α-synuclein–lipoprotein interactions and elevated ApoE level in cerebrospinal fluid from Parkinson’s disease patients

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The progressive accumulation, aggregation, and spread of α-synuclein (αSN) are common hallmarks of Parkinson’s disease (PD) pathology. Moreover, numerous proteins interact with αSN species, influencing its toxicity in the brain. In the present study, we extended analyses of αSN-interacting proteins to cerebrospinal fluid (CSF). Using coimmunoprecipitation, followed by mass spectrometry, we found that αSN colocalize with apolipoproteins on lipoprotein vesicles. We confirmed these interactions using several methods, including the enrichment of lipoproteins with a recombinant αSN, and the subsequent uptake of prepared vesicles by human dopaminergic neuronal-like cells. Further, we report an increased level of ApoE in CSF from early PD patients compared with matched controls in 3 independent cohorts. Moreover, in contrast to controls, we observed the presence of ApoE-positive neuromelanin-containing dopaminergic neurons in substantia nigra of PD patients. In conclusion, the cooccurrence of αSN on lipoprotein vesicles, and their uptake by dopaminergic neurons along with an increase of ApoE in early PD, proposes a mechanism(s) for αSN spreading in the extracellular milieu of PD.

Significance

Two of the most important issues in Parkinson’s disease (PD) research are the identification of mechanisms underlying α-synuclein cell-to-cell transfer in the nervous system and the discovery of early diagnostic biomarkers. Both of these issues are addressed in our current manuscript. Using multiple approaches, we present that α-synuclein interacts with lipoproteins within human cerebrospinal fluid and can be taken up by cells in such a state. Moreover, using cerebrospinal fluid samples from 3 large and independent cohorts of patients, we demonstrate that apolipoprotein E is elevated in early, not yet medicated, patients with PD. Finally, using postmortem brain tissue, we provide preliminary histological evidence that apolipoprotein E is enriched in a subpopulation of dopaminergic neurons of human substantia nigra.

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were discarded, and between 10 and 12 mL of CSF was collected. Cell counts were measured, samples were centrifuged, aliquoted, frozen on dry ice, and stored. Time between sample collection and freezing was maximum 30 min. Blood-contaminated samples were excluded. Formalin-fixed, paraffin-embedded 5-μm human brain sections, from subjects without neurological disorders (n = 2; male, age 77 y and male, age 73 y) or PD (n = 2; male, age 78 y, disease duration 12 y and male, age 75 y, disease duration 12 y), were obtained from the Brain Bank at Karolinska Institutet.

Chemicals and Antibodies. Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Merck KGaA) and were of an analytical grade. All solutions were prepared using Milli-Q deionized water (Millipore). The recombinant ApoE was purchased from Sigma-Aldrich (SRP4696, Merck KGaA). The list of antibodies, and methods in which they were used, can be found in SI Appendix, Table S1.

Coimmunoprecipitation. Protein A/G agarose beads (Abcam) were pretreated with 1% BSA, dissolved in phosphate-buffered saline (PBS) buffer (20 mM phosphate, 150 mM NaCl, pH 7.4), to block any unspecific interactions. A large volume of CSF (50 mL) was pulled down from healthy controls, and endogenous immunoglobulins were depleted from the sample during 2-h incubation with protein A/G agarose beads, followed by elimination of beads via centrifugation. The antibody recognizing αSN, used for similar approaches previously (25–27), was added to the obtained solution and incubated overnight in 4 °C. Thereafter, the sample was incubated for 6 h with protein A/G agarose beads at room temperature (RT) and spun. The beads were washed, and protein elution was conducted by 0.2 M glycine pH 2.6. The sample was dialyzed, lyophilized, and stored at −20 °C for further analysis. In parallel, separate positive and negative control samples were prepared. Recombinant αSN served as a positive control. The negative control was a CSF sample without addition of the primary anti-αSN antibody.

ApoE Depletion. Depletion of ApoE-positive vesicles from human CSF was performed using the aforementioned coimmunoprecipitation (co-IP) approach; 200 μL of CSF was used as a starting material. The procedure was terminated after collecting the supernatant obtained after removal of protein A/G agarose beads bound to the anti-ApoE antibody.

ELISA. A microtiter plate was coated with the capture antibody at 10 μg/mL concentration in carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Next, the plate was washed with PBS containing 0.1% (vol/vol) Tween-20 (PBS-T), and remaining protein-binding sites were blocked for 2 h at RT with 5% nonfat dry milk in PBS. The plate was washed with PBS-T, and 10 μL of each sample diluted in PBS, with or without addition of the detergent, was incubated for 2 h at 37 °C and washed with PBS-T. Subsequently, a detection antibody was added to each well, incubated for 2 h at RT, and washed with PBS-T. A secondary antibody was added to each well, incubated for 1 h at RT and washed with PBS-T. Then 3,3′,5,5′-Tetramethyldibenzamidine solution (10 μg/mL in 0.05 M phosphate-citrate buffer, pH 5.0) was added to each well and incubated for 30 min. Equal volume of a stopping solution (2 M H$_3$SO$_4$) was added to each well and the optical density was read at 450 nm. For detection of αSN before and after depletion of ApoE-positive vesicles cholineinsubstrate (Thermo Fisher Scientific SuperSignal ECLISA Pico Substrate, Thermo Fisher Scientific) was used instead of 3,3′,5,5′-Tetramethylbenzidine solution.

Mass Spectrometry. IP samples were digested using modified trypsin (Promega). Obtained peptides were purified using C18 resin ZipTips (Millipore). All samples were dried down to approximately 10 μL using a vacuum centrifuge. Thereafter, samples were analyzed on a nanoLC system (Easy-NLC II; Thermo Fisher Scientific) coupled to an electrospray linear ion trap (LTQ; Thermo Fisher Scientific) mass spectrometer. The acquired mass spectrometry (MS/MS) data were converted into a combined mgf-file. The mgf-files were searched against Homo sapiens Database using a X!Tandem search engine.

Immunogold Labeling and Transmission Electron Microscopy. To remove excess proteins and analyze mostly vesicles, the CSF sample was initially filtered, and the retained solution was collected for transmission electron microscopy (TEM) analysis. An aliquot from each sample was added to a grid, and the excess solution was soaked off by a filter paper. The samples were incubated with primary antibodies for 1.5 h, grids were rinsed, and the secondary antibodies were added: goat polyclonal anti-mouse (5 nm, anti-ApoE antibody detection) or goat polyclonal anti-rabbit (10 nm, anti-αSN antibody detection) antibodies at a final dilution of 1:100 each. The samples were examined at 80 kV.

SDS/PAGE. SDS/PAGE was performed as described before (28). Briefly, the sample was mixed with a denaturing loading buffer and boiled at 95 °C for 5 min. For analysis of samples containing αSN oligomers or fibrils, the denaturing loading buffer was additionally supplemented with urea to 10 M final concentration, and incubation in 95 °C for 5 min was changed to 65 °C for 60 min to allow dissociation of aggregates. Next, samples were separated using a bis-Tris acrylamide gel using 2-μ morpholinol)ethanesulfonic acid running buffer. If necessary, the gel was stained using Coomassie Brilliant Blue (CBB) R-250 solution.

Western Blotting. After SDS/PAGE, gels were assembled with 0.45-vol. 116 μL of each sample diluted in PBS, with or without addition of the degrading buffer. If necessary, the gel was stained using Coomassie Brilliant Blue (CBB) R-250 solution.

| Sample | log(e) | log(I) | PCM | PCC | UP | MW | Description |
|--------|-------|-------|-----|-----|----|----|-------------|
| CSF IP | −188.3| 7.05  | 52  | 67  | 14 | 36.1| APOE:p, apolipoprotein E |
| −141  | 5.62  | 71   | 82  | 11  | 11.4| SNCA:p, synuclein alpha |
| −132.15| 5.28  | 38   | 21  | 10  | 69.2| Serum albumin, bovine; BSA |
| −104.2 | 6.84  | 35   | 44  | 9   | 30.8| APOA1:p, apolipoprotein A1 |
| −43.9  | 7     | 12   | 29  | 4   | 52.5| ApoJ:p, apolipoprotein J |
| −20.2  | 4.8   | 12   | 15  | 2   | 24.4| Trypsin; EC 3.4.21.4 |
| −14.8  | 4     | 24   | 33  | 2   | 12.6| IGHAV@:p, Ig heavy variable 3-23 |
| −52    | 4.47  | 8.8  | 16  | 5   | 41.3| Ig heavy constant gamma 3 |
| −4.7   | 4.11  | 4.7  | 7   | 1   | 24.5| Bovine Alpha-S1-casein |
| −4.6   | 3.28  | 2.6  | 4   | 36.6| IGH@:p, Ig heavy constant α2 |
| −145   | 5.15  | 71   | 82  | 11  | 11.4| SNCA:p, synuclein alpha |

Proteins identified in CSF samples, a specificity control, i.e., the sample without the αSN antibody and a positive control, i.e., samples spiked with recombinant αSN; log(e), base-10 log of the expectation that this assignment is stochastic; log(I), base-10 log of the sum of the intensities of the fragment ion spectra; PCM, protein coverage measured; PCC, protein coverage corrected; UP, number of unique peptides found; MW, molecular weight (kilodaltons).
unspecific interactions were blocked using 5% goat normal serum followed by overnight incubation with the anti-ApoE antibody. Thereafter, sections were incubated with the HRP-conjugated secondary antibody, and the peroxidase labeling was visualized with a mix of 3,3-diaminobenzidine and nickel (SK-4100; Vector Laboratories), which yielded a blue/gray reaction product.

**Densitometry.** The optical density of ApoE immunoreactivity was performed on representative light microscopy pictures. The digital images of SN were obtained by a high-power objective lens (40×, CFI Plan Apo Lambda; Nikon) and analyzed by ImageJ in grayscale. From each picture, 6 random neuromelanin-positive cells were selected. The intracellular signal was measured within the cell body in the region where no neuromelanin was present. The extracellular signal was measured in the parenchyma just next to the cell of interest. The optical density for each cell was corrected for nonspecific background density. The data are presented as arbitrary units or intracellular/extracellular signal ratio.

**Preparation of the Recombinant αSN Monomers, Oligomers, and Fibrils.** The recombinant human αSN was expressed in Escherichia coli and purified as described before (11). Briefly, cells were harvested and lysed. The majority of unwanted proteins were precipitated by acidification. The solution was fractionated on a Q-Sepharose column. Fractions containing αSN were identified by SDS/PAGE and pulled together, and high molecular weight aggregates were removed by filtration. αSN oligomers were prepared by dissolving αSN monomers at 12 mg/mL followed by incubation at 37 °C with shaking. Insoluble material was removed, and supernatant was fractioned using Superpose 6 column (GE Healthcare). Oligomer fractions were collected, concentrated, and stored at 4 °C. The recombinant αSN was fibrillated by dissolving αSN monomers at 1 mg/mL and incubated at 37 °C with shaking for 5 d. Obtained samples were centrifuged, obtained pellet was suspended in PBS buffer, and preformed fibrils (PFF) were prepared by sonicating the sample to obtain unified length of fibrils. For aggregation analysis, samples were incubated with or without addition of ApoE, at a final concentration of 1 mg/mL for αSN and 0.25 mg/mL for ApoE with 40 μM ThT in a Tecan Spark 10 M (Tecan Nordic AB) plate reader at 37 °C with shaking. The ThT signal was monitored at 448-nm excitation and 485-nm emission.

**Preparation of Enriched Lipoprotein Vesicles.** Human plasma high-density lipoprotein (HDL) (437647) and very low-density lipoprotein (VLDL) (437641)
vesicles were purchased from Merck Millipore. For the enrichment, 550 μg/mL (cholesterol content) lipoproteins were mixed with αSN or ApoE (11 μM final concentration each) and incubated for 1 h at 37 °C followed by 1-h incubation with ApoE. Unbound proteins were removed by passing the solution through 100-kDa or 50-kDa Amicon Ultra-0.5 Centrifugal Filter Units (Millipore). Finally, the sample was washed 3 times by adding PBS to the retained fraction and passing the solution through 100-kDa or 50-kDa Centrifugal Filter Units.

Lipoprotein Uptake by Dopaminergic Cells. SH-SY5Y human neuroblastoma cells were routinely maintained in a Dulbecco’s Modified Eagle Medium (DMEM) modified medium supplemented with fetal bovine serum (FBS) (10%), l-αlanyl-l-glutamine (2 mM), penicillin (100 μg/mL), and streptomycin (100 μg/mL). Cultures were maintained at 37 °C in 5% CO2/humidified air. For the uptake screening, cells were cultured in 24-well plates on laminin-coated cover glasses at a seeding density of 1 × 10^5 cells per well in a differentiating medium (DMEM modified medium supplemented with FBS (10%), l-αlanyl-l-glutamine (2 mM), penicillin (100 μg/mL), and 10 μM retinoic acid) for 4 d. Human plasma HDL (437647; Merck Millipore), VLDL (437641; Merck Millipore), and the recombinant αSN were marked with Alexa Fluor 568 NHS-ester (A20003; Thermo Fisher Scientific) or Alexa Fluor 488 NHS-ester (A20000; Thermo Fisher Scientific) according to the manufacturer protocol. For uptake study, cell medium was changed to DMEM modified medium supplemented with FBS (0.1%), l-αlanyl-l-glutamine (2 mM), penicillin (100 μg/mL), streptomycin (100 μg/mL), and 10 μM retinoic acid. Labeled human plasma HDL and VLDL, with or without enrichment with the labeled monomeric αSN (enrichment protocol described above), the labeled monomeric αSN, and vehicle controls were added to cells (final concentrations: 20 μM cholesterol and 1 μM αSN) and incubated for 4 h. Next, cells were washed with PBS and fixed with 4% paraformaldehyde. Subsequently, cells were stained with the Alexa Fluor 647 phallolidin dye (A22287; Thermo Fisher Scientific) according to the manufacturer protocol followed by a DAPI counterstaining. The single-plane images of cells were obtained using a confocal microscopy (LSM880; Zeiss).

Separation of Lipoproteins by Density-Gradient Ultracentrifugation. Lipoproteins were isolated from 0.1 mL of αSN–lipoprotein mix by KBr-density gradient ultracentrifugation according to Redgrave et al. (30). Briefly, after incubation, density and volume of each sample was adjusted, respectively, to 1.21 g/mL and 1 mL with KBr and transferred into centrifuge tubes. A discontinuous density gradient was formed by layering 3 mL of 1.063 g/mL salt solution above the sample, followed by 3 mL of 1.019 g/mL salt solution and 3 mL of 1.006 g/mL salt solution. Salt solutions were prepared from KBr and NaCl and contained 0.1 mg/mL 2,2′,2′′-tris(ethane-1,2-diyldinitriilo)tetracetic acid. The samples were centrifuged at 386,000 × g for 24 h at 20 °C. The 1.25-mL fractions were collected and analyzed using the Western blotting (WB) method.

Size-Exclusion Chromatography. The αSN–lipoprotein samples were analyzed and fractionated using Superose 6 Increase 10/300 GL column connected to an ÄKTA Explorer system (GE Healthcare) using PBS as a mobile phase. The appearance of proteins was monitored by reading the absorbance at 280 nm. Obtained fractions were collected and analyzed using the WB method.

Sample Randomization. For each patient cohort, all available samples were chosen taking age and gender matching into consideration. Obtained CSF and plasma samples were coded and analyzed blindly. No sample was excluded from the final analysis. Samples were decoded after experimental data were obtained.

Statistics. Statistical analyses were performed using a GraphPad Prism software (GraphPadInc). Comparison between 2 groups was performed using a Mann–Whitney U test. Multiple comparison was performed using a Kruskal–Wallis H test with a Dunn’s correction. A Spearman’s rank correlation coefficient was used to analyze dependence between 2 sets of data. All statistical tests were 2-tailed, and P value < 0.05 was considered statistically significant.

Data Availability. The data are available upon reasonable request. Due to sensitive nature of the patients’ clinical information, the ethics protocol does not allow open data sharing.

For more detailed description of the methods, see SI Appendix.
62.65 ± 12.72 y). We observed a decrease of αSN in samples from both PD and control subjects (Fig. 1C and SI Appendix, Fig. S5). Moreover, the amount of αSN bound to ApoE-positive vesicles was significantly higher in PD cases compared with healthy controls (Fig. 1C).

To investigate whether the interaction between αSN and ApoE is direct or indirect, we analyzed kinetics of αSN fibril formation after addition of recombinant ApoE. We did not observe any significant changes in the aggregation profile of the recombinant αSN, suggesting indirect contact between these proteins (Fig. 1D).

To further examine the character of interactions observed between αSN and ApoE, we performed TEM analysis on CSF samples. Indeed, αSN and ApoE double immune-gold labeling revealed a colocalization of αSN and ApoE on larger (>30 nm) vesicular structures in CSF (Fig. 1E and SI Appendix, Fig. S6A). However, it is possible that the αSN and ApoE colocalization also occurred on smaller vesicles, but, due to a steric hindrance, caused by a relatively large size of immunogold particles and antibodies, only a single protein could be detected on vesicles smaller than 30 nm in size (SI Appendix, Fig. S6B).

No clear lipid bilayer was observed on αSN- and ApoE-positive vesicular structures in TEM analysis (Fig. 1E), suggesting that they are lipoprotein vesicles. This is in accordance with the data from the MS analysis identifying only lipoproteins' markers (Table 1, Fig. L4, and SI Appendix, Fig. S3). We therefore investigated the ability of αSN to interact with lipoprotein vesicles in vitro. Considering that CSF lipoproteins are predominantly of the size and density of plasma HDL, human plasma HDLs were included in the study. Moreover, due to fact that CSF HDLs are mainly ApoE-positive, contrary to plasma HDL which are mainly ApoAI-positive (31), human plasma VLDL were also used to address the role of ApoE-positive lipoproteins. Accordingly, we incubated human plasma HDL or VLDL vesicles with recombinant monomeric αSN and passed the obtained samples through a size-exclusion filter. Subsequent WB analysis of eluates showed that the recombinant monomeric αSN without vesicles passed entirely through the filter, while a large amount of αSN incubated with either HDL or VLDL vesicles was retained on the filter. This confirmed αSN binding ability to lipoprotein vesicles (Fig. 2A).

To verify that αSN and ApoE might colocalize on the lipoproteins resembling CSF lipoproteins, we performed double enrichment of plasma HDL and examined whether αSN- and/or ApoE-enriched vesicle can be detected by ELISA settings applied in the CSF study. When using both capture and detection antibodies against ApoE, we obtained signal for VLDL, VLDL+αSN, and HDL+αSN+ApoE vesicles (Fig. 2B). For the anti-ApoE capture antibody and the anti-αSN detection antibody, the signal was observed for VLDL+αSN and HDL+αSN+ApoE vesicles (Fig. 2C). For the anti-αSN capture antibody and the anti-ApoE detection antibody, the ApoE signal was observed for VLDL+αSN and HDL+αSN+ApoE vesicles (Fig. 2D). When using both capture and detection antibodies against αSN, we obtained signal for VLDL+αSN, HDL+αSN, and HDL+αSN+/ApoE vesicles (Fig. 2E). We extended our examination of αSN and lipoproteins interactions to include not only monomeric αSN but also oligomeric and PFF αSN, using 2 additional methods. First, instead of filtration technique, we performed density gradient ultracentrifugation separation followed by WB analysis (SI Appendix, Fig. S7). In this experiment, we were able to detect the 3 forms of αSN both in protein and lipoprotein fractions. We conclude that not only monomeric but also oligomeric and PFF forms of αSN can interact with lipoproteins. The fact that ultracentrifugation detached apolipoproteins from vesicles is in agreement with previous reports (32) (SI Appendix, Fig. S7). Therefore, to omit detachment of proteins from vesicles, we decided to use a size-exclusion chromatography (SEC) approach, which does not introduce any excessive forces to the sample separation. As described above, human plasma HDL or VLDL vesicles were incubated with each of the 3 forms of αSN separately and injected to the column for SEC analysis. We observed a shift in retention volume after αSN and lipoprotein coinuculation, indicating formation of larger-size complexes (SI Appendix, Fig. S8). We further confirmed that both αSN and lipoproteins are present in fractions collected from shifted peaks, using the WB method, verifying interactions of monomeric, oligomeric, and PFFs forms of αSN with lipoproteins.

Moreover, using a filtration method with spin filters, presented in Fig. 2, and SEC, we confirm that such isolation of lipoproteins enriched with monomeric αSN does not affect the interaction between monomeric αSN and lipoproteins (SI Appendix, Fig. S9).

Based on the obtained results, we decided to investigate whether ApoE, and other identified apolipoproteins, are changed in the CSF and plasma from PD patients and examine their biomarker potential.

**Table 2. Results from the statistical analysis of differences in apolipoproteins levels in CSF and plasma samples, between PD patients and matched controls from the Stockholm cohort**

| Analyzed apolipoprotein | Mann–Whitney Control vs. PD | CSF | Plasma | Kruskal–Wallis Control vs. PD treated vs. PD untreated | Control vs. PD treated vs. PD untreated |
|-------------------------|-----------------------------|-----|--------|-----------------------------------------------|----------------------------------------|
| ApoAI                   | 0.7995                      | 0.0048 |        | >0.9999                                      | >0.9999                                 |
| ApoCl                   | 0.4245                      | 0.6176 |        | 0.0807                                       | 0.0439                                 |
| ApoE                    | <0.0001                     | 0.8194 |        | 0.0023                                       | 0.2830                                 |
| ApoJ                    | 0.0116                      | 0.639  |        | 0.0413                                       | 0.9999                                 |

The left part of the table represents *P* values obtained after analyzing apolipoproteins' levels in PD (PD) patient set vs. healthy controls (the Mann–Whitney *U* test). The right part of the table represents *P* values obtained after splitting the PD group into treated and untreated patients’ subsets and analyzing apolipoproteins’ levels between them and vs. healthy controls (the Kruskal–Wallis *H* test with a Dunn’s correction). *P* values < 0.05 are considered significant and are marked with a bold font.

**Increased Level of ApoE in CSF, but Not in Plasma, from PD Patients.**

ApoE is the main apolipoprotein in central nervous system (CNS), and its allelic variants are strongly implicated in the pathogenesis of Alzheimer’s disease (33, 34). There is also some evidence that allelic variants of ApoE predispose to dementia in PD (35). However, no study has reported changes in ApoE protein level in CSF or plasma from PD patients. Therefore, we measured ApoE in biofluids from PD patients and from matched controls (SI Appendix, Tables S2 and S3). Throughout the ELISA optimization process, we realized that the level of ApoE detected by ELISA in the human CSF rises with increasing concentration.
of detergents in a buffer, up to the point where the detergent concentration is too high for the method to work at all. At the same time, the level of recombinant ApoE, detected with ELISA method, was independent of a buffer's composition (SI Appendix, Fig. S10). This effect was probably caused by the strong affinity of ApoE to lipoproteins and the steric hindrance caused by the large size of an antibody compared with the small size of lipoproteins (SI Appendix, Fig. S6B), consequently making ApoE hard to analyze without usage of strong detergents, and therefore not suitable for high-throughput methods like ELISA. Subsequently, we decided to analyze ApoE protein levels with SDS/PAGE followed by WB (SI Appendix, Fig. S11). We found a significant increase in the ApoE level in CSF from PD patients compared with matched controls in 3 independent cohorts (Table 2 and Fig. 3 A–F). Using the recombinant ApoE protein, we estimated that the average concentration of ApoE in Stockholm cohort controls was 8.0 μg/mL, while, in PD patients, the average level was 12.6 μg/mL (SI Appendix, Fig. S12), nearly 57% more. In contrast, we found no difference in the plasma level of ApoE between controls and PD patients from Stockholm cohort (Fig. 3 G and H).

The CSF level of ApoE was negatively correlated only with Levodopa Equivalent Daily Doses score (SI Appendix, Table S4). No significant correlations were observed between the ApoE level and disease duration, or rating scores from the unified PD rating scale part 3, the Hoehn &Yahr scale, the nonmotor symptoms scale, Beck's depression inventory (BDI), and Montreal cognitive assessment (SI Appendix, Table S4). Similarly, there were no correlations between the plasma ApoE level and patient age or any of the PD rating scores (SI Appendix, Tables S4 and S5). There was no correlation between plasma and CSF ApoE levels (SI Appendix, Table S6).

To investigate the ApoE biomarker potential for PD diagnosis, we performed a receiver operating characteristic (ROC) curve analysis (36, 37) for data obtained from Stockholm cohort. Our analysis showed that, at the level of 0.8 sensitivity, ~0.6 specificity was achievable (SI Appendix, Fig. S13A, solid black line). Interestingly, with the same sensitivity of 0.8, we could obtain around 0.7 specificity when only untreated patients were analyzed (SI Appendix, Fig. S13A, dashed black line).

ApoE as a Potential Biomarker for Atypical Parkinsonism. To check whether the ApoE increase is specific for PD, we analyzed the level of ApoE in CSF from patients with MSA or PSP from Umeå and Lund cohorts. The ApoE level in MSA patients, another synucleinopathy, was significantly higher compared with controls, whereas its level in PSP patients, a tauopathy, was not significantly changed (SI Appendix, Fig. S14). These results further strengthen the relationship between ApoE and αSN.

Uptake of αSN-Enriched Lipoprotein Vesicles in Dopaminergic Neuronal-like Cells. To investigate whether αSN-enriched lipoprotein vesicles can be a possible mechanism of αSN uptake and spreading, we examined the uptake of HDL and VLDL along with αSN-enriched lipoprotein vesicles in neuronal-like cells. We thus differentiated SH-SY5Y human dopaminergic neuroblastoma cells to a neuronal-like state and incubated them with a fluorescently labeled monomeric αSN, HDM, and VLDL, and with double-labeled complexes of αSN-enriched lipoprotein vesicles. We also checked that the labeled αSN does not undergo aggregation or degradation/fragmentation, using both WB and SEC approaches (SI Appendix, Fig. S15). We decided not to use unlabeled recombinant αSN, due to the fact that SH-SY5Y cells, which are of human origin, express endogenous αSN. Performing αSN staining in such conditions will not allow discrimination between endogenous and recombinant αSN. Furthermore, we decided to use cells, recombinant proteins, and lipoproteins of human origin to omit any concerns coming from receptor differences across species. After 4-h incubation, we observed that the monomeric αSN alone, as well as HDL and VLDL, easily entered neuronal cells. Interestingly, we observed the same effect for αSN-enriched HDL and VLDL vesicles (Fig. 4), confirming αSN couptake during lipoprotein internalization as a possible mechanism of αSN uptake and spreading.

Immunohistochemical Detection of ApoE in the Human SNC. To examine the distribution of ApoE in the human SN, we performed immunohistochemistry analysis on postmortem human brain tissue from SNC of PD patients and healthy controls. Similarly to previous reports (38, 39), we detected ApoE in paravascular spaces (SI Appendix, Fig. S16 A and B). Moreover, we observed a strong region-specific ApoE signal mostly in periaqueductal gray, intercollicular nucleus, and, most importantly, SNC (SI Appendix, Fig. S17 A–H). Interestingly, in contrast, most of neuromelanin-positive cells (dopaminergic neurons) in SNC were negative (or exceedingly
low) for the ApoE staining (Fig. 5A). In contrast to controls, in sections from PD patients, most of the dopaminergic neurons were clearly positive for ApoE (Fig. 5A). Additionally, to confirm observed changes and rule out optical illusion, we performed densitometric analysis of ApoE signal distribution between the intracellular compartment of dopaminergic cells and extracellular space (Fig. 5 B–D). We detected a significantly higher signal for extracellular than intracellular ApoE in controls (Fig. 5B), while, in PD patients, ApoE signal was distributed equally (Fig. 5C). Finally, we compared the ratio of intracellular and extracellular ApoE signal in controls and PD samples and observed a significantly lower ratio for controls, indicating changes in the ApoE gene expression and/or an increased uptake of ApoE in dopaminergic neurons of PD patients. Importantly, we did not observe any ApoE-negative dopaminergic neurons in PD patients, while close cooccurrences of both negative and positive ApoE dopaminergic neurons were detected in controls (SI Appendix, Fig. S17 A–K). Finally, we did not observe any signal on negative control sections in which the primary antibody was omitted (SI Appendix, Fig. S16C).

Decreased Level of ApoAI in Plasma, but Not in CSF, from PD Patients.

We have identified ApoAI as one of the αSN-interacting proteins in CSF (Table 1). The level of ApoAI in CSF was not changed between controls and PD patients (SI Appendix, Fig. S18 A and B and Table 2). However, in agreement with a previous study (40), we observed a decreased plasma level of ApoAI in PD patients compared with controls (SI Appendix, Fig. S18 C and D and Table 2). We did not observe any correlation between the level of ApoAI and PD severity scores, disease duration, or age, either in CSF or in plasma (SI Appendix, Tables S4 and S5). However, the CSF level of ApoAI significantly correlated with its IgG and albumin content (SI Appendix, Table S5). At the same time, we did not observe any significant changes in albumin and IgG at a group level, either in CSF or in plasma of PD patients and controls (SI Appendix, Fig. S19 A–D). However, the number of mononuclear cells in CSF was significantly higher in PD patients compared with controls (SI Appendix, Fig. S19 E and F), suggesting a mild inflammatory response in PD.

Elevated Levels of ApoJ and ApoCI in CSF, but Not in Plasma, from PD Patients.

We also analyzed CSF and plasma levels of 2 other apolipoproteins: ApoJ and ApoCI. The level of ApoJ was significantly increased in CSF (SI Appendix, Fig. S20 A and B and Table 2), but not in plasma (SI Appendix, Fig. S20 C and D and Table 2), of PD patients compared with controls. We did not find correlations between the ApoJ level and any PD severity score (SI Appendix, Table S4). However, the plasma ApoJ level negatively correlated with age and the albumin content and positively correlated with IgG levels in PD patients (SI Appendix, Table S5).

Additionally, we decided to investigate the ApoCI level, due to its high abundance in CSF. Although the level of CSF ApoCI in PD patients did not differ from controls (SI Appendix, Fig. S21 A and B and Table 2), it was significantly higher in treated compared with untreated PD patients (SI Appendix, Fig. S21 A and B and Table 2). Furthermore, the ApoCI level in CSF positively correlated with the disease duration, and the plasma level positively correlated with the BDI score (SI Appendix, Table S4). We did not observe any difference in the level of ApoCI in plasma between controls and PD patients (SI Appendix, Fig. S21 C and D). The ApoCI level from controls correlated with albumin and IgG levels in CSF and plasma. For PD patients, ApoCI levels correlated with albumin and IgG levels in CSF and the albumin level in plasma (SI Appendix, Table S5). These data suggest a possible involvement of ApoCI in neuroinflammation rather than a specific contribution to PD.

Correlations between Apolipoproteins Levels in Plasma and CSF and Their Combined Accuracy for PD Detection. As mentioned above, our data strongly indicate differences in levels of various types of apolipoproteins in CSF and plasma of PD patients. Therefore, we investigated whether there is any correlation between levels of different types of apolipoproteins within and between plasma and CSF. We observed that the increased level of ApoE in CSF corresponded to the decreased level of ApoAI in plasma (SI Appendix, Table S6). Moreover, we noticed positive correlations...
between plasma levels of ApoCI and ApoE as well as between CSF levels of ApoCI and ApoAI. Furthermore, we detected a correlation between plasma levels of ApoJ and ApoAI (SI Appendix, Table S6).

In addition, we examined whether a combined ROC analysis of several apolipoproteins would increase the diagnostic accuracy compared with the analysis of ApoE alone. Thus, we combined the level of ApoE in CSF with the level of ApoAI in plasma and recalculated the ROC curve (SI Appendix, Fig. S13B). We also performed the ROC analysis combining the level of ApoAI in plasma with levels of ApoJ and ApoE in CSF (SI Appendix, Fig. S13C). Our analysis showed that, with the sensitivity of 0.8, combined levels did not increase the specificity (SI Appendix, Fig. S13D). However, when the sensitivity was set to 0.71, we could increase the specificity from 0.70 for ApoE alone to 0.83 when combining ApoE, ApoAI, and ApoJ levels (SI Appendix, Fig. S13D).

Discussion

During the last decade, a number of studies reported that αSN possesses a membrane-binding capacity and can interact not only with artificial lipid vesicles but also with cell membranes and extracellular vesicles, including exosomes (41). Here, using a co-IP approach followed by MS analysis, we discovered a robust interaction between αSN and apolipoproteins in the human CSF. We observed the strongest signal for ApoE, and we further confirmed this interaction using an ELISA method relying on different sets of antibodies. Our data suggest that αSN–apolipoprotein interactions are indirect, since the ApoE protein was not able to interfere with the αSN aggregation, and we also observed an interaction of αSN with ApoE-negative lipoproteins in vitro. This interaction rather depends on the αSN propensity to bind to the negatively charged lipid particles (42, 43). Importantly, while it is vital to note that both ApoE (44) and αSN (45–47) were found on exosomes, our TEM analysis revealed structures without a clear lipid bilayer. More importantly, we did not identify any exosome markers during the MS analysis, while we observed several apolipoproteins. Our data suggest that αSN associates with lipoprotein particles both in vitro and in human CSF. Approximately 45% of CSF αSN is bound to ApoE-positive lipoproteins. The percentage of αSN bound to ApoE-positive vesicles in CSF is significantly higher in PD patients compared with healthy controls. This is in accordance with the higher level of CSF ApoE in PD patients. It is therefore a rather common event and could be a major mechanism of αSN uptake. At the same time, combining the facts that αSN concentration in CSF is estimated to be around 1 to 2 ng/mL and ApoE concentration is in the range of 5 to 15 μg/mL, we can conclude that this is a rare event from the perspective of lipoproteins. Furthermore, it is likely the amount of αSN–lipoproteins particles taken up by cells will be determined by cellular requirements for lipoprotein cargo. Therefore, αSN–lipoproteins particle uptake will differ between the nonmitotic cell line and fully mature dopaminergic neurons. Moreover, we determined that not only αSN monomers but also oligomers and fibrils can interact with human plasma-derived HDL and VLDL vesicles in vitro. Likewise, during the preparation of this manuscript, it was reported that αSN can interact with lipoproteins also in human plasma (48), further confirming our observations. Finally, we are showing that not only HDL and VLDL alone, but...
also αSN-enriched lipoprotein vesicles, undergo a cellular uptake by dopaminergic neuronal like cells in vitro. Additionally, we observed compartmentalization of αSN after cellular uptake. This is likely due to the fact that the uptake of lipoproteins, similar to other endocytosis mechanisms, results in trafficking of lipoproteins to endosomes and then to lysosomes (49, 50). Consequently, enrichment of staining in specific cellular compartments is expected.

Taken together, we believe that our present data on αSN–lipoprotein interactions, and their cellular uptake, raise a plausible explanation for the αSN uptake and spreading in the brain. It is possible that the monomeric αSN, as well as hydrophobic aggregates, binds to extracellular lipoprotein particles, which are then taken up by other cells through a lipoproteins endocytosis pathway (Fig. 6). This lipoprotein-dependent mechanism may cooperate with others, including exosome-based and nanotube models, to propagate αSN pathology.

Apolipoproteins themselves are key components of lipoprotein particles and are responsible for the lipid homeostasis as they transport lipids and lipid-soluble compounds from one cell to another (51). The major apolipoprotein in CSF is ApoE, followed by ApoAI, ApoAII, ApoCs, ApoJ, and ApoD (52). The ApoE concentration in the brain is the second highest, followed that of liver. In the CNS, ApoE is synthesized mainly by astrocytes and microglia, and, to a certain extent, by immature neurons (53). The ApoE content is high in the paravascular (glymphatic) space, a CSF flow pathway through the brain (39, 54, 55). Moreover, its presence and colocalization with amyloidβ in perivascular drainage channels was linked to the ApoE-dependent amyloidβ clearance through the blood–brain barrier (56–58).

ApoE levels are increased in injured or stressed neurons and therefore postulated to play a role in the growth and repair of cells in the CNS (59). Furthermore, ApoE allelic variants are linked to an increased risk and earlier age of onset for Alzheimer’s disease (34) and cognitive decline in PD (35). While the influence of ApoE genotypes was widely studied in PD, little work has been performed at the protein level (60, 61). We realized that the use of strong detergents is necessary to isolate ApoE molecules for a proper quantification. For this reason, we analyzed apolipoproteins levels in CSF and plasma with SDS/PAGE followed by WB. Our analysis demonstrated a significant increase of CSF ApoE in PD patients in 3 separate cohorts. Further, by performing the ROC curve analysis, we conclude that measuring apolipoproteins in CSF and plasma can be clinically meaningful, especially when used in combination with other known biomarkers.

ApoE was found to be enriched in the human SN, mostly localized in extracellular or paravascular spaces rather than in the cytosol of dopaminergic neurons. Furthermore, in preliminary studies, we distinguished 2 different populations of neuromelanin-containing dopaminergic neurons: ApoE-positive and ApoE-negative. In controls, the number of ApoE-negative dopaminergic neurons appears higher than the number of ApoE-positive ones. Conversely, in PD patients, the vast majority of remaining neuromelanin-containing neurons appear to be ApoE-positive. However, further analyses are needed to elucidate whether this effect is due to a higher uptake/expression of lipoproteins and/or ApoE-positive neurons being more resistant to neurodegeneration. Furthermore, the results need to be validated in additional PD populations. Nevertheless, our initial observations suggest a functional involvement of ApoE in PD pathology and/or defense against it.

We also studied other apolipoproteins in CSF as well as in plasma. We did not observe any changes in the CSF ApoAI level from PD patients, but, consistent with previous studies, the plasma ApoAI level was decreased (40). Contrary to ApoE, ApoAI in the brain is believed to be plasma-derived (52), while a small amount is synthesized by endothelial cells in the cerebral vasculature (62). This, together with the identified ApoAI–αSN interaction, suggests that toxic αSN species might be transported with ApoAI lipoprotein particles from the periphery and then distributed throughout the brain.

Similar to ApoE, we observed a significant increase in ApoJ levels in CSF from PD patients. Similar to other apolipoproteins, ApoJ, also known as clusterin, is involved in a lipid transportation, but it also serves a chaperone role during the cellular stress response (63). Besides, it stabilizes a range of misfolded proteins (64). Interestingly, ApoJ is involved in the amyloidβ clearance through blood–brain barrier (55, 65), and the level of brain ApoJ increases in numerous neurological disorders such as Alzheimer’s disease, MSA, ischemia, and epilepsy (66).

Finally, we observed a change in the CSF ApoCI level, but only in treated PD patients. Moreover, CSF and plasma ApoCI levels positively correlated with albumin and IgGs levels, indicating an association of ApoCI with blood–brain barrier integrity and a mild ongoing inflammation.

In summary, the presence of αSN on lipoprotein vesicles in the human CSF, along with the presence of ApoE in some dopaminergic neurons and the cellular uptake of αSN-enriched lipoproteins, represents a possible mechanism for αSN uptake and spreading in the brain. Finally, changes in apolipoproteins’ levels in human CSF represent an aspect of PD pathology and can be become clinically meaningful, especially when used in combination with other biomarkers.

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Fig. 6. Schematic representing the ApoE and αSN interaction in the human CNS and a possible spreading of a pathological αSN between cells. The pathological and the normal αSN forms might be released form healthy and/or dying neurons to the extracellular space, where they start interacting with lipoprotein particles released by microglia and astrocytes. Further, probably after recognition by lipoprotein receptors, they are taken up by healthy neurons. Possibly, pathological αSN aggregates, resistant to proteolytic cleavage, remain in the cell undigested and become the seed elongated by the normal αSN, which further might lead to neurodegeneration and disease spreading. Image prepared using Smart Servier Medical Art (https://smart.servier.com/), which is licensed under CC BY 3.0.
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