treated WT and *Fabp3*$^{-/-}$ mice, significantly higher levels of αSyn oligomers were seen in nondenatured substantia nigra extracts in WT compared with *Fabp3*$^{-/-}$ mice following MPTP treatment ($p < 0.01$, $n = 5$) (Fig. 4*A*). In denatured samples, levels of αSyn monomer and FABP3 were significantly up-regulated in MPTP-treated WT mice but not in *Fabp3*$^{-/-}$ mice (Fig. 4*B*) (αSyn ($t = 4.1$, $p < 0.01$) and FABP3 ($t = 2.44$, $p < 0.05$)). In confocal microscopic analysis, consistent with our result in Fig. 1, the TH immunoreactivity in the substantia nigra was markedly reduced in MPTP-treated WT mice. In addition, immunolabeling with αSyn antibody showed significant αSyn accumulation in DA cell bodies in the substantia nigra of MPTP-treated WT mice. In contrast, only mild αSyn immunoreactivity was detected in DA cell bodies of MPTP-treated *Fabp3*$^{-/-}$ mice (saline-treated WT, 34 cells; MPTP-treated WT, 248 cells; saline-treated *Fabp3*$^{-/-}$, 42 cells; MPTP-treated *Fabp3*$^{-/-}$, 89 cells; $n = 5$ each) (Fig. 4*C*).

FABP3 Makes Complexes with αSyn Oligomers—In previous reports, purified recombinant human αSyn could bind radiolabeled oleic acid (^{14}C-18:1) and decosahexaenoic acid (22:6) *in vitro*, and these fatty acids promote αSyn oligomerization (16, 18). We now asked whether the direct binding of AA to αSyn promotes its oligomerization. After 30 min of incubation without AA, the αSyn was only detected as monomeric form (15 kDa). In contrast, incubation with AA clearly promoted the oligomerization of recombinant αSyn (60–100 kDa). Importantly, the αSyn oligomerization was enhanced by adding recombinant human FABP3 (50 μM AA $p = 0.0016$ and 100 μM AA $p = 0.024$, in FABP3 absence *versus* presence, $n = 3$ each) (Fig. 5*A*). To confirm the interaction between αSyn and FABP3 *in vitro*, we performed chemical cross-linking, a well established biochemical method to identify αSyn oligomerization (29). Consistent with the previous report, we detected cross-linker-induced αSyn oligomers with 60 and 90 kDa. The 60-kDa formation was FABP3 concentration-dependent manner (Fig. 5*B*, *left*, *arrow*). In addition, we found a few minor immunoreactive bands with 70 and 95 kDa, which correspond to FABP3 immunoreactive bands with 70 and 95 kDa (Fig. 5*B*, *right*, *arrowhead*). Using FABP3 antibody, we also observed a few minor αSyn-FABP3 complexes with 60 and 90 kDa, which likely contain αSyn oligomers (Fig. 5*B*, *left and right*, *arrow*). To further confirm an interaction between αSyn and FABP3 *in vivo*, we performed immunoprecipitation of αSyn from substantia nigra extracts using αSyn antibody. The immunoprecipitates were then immunoblotted with FABP3 antibody. αSyn-FABP3 oligomeric complexes with 65 and 90 kDa were observed in MPTP-treated WT mice but not in *Fabp3*$^{-/-}$ mice (Fig. 5*C*). In addition, most FABP3 immunoreactivity colocalized with αSyn accumulation in DA cell bodies of MPTP-treated WT mice (Fig. 5*D*, *arrow*), suggesting that FABP3 makes complexes with αSyn oligomers and promotes αSyn oligomerization.

FABP3 Accelerates AA-induced αSyn Oligomerization and Cell Death—Next, we addressed whether FABP3 overexpression accelerates αSyn oligomerization. We investigated MPP^+-induced αSyn oligomerization in αSyn-transfected PC12 cells, with or without FABP3 overexpression. MPP^+ treatment clearly increased the oligomer-to-monomer ratio in αSyn- and FABP3-cotransfected cells compared with cells expressing αSyn only (Fig. 6*A*) (213.8 ± 1.8% of αSyn only cells without MPP^+, $p < 0.01$, $n = 3$ each). In addition, FABP3 immunoreactivity colocalized with αSyn inclusions in MPP^+-treated PC12 cells (Fig. 6*B*). MPP^+-induced αSyn/FABP3 aggregates colocalized with ubiquitin, a common marker in α-synucleinopathy (Fig. 6, *C* and *D*).

Finally, we addressed whether AA promotes FABP3-induced αSyn aggregation. Interestingly, 100 μM AA treatment enhanced levels of αSyn oligomerization in FABP3-transfected cells (202.6 ± 10.2% of αSyn- and FABP3-cotransfected cells with MPP^+, $p < 0.01$, $n = 3$ each), and oligomerization was markedly attenuated in cells transfected with the FABP3(F16S) construct, a mutant lacking fatty acid binding capacity (Fig. 7*A*) (27). These results indicate that AA-bound FABP3 increases αSyn oligomerization. More importantly, exposure of FABP3-overexpressing cells to AA significantly promoted cell death in response to MPP^+ compared with cells expressing αSyn alone, and FABP3(F16S) overexpression significantly rescued cells from AA-potentiated FABP3-induced cell death (Fig. 7*B*) (mock, 25.0 ± 4.7%; FABP, 49.7 ± 5.9%; FABP3(F16S), 22.7 ± 6.1%; FABP3+AA, 41.7 ± 4.4%; FABP3(F16S), 84.0 ± 7.4%; FABP3(F16S) + AA, 54.3 ± 6.1% of total cells, $n = 3$ each).

DISCUSSION

In this study, we report that FABP3 is implicated in the MPTP-induced neuronal toxicity and αSyn accumulation. We first observed that *Fabp3*$^{-/-}$ mice were more resistant to neu-

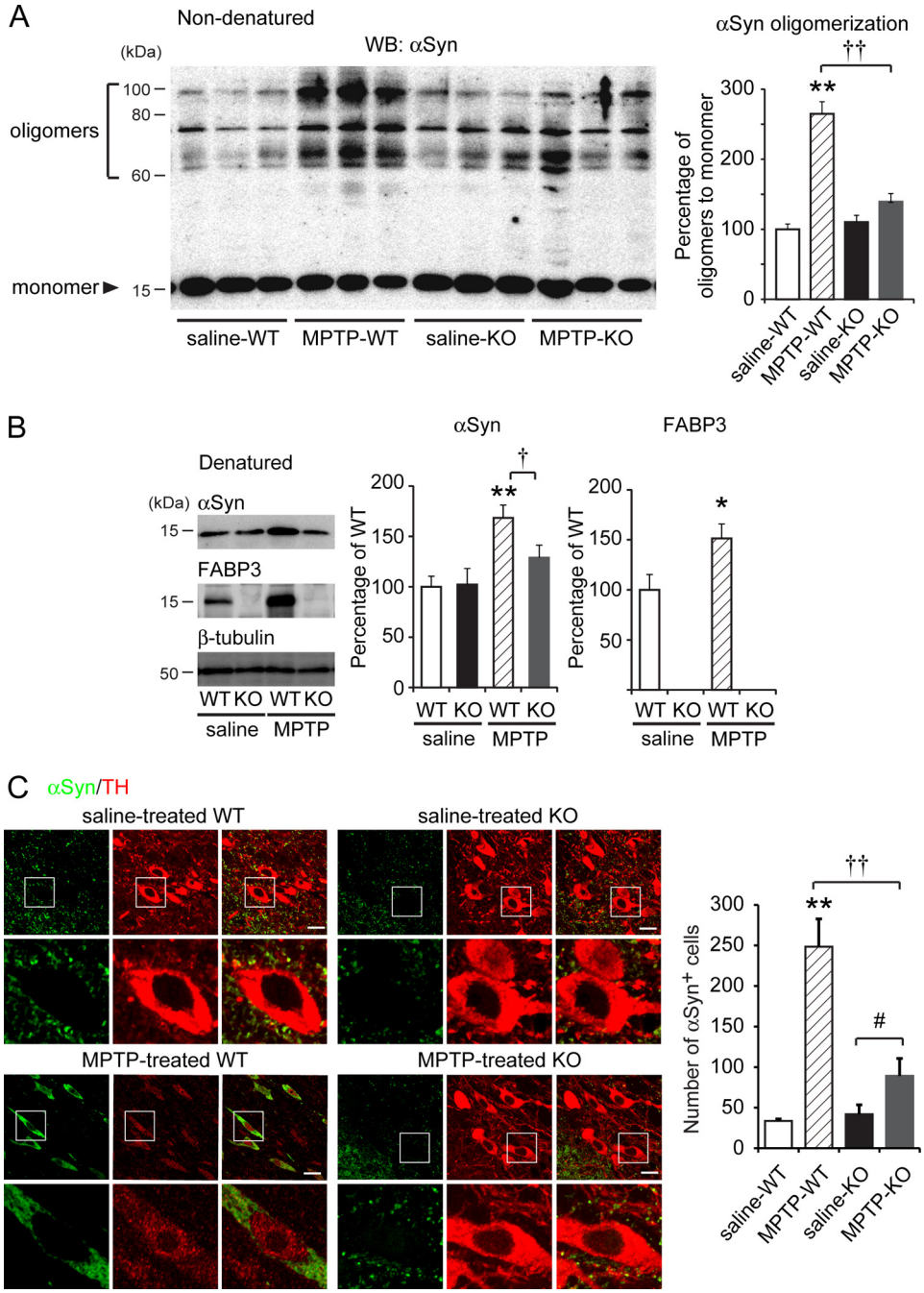
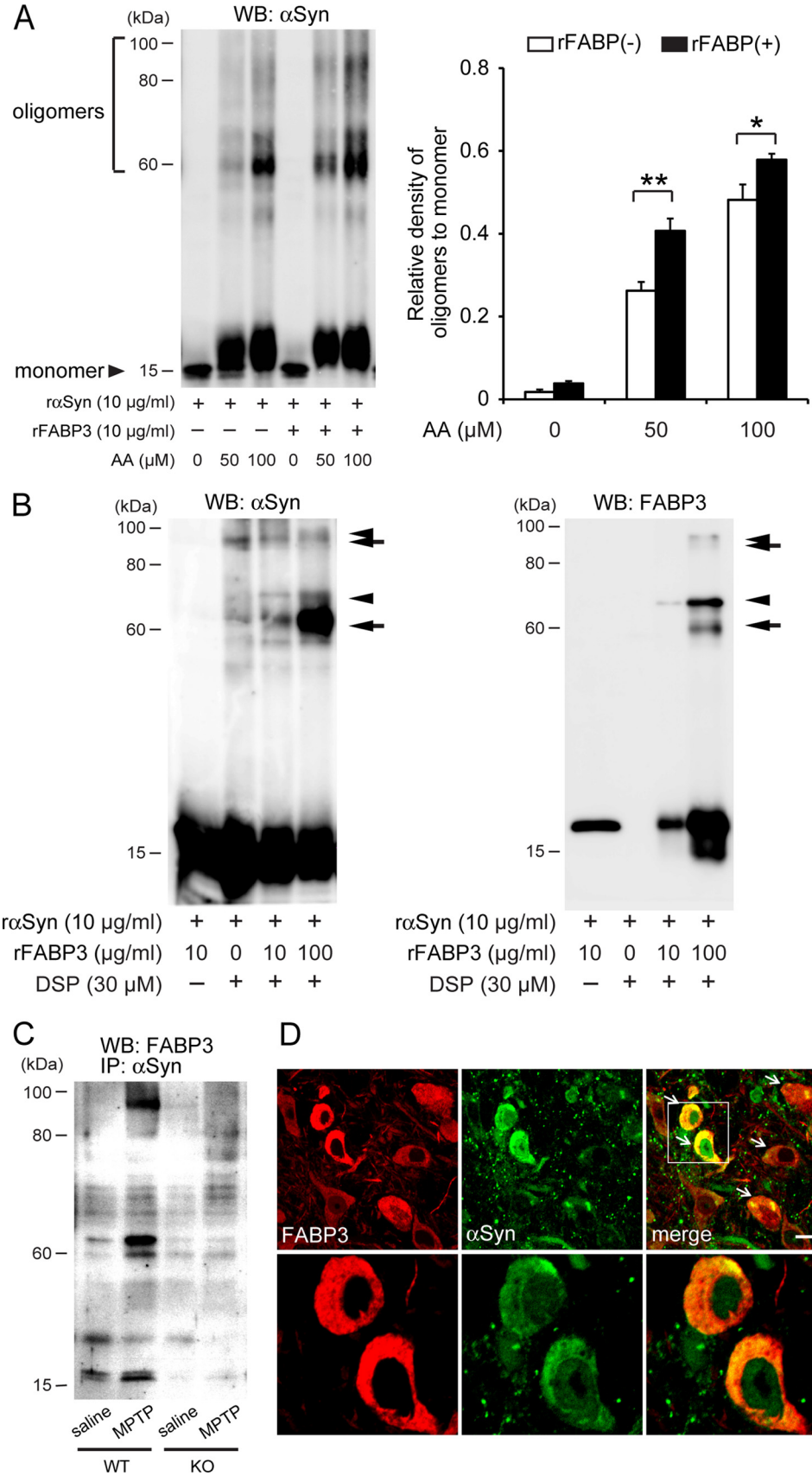


FIGURE 4. **FABP3 deficiency attenuates MPTP-induced αSyn oligomerization in the SNpc.** *A* and *B*, representative immunoblots (*left*) and quantitative densitometry analyses (*right*) of proteins in a lysate from substantia nigra probed with various antibodies. *, $p < 0.05$; **, $p < 0.01$ in saline-treated WT *versus* MPTP-treated WT; †, $p < 0.05$; ††, $p < 0.01$ in MPTP-treated WT *versus* MPTP-treated KO. *C*, *left*, confocal images showing localization of αSyn (*green*) and TH (*red*) in the substantia nigra. At the *bottom*, enlarged images correspond to *boxed areas*. *Right*, quantitative analysis of the number of αSyn-positive neurons in the SNpc. **, $p < 0.01$ in saline-treated WT *versus* MPTP-treated WT; #, $p < 0.05$ in saline-treated KO *versus* MPTP-treated KO; ††, $p < 0.01$ in MPTP-treated WT *versus* MPTP-treated KO. *Scale bar,* 20 μm. *WT,* wild-type mice; *KO, Fabp3*$^{-/-}$ mice; *WB*, Western blot.

rotoxin-induced DA neurodegeneration and motor deficits in the murine PD model. The ameliorating effects seen in *Fabp3*$^{-/-}$ mice were highly correlated with a reduction in αSyn oligomerization in the SNpc. We then confirmed enhanced αSyn oligomerization in response to up-regulated FABP3 expression and FABP3-mediated AA incorporation following neurotoxin exposure. Based on these observations, we suggest that FABP3 up-regulation by MPTP accelerates αSyn oligomerization and accumulation, leading to DA neurodegeneration.

Interestingly, others have proposed that αSyn could function as an FABP, as it exhibits an α-helical lipid-binding motif similar to class A2 lipid-binding domains seen in apolipoproteins and which accounts for binding to membrane phospholipids (16). However, titration microcalorimetry analysis indicates that αSyn binds monomeric AA and decosahexaenoic acid with only low affinity ($K_d = 1–4$ μM) (31), which is about 2 orders of magnitude less affinity than classical FABPs, including FABP3 (32). In addition, unlike the case with classical FABPs, NMR spectroscopy has not identified specific fatty acid-binding sites



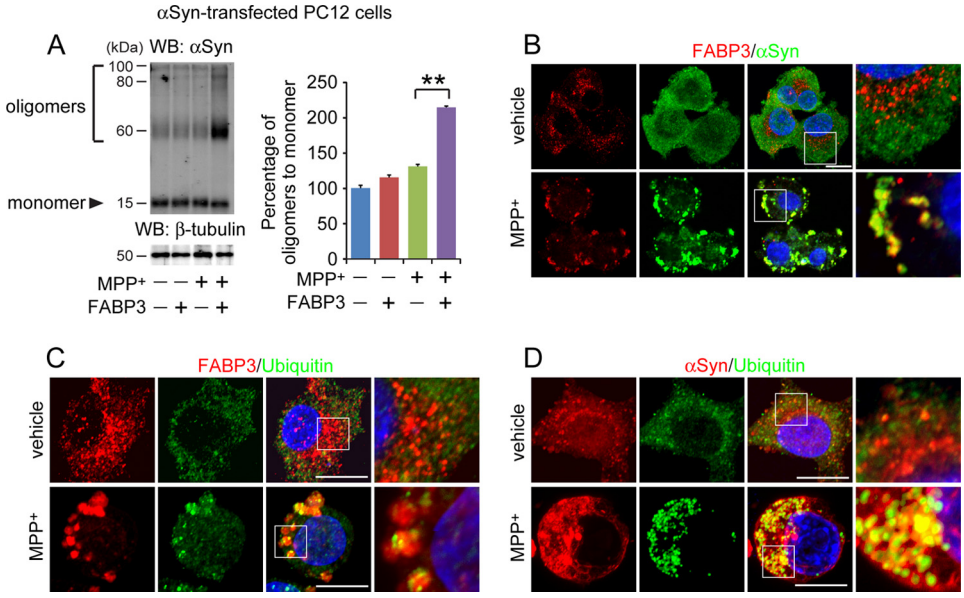


FIGURE 6. **FABP3 overexpression accelerates αSyn oligomerization in PC12 cells.** *A,* representative immunoblots (*left*) and quantitative densitometry analysis (*right*) of PC12 cell extracts probed with various antibodies. **, $p < 0.01$ in mock cells plus MPP^+ *versus* FABP3-transfected cells plus MPP^+. *WB*, Western blot. *B,* confocal images showing localization of αSyn (*green*) and FABP3 (*red*) in PC12 cells with or without MPP^+. At *right*, enlarged images correspond to *boxed areas*. *C,* confocal images showing localization of ubiquitin (*green*) and FABP3 (*red*) in PC12 cells with or without MPP^+. At *right*, enlarged images correspond to *boxed areas*. *D,* confocal images showing localization of ubiquitin (*green*) and αSyn (*red*) in PC12 cells with or without MPP^+. At *right*, enlarged images correspond to *boxed areas*. *Scale bars, B–D,* 20 μm.

or similarities in tertiary structure between αSyn and FABP (33). Thus, regulation of αSyn by PUFAs may require a specific lipid composition or the presence of neuron-specific lipid-binding partners. FABP3 would interact with αSyn and AA to promote αSyn oligomerization.

More importantly, FABP3 is highly expressed in DA neurons in SNpc and plays critical roles in DA neurotoxicity *in vivo*. Because *Fabp3*$^{-/-}$ mice exhibit markedly reduced incorporation of AA into brain tissue plasma membranes (23), we hypothesized that DA neuroprotection in *Fabp3*$^{-/-}$ mice is elicited by AA-dependent production of prostaglandin E2 (PGE_2), which others have shown to be responsible for cyclooxygenase-2 (COX-2)-mediated neurotoxicity in neuroinflammatory events (34). To investigate potential roles for PGE_2 production in *Fabp3*$^{-/-}$ mice, we determined levels of released PGE_2 in mesencephalic cultures treated with MPP^+. Unexpectedly, we observed no significant difference in PGE_2 production between WT and *Fabp3*$^{-/-}$ mesencephalic cells.[3] This observation indicates that neurotoxin-induced PGE_2 production does not account for inhibition of DA neuronal death seen in *Fabp3*$^{-/-}$ mice.

MPP^+, a toxic metabolite of MPTP, is an inhibitor of complex I in the mitochondrial electron transport chain and a substrate for the dopamine transporter, therefore accumulating in DA neurons and eliciting neurodegeneration (35). Interestingly, the N-terminal 32 amino acids of human αSyn contain a cryptic mitochondrial targeting signal (36), and αSyn is accumulated in the mitochondria of post-mortem PD brains (36). FABP3 overexpression causes mitochondrial dysfunction and induces apoptosis in the P19 mouse teratocarcinoma cell line (37); FABP3 may also induce a mitochondrial dysfunction and is implicated in oxidative stress induced by MPTP toxicity.

In our study, we found the significant reduction of the number of αSyn-accumulated cells in DA cell bodies of MPTP-treated *Fabp3*$^{-/-}$ mice compared with MPTP-treated WT mice (Fig. 4*C*). However, MPTP-treated mouse models do not induce αSyn-containing inclusions, similar to LBs (2). Further study will be required to investigate some differences in formation of αSyn-containing inclusions between the MPTP treatment model and the PD model by rotenone treatment (38) or ubiquitin-proteasome inhibitor treatment (39).

Increased AA intake is reportedly correlated with PD risk (40), and higher levels of AA and total *n*-6 PUFAs have been observed in post-mortem PD brains than healthy controls (41). Proteomic analysis of human substantia nigra indicated higher levels of FABP3 protein in PD patients than in control subjects (42). Higher FABP3 levels have been reported in the sera of patients with dementia accompanied by LBs (43) and of PD patients (44) compared with Alzheimer disease patients and nondemented controls. Although further studies are war-

[3] N. Shioda and K. Fukunaga, manuscript in preparation.

FIGURE 5. **FABP3 makes complexes with αSyn oligomers.** *A, left*, recombinant human αSyn (*rαSyn*) was incubated with or without AA at the indicated concentrations at 37 °C for 30 min. Samples were immunoblotted with anti-αSyn antibody. Of note, αSyn oligomerization was enhanced by adding recombinant human FABP3 (*rFABP3*). *Right*, quantitative densitometry analysis. *, $p < 0.05$; **, $p < 0.01$ in the presence *versus* absence of rFABP3. *B,* recombinant human αSyn at a concentration of 10 μg/ml was cross-linked using 30 μM DSP in the presence or absence of recombinant human FABP3 (10 or 100 μg/ml). Samples were immunoblotted with anti-αSyn (*left*) or with anti-FABP3 (*right*) antibody. *C,* coimmunoprecipitation of αSyn and FABP3 in total lysates from the substantia nigra. Extracts were immunoprecipitated (*IP*) with anti-αSyn antibody, and immunoprecipitates were then immunoblotted (*WB*) with anti-FABP3 antibody. *D,* confocal images showing localization of αSyn (*red*) and FABP3 (*green*) in the substantia nigra. αSyn and FABP3 are colocalized in DA cell body (*arrow*). *Scale bar, D,* 20 μm. *WT,* wild-type mice; *KO, Fabp3*$^{-/-}$ mice.

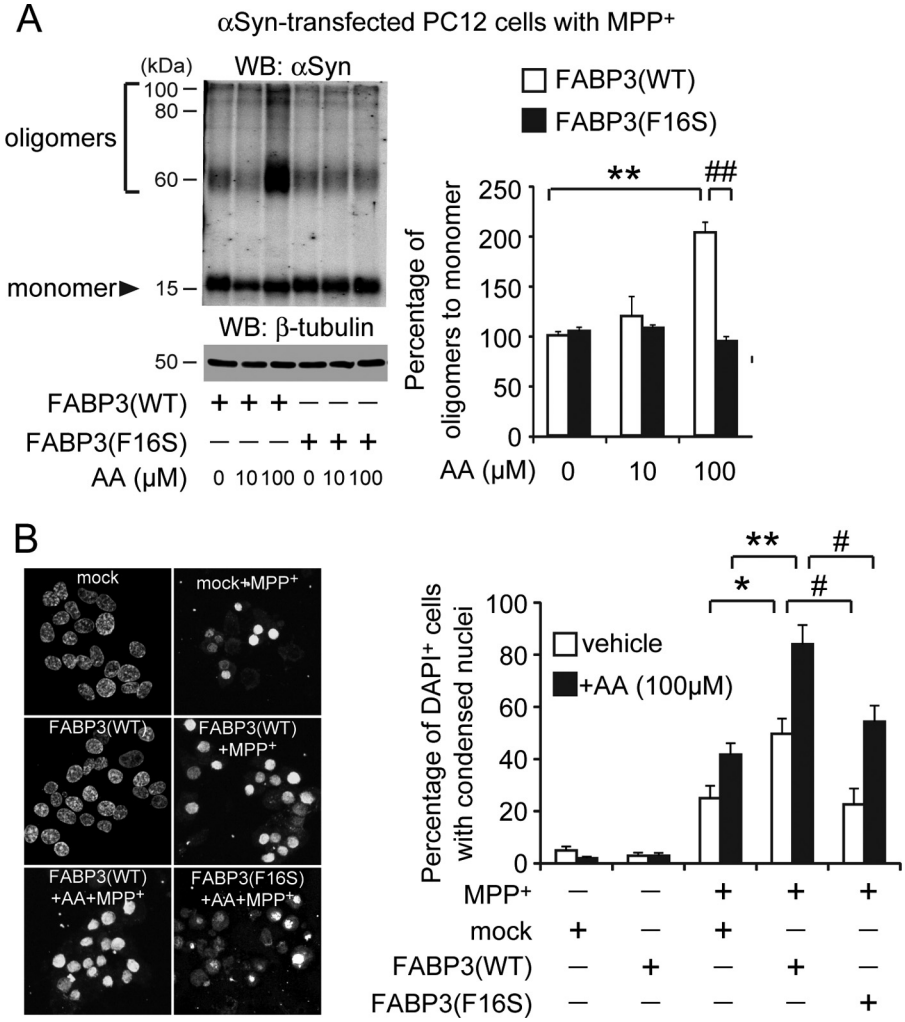


FIGURE 7. **FABP3-mediated AA incorporation accelerates αSyn oligomerization and cell death.** *A,* representative immunoblots (*left*) and quantitative densitometry analyses (*right*) of PC12 cell extracts probed with various antibodies. **, $p < 0.01$ in FABP3-transfected cells plus MPP^+ *versus* FABP3-transfected cells plus MPP^+ and 100 μM AA. ##, $p < 0.01$ in FABP3-transfected cells plus MPP^+ and 100 μM AA *versus* FABP3(F16S)-transfected cells plus MPP^+ and 100 μM AA. *WB,* Western blot. *B,* PC12 cells were transfected with indicated plasmids and treated with MPP^+. Condensed nuclei, as indicators of cell death, were counted using DAPI -staining. *, $p < 0.05$; **, $p < 0.01$ in mock cells plus MPP^+ *versus* FABP3-transfected cells plus MPP^+; #, $p < 0.05$ in FABP3-transfected cells plus MPP^+ *versus* FABP3(F16S)-transfected cells plus MPP^+.

ranted, our findings suggest that up-regulation of FABP3 protein and increased AA/PUFA incorporation likely function in LB formation in PD. These results also provide an intriguing clue with respect to a potential molecular target for neurodegeneration in human α-synucleinopathies, including PD.

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Neurobiology:

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