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Genetics

The effects of apolipoprotein E genotype, α-synuclein deficiency, and sex on brain synaptic and Alzheimer's disease–related pathology

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Abstract

Introduction: Alzheimer's disease (AD) and synucleinopathies share common pathological mechanisms. Apolipoprotein E4 (apoE4), the most prevalent genetic risk factor for AD, also increases the risk for dementia in pure synucleinopathies. We presently examined the effects of α-synuclein deficiency (α-syn−/−) and sex on apoE4-driven pathologies.

Methods: AD-related, synaptic, and vascular markers were analyzed in female and male α-syn−/− and α-syn+/+ apoE4, apoE3, and apoE3/E4 mice.

Results: ApoE4 was hypolipidated, and this effect was unchanged by α-syn−/− and sex. The levels of synaptic markers were lower, and the levels of AD-related parameters were higher in female α-syn−/− apoE4 mice compared with the corresponding apoE3 mice. By comparison, apoE4 had small effects on the AD parameters of male and female α-syn+/+ apoE4 mice.

Discussion: Although α-syn−/− does not affect the upstream lipidation impairment of apoE4, it acts as a "second hit" enhancer of the subsequent apoE4-driven pathologies.

Keywords: Alzheimer's disease; Apolipoprotein E4 (apoE4); apoE-targeted replacement mice; Sex; α-Synuclein deficiency

1. Introduction

The apolipoprotein E (*APOE*) gene, which codes for the most prevalent brain lipoprotein, is associated with increased risk for late-onset Alzheimer's disease (AD) [1–3]. There are three major alleles of *APOE*: ε2, ε3, and ε4, of which the ε4 allele is the strongest genetic risk factor for sporadic AD. The frequency of *APOE4* carriers in sporadic AD is in general about 60%; it increases the risk for AD by lowering the age of onset of the disease by 7 to 9 years per allele copy [2]. Although the association of apoE4 with AD is generally observed throughout the globe, the quantitative association between apoE4 and AD varies somewhat between regions such that, for example, it is highest in northern Europe and lowest in southern Europe and Asia [4,5]. The prevalence of AD in apoE4 carriers is higher in females than in males [6]; it is reduced by education [7] and can be modified by diet [8]. Taken together, these observations show that the phenotypic expression of the apoE4 genotype is affected by sex and genetic background and that it can be modulated by environmental conditions.

Pathologically, apoE4 is associated in AD with impaired synaptic plasticity [9] and with increased hippocampal atrophy and loss of dendritic spines [10]. ApoE4 is also associated with increased levels of neuritic plaques and neurofibrillary tangles [11,12]. In addition to these neuronal and AD-related pathologies, apoE4 is associated with increased vascular pathology in AD [13] and is a risk factor for vascular diseases [14,15]. Although there is no consensus in the field regarding the mechanisms underlying the pathologic effects of apoE4, the field has benefited tremendously from the development of several mouse models that express key apoE4-related pathologies. One of the most widely used models is targeted replacement (TR) mice in which the mouse apoE is replaced by either human apoE4 or its AD benign isoform, apoE3, both of which are expressed under the control of the endogenous mouse

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apoE promoter [16]. These apoE4 mice have impaired learning and memory, which are associated with neuronal and synaptic pathology and the accumulation of amyloid beta (Aβ) and hyperphosphorylated tau in hippocampal neurons [17,18]. Furthermore, like in AD, the brain and cognitive effects of apoE4 in these mice are more pronounced in females than in males [19] and can be modulated by diet. ApoE4-driven vascular and cerebral blood flow impairments have also been reported in the apoE4 mice [20], but they have been studied less extensively.

The apoE4-TR mice, which were originally developed more than 10 years ago by Sullivan [16], are now available commercially from Taconic Laboratories (Germantown, NY), where the apoE4 and apoE3 mice are kept as closed homozygous colonies. One of the drawbacks of prolonged maintenance in closed colonies is that spontaneous genetic drift could introduce differences between the colonies, which are not related to their apoE genotype. We thus backcrossed the Taconic apoE3 and apoE4 mice to C57Bl mice. This was performed by using C57Bl control mice from Harlan, which were maintained in our animal facility (line C57Bl/6JOlaHsd). After performing these backcrosses, we realized that the C57Bl mice from Harlan are α-synuclein deficient (α-syn−/−) [21], which led to the formation of apoE3 and apoE4-TR mouse colonies that were either deficient or haplodeficient for the α-syn gene. To generate homogeneous colonies, we initiated an inner-colony backcross, which resulted in the foundation of a large breeding nucleus of apoE4 and apoE3 mice on α-syn−/− background. These mice were then used to germinate the apoE3 and apoE4-TR α-syn−/− colonies.

The α-syn protein is a key player in the pathology of Parkinson's disease (PD) [22], and apoE4 has also been reported to be associated with PD and with PD dementia [23]. Furthermore, α-syn deficiency was shown to increase the accumulation of amyloid in a transgenic mouse model of AD [24], and both α-syn and apoE4 share common lipid-related functions [25,26]. However, the extent to which α-syn plays a role in mediating the pathologic effects of apoE4 is not known. Accordingly, the previous serendipitous course of events now provides us with the means to study the possible role of α-syn in mediating the pathologic effects of apoE4.

Hence, the overall objective of this study was to determine the effects of α-syn deficiency on the neuronal and vascular pathologic phenotypes of apoE4 in male and female mice. This was pursued by using the previously mentioned α-syn−/− apoE4 and apoE3 mice and corresponding apoE3 and apoE4 α-syn+/+ mice, which were obtained by backcrossing the apoE3 and apoE4 α-syn−/− mice to α-syn+/+ C57Bl mice (C57BL/6J RccHsd strain from Harlan).

2. Materials and methods

2.1. Mice

ApoE-TR mice, in which the endogenous mouse apoE was replaced by either human apoE3 or apoE4, were created by gene targeting [27] and were purchased from Taconic Laboratories (Germantown, NY). These mice were backcrossed at Taconic for eight generations after their preparation. To minimize possible genetic drifting between the apoE4 and apoE3 mice, which were offspring of the homozygous apoE4 and apoE3 mice generated by Taconic around 2001, they were further crossed by us with Harlan C57Bl/6JOlaHsd mice, which unlike the standard Jackson laboratory C57Bl/6J ApoE$^{tm1.1(APOE*4)Adpmc}$ mice (Jackson Laboratories, Bar Harbor, ME) turned out to be α-syn−/−. The resulting mice were then crossbred to yield apoE4 and apoE3 homozygous mice on α-syn−/− background. These were then further crossed with control C57Bl α-syn+/+ mice (Harlan C57BL/6J RccHsd) to produce apoE3 and apoE4-TR mice, which were α-syn+/+. The homozygous apoE3 and apoE4 mice are referred to in the text as apoE3 and apoE4 mice, whereas heterozygous mice obtained by the breeding of these mice are denoted as apoE3/E4. The apoE genotype of the mice was confirmed by polymerase chain reaction (PCR) analysis [28]. All the experiments were performed on 4-month-old male and female mice and were approved by the Tel Aviv University Animal Care Committee.

2.2. Immunohistochemistry and immunofluorescence confocal microscopy

Mice were anesthetized with ketamine and xylazine and perfused transcardially with phosphate-buffered saline. Their brains were then removed and halved, and each hemisphere was further processed for either histologic or biochemical analysis, as previously described [18]. Free-floating sections were immunostained with the following primary antibodies (Abs): rabbit anti-collagen IV (1:1000, Abcam); rabbit anti-synaptophysin (1:200, Santa Cruz); rabbit anti-glial fibrillary acidic protein (GFAP) (1:1000, Sigma); rabbit anti-apoE receptor 2 (ApoER2, 1:1000, kindly provided by Prof. Joachim Herz, UT Southwestern); rabbit anti-Aβ42 (1:500; Chemicon, Temecula, CA); rabbit anti-202/205 phosphorylated tau (AT8, 1:200, Innogenetics); guinea-pig anti-vesicular glutamate transporter 1 (VGluT1) (1:2000; Millipore); and mouse anti-vesicular GABA transporter (VGaT) (1:200, Synaptic Systems). The Aβ42 and AT8 diaminobenzidine (DAB)-immunostained sections were viewed using a Zeiss light microscope (Axioskop, Oberkochen, Germany) interfaced with a charge-coupled device (CCD) video camera (Kodak Megaplus, Rochester, NY). Pictures of stained brains were obtained at ×10 magnification. GFAP, VGluT1, VGaT, ApoER2, collagen IV, and synaptophysin staining were performed using immunofluorescence staining. Immunofluorescence was visualized using a confocal scanning laser microscope (Zeiss, LSM 510). Images (×20 magnification 1024 × 1024 pixels, 12 bit) were acquired by averaging four scans. Analysis and quantification of the staining in CA3 (in which the effects of the apoE genotype were previously shown to be most pronounced) [18] were performed using the Image-Pro plus system for image analysis

(v. 5.1, Media Cybernetics, Silver Spring, MD). The intensities of DAB staining or immunofluorescence staining were expressed as the percentage of the area stained, as previously described [28]. All images for each immunostaining were obtained under identical conditions, and their quantitative analyses were performed with no further handling.

2.3. Immunoblots

The hippocampus was rapidly removed from one freshly excised hemisphere and stored frozen at −70°C until use. Frozen hippocampi were thawed, homogenized with a Teflon-glass homogenizer in cold Tris-buffered saline containing a protease inhibitor mixture (P8340; Sigma) and a phosphatase inhibitor mixture (P5726; Sigma), and then aliquoted and frozen at −70°C until use.

For apoE and α-syn sodium dodecyl sulfate (SDS)-electrophoresis, the hippocampal homogenates were boiled for 10 minutes with 0.5% SDS and immunoblotted as described previously [28,29]. Gels were then transferred to a nitrocellulose membrane and stained with goat anti-apoE Ab (1:10,000; Millipore) and rabbit anti–α-syn Ab (1:10,000; Abcam). The immunoblot bands were all visualized using the enhanced chemiluminescence (ECL) substrate (Pierce), after which their intensity was visualized and quantified using ImageLab Inc Software (Bio-Rad, CA). β-Tubulin levels (mouse anti-β-tubulin, 1:1000; Sigma) were used as gel-loading controls and the results are presented relative to the control apoE3 mice.

2.4. Assessment of apoE lipidation

Brain extracts were run on 4% to 16% gels purchased from Novex in the NativePAGE Novex Bis-Tris Gel System according to the manufacturer's instructions and as previously described [30]. Gels were then transferred to polyvinylidene difluoride (PVDF) membranes and stained with goat anti-apoE Ab (1:10,000; Millipore). The immunoblot bands were all visualized using the ECL chemiluminescent substrate (Pierce).

2.5. Quantitative reverse transcription PCR (qRT PCR) analysis

qRT-PCR analysis was performed as described previously [31]. In brief, the hippocampus was rapidly excised from one freshly removed hemisphere and stored frozen at −70°C until use. RNA was extracted from the tissue using the EZ-RNA total RNA isolation kit (Biological Industries). RNA was transformed into complementary DNA using the High Capacity complementary DNA reverse transcription kit (Applied Biosystems). TaqMan qRT-PCR assays were conducted according to the manufacturer's specifications (Applied Biosystems). Oligonucleotides (probes) for TaqMan qRT-PCR were attached to 6-carboxyfluorescein at the 5′-end and a quencher dye at the 3′-end. ApoE, α-syn, synaptophysin, ApoER2, VGaT, and VGluT1 gene expression levels were determined using TaqMan qRT-PCR specific primers (Applied Biosystems, Foster City, CA, USA). Analysis and quantification were conducted using the 7300 system software and compared with the expression of the housekeeping HPRT-1 gene.

2.6. Novel object recognition test

This was performed as described previously [32]. In brief, the mice were first placed in an arena (60 × 60 cm with 50 cm walls) in the absence of objects, after which two identical objects were added. Twenty-four hours later, the mice were reintroduced to the arena in which one of the objects was replaced by a novel one. The behavior of the mice was then monitored using the EthoVision XT 11.5 program for 5 minutes, and the time and number of visits that the mice paid to each of the objects were measured. The results are presented as the ratio in the percentage of the number of visits to the novel object relative to the total number of visits to both new and old objects.

2.7. Statistical analysis

The experimental design consisted of three genotypes (apoE3, apoE4, and apoE3/E4) and sex (male and female) on the background of either α-syn+/+ or α-syn−/−. The results were analyzed using one-way analysis of variance (ANOVA) for the three apoE genotypes and two-way ANOVA for the comparison of homozygous apoE3 and apoE4 males or females, with or without α-syn gene. The analysis was performed using STATISTICA software (Version 8.0 StatSoft, Inc, Tulsa, OK). Only after the ANOVA analysis retrieved significant results, was further post hoc Tukey analysis performed to test for individual effects, and these findings are depicted in the figures. A minimum of two cohorts containing all apoE isoforms were used for each condition (sex and α-syn background); each group contained either five to nine or eight to 10 mice. The histologic and biochemical results obtained with the different cohorts were similar, and they are presented jointly after normalization of each of the experiments relative to the apoE3 control group. Similar results were obtained when the cohorts were analyzed separately, and thus no covariance was conducted. The histologic and biochemical results obtained were normalized to the apoE3 control group.

3. Results

Immunoblot measurements revealed that the levels of brain apoE were similar in the α-syn+/+ and α-syn−/− mice and that, in accordance with previous observations, they were lower in the apoE4 than in the apoE3 mice (Fig. 1). Two-way ANOVA of these results revealed a significant effect of apoE genotype ($P < .0001$) in both males (Fig. 1A) and females (Fig. 1B). Further post hoc analysis revealed that the levels of apoE were significantly lower in the apoE4 mice compared with the apoE3 mice ($P < .05$ for α-syn−/− and α-syn+/+ males, $P \leq .02$ for α-syn−/− and α-syn+/+ females). qRT-PCR analysis of

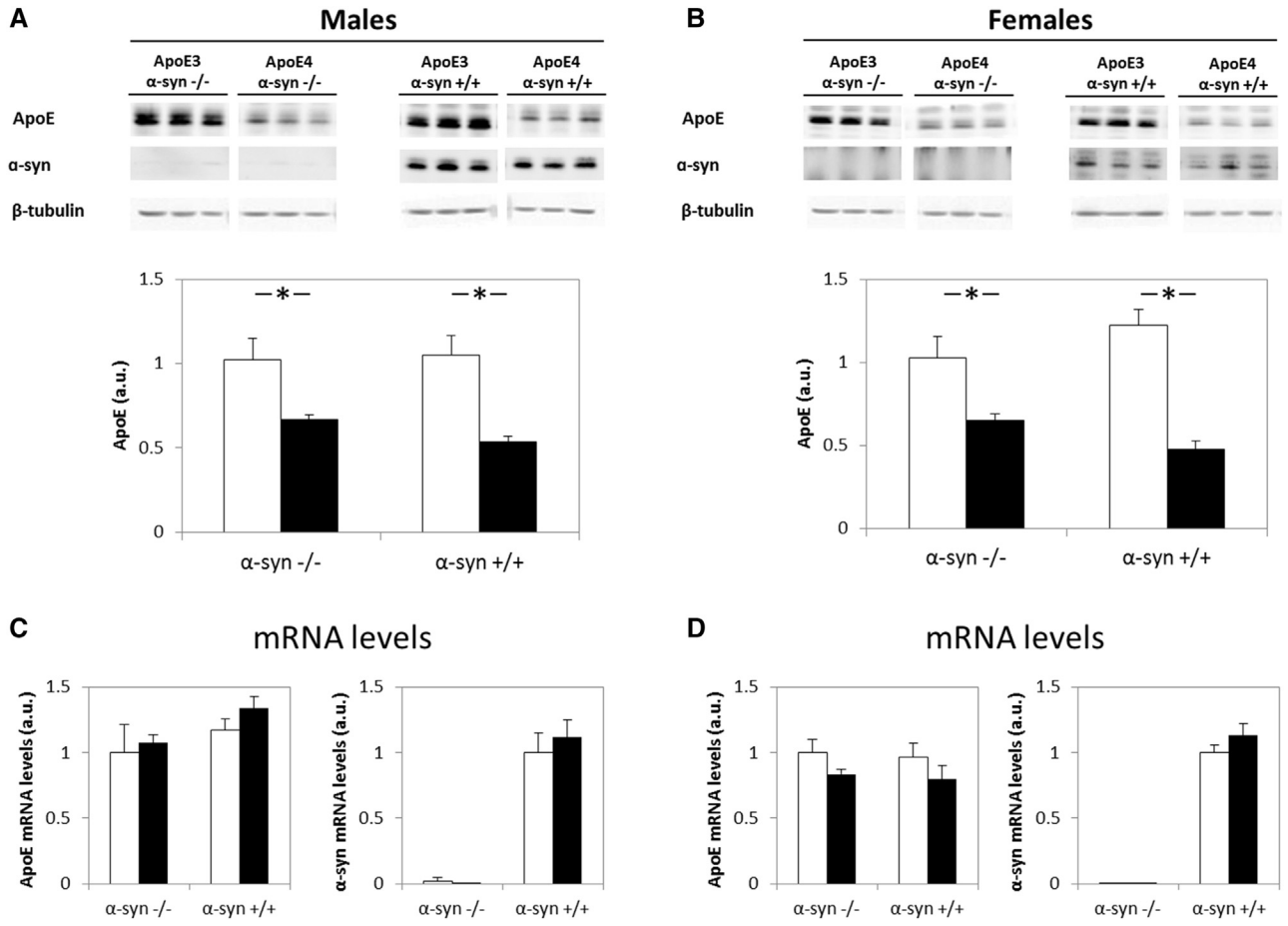

Fig. 1. The levels of apoE and α-syn in the hippocampus of apoE4 and apoE3-TR mice. Hippocampi of 4-month-old male and female apoE3 and apoE4 mice with or without α-syn were excised, homogenized, and subjected to apoE and α-syn immunoblotting and qRT-PCR, as described in Section 2. Representative apoE, α-syn, and loading standard β-tubulin bands of α-syn–deficient or normal α-syn males (A) and females (B) apoE3 (white bars) and apoE4 (black bars) mice are presented. Quantitations of the apoE protein levels (mean ± SEM; $n = 5$ per group) are normalized relative to the α-syn−/− apoE3 mice of each sex. As can be seen, the levels of apoE were higher in the apoE3 than the apoE4 mice, regardless of α-syn status or sex. qRT-PCR measurement of α-syn and apoE in males (C) and females (D) show no difference in mRNA levels of α-syn and apoE between the different genotypes (mean ± SEM; $n = 4$ per group). Abbreviations: apoE, apolipoprotein E; α-syn, α-synuclein; mRNA, messenger RNA; SEM, standard error of the mean. *P <.05 for the effect of genotype on apoE levels.

the apoE and α-syn gene levels shows no difference in gene expression between apoE3 and apoE4 mice. As expected, α-syn expression is null in the α-syn−/− mice.

It has previously been shown that apoE4 is hypolipidated relative to apoE3 and that the pathologic effects of apoE4 can be counteracted by treatments that reverse this hypolipidation [30,33,34]. We therefore next examined the extent to which this "upstream" effect of apoE4 is affected either by sex or by α-syn deficiency. As can be seen in Fig. 2, apoE4 mice present with lower molecular apoE species compared with apoE3, in both females and males, and this was not affected by the α-syn background.

The possibility that the secondary downstream effects of apoE4 are affected by the presence or absence of α-syn was next examined. This was pursued by focusing on AD and neuronal-related parameters that have previously been shown to be affected by apoE4 in the hippocampal CA3 area [18]. As shown in Fig. 3, the levels of the general presynaptic marker synaptophysin and of the glutamatergic and gabaergic presynaptic markers, VGluT1, and VGaT, as well as those of the apoE receptor ApoER2, were significantly downregulated in female apoE4 α-syn−/− mice compared with the corresponding apoE3 mice. The same decrease was observed in apoE3/E4 heterozygous mice, suggesting that the effects of apoE4 are already saturated in the heterozygous mice. One-way ANOVA of these results revealed a significant effect of genotype ($P < .05$ for synaptophysin and VGaT and $P < .005$ for VGluT1 and ApoER2). Further post hoc analysis revealed that the levels of these markers were significantly lower in the apoE4 and apoE3/E4 mice compared with the apoE3 mice ($P \leq .05$ for synaptophysin; $P = .0001$ for VGluT1; $P \leq .05$ for VGaT, and $P \leq .02$ for ApoER2). Gene expression levels of these neuronal and synaptic markers were analyzed using qRT-PCR. As can be seen in Fig. 3E, no difference was observed in gene expression levels between the apoE3 and apoE4 female mice, suggesting that the apoE4-driven effects are apparent only on the protein level.

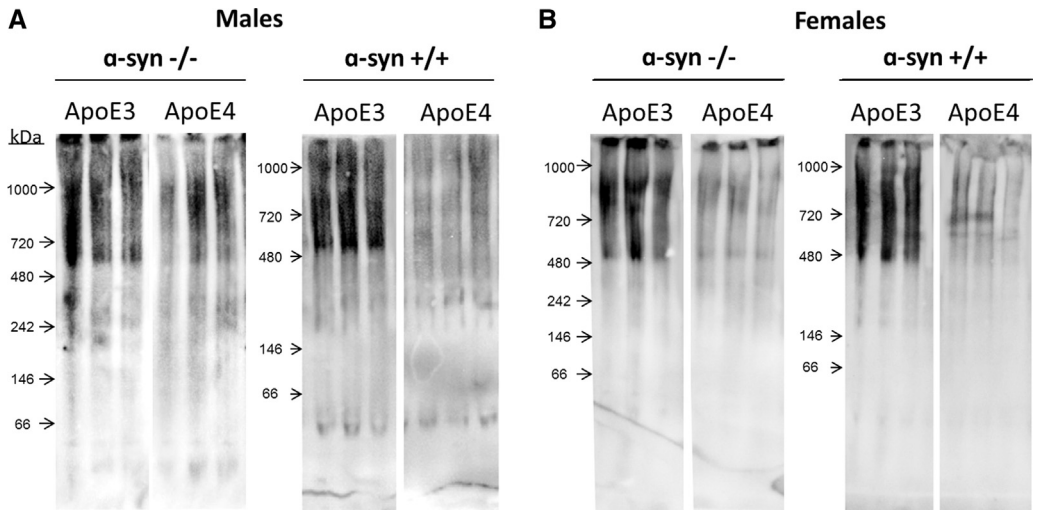

Fig. 2. The effects of apoE genotype and α-syn on the lipidation of apoE. Hippocampal homogenates of α-syn–deficient or normal α-syn apoE3 and apoE4 male (A) and female (B) mice were subjected to a blue native gel and stained with anti-apoE Ab as described in Section 2. Representative immunoblots of three mice per group are presented. As can be seen, the apoE3 mice, both male and female, contain higher levels of high molecular weight apoE and this effect is maintained independently of the presence or absence of α-syn. Abbreviations: Ab, antibody; apoE, apolipoprotein E; α-syn, α-synuclein.

The effects of apoE4 on the AD hallmarks, Aβ42, and hyperphosphorylated tau, as well as on the glial and vascular markers GFAP and collagen IV, respectively, of female α-syn−/− mice are depicted in Fig. 4. As can be seen, apoE4 was associated with increased accumulation of Aβ42 and AT8, as well as increased levels of GFAP positive astrocytes in hippocampal CA3 neurons. One-way ANOVA of these results revealed a significant effect of genotype

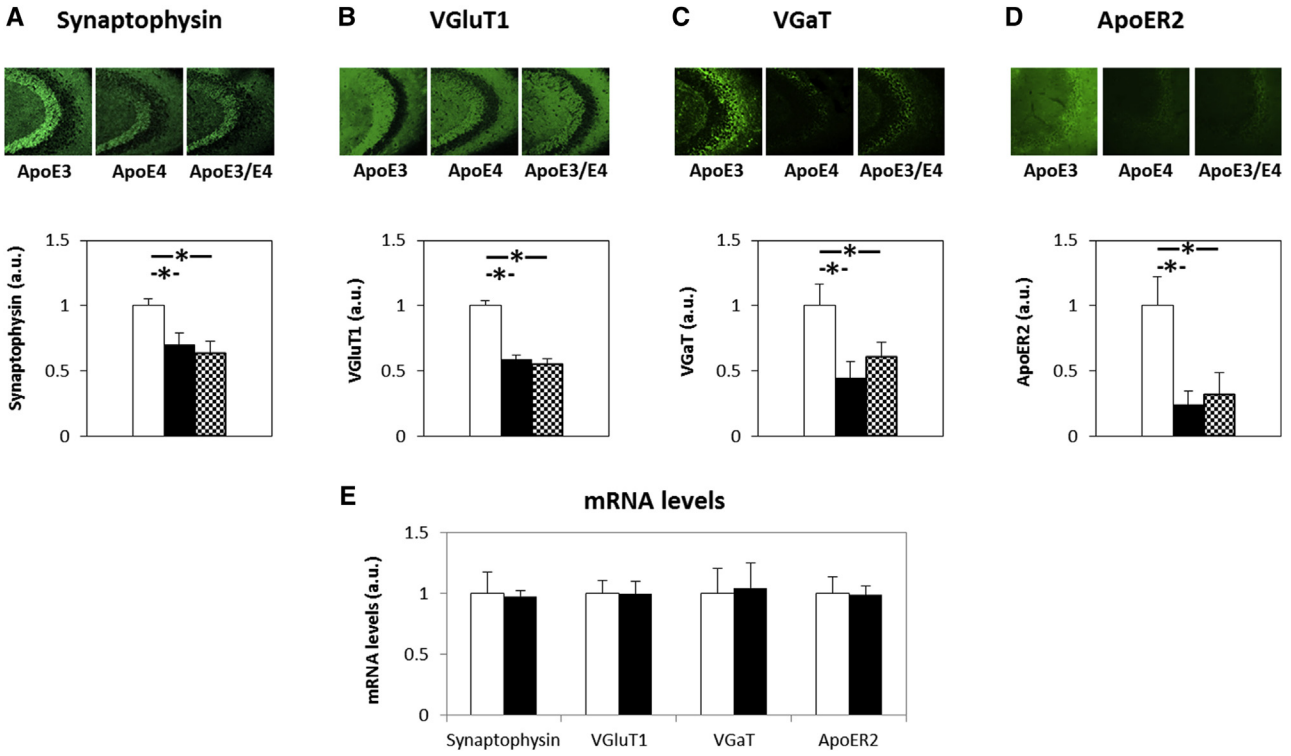

Fig. 3. The effects of apoE genotype on the levels of the synaptic marker synaptophysin, VGluT1, VGaT, and ApoER2 in α-syn–deficient female mice. Brains of apoE3, apoE4 homozygous, and apoE3/E4 heterozygous female mice were subjected to histologic staining with anti-synaptophysin (A), anti-VGluT1 (B), anti-VGaT (C), and anti-ApoER2 (D) Abs. Representative images (20 × magnification) of the CA3 hippocampal subfield are presented in the upper part of each panel and show reduced levels in both apoE4 and apoE3/E4 mice compared with apoE3 mice. The results (mean ± SEM; n = 7–10 per group) of apoE3 mice (white bars), apoE4 mice (black bars), and apoE3/E4 (checkered bars) were quantified by computerized image analysis. qRT-PCR of the synaptic parameters (E) show no difference between apoE genotypes in the mRNA expression levels. The results shown were all normalized relative to control apoE3 mice (mean ± SEM; n = 4 per group). Abbreviations: Abs, antibodies; apoE, apolipoprotein E; α-syn, α-synuclein; mRNA, messenger RNA; SEM, standard error of the mean. *P <.05 for the effect of genotype on the levels of the markers.

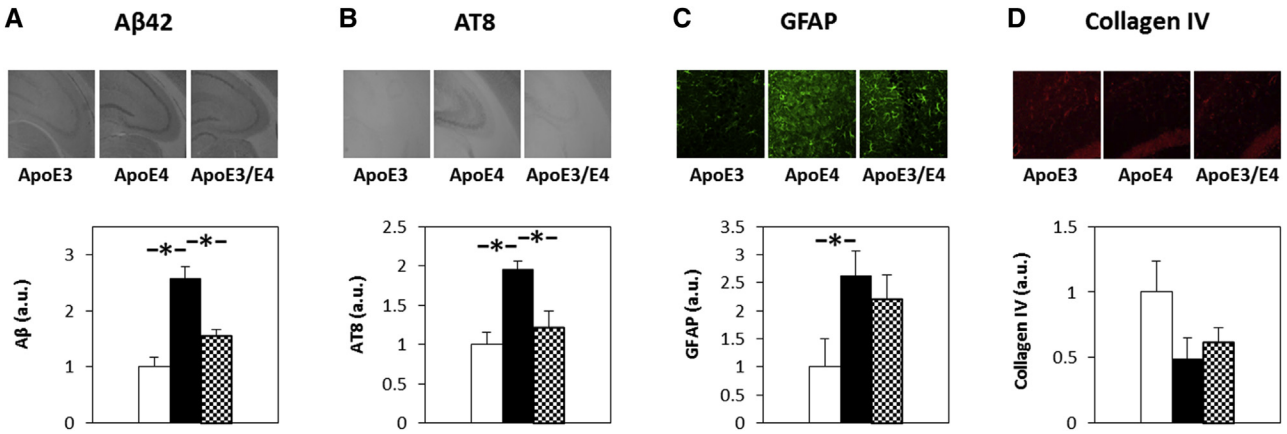

Fig. 4. The effects of apoE on the levels of Aβ42, phosphorylated tau, glial, and vascular marker genotype in α-syn–deficient female mice. Brains of apoE3, apoE4 homozygous, and apoE3/E4 heterozygous female mice were subjected to histologic staining with anti-Aβ42 (A), anti-AT8 mAb (B), which specifically recognizes the phosphorylated Ser202/Thr205 tau epitope, anti-GFAP (C), and anti-collagen IV (D) Abs. Representative images (10 ×magnification) of the CA3 hippocampal subfield are presented for Aβ42, AT8, and GFAP, whereas the collagen IV staining and analysis were performed in the stratum lacunosum molecular area of the hippocampus. The results (mean ± SEM; n = 7–10 per group) of apoE3 mice (white bars), apoE4 mice (black bars), and apoE3/E4 (checkered bars) were quantified by computerized image analysis as described in Section 2. As seen, the AD-related phenotypes of the apoE4 mice, namely, high levels of Aβ42, tau phosphorylation, and the glial marker GFAP, are not present in the heterozygous mice. The results shown are normalized relative to control apoE3 mice. In contrast, the levels of the vascular marker collagen IV are reduced in both apoE4 and apoE3/E4 mice. Abbreviations: Abs, antibodies; apoE, apolipoprotein E; α-syn, α-synuclein; SEM, standard error of the mean. *$P \leq$.05 for the effect of genotype on the levels of the markers.

($P < .0001$ for Aβ42, $P = .001$ for AT8, and $P = .05$ for GFAP). Further post hoc analysis revealed that the levels of these markers were significantly higher in the apoE4 compared with the apoE3 and apoE3/E4 mice ($P \leq .003$ for Aβ42, $P \leq .007$ for AT8, and $P = .05$ for GFAP). The levels of the vascular marker, collagen IV, were also affected by the apoE genotype in the female α-syn−/− mice except that its levels were reduced in the apoE4 mice, although not significantly (Fig. 4D). Furthermore, unlike the synaptic-related phenotypes (Fig. 3), the effects of apoE4 on the AD parameters and on GFAP (Fig. 4A–C) in the apoE3/E4 heterozygote α-syn−/− mice were in general intermediate to those observed in the apoE4 and apoE3 homozygous mice. The levels of collagen IV were similarly reduced in the apoE4 and apoE3/E4 mice (Fig. 4D). Taken together, these experiments show that the vascular and synaptic effects of apoE4 in the α-syn−/− mice (Figs. 3 and 4D) share the same gene dose dependency and are already maximal in the apoE3/E4 heterozygous mice, whereas the effects of apoE4 on Aβ42, tau, and GFAP are only partial under these conditions. Complementary experiments using male apoE4 and apoE3 homozygous mice on α-syn−/− background revealed a similar apoE4 phenotype, except that the magnitude of the effect was smaller and with greater variability.

The extent to which the expression of the apoE4 phenotype is affected by α-syn deficiency was next examined using female apoE4 and apoE3 mice on either α-syn+/+ or α-syn−/− background. As can be seen in Fig. 5, apoE4 had a much smaller effect in the female α-syn+/+ mice than the apoE4 α-syn−/− mice, when compared with the corresponding apoE3 mice. Specifically, the difference in the hippocampal levels of the AD-related phenotypes Aβ42 and AT8, as well as the synaptic and neuronal markers, VGluT1 and ApoER2, between apoE4 and apoE3 mice was less pronounce in the α-syn+/+ mice than that observed with the mice on the α-syn−/− background, suggesting that apoE4 and α-syn ablation have a synergistic effect.

Finally, female apoE3 and apoE4 mice either expressing α-syn or α-syn−/− were subjected to the novel object recognition test, in which their tendency to approach a novel object was measured. ApoE3 α-syn−/− mice showed a preference to the novel object, whereas the corresponding apoE4 mice showed no such preference, suggesting their inability to discriminate between old and new objects (Fig. 6). In contrast, there was no significant effect of apoE genotype on the performance of the corresponding α-syn+/+ female mice in this test. Two-way ANOVA of these results revealed a significant effect of genotype ($P = .05$). Further post hoc analysis revealed that the apoE3 α-syn −/− made significantly more visits to the novel object compared with the corresponding apoE4 mice ($P = .04$).

Taken together, these results suggest that the effects of apoE4 are accentuated by α-syn deficiency and that these effects are not mediated by direct effects on the apoE4 molecule but rather by a second hit mechanism, which affects downstream targets of apoE4.

4. Discussion

ApoE4 and apoE3-TR mice were used to investigate the effects of α-syn deficiency and sex on the phenotypic expression of apoE4. This revealed that the hypolipidation of apoE4 is not affected by α-syn deficiency and that it is the same in female and male apoE4 mice. In contrast, the brain pathological effects of apoE4, namely, reduced levels of the synaptic markers, synaptophysin, VGluT1, VGaT, and of the

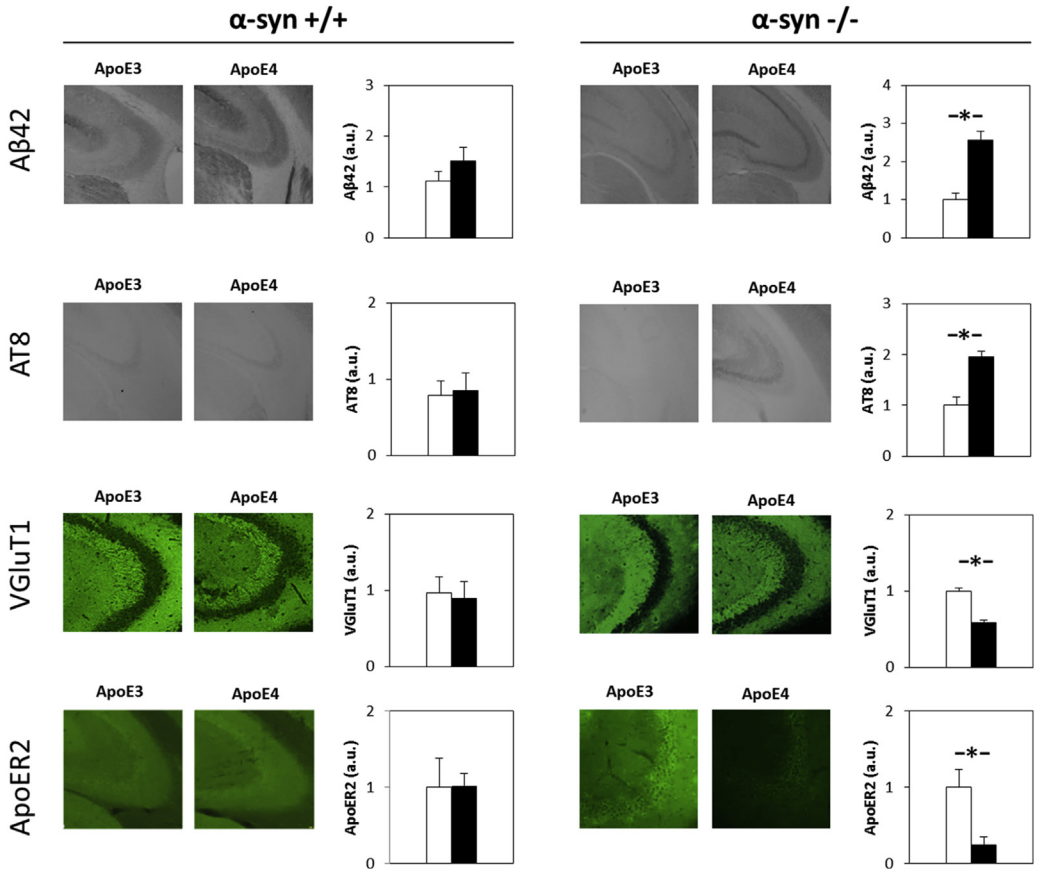

Fig. 5. The effects of apoE genotype on the levels of Aβ42, phosphorylated tau, VGluT1, and ApoER2 in α-syn expressing and α-syn–deficient female mice. Brains of apoE3 and apoE4 homozygous female mice that express normal levels of α-syn (left panel) or are α-syn–deficient (right panel) were subjected to histologic staining with anti-Aβ42, anti-AT8 mAb, which specifically recognizes the phosphorylated Ser202/Thr205 tau epitope, anti-VGluT1, and anti-ApoER2 Abs, as described in Section 2. The results (mean ± SEM; n = 5–9 per group) of apoE3 mice (white bars) and apoE4 mice (black bars) were quantified by computerized image analysis and are presented relative to the apoE3 mice of the corresponding α-syn background. This revealed that in α-syn+/+ mice, unlike the α-syn−/− mice, no statistically significant difference between apoE3 and apoE4 mice was observed. Abbreviations: Abs, antibodies; apoE, apolipoprotein E; α-syn, α-synuclein; SEM, standard error of the mean. *P <.05 for the effect of genotype on the levels of the markers.

apoE receptor ApoER2, as well as increased levels of the AD hallmarks Aβ42, and phosphorylated tau and the astrocytic marker GFAP in hippocampal neurons were much more pronounced in α-syn−/− apoE4 mice than in corresponding apoE4 α-syn+/+ mice. These effects were more pronounced in homozygous α-syn−/− apoE4 mice than in corresponding apoE3/E4 heterozygous mice and in female more than in male mice. Taken together, these findings show that α-syn deficiency does not affect the upstream lipidation-related impairments of apoE4, but that it acts as a "second hit" enhancer of the subsequent pathologic effects of apoE4. The second hit hypothesis proposes that apoE4-driven pathologies are exacerbated in the presence of additional risk factors or stressors, such as old age [35,36], environmental factors [37,38], female sex [39,40], and other genetic modulation [41]. In the present study, we suggest α-syn deficiency as such a second hit. α-Syn absence on its own is insufficient to exert the pathologic phenotypes observed in our model, as indicated by several lines of α-syn knockout mice that show only a very mild phenotype [42–44], which could be attributed to functional redundancy among α-syn, β-synuclein, and γ-synuclein [45,46]. Thus, the coexistence of apoE4 α-syn deficiency may bring to light pathologic phenotypes that have been previously undetected. Accordingly, it should be noted that recent studies from our laboratory were performed using the Harlan mice, namely, male apoE3 and apoE4 α-syn−/−, and therefore the concept of the double-hit hypothesis is relevant to them as well.

4.1. α-syn deficiency and apoE4

This apoE4-α-syn deficiency double-hit hypothesis described previously could stem from multiple synergistic mechanisms, such as impaired synaptic transmission, proinflammatory and phagocytic regulation, and lipid binding.

Although previous studies of α-syn−/− mice are inconsistent with regard to neuropathologic or behavioral phenotypes, further detailed analysis revealed aberrations in the presynaptic function of these mice. Accordingly, α-syn deficiency results in deficits in the nigrostriatal system and impaired activity-dependent regulation of dopaminergic

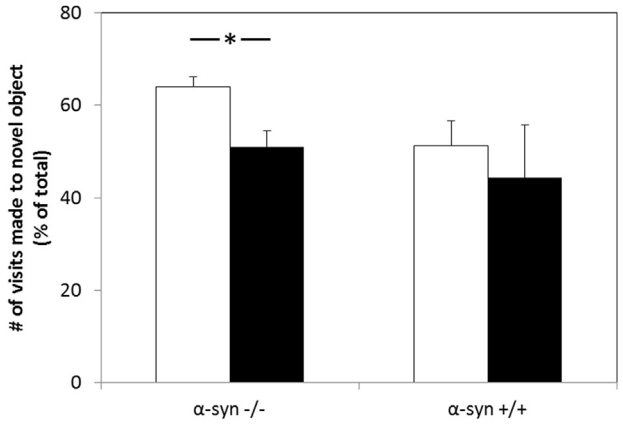

Fig. 6. The effects of apoE genotype on performance of α-syn expressing and α-syn–deficient female mice in the novel object recognition test. ApoE3 and apoE4 homozygous female mice that are α-syn–deficient or express normal levels of α-syn were first exposed to two identical objects, followed by a delay of 24 hours, after which the mice were exposed to an old and a new object. The preference of the mice to the different objects was monitored, as described in Section 2. The results obtained are depicted as the percent of visits made to the novel object out of the total number of visits to both familiar and novel objects. White bars correspond to apoE3 mice, whereas black bars correspond to apoE4 mice (mean ± SEM; $n = 10$ mice). As seen, the α-syn–deficient apoE4 mice are unable to discriminate between old and novel objects. Abbreviations: apoE, apolipoprotein E; α-syn, α-synuclein; SEM, standard error of the mean. *$P < .05$ for the effect of genotype.

and nondopaminergic transmission [42], as well as the loss of synaptic proteins, such as synaptotagmin, during aging [47]. Furthermore, α-syn deficiency impairs the synaptic response to repetitive stimulation, which was associated with a marked reduction in the pool of reserve synaptic vesicles, specifically diminished mobilization of glutamate from the reserve pools [48]. These findings and the observation that α-syn binds specifically to distinct presynaptic proteins such as VAMP2/synaptobrevin and Rab3 suggest that α-syn plays an important role in the maintenance and function of the nerve terminal and the release machinery [49].

Previous in vivo studies and corresponding cell culture studies revealed that nerve terminals are particularly susceptible to apoE4 [50,51]. Accordingly, the present findings that the synaptic pathologic effects of apoE4 are specifically enhanced by α-syn deficiency are very likely driven by an interaction between apoE4 and α-syn deficiency, which synergistically affects presynaptic mechanisms.

With regard to the Aβ accumulation, it has been shown that amyloid plaques are increased in the absence of α-syn at old age, suggesting that α-syn may serve as a chaperone helping the cells to clear protein deposits [24]. This is consistent with the accumulation of Aβ42 presently observed in the apoE4 mice lacking α-syn, which may be the synergistic result of two faulty Aβ-clearance mechanisms. This in turn may lead to the hyperphosphorylation of tau either by apoE4-driven mechanisms [52] or through the Aβ peptide [53].

Another possible mechanism of interaction between apoE and α-syn is based on their effects on microglial proinflammatory activation. The lack of α-syn has been shown to affect the morphology and activation of microglia. Specifically, α-syn−/− microglia have a vacuolar morphology with increased cytokine secretion and decreased phagocytic ability [54], which has been suggested to be mediated via α-syn–driven regulation of microglial secretory behavior and expression of a subset of lipid-signaling–associated enzymes [55]. Proinflammatory pathways have also been shown to be induced by apoE4. Specifically, the proinflammatory PGE2 pathway was shown to be robustly activated in apoE4-TR mouse microglia. Furthermore, the innate immune suppressor, *TREM2*, showed highly decreased microglia expression in apoE4 cells compared with apoE3 [56]. Together, these data demonstrate that both α-syn absence and apoE4 contribute to proinflammatory activation of microglia, which may in turn lead to neurotoxicity and neuronal impairment. However, preliminary results from our laboratory show no significant difference in the levels of the microglial marker Iba1 between apoE3 and apoE4 α-syn−/− mice (data not shown), suggesting that this perhaps is not the main mechanism by which apoE4 and α-syn exert their synergistic effect.

The lipid-protein interaction of both α-syn and apoE plays an important role in their biological function. α-Syn interacts with membranes and the binding strength is related to the specificity of the lipid environment [25]. It has been shown that α-syn gene ablation in astrocytes significantly effects cellular fatty acid incorporation, fractional distribution, and esterification that are fatty acid dependent [57]. Because apoE is the main brain lipid transporter [26] and the lipidation of apoE4 is impaired relative to that of its AD benign isoform apoE3 [31,33,34], it is possible that the presently observed synergistic synaptic effects of apoE4 and α-syn deficiency are lipid dependent and take place at the membrane level. However, it cannot be excluded that the effects of α-syn deficiency on the composition of fatty acids in the membrane may affect the composition of the apolipoprotein particle itself, thus suggesting a more direct role of α-syn deficiency on the apoE4-driven pathologies.

Additional studies are required to better understand the mechanisms underlying the cross talk between apoE4 and α-syn.

4.2. Sex

The three greatest risk factors for AD are age, apoE4 genotype, and female sex [58]. The present findings that α-syn−/− female mice have a more pronounced apoE4 phenotype than the corresponding male mice is consistent with previous findings that were observed with α-syn+/+ apoE4 mice [59,60], suggesting that these apoE4-driven phenotypes are independent of synuclein background status. These findings are also in accordance with human studies, which revealed that the apoE4-driven risk for AD is substantially higher in women relative to men [6]. The fact that many of the apoE4 pathologies are synaptic is consistent with previous studies, showing that presynaptic density in the dentate gyrus (DG) subsection of the hippocampus is decreased only in female apoE4 mice,

and that they also present with synaptic mitochondrial damage [61,62]. Although the finding that female sex and apoE4 interact is well established, the mechanism underlying it is still unknown and remains to be determined [6].

4.3. Heterozygosity in α-syn−/− mice

Similarly to AD, the presently observed effects of apoE4 in the mice were allele dose dependent. Assessment of these results in the female α-syn−/− mice, where the effects of apoE4 were most pronounced, revealed differences in the apoE4 gene dosage dependency of the apoE4 phenotypes. Accordingly, the AD-related phenotypes, namely, the accumulation of Aβ and hyperphosphorylated tau in hippocampal neurons, were apparent in the apoE4 homozygous mice but not in the corresponding apoE3/E4 heterozygous mice (Fig. 4). In contrast, the effects of apoE4 levels on the neuronal presynaptic parameters, synaptophysin, VGluT1, VGaT, ApoER2, the glial marker GFAP, and the vascular-related parameter, collagen IV, were already maximal in the apoE3/E4 mice. The presently observed allele dependency of the effect of apoE4 on Aβ42 is in accordance with a previous finding obtained in apoE × APP mice where apoE4 homozygosity stimulates the accumulation of Aβ in the hippocampus, and this effect is abolished by halving the levels of apoE4 [63]. The findings that the neuronal parameters, but not tau and Aβ42, are already maximal in the apoE3/E4 mice could be because of differences in the apoE4-dose response of these phenotypes. Alternatively, because α-syn is a presynaptic protein, the increased susceptibility of the synaptic parameters to apoE4 may be because of a deficiency of this protein. Further studies are needed to unravel the mechanisms underlying the difference in the apoE4 allele dose dependency of the synaptic and AD hallmark-related apoE4 phenotypes and the extent to which they are because of the involvement of various molecular pathways.

4.4. Clinical implications

A link between apoE4, which is associated with earlier onset of PD and a higher risk of dementia [64], and PD dementia, which is associated with both α-syn and AD-type pathologies [65], is now well recognized. This was initially thought to be mediated via the interaction of apoE4 with the AD-like pathologic aspects of PD dementia [66]. However, the recent report that apoE4 increases the risk for dementia in pure synucleinopathies [67] suggests that a more direct pathologic cross talk between apoE4 and α-syn occurs. The molecular mechanisms underlying the role of α-syn in synucleinopathies are not fully understood. There is compelling evidence that the pathologic effects of α-syn are produced via a gain-of-function toxic mechanism driven by the aggregation of this molecule. It has, however, also been suggested that loss of the normal physiological functions of α-syn may play a critical role [45]. The present study provides a model for studying the mechanisms underlying loss-of-function–related effects of α-syn and apoE4. Integrating these results with future double transgenic studies using mice that express the human apoE isoforms together with PD-related mutants of α-syn [68] will enable the in vivo modeling of both the gain- and loss-of-function aspects of the interaction of α-syn with apoE4.

In conclusion, the present findings show that the pathologic effects of apoE4 in TR mice are accentuated by α-syn deficiency and that the effects of apoE4 are gene dose dependent and are more pronounced in females. These findings have practical implications, in that they describe a new apoE4 mouse model in which the pathologic effects of apoE4 are robust and can be studied at a young age. They also pave the way for future model studies of the mechanisms underlying the cross talk between apoE4 and α-syn in PD and related synucleinopathies.

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RESEARCH IN CONTEXT

1. Systematic review: Apolipoprotein E4 (apoE4) is the most prevalent genetic risk factor for Alzheimer's disease (AD), whereas α-synuclein (α-syn) is a key player in synucleinopathies. Because these diseases share common pathologies, we presently examined the extent to which α-syn affects the phenotypic expression of apoE4. Experiments using mice that express apoE4 with or without α-syn revealed that the pathologic phenotype of apoE4, namely, its hypolipidated state, was not affected by α-syn, but that α-syn deficiency increased the associated neuropathologic effects of apoE4 when compared with apoE3.
2. Interpretation: These findings suggest that the trigger for apoE4-mediated pathology, which is driven by hypolipidation of apoE4, is not affected by α-syn deficiency, which, however, serves as a downstream second hit that amplifies the pathologic consequences of apoE4.
3. Future directions: The α-syn and apoE4 interaction will be further studied by crossing the apoE4 mice with transgenic models of synucleinopathies expressing human, mutated, or truncated α-syn.

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