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Bidirectional modulation of Alzheimer phenotype by alpha-synuclein in mice and primary neurons

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Abstract

α -Synuclein (α Syn) histopathology defines several neurodegenerative disorders, including Parkinson's disease, Lewy body dementia, and Alzheimer's disease (AD). However, the functional link between soluble α Syn and disease etiology remains elusive, especially in AD. We therefore genetically targeted α Syn in APP transgenic mice modeling AD and mouse primary neurons. Our results demonstrate bidirectional modulation of behavioral deficits and pathophysiology by α Syn. Overexpression of human wild-type α Syn in APP animals markedly reduced amyloid deposition but, counter-intuitively, exacerbated deficits in spatial memory. It also increased extracellular amyloid- β oligomers (A β Os), α Syn oligomers, exacerbated tau conformational and

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Conflicts of Interests

The authors have no conflicts of interests in relation to this manuscript.

phosphorylation variants associated with AD, and enhanced neuronal cell cycle re-entry (CCR), a frequent prelude to neuron death in AD. Conversely, ablation of the *SNCA* gene encoding for α Syn in APP mice improved memory retention in spite of increased plaque burden. Reminiscent of the effect of *MAPT* ablation in APP mice, *SNCA* deletion prevented premature mortality. Moreover, the absence of α Syn decreased extracellular A β O_s, ameliorated CCR, and rescued postsynaptic marker deficits. In summary, this complementary, bidirectional genetic approach implicates α Syn as an essential mediator of key phenotypes in AD and offers new functional insight into α Syn pathophysiology.

Keywords

Amyloid- β ; α -Synuclein; Tau; Alzheimer's disease; Spatial memory; Neuronal cell-cycle re-entry

Introduction

Alzheimer's disease (AD) is neuropathologically defined by extracellular amyloid- β (A β) plaques and intracellular neurofibrillary tangles of tau. At the molecular level, the soluble A β and tau aggregates that precede plaques and tangles are potently cytotoxic and are therefore considered to be the primary drivers of neurodegeneration. Soluble multimeric A β and tau assemblies, or oligomers, coordinately promote synapse loss, memory deficiencies, and ectopic neuronal cell cycle re-entry (CCR), a precursor to neuron death in AD [6, 37, 44, 45, 48]. Alongside A β and tau, soluble alpha-synuclein (α Syn) is also strongly linked to memory deficits in AD, as well as in Parkinson's disease (PD) and Lewy body dementia (LBD) [1, 3, 18, 30, 40], suggesting an intrinsic contribution of α Syn to brain pathophysiology. However, the conformational state(s) and functions of soluble α Syn is particularly unclear in AD. Furthermore, the relationship of α Syn to A β oligomer (A β O)- and/or tau-induced neuronal dysfunction remains controversial, primarily due to conflicting observations.

To develop a better understanding of the contribution of α Syn to the core phenotypic features of AD (i.e., amyloid deposition, synaptic dysfunction, and subsequent cognitive dysfunction), we applied a bidirectional genetic approach to rigorously determine the role of α Syn in a widely-used amyloid precursor protein (APP) transgenic mouse model of AD and in mouse primary cortical neuron cultures. This approach is routinely used in genetic studies with simpler organisms, such as worms and flies, to examine genes modifying a given phenotype, but rarely in mammals due to the time and effort commitment it requires. We generated bigenic mice that either co-express human APP with human wild-type α Syn (APP/ α Syn), or APP transgenic animals lacking α Syn by ablating the murine *SNCA* gene (APP/ α Syn-KO). We then performed behavioral, histopathological, candidate-driven protein expression and cell biological analyses to unravel functional roles of α Syn in AD pathogenesis. Our results demonstrate that human wild-type α Syn expression in APP mice exacerbates spatial learning and memory deficits, increases soluble A β O_s, pathological α Syn and tau proteins, and potentiates both synaptic protein loss and ectopic neuronal CCR. By contrast, ablation of α Syn in APP mice rescues premature mortality, prevents learning and memory deficits, decreases pathological tau species, prevents loss of postsynaptic

GluN2A and Drebrin proteins, and ameliorates neuronal CCR. Thus, our results reveal α Syn as a core modulator of AD pathogenesis and have direct implications for other α -synucleinopathies.

Materials and Methods

Transgenic animals

Three transgenic lines were used: (i) TgI2.2 mice expressing the wild type form of human α -synuclein under the control of the mouse prion promoter [32], (ii) *SNCA*-null mice [2] and (iii) J20 (originally called hAPPJ20) mice [36]. *SNCA*-null mice were obtained from Jackson laboratories and backcrossed to C57BL6/J for greater than 10 generations. Every 6 months, the homozygous KO mice are outbred to wild-type C57BL6/J and homozygous KO mice are reconstituted from mating of heterozygote animals. Animals were then transferred from Michael K. Lee, University of Minnesota to Sylvain Lesné, University of Minnesota. Bigenic J20xTgI2.2 mice resulted from the mating of TgI2.2 and J20 mice. All lines used were in the C57BL6 background strain. Both male and female animals were used in equal numbers for biochemical studies and Barnes Maze behavioral testing. All animal procedures and studies were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee and Institutional Review Board.

Protein extractions

Soluble aggregation-prone protein levels in brain tissue were analyzed using the extraction protocol previously described [34, 50], with a detailed 32-step-protocol explained in the latter. The lysis process fractionates proteins based on their cellular compartmentalization. The sequential separation allows the recovery of a predicted protein in its compartment of 75–90% [31, 34]. Briefly, dissected frozen hemi-forebrain tissues (125–200 mg) are gently dissociated in NP40-lysis buffer (50 mM Tris-HCl [pH 7.6], 0.01% NP-40, 150 mM NaCl, 2mM EDTA, 0.1% SDS) and centrifuged at $800 \times g$, to separate extracellular proteins contained in the supernatant. The remaining loose pellet is then lysed with TNT-lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100), and centrifuged at $16,100 \times g$, to separate intracellular proteins present in the aqueous phase. The subsequent pellet is finally dissociated in RIPA-lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 3% SDS, 1% deoxycholate) and centrifuged at $16,100 \times g$, to separate membrane-bound proteins present in the supernatant. All supernatants were ultra-centrifuged for 20 minutes at $100,000 \times g$. Before analysis, fractions were depleted of endogenous immunoglobulins by incubating lysates with 50 μ L of Protein A-Sepharose, Fast Flow® beads for one hour at 4° C, followed by 50 μ L of Protein G-Sepharose, Fast Flow® beads (GE Healthcare Life Sciences). Protein amounts were determined with the Bicinchoninic acid protein assay (BCA Protein Assay, Pierce™).

Antibodies

The following primary antibodies were used in this study: 6E10 [1:2,000], 4D6 anti- α -Synuclein [1:500], LB509 [1:5,000–10,000], and Tau-5 (Catalog nos. SIG-803003, SIG-39720, SIG-39725 and SIG-39413, BioLegend), anti-MAP2 [1:500] (Catalog no. NB300-213, Novus), anti-Cyclin D1 [1:120], anti-MAP2 [1:2000] (Catalog nos. ab16663

and ab92434, Abcam), anti- β -III-Tubulin (TUJ1) [1:5000] (gift from Anthony Spano, University of Virginia), anti-NeuN [1:500], anti-actin (C4) [1:10,000], anti-SYP [1:25,000] (Catalog nos. MAB377 and MAB1501, EMD Millipore). anti-PCNA [1:200] anti-PSD95 [1:200], anti-GluN1 [1:1000], anti-GluN2A [1:1000], anti-GluN2B [1:1000] (Catalog nos. sc-56, sc-8575, sc-1467, sc-9058, and sc-9056, Santa Cruz Biotechnology), Phospho-Retinoblastoma (pS780) and Rab3A (Catalog nos. 8180S and 3930S, Cell Signaling Technologies), anti- α -Synuclein [1:100] (Catalog no. PA5-16738, ThermoFisher Scientific) pS202-tau (CP13) [1:500], PG5 [1:500], MC1-tau [1:500], and PHF1 [1:500] (gifts from P. Davis, Albert Einstein College of Medicine, Yeshiva University), A11 [1:1000], OC [1:2,000], Syn33 [1:500], and F8H7 [1:500] (gift from R. Kayed, University of Texas Medical Branch), DW6 [1:500] (gift from D. Walsh, Harvard University). D. Walsh, Harvard University).

The following secondary antibodies were used in this study: Alexa Fluor™ (Molecular Probes, Invitrogen) Goat-anti-Chicken 488 (Catalog no. A-11039), 568 (Catalog no. A-11041), 647 (Catalog no. A-21449), Goat-anti-Mouse 568 (Catalog no. A-11004), 647 (Catalog no. A-21235), Goat-anti-Rabbit 488 (Catalog no. A-11034), 555 (Catalog no. A-21435), 568 (Catalog no. A-11036), DyLight® Goat-anti-Mouse 405 (Catalog no. 35501BID), IRDye® (Li-COR) 800cw Goat anti-Rabbit (Catalog no. 925-32211), IRDye® (Li-COR) 680LT Goat anti-Mouse (Catalog no. 925-68020).

A β immunofluorescent staining and confocal imaging

A series of mouse brain sagittal sections (30 μ m thick, n = 8 sections/animal) spaced at 400 μ m intervals was stained for deposited A β plaques. Briefly, sections were rinsed with PBS, pretreated with 80% formic acid for 1 min at room temperature, pretreated with 0.1% TWEEN®20-containing PBS, and blocked with PBS containing 5% normal goat serum before incubation at 4° C with 6E10 antibodies in blocking solution. Detection was performed as previously described [29, 31, 33] using Alexa Fluor™ conjugated secondary antibodies (Molecular Probes, Invitrogen), treated for autofluorescence with 1% Sudan Black solution [46] and coverslipped with ProLong-DAPI mounting medium (Molecular Probes). Digital images were obtained using an Olympus IX81 FluoView1000 microscope. Raw image z-stacks were analyzed using Imaris8.0 software suite (Bitplane Scientific Software, USA).

Barnes circular maze

The apparatus used was an elevated circular platform (0.91 m in diameter) with 20 holes (5 cm diameter) around the perimeter of the platform, one of which was connected to a dark escape recessed chamber (target box) (San Diego Instruments, USA). The maze was positioned in a room with large, simple visual cues attached on the surrounding walls. The protocol used here was published elsewhere [31, 53] (<http://www.nature.com/protocolexchange/protocols/349>). Briefly, mice were habituated to the training room prior to each training day for 30 minutes in their cages. In addition, on the first day mice were placed at the center of the maze in a bottomless opaque cylinder for 60 sec to familiarize the animals with the handling. Training sessions started 15 minutes later. Acquisition consisted of 4 trials per day for 4 days separated by a 15-minute-intertrial interval. Each mouse was

positioned in the center of the maze in an opaque cylinder, which was gently lifted and removed to start the session. The mice were allowed 180 seconds to find the target box on the first trial; all trials were 3 minutes long. At the end of the first 3 minutes, if the mouse failed to find the recessed escape box, it was gently guided to the chamber and allowed to stay in the target platform for 60 seconds. The location of the escape box was kept constant with respect to the visual cues, but the hole location of the target platform was changed randomly. An animal was considered to find the escape chamber when its back legs crossed the horizontal plane of the platform. An animal was considered to enter the escape chamber when the animal's entire body was in the escape chamber and no longer visible on the platform. Memory retention was tested 24 hours after the last training session (Probe trial day 5). The same parameters were collected during acquisition and retention phases using the ANY-maze software (Stoelting Co., USA).

Primary neurons

Primary neuron cultures were prepared from wildtype and tau-KO mice (C57BL6 strain) as previously described [37, 48].

Preparation of Amyloid- β oligomers

A β Os were prepared as previously described [37]. Briefly, Lyophilized, synthetic A β _{1–42} (AnaSpec) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich) to ~1 mM and evaporated overnight at room temperature. The dried powder was resuspended for 5 minutes at room temperature in 40–50 μ l dimethylsulfoxide to ~1 mM and sonicated for 10 minutes in a water bath. To prepare oligomers, the dissolved, monomeric peptide was diluted to ~400 μ l (100 μ M final concentration) in Neurobasal medium (GIBCO), incubated 24–48 hours at 4° C with rocking, and then centrifuged at 14,000 g for 15 minutes to remove fibrils. For all experiments, A β Os were diluted into tissue culture medium to a final concentration of ~1.5 μ M total A β _{1–42}.

Complementary DNA constructs and shRNA sequences

The control shRNA plasmid contained a scrambled sequence and was purchased from Addgene (Plasmid 1864; deposited by Dr David Sabatini). α Syn shRNAs (shRNA 1: TRCN000003736, shRNA 2: TRCN0000366590) were purchased from the RNAi consortium. Lentiviral shRNA efficiency was monitored by immunoblotting. An expression vector for human wild type α Syn, under control of the synapsin-1 promoter, was generated using the FSW plasmid. Primers used for insertion of the α -Syn DNA were Forward: 3'-GG A CCG GTA TGG ATG TAT TCA TGA AAG G-5' and Reverse: 3'-AAG GCT AGC TTA GGC TTC AGG TTC GTA G-5'. Plasmids were validated by DNA sequencing, immunofluorescence, and western blotting. The vector FSW (with synapsin promoter) was kindly provided by Thomas Südhof from Stanford. Human wildtype α Syn expression was monitored by immunofluorescence or immunoblotting.

Lentivirus production and infection

Lentiviruses were prepared as previously described [37] with some modifications. Briefly, HEK293T17 cells were plated on 15 cm dishes until they reached 60–70% confluency. The

cells then received a full media change using Opti-MEM lentiviral packaging reduced growth serum. The next day, the cells were transfected with 15 µg total plasmid DNA at a ratio of expression/shRNA vector (50%), packaging (pspax2) (37.5%) and envelope (pMD2.G) (12.5%) vectors, with 30µL each of P3000 Reagent and Lipofectamine 3000™ Reagent (ThermoFisher Scientific). Packaging (pspax2) and envelope (pMD2.G) vectors were obtained from Addgene. After 6 hours of incubation the media were replaced with full serum media and every 24 hours the lentiviral containing media were collected and stored at 4° C. The lentiviral containing media were then concentrated by centrifugation at 23,000 rpm for 2 hours at 4° C using a Beckman SW28 swinging bucket rotor. Cells were infected at least 3 days before AβO treatment at a 1/25 viral dilution. Transduction efficiency was monitored by immunoblot or immunofluorescence.

Confocal Imaging

Triple-label immunofluorescence was performed as previously described [30] using Alexa Fluor™-488, -555, -647–conjugated secondary antibodies (Molecular Probes, Invitrogen), treated for autofluorescence with 0.1% Sudan Black solution, and coverslipped with ProLong-DAPI mounting medium (Molecular Probes). Digital images were obtained using an Olympus IX81 FluoView1000 microscope. Raw image z-stacks were analyzed using Imaris7.x software suite (Bitplane Scientific Software).

Western blotting

Primary Neuron Sample Preparation—Cultured neurons were lysed using N-PER™ Neuronal Protein Extraction Reagent (ThermoFisher Scientific) following the manufacturer's instructions. N-PER™ was supplemented with Halt™ protease and phosphatase inhibitors (ThermoFisher Scientific). The protein concentrations were determined by using the Pierce™ BCA protein assay.

Electrophoresis—Protein separation was done using SDS-PAGE on freshly prepared 12% SDS-polyacrylamide gels, pre-cast 10–20% SDS-polyacrylamide Tris-Tricine gels, or 10.5–14% or 4–10.5% Tris-HCl gels (Bio-Rad). Protein levels were normalized by using 2–100 µg of protein per sample (depending on the targeted protein). The samples were resuspended with 4X Tricine loading buffer and boiled for 5 minutes prior to loading.

Western blotting—Proteins were transferred to 0.2 µm nitrocellulose membrane (Bio-Rad) following electrophoresis. For primary neuron experiments, membranes were blocked and antibodies were diluted into Odyssey Blocking Buffer (TBS version; LI-COR Biosciences, USA). For all other experiments, membranes were blocked in TBS containing 5% bovine serum albumin (BSA; Sigma) for 1–2 hours at room temperature, and probed with the appropriate antisera/antibodies diluted in 5% BSA-TBST (TBS with 0.1% Tween-20). Primary antibodies were probed with either anti-IgG immunoglobulins conjugated with biotin, HRP or IR dyes (LI-COR Biosciences). When biotin-conjugated secondary antibodies were used, HRP- or IR-conjugated Neutravidin® (Pierce) or ExtrAvidin® (Sigma) was added to amplify the signal. Blots were revealed on a LI-COR Odyssey imaging platform (Li-Cor Biosciences).

Stripping—For reprobing, membranes were stripped using Restore™ Plus Stripping buffer (Pierce) for 5–180 min at room temperature, depending on the antibody affinity.

Quantification—Densitometry analyses were performed using the LI-COR Odyssey software. Each protein of interest was probed in 3 individual experiments under the same conditions. Quantification by software analysis, expressed as DLUs, followed determination of experimental conditions ascertaining linearity in the detection of the signal. This method allows for a dynamic range of ~100-fold above background. Respective averages were then determined across the triplicate Western blots. Normalization was performed against actin, β III-tubulin or NeuN, which were also measured in triplicate. The color of the signal detected at 680 nm (red by default on the Odyssey) was modified to magenta to allow colorblind individuals to distinguish both channels.

Dot Blotting

Two μ g of extracellular-enriched or membrane-associated protein lysates were mixed with sterile filtered deionized water in a total volume of 2.5 μ L. Each sample was then adsorbed onto a nitrocellulose membrane until dry. Following a brief activation in 10% methanol/TBS, the membrane was boiled in PBS to enhance antigen detection as previously described [50]. Membranes were blocked in TBS containing 5% BSA for 60 minutes, then moved to the appropriate primary antibodies for overnight incubation at 4° C. Following washes, anti-mouse IgG-IR800 (1:100,000) and anti-rabbit IgG-IR680 (1:150,000) secondary antibodies were used for detection with a LI-COR Odyssey imager. All steps were performed without detergent to enhance A11/OC binding of oligomeric species as previously reported [4, 15, 34].

Immunocytofluorescence microscopy

Cultured neurons were labeled as previously described [37, 48] with the following modifications. Depending on antibody vendors' recommendations, cells were fixed with either freshly made 4% paraformaldehyde for 15 minutes at room temperature or with methanol for 15 minutes at – 20° C. After 3 washes with PBS, samples were blocked in PBS containing 5% normal goat serum and 0.25% Tween-20 for one hour. After blocking, samples were incubated with primary antibodies at 4° C overnight. The next day, samples were washed 3 times with PBS, and then incubated for 1 hour in Alexa Fluor®-tagged goat anti-mouse, anti-rabbit, or anti-chicken IgG secondary antibodies (ThermoFisher Scientific). For some experiments 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI; ThermoFisher Scientific) counterstaining was used between subsequent washes. Coverslips were then mounted onto microscope slides and allowed to dry overnight. Samples were then imaged using a Nikon Eclipse Ti inverted microscope equipped with a Yokogawa CSU-X1 spinning disk head, a 60x 1.4 NA Plan Apo objective; 405 nm, 488 nm, 561 nm and 640 nm lasers; and a Hamamatsu Flash 4.0 scientific CMOS camera. Analysis was performed using the Nikon software and ImageJ (<https://imagej.nih.gov/ij/plugins/cell-counter.html>).

Brain tissue sections were labeled for immunohistochemistry as previously described[37].

Statistical Analyses

When variables were non-normally distributed, nonparametric statistics were used (Spearman rho correlation coefficients, Kruskal-Wallis non-parametric analysis of variance followed by Bonferroni-corrected two-group *posthoc* Mann-Whitney U tests). When variables were normally distributed, the following parametric statistics were used (one/two-way ANOVA followed by Bonferroni-corrected two-group *posthoc* Student t tests). Sample size was determined by power analysis to be able to detect statistically significant changes within a 20% variation of measured responses. Analyses were performed using JMP 12 or JMP 13 (SAS Institute, USA).

Results

α Syn overexpression decreases amyloid deposition but exacerbates behavioral deficits in APP mice

Mutant α Syn (α Syn^{A30P} and α Syn^{A53T}) overexpression in APP mice has conflicting effects on amyloid burden [5, 12] and therefore the contribution of α Syn to this phenotype is unclear. Importantly, mutant α Syn displays unique properties relative to its wildtype form (α Syn^{WT}) [8], so whether human α Syn^{WT} alters amyloid load remains unknown. To investigate interactions between α Syn^{WT} and A β , we crossed mice overexpressing α Syn^{WT} (TgI2.2 line [32]) with APP mice (J20 line [36]) hereafter denoted APP/ α Syn. Overexpression of α Syn did not alter early mortality in APP mice (Fig. 1a) nor did it alter forebrain full-length APP (fl-APP) and APP carboxyl terminal fragment (APP-CTF) protein abundance when compared to age-matched APP mice (Suppl. Fig. 1 (Online Resource 1); as previously reported [30]). However, examination of amyloid burden at 6 months of age, when amyloid deposition is limited to the hippocampus in APP mice [36], revealed striking differences using two antibodies detecting A β (Fig. 1b and Suppl. Fig. 1c, arrows (Online Resource 1)). Quantitation of plaque burden and density confirmed a reduced plaque load in APP/ α Syn mice when compared to APP mice (Fig. 1c, d) with the majority of plaque load reduction arising from fewer, small amyloid deposits <200 μ m² in size (Fig. 1e). Notably, α Syn overexpression did not cause α Syn deposition as Lewy bodies (Suppl. Fig. 1c (Online Resource 1)). Thus, these results show diminished amyloid plaque formation by α Syn^{WT} *in vivo*, and also support previous observations using α Syn^{A30P} mice [5].

Although APP/PS1 α Syn^{A30P} bigenic animals displayed synaptic abnormalities suggestive of synapse loss [5], it remained unknown whether these changes translate into cognitive deficits. In 6-month-old APP/ α Syn mice, the reduction in amyloid load did not alleviate memory deficits assessed by the Barnes circular maze. Rather, APP/ α Syn animals displayed striking learning deficits (Fig. 1f) while APP and α Syn single transgenic mice were comparable to wildtype controls. Furthermore, while 6-month-old APP and α Syn transgenic mice displayed subtle deficits in memory retention during the probe trial (as previously reported [31]), APP/ α Syn mice suffered more pronounced impairment than both of these groups, with lower target quadrant occupancy and path efficiency (Fig. 1g and Suppl. Figs. 2–3 (Online Resources 2 and 3)). Beyond memory impairment, hyperactivity and a higher frequency of freezing episodes were unremarkably similar between APP/ α Syn mice and APP littermates during the task (Suppl. Fig. 3 (Online Resource 3)). Altogether, these

findings indicate that increased expression of α Syn^{WT} exacerbates cognitive deficits in APP mice in absence of α Syn deposition.

α Syn ablation increases plaque load but rescues behavioral deficits

Because APP/ α Syn mice displayed attenuated amyloid deposition accompanied with enhanced memory deficits, we hypothesized that α Syn ablation would also modify these phenotypical features. Hence, we crossed APP mice with α Syn knockout (α Syn-KO) animals, hereafter denoted APP/ α Syn-KO. In contrast to APP/ α Syn mice, in which the human α Syn^{WT} did not modify premature lethality, early mortality in APP mice was strikingly rescued in APP/ α Syn-KO mice (Fig. 2a). Unexpectedly, analyses of amyloid burden and plaque density revealed increases of 24% and 35% respectively in age-matched APP/ α Syn-KO mice when compared to APP mice (Fig. 2b–d). These changes were the result of an increased frequency in amyloid plaques $< 200\mu\text{m}^2$ in size (Fig. 2e). Finally, spatial reference memory was assessed in these mice at 6-months of age using the Barnes circular maze. Although all groups learned the task similarly (Fig. 2f), only APP mice showed impaired spatial memory retention during the probe trial with lower target quadrant occupancy and poorer path efficiency compared to wildtype controls (Fig. 2g and Suppl. Fig. 4d (Online Resource 4)). This deficit was absent in APP/ α Syn-KO mice (Fig. 2g and Suppl. Fig. 2 (Online Resource 2)). Furthermore, both hyperactivity and freezing behavior characterizing APP mice were significantly attenuated in APP/ α Syn-KO mice (Suppl. Fig. 4a–c (Online Resource 4)). Of note, α Syn-KO mice were indistinguishable from wildtype controls in each metric assessed (Fig. 2a, f, g and Suppl. Figs. 2, 4 (Online Resources 2 and 4)). Overall, these results indicate that ablation of *SNCA* in APP mice potentiates A β deposition and prevents memory impairment. These phenotypic alterations sharply oppose the effect caused by α Syn overexpression in APP animals. Beyond amyloid deposition and cognitive function, α Syn reduction also protected APP mice against premature death and ameliorated motor activity and aversive behavior, causing a profound rescue of diverse behavioral components.

α Syn modulates the production and cellular distribution of soluble A β Os

The dramatic differences in amyloid burden and behavioral deficits observed among APP/ α Syn, APP and APP/ α Syn-KO mice, in the absence of apparent changes in APP expression or processing (Suppl. Fig. 1 (Online Resource 1)), led us to hypothesize that soluble A β Os would also be altered. A11 and OC antibodies were used to detect soluble type I (non-fibrillar) and type II (pre-fibrillar) A β Os by non-denaturing dot blotting [35]. 6E10 (anti APP/A β) and anti-actin antibodies were used as internal controls. Our analysis revealed consistent differences in A β Os among APP, APP/ α Syn and APP/ α Syn-KO mice that complement the changes we observed for deposited A β (Fig. 3). In APP/ α Syn mice, forebrain lysates contained elevated OC+ pre-fibrillar A β Os in the extracellular-enriched fraction (EC) and decreased OC+ pre-fibrillar A β Os in the membrane-enriched fraction (MB). These changes indicate a shift in the compartmentalization of these A β assemblies between APP/ α Syn and APP animals. The opposite shift occurred in APP/ α Syn-KO mice. We found decreased detection profiles for A11, OC and 6E10 in the EC fractions of APP/ α Syn-KO forebrain lysates compared to APP mice. By contrast, OC and 6E10 immunoreactivity were elevated in the corresponding MB fraction of these animals, showing

a redistribution of OC+ pre-fibrillar A β O_s associated with enhanced plaque deposition. (Fig. 3a, b). To address this issue further, brain sections were labeled with OC and 4D6, an antibody raised against α Syn, for confocal analyses which revealed a marked redistribution of OC+ A β O_s in APP/ α Syn mice compared to APP and APP/ α Syn-KO animals (Fig. 3c). In the latter, OC readily detected several morphologies of A β deposits. In APP/ α Syn hippocampi, nearly all OC immunoreactivity was in sparse punctae in the stratum radiatum, stratum oriens and stratum pyramidale (white box), consistent with a synaptic and somatic localization of this class of A β O_s (Fig. 3c, left panel). Taken together, these results show modulation of soluble A β O_s in APP mice by α Syn.

Analogous to A β , the conformational state of α Syn dictates its toxicity. Given the phenotypical changes observed following human α Syn expression in APP mice, we next sought to biochemically characterize the species of α Syn present in our mice (Fig. 3d, e and Suppl. Figs. 5, 6 (Online Resources 5 and 6)). As previously described [30], the conformational state of α Syn in intracellular-enriched fractions was assayed by dot blotting using two α Syn antibodies, Syn33 and F8H7, each of which recognizes a distinct set of oligomers. Total and human-specific α Syn were also assayed on dot blots using 4D6 and LB509 antibodies, respectively (Fig. 3d, e). Briefly, we found that Syn33-positive α Syn species were most abundant in APP/ α Syn mice surpassing the measurements in α Syn mice. In contrast to APP/ α Syn and α Syn mice, Syn33-positive α Syn species were virtually absent in APP, APP/ α Syn-KO and WT mice. F8H7-positive α Syn assemblies were undetected across all groups (Fig 3e and Suppl. Fig. 5 (Online Resource 5)). Dot blotting analyses with 4D6 and LB509 antibodies confirmed an elevation in α Syn immunoreactivity in forebrain lysates from APP/ α Syn bigenic mice compared to α Syn transgenic animals. Of note, the relative abundance of 4D6-positive α Syn species was similar between APP and WT mice, and non-existent in APP/ α Syn-KO lysates (Fig. 3d, e and Suppl. Fig. 5a, b (Online Resource 5)). Since prior studies suggested the existence of heterologous α Syn:A β hybrid oligomers, we performed co-immunoprecipitations with LB509 which were revealed with 6E10, an antibody against the N-terminus of the A β peptide. Although human α Syn was readily pulled down, we could not reveal the presence of A β within putative complexes using forebrain lysates from APP/ α Syn mice (Suppl. Fig. 5c (Online Resource 5)), suggesting that α Syn and A β species recognized by these broad-spectrum antibodies were not interacting. Finally, to determine the relative size of α Syn species contributing to the increased signal with 4D6 and LB509 antibodies, we performed western blotting analyses as recently described by our group [30]. In our hands, Syn33 and F8H7 were not compatible for western blot analyses (they both fail to differentiate α Syn-KO from other mouse lines; data not shown) and were therefore omitted by design. Using both 4D6 and LB509, the relative protein abundance of monomeric α Syn and of the α Syn-72kDa assembly was not different when comparing APP/ α Syn and α Syn animals. However, the forebrain abundance of putative α Syn dimers and tetramers (α Syn-28kDa, α Syn-35kDa and α Syn-56kDa) was increased markedly (Suppl. Fig. 6 (Online Resource 6)). Together, these results demonstrate an increase in α Syn oligomers in 6-month-old APP/ α Syn mice.

Specific postsynaptic markers are modulated by α Syn

Synapse loss constitutes an early event that defines AD pathogenesis [47] and since A β Os potentially induce synapse loss in APPJ20 mice, we next assessed synaptic protein integrity in our bigenic animals. We selected several pre-synaptic (i.e. α Syn, synaptophysin [SYP] and Ras-related protein Rab3A,) and post-synaptic (i.e. postsynaptic density protein 95 [PSD95], Drebrin and the N-Methyl-D-Aspartate receptor subunit GluN2A) proteins that are strongly implicated in AD pathophysiology (Fig. 4). No changes in SYP and Rab3A protein expression were observed in α Syn mice Suppl. Fig. 7 (Online Resource 7)), consistent with previous reports [30, 31]. We found no significant changes in SYP, Rab3A, GluN2A and Drebrin in forebrain lysates from APP mice compared to non-transgenic mice despite a downward trend (a ~20% reduction) in SYP abundance (Fig. 4). This profile is consistent with the ~20% lowering of SYP in 5-month-old APP mice [11] and the absence of GluN2B changes in 5 to 8-month-old APP mice [41, 42]. However, the expression of human α Syn^{WT} in APP mice synergistically lowered the forebrain abundance of all synaptic proteins tested when compared to the APP parental line (Fig. 4a, b). Contrasting with APP/ α Syn mice, α Syn ablation did not alter the protein expression of SYP, Rab3, or PSD95 compared to age-matched APP mice. Rather, forebrain lysates from APP/ α Syn-KO mice displayed higher protein amounts of postsynaptic markers Drebrin and GluN2A relative to APP mice. These observations were further supported by immunofluorescent staining of synaptic markers SYP, GluN1 and GluN2B which revealed a qualitative reduction in SYP immunoreactivity in the hippocampus of APP/ α Syn mice whereas fluorescent detection of NMDA receptor subunits GluN1 and GluN2B appeared unchanged (Fig. 4c). Thus, these results demonstrate that human α Syn^{WT} compromises synaptic protein integrity in APP mice, and that α Syn selectively affects GluN2A and Drebrin abundance.

Early pathological features of tau are α Syn-dependent

Because substantial evidence suggests that A β -induced toxicity is mediated by tau [4, 7, 21, 31, 38, 43, 45, 48, 49], we next investigated the state of tau in these animals. Using a well-established panel of antibodies against various pathological forms of tau (kind gift from Dr. Peter Davies), we assessed phosphorylation and conformational changes of tau in intracellular-enriched (IC) and membrane-enriched forebrain fractions as described earlier [4, 31, 49]. Here, western blot analysis revealed subtle pathological changes in MC1-tau immunoreactivity within the intracellular-enriched fractions across genotypes, which was reduced in APP/ α Syn-KO compared to APP and APP/ α Syn mice (Fig. 5a, b). MC1 recognizes an early AD conformation requiring interaction between the N and C termini of tau [23, 55]. By contrast, APP/ α Syn mice displayed increased anti-tau immunoreactivity of MC1 and also CP13, which recognizes tau phosphorylated at S202 [55], by ~2- and ~1.5-fold, respectively, in the membrane-enriched fraction (Fig. 5c, d and Suppl. Fig. 8 (Online Resource 8)). These biochemical changes were further supported by confocal image analysis (Fig. 5e and Suppl. Fig. 8 (Online Resource 8)). Overall, these results show that α Syn alters conformational and phosphorylated tau molecules detected by MC1 and CP13 in APP mice.

A β -induced ectopic cell-cycle re-entry depends on α Syn

In addition to A β -mediated synaptic and cognitive deficits, A β O_s induces ectopic CCR in post-mitotic neurons thereby initiating an early signaling cascade that results in dendritic abnormalities and precedes neuron loss in AD [6]. We previously reported that neuronal CCR is a phenotypic feature of 6-month-old J20 mice [48], which led us to test whether α Syn expression modulated A β O-induced CCR in APP mice. CCR was determined by measuring the percentage of cortical neurons that also expressed nuclear cyclin D1, a protein that is required for G1/S phase transition during the cell cycle, as a surrogate for CCR (Fig. 6). We observed a robust increase in cyclin D1-positive cortical neurons from J20 mice ($n = 1092/3424$, 31.06%) relative to their wildtype controls ($n = 253/4966$, 5.09%; Fig. 6b), which is consistent with our previous report. By contrast, neuronal cyclin D1 in APP/ α Syn^{WT} mice was markedly enhanced by ~1.5-fold ($n = 1959/4064$, 47.34%) relative to APP mice. Importantly, neuronal cyclin D1 in TgI2.2 α Syn^{WT} mice ($n = 147/3209$, 4.6%) did not significantly differ from wildtype animals, suggesting that the enhancement of CCR in the APP/ α Syn mice was not due to an additive effect (Fig. 6b and Suppl. Fig. 9a (Online Resource 9)). On the other hand, genetic ablation of endogenous α Syn in APP mice ameliorated neuronal CCR, with cyclin D1-positive neuron counts ($n = 210/3835$, 6.18%) indistinguishable from that of wildtype controls (Fig. 6a, b). Thus, these results demonstrate a novel modulation of neuronal CCR by α Syn in vivo.

To further assess the cellular consequences of α Syn reduction on neuronal CCR, we next quantified A β O-induced neuronal cyclin D1 after lentiviral depletion of α Syn in primary cortical neuron cultures from wildtype C57BL6 mice (Suppl. Fig. 9b, c (Online Resource 9)). Strikingly, viral knockdown of α Syn transcripts by two different shRNAs protected cultured neurons from A β O-induced CCR, as determined by quantitative confocal imaging (Fig. 6c, d) and immunoblotting (Fig. 6e–h). Contrary to previous observations [52] this effect was independent of Rab3A, as A β O exposure did not alter the protein abundance of Rab3A under our experimental conditions (Suppl. Figs. 7, 9 (Online Resources 7 and 9)). During the G1/S phase cell cycle transition, cyclin D1 forms a complex with the cyclin-dependent kinases CDK4/6, thereby activating the cyclin-CDK complex and relieving the repressor action of the retinoblastoma protein (Rb) via phosphorylation at serine 780 (S780). Consistent with this canonical Cyclin D-CDK4/6 signaling cascade, α Syn knockdown prevented the abnormal elevation of Rb phosphorylation at S780 (pRb) induced by A β O_s (Fig. 6g). Finally, we also measured the protein abundance of the proliferating cell nuclear antigen (PCNA) following A β O treatment, which is active during periods of DNA replication and synthesis. In agreement with independent studies [6], we found that A β O_s elevated PCNA protein amounts. By contrast, α Syn knockdown prevented the increase in PCNA expression by A β O_s in neuronal cultures (Fig. 6h). Together, these surprising results demonstrate that α Syn is required for A β O-induced neuronal CCR progression, likely by preventing the initial increase in cyclin D1 expression levels.

Enhancement of CCR by α Syn in primary neurons is tau-dependent

Based on the findings that α Syn modulates MC1-immunoreactive pathological tau conformers in vivo (Fig. 5 and Suppl. Fig. 8 (Online Resource 8)), we investigated the contribution of tau to the enhancement of A β O-induced CCR by α Syn in vitro and our

primary mice. Using wildtype primary cortical neurons, we found that expression of human α Syn^{WT}, virally driven by the neuron-specific synapsin-1 promoter, exacerbated MC1-tau accumulation by A β O_s whereas lentiviral knockdown of α Syn had the contrasting effect (Fig. 7a and Suppl. Fig. 10 (Online Resource 10)). These observations are consistent with the biochemical changes revealed in APP/ α Syn and APP/ α Syn-KO mice, further supporting a central role of α Syn in modulating A β -induced phenotypes. To assess if the enhancement of A β -induced CCR by α Syn was tau-dependent, we next overexpressed human α Syn^{WT} in primary neurons from wildtype and tau-KO mice, and quantified Cyclin D1-positive neurons following fluorescent immunostaining (Fig. 7b–d). Consistent with the CCR analysis of APP/ α Syn mice, lentiviral delivery of α Syn in wildtype primary neurons resulted in a substantial increase in Cyclin D1-positive neurons (29%) compared to neurons exposed to A β O treatment alone (19.83%) or to viral control groups expressing eGFP (20.36%). In stark contrast to primary cultures derived from wildtype mice, we found virtually no cyclin D1-positive neurons in tau-KO mice in all treatment conditions, including in neurons transduced with α Syn and treated with A β O_s (Fig. 7c, d and Suppl. Fig. 11 (Online Resource 11)). Taken together, these results indicate that α Syn, like tau, is required for A β O-induced neuronal CCR.

Discussion

Our findings provide novel functional insights into how α Syn modulates the phenotype of APP mice and A β O-treated primary neurons. Our study sets itself apart by directly demonstrating that bigenic APP/ α Syn animals display: 1) reduced hippocampal amyloid load, 2) exacerbated cognitive deficits, consistent with the faster cognitive decline seen in individuals with mixed DLB [39]; 3) alterations of oligomeric A β and α Syn profiles; 4) enhanced accumulation of soluble tau; and 5) exacerbated neuronal CCR. Importantly, we also utilized the converse approach by ablating *SNCA* in APP mice to better comprehend the role of α Syn in AD phenotypes. These APP/ α Syn-KO animals display: 1) complete rescue of early mortality; 2) elevated amyloid load, 3) rescued spatial memory learning and retention; 4) reduced accumulation of soluble tau; and 5) abolished neuronal CCR. In particular, the observations documenting that *SNCA* deletion rescues early mortality in APP mice and that α Syn is a novel phenotypic modifier of neuronal CCR in vivo and in vitro are profound and unexpected.

Beyond the uncertainties surrounding the functions of α Syn in AD, the contribution of α Syn to changes in amyloid load itself is unresolved. Moreover, the relationship between changes in amyloid burden mediated by α Syn and other symptoms, such as synaptic and behavioral deficits when reported, have also been unclear. Specifically, APP/PS1 mice overexpressing mutant α Syn^{A30P} displayed decreased amyloid burden [5], whereas overexpression of mutant α Syn^{A53T} in 3xTg-AD mice led to an acceleration of amyloid deposition [12]. Conversely, deletion of *SNCA* in Tg2576 animals led to increased amyloid load at old ages [27], *SNCA* ablation in APP₇₅₁^{Swe/Lon} mice did not alter amyloid loads [52]. Here, we found that modest overexpression of human α Syn^{WT} in APP mice resulted in decreased A β plaque load, but importantly, higher levels of extracellular A β O_s. By contrast, and in spite of an increase in A β plaque load, spatial memory impairment was dramatically improved in APP/ α Syn-KO mice. Moreover, the behavioral rescue of APP/ α Syn-KO mice was

associated with decreased amounts of extracellular A β Os. These findings support earlier observations that increased A β fibril formation, and subsequent lowering of A β oligomers, reduce functional deficits in APP mice [10], and thereby emphasize the far more toxic nature of oligomeric, as opposed to fibrillar A β .

By analyzing both pre- and post-synaptic proteins, we unexpectedly identified novel modulatory roles of α Syn on two postsynaptic markers, GluN2A and Drebrin. The measurements of SYP protein abundance in APP/ α Syn support similar findings observed in APP/PS1 $\times$ α Syn^{A30P} animals reported by Bachhuber and coworkers [5], revealing that overexpression of WT or mutant α Syn exacerbates pre-synaptic injury in APP mice. Our study further expands this interpretation to additional synaptic candidate proteins, including Rab3A and PSD95, indicating that excess α Syn can lead to a general loss of synaptic machinery. Surprisingly, the relative protein abundance of SYP and PSD95 was indistinguishable between APP and APP/ α Syn-KO mice. However, the protein abundance of GluN2A and Drebrin in APP/ α Syn-KO mice surpassed that of APP mice. The effects of α Syn ablation on GluN2A and the NMDAR anchor, Drebrin, are compelling considering that both postsynaptic proteins are implicated in brain executive function and synaptic plasticity [22, 28]. Loss of Drebrin has long been associated with memory impairment [14] and AD [17]. Because Drebrin is a key regulator of dendritic spine morphogenesis [26], increased Drebrin may contribute to the protective effects on memory retention in APP/ α Syn-KO mice by expanding spine size and receptor integration at the postsynaptic membrane. Altogether, the results pertaining to Drebrin and GluN2A may therefore offer molecular insights into the behavioral rescue conferred by *SNCA* ablation in APP mice.

In addition to the bidirectional changes in amyloid burden caused by α Syn^{WT}, notable differences exist between our work and the studies from Spencer and colleagues (2016). For instance, in contrast to the rescue of A β -induced Rab3 depletion observed in APP₇₅₁^{Swe/Lon}/ α Syn-KO mice and in primary neurons, we found no difference in Rab3A following α Syn ablation in vivo and in vitro. Instead, forebrain Rab3A protein amounts were lowered by half only following α Syn overexpression in APP mice. Our results are thus inexplicably inconsistent with the aforementioned published data [52]. However, our results are consistent with earlier work demonstrating that neither α Syn^{WT} overexpression nor α Syn ablation alters Rab3A protein abundance in mouse synaptosomes [9]. Notable behavioral differences were also striking between the two studies. For example, we found that *SNCA* deletion profoundly impacted cognitive function in APP mice rescuing acquisition and retention of spatial reference memory as well as freezing behavior. By contrast, deficits in spatial memory retention were similar between APP₇₅₁^{Swe/Lon} and APP₇₅₁^{Swe/Lon}/ α Syn-KO mice [52]. We speculate that these sharp differences are due to the uncontrolled heterogeneity of the background strains of the animals used in this study (C57BL/6 for APP₇₅₁^{Swe/Lon}, 129x1SvJ for α Syn-KO and C57BL/6; 129x1SvJ for APP₇₅₁^{Swe/Lon}/ α Syn-KO mice) compared to our studies (C57BL/6 for all lines).

There is substantial evidence to support a role for tau in mediating A β -induced toxicity [21, 24, 31, 38, 43, 45, 48, 49]. We therefore assessed whether overexpression or deletion of α Syn affects pathological tau changes caused by A β in vivo and in vitro. We identified distinct differences across APP, APP/ α Syn and APP/ α Syn-KO mice in two early markers of

tau pathology, CP13 (pS202-tau) and MC1. The accumulation of pS202-Tau and misfolded (MC1-positive) tau in membrane-enriched lysates from α Syn-overexpressing APP mice is notable because it is consistent with subsequent synaptic dysfunction [19]. Confocal imaging of both pathological tau forms confirmed prominent dendritic labeling of pyramidal neurons in the hippocampi from APP/ α Syn compared to APP littermates. However, only conformationally altered tau molecules reactive with MC1 were bidirectionally controlled by α Syn. Previous studies have described interactions between α Syn and tau at multiple levels [13, 16, 51, 54]. Based on these observations, it is tempting to advance the possibility that α Syn regulates or stabilizes the misfolded state of tau, or vice versa. Considering the rapid accumulation of evidence linking α Syn to tau, future studies will be required to decipher the mechanistic and functional details of this molecular interaction.

Finally, this work also makes the unexpected and novel observation that α Syn expression is required for A β O-induced neuronal CCR. In line with our in vivo behavioral and synaptic protein results, α Syn^{WT} overexpression in APP transgenic mice and in cultured neurons exposed to A β O exacerbated neuronal CCR. By contrast, we also found that genetic ablation of α Syn, or α Syn lentiviral knockdown using RNA interference, lowered or prevented A β O-induced CCR in cultured neurons. Our findings thus suggest that α Syn is necessary to induce A β O-mediated neuronal CCR in vivo and in vitro, and is thereby a crucial factor in this major neuron death pathway in AD. Unlike our observations in WT neurons, when we tested for enhancement of A β O-induced CCR by α Syn in tau-KO neurons, we found that α Syn^{WT} overexpression, in combination with A β O exposure, did not promote neuronal CCR. Therefore, our results indicate that α Syn and tau might work coordinately to modulate neuronal CCR. By extension, our work may have implications for other α -synucleinopathies in which neuronal CCR has been detected [20, 25].

In conclusion, the findings reported here highlight novel contributions of α Syn to AD pathogenesis. Considering the bidirectional effects of α Syn on both A β and tau, targeting α Syn in AD may prove a viable therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	Alzheimer's disease
CCR	Neuronal Cell Cycle Re-Entry

WT	wild-type
APP	J20 APP transgenic mice
αSyn	TgI2.2 transgenic mice
αSyn-KO	<i>SNCA</i> -null mice

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Fig. 1. Effects of human $\alpha\text{Syn}^{\text{WT}}$ overexpression on the phenotype of APP transgenic mice (a) Kaplan-Meier survival curves showing effect of the overexpression of human $\alpha\text{Syn}^{\text{WT}}$ on premature mortality in APP transgenic mice. All genotyped mice in the colony were included in the analysis ($N = 378$, $n_{\text{WT}} = 70$, $n_{\text{APP}} = 92$, $n_{\alpha\text{Syn}} = 87$, $n_{\text{APP}/\alpha\text{Syn}} = 99$ mice). By Log-Rank comparison, both APP and APP/ αSyn mice differed from all other groups ($\chi^2_{(3)} = 22.101$, $\star P = 0.0001$ vs. WT). (b) Anti-A β immunofluorescent labeling (6E10) was used to assess amyloid burden of the hippocampus of APP transgenic mice harvested after behavioral testing (6 months of age). White arrows indicate amyloid deposits. (c, d) Quantitation of the area covered by 6E10-immunoreactive deposits (c) and the number of amyloid plaques per section (d) detected in APP and APP/ αSyn mice (Bars represent the mean $\pm$ S.D.; t test, $\star P < 0.05$, $n = 8$ sections/animal, $N = 10$ –12 animals/genotype). (e) Comparison of plaque distribution between APP and APP/ αSyn mice binned by covered area ($100 \mu\text{m}^2$ increments). (Bars represent the mean $\pm$ S.D.; t test, $\star P < 0.05$, $n = 8$ sections/animal, $N = 10$ –12 animals/genotype). (f, g) Influence of αSyn overexpression on spatial reference memory in young mice. Six-month-old WT, APP, αSyn and APP/ αSyn mice ($n = 8$ –11 mice/genotype) were trained in the Barnes circular maze for 4 days. A probe trial (escape platform removed) was conducted 24 h after the last training session. During acquisition of the task, escape latency (f) was recorded. Two-way repeated-measures ANOVA revealed a significant effect of training ($F_{(3,540)} = 34.228$, $P < 0.0001$), of the transgene ($F_{(3,540)} = 19.626$, $P < 0.0001$) and a significant day*transgene interaction ($F_{(9,540)}$

= 3.688, $P=0.0002$) for all mice. APP/ α Syn mice showed little ability to learn the task well ($F_{(4,44)}=8.56$, $P<0.0001$). During the probe trial on day 5 (g), APP mice showed poorer memory retention than any other group as confirmed by one-way ANOVA analysis ($F_{(3,34)}=24.596$, $P<0.0001$) followed by Student t test with Bonferroni correction, $P<0.0001$. Data represent mean $\pm$ S.E.M. (f) or S.D. (g) (n = 8–11 mice/age/genotype)

Fig. 2. Effects of α Syn gene deletion on the phenotype of APP transgenic mice

(a) Kaplan-Meier survival curves showing effect of the ablation of endogenous α Syn on premature mortality in APP transgenic mice. All genotyped mice in the colony were included in the analysis ($N = 438$, $n_{WT} = 70$, $n_{APP} = 92$, $n_{\alpha Syn-KO} = 118$, $n_{APP/\alpha Syn-KO} = 158$ mice). By Log-Rank comparison, only APP mice differed from all other groups ($\chi^2_{(3)} = 18.784$, $\star P = 0.0001$ vs. WT, $\star P = 0.0001$ vs. APP). (b) Anti-A β immunofluorescent labeling (6E10) was used to assess amyloid burden of the hippocampus of APP transgenic mice harvested after behavioral testing. White arrows indicate amyloid deposits. (c, d) Quantitation of the area covered by 6E10-immunoreactive deposits (c) and the number of amyloid plaques per section (d) detected in APP and APP/ α Syn-KO mice (Bars represent the mean $\pm$ S.D.; t test, $\star P < 0.05$, $n = 8$ sections/animal, $N = 10$ –12 animals/genotype). (e) Comparison of plaque distribution between APP and APP/ α Syn-KO mice binned by covered area (100 μm^2 increments). (Bars represent the mean $\pm$ S.D.; t test, $\star P < 0.05$, $n = 8$ sections/animal, $N = 10$ –12 animals/genotype). (f, g) Influence of *SNCA* deletion on spatial reference memory in young mice. Six-month-old WT, APP, α Syn-KO and APP/ α Syn-KO mice ($n = 8$ –11 mice/genotype) were trained in the Barnes circular maze for 4 days. A probe trial (escape platform removed) was conducted 24 h after the last training session. During acquisition of the task, escape latency (F) was recorded. Two-way repeated-measures ANOVA revealed a significant effect of training ($F_{(3,544)} = 74.124$, $P < 0.0001$), no effect of genetic modification ($F_{(3,544)} = 1.008$, $P = 0.3886$), and a significant day*transgene interaction ($F_{(9,544)} = 2.796$, $P = 0.0033$) for all four groups. During the probe trial on day 5

(g), APP mice showed poorer memory retention than any other group as confirmed by one-way ANOVA analysis ($F_{(3,34)} = 7.354$, $P = 0.0008$) followed by Student t test with Bonferroni correction, $p < 0.0001$. Data represent mean $\pm$ S.E.M. (f) or S.D. (g) ($n = 8-11$ mice/age/genotype)

Fig. 3. Bidirectional redistribution of AβOs caused by αSyn in APP mice

(a) Detection of oligomeric amyloid conformers in extracellular-enriched (EC) and membrane-enriched (MB) lysates from APP/αSyn, APP and APP/αSyn-KO mice by dot blot analysis using A11 and OC antibodies. 6E10 was also used to measure APP/Aβ abundance and actin was used as internal control. ($n = 6$ animals/age/genotype). Note that only 3 (out of 6) APP/αSyn specimens were adsorbed onto the presented nitrocellulose membranes shown for A11, OC and Actin. Grey and teal rectangles correspond to WT and αSyn control lysates respectively. Note that Actin is not present in extracellular-enriched lysates as expected. (b) Normalized abundance of oligomeric species indicated a bidirectional redistribution of A11 and OC conformers in EC and MB extracts of APP animals. (Histograms represent the mean $\pm$ S.D.; One-way ANOVA [$F_{(2,18)}^{A11-EC} = 12.4110$, $P = 0.0008$, $F_{(2,18)}^{OC-EC} = 22.6278$, $P < 0.0004$, $F_{(2,18)}^{6E10-EC} = 8.5928$, $P = 0.0037$ and $F_{(2,18)}^{A11-MB} = 5.8887$, $P = 0.0139$, $F_{(2,18)}^{OC-MB} = 53.8985$, $P < 0.0001$, $F_{(2,18)}^{6E10-MB} = 5.131$, $P = 0.0123$ respectively] followed by Student t test with Bonferroni correction; $\star P < 0.05$ vs. 6-month-old APP mice, $n = 6$ animals/age/genotype). (c) Representative confocal images of hippocampi labeled for αSyn (magenta; 4D6 antibody) and prefibrillar AβOs (green, OC antibody) from 6-month-old APP/αSyn, APP and APP/

α Syn-KO mice ($n = 6$ animals/age/genotype). Arrows indicate OC-positive A β deposits in APP and APP/ α Syn-KO mice. Note the synaptic pattern of OC+ A β Os in APP/ α Syn mice (empty white box). Scale bars = 200 μ m. **(d)** Detection of oligomeric α Syn in intracellular enriched (IC) lysates by dot blot analysis using Syn33 and F8H7 antibodies. 4D6 and LB509 were used to detect total or human α Syn respectively and actin was used as an internal control. ($n = 8$ animals/age/genotype). Grey and teal rectangles correspond to WT and α Syn control lysates respectively. **(e)** Quantitation Syn33 oligomeric species in IC extracts. Histograms represent the mean $\pm$ S.D.; One-way ANOVA [$F_{(4,40)} = 304.3331$, $P < 0.0001$ for Syn33] followed by Student t test with Bonferroni correction; $\star P < 0.05$ vs. 6-month-old APP mice, $\star P < 0.0001$ vs. α Syn. ($n = 8$ animals/age/genotype)

Fig. 4. Synaptic marker changes in APP/αSyn, APP and APP/αSyn-KO mice

(a, b) Representative Western blots (a) and quantitation (b) of pre- and postsynaptic proteins detected in membrane (MB)-enriched fractions from 6-month-old APP/αSyn, APP and APP/αSyn-KO mice. Histograms show mean $\pm$ S.D.; One-way ANOVA [$F_{(2,21)}^{\alpha\text{Syn}} = 83.5965$, $P < 0.0001$, $F_{(2,21)}^{\text{SYP}} = 21.0623$, $P < 0.0001$, $F_{(2,21)}^{\text{Rab3A}} = 18.6904$, $P < 0.0001$, $F_{(2,21)}^{\text{PSD95}} = 29.3079$, $P < 0.0001$, $F_{(2,24)}^{\text{Drebrin}} = 15.1153$, $P = 0.0001$ and $F_{(2,23)}^{\text{GluN2A}} = 27.7055$, $P < 0.0001$ respectively] followed by Student's t test, $\star P < 0.05$ vs. age-matched APP mice; $\star P < 0.05$ vs. APP/αSyn mice; $n = 6-9$ mice/group. (c) Representative confocal images of CA1 hippocampal neurons immunostained for synaptophysin (SYP, yellow), NMDA receptor subunits GluN1 (green) and GluN2B (magenta) and MAP2 (blue) revealed a marked reduction in SYP density in 6-month-old APP/αSyn mice compared to APP or APP/αSyn-KO animals. Scale bar = 20 μm ; $n = 6$ sections per animal; $N = 6$ animals/age/genotype

Fig. 5. Forebrain abundance of conformationally-altered tau molecules is bidirectionally controlled by αSyn expression in APP mice

(a, b) Representative Western blots (a) and quantitation (b) of soluble tau species detected in intracellular (IC)-enriched fractions from 6-month-old APP/αSyn, APP and APP/αSyn-KO mice. Histograms show mean ± S.D.; One-way ANOVA [$F_{(2,23)} = 12.3071$, $P = 0.0007$ for MC1-tau] followed by Student's t test, $\star P < 0.05$ vs. age-matched APP mice; $n = 6-9$ mice/group. (c, d) Representative Western blots (c) and quantitation (d) of soluble tau species detected in membrane (MB)-enriched fractions from 6-month-old APP/αSyn, APP and APP/αSyn-KO mice. Histograms show mean ± S.D.; One-way ANOVA [$F_{(2,24)} = 8.7025$, $P = 0.0042$ and $F_{(2,24)} = 19.1536$, $P < 0.0001$ for CP13- and MC1-tau respectively] followed by Student's t test, $\star P < 0.05$ vs. age-matched APP mice; $n = 6-9$ mice/age/genotype. (e) Representative confocal images of CA3 hippocampal neurons immunostained for Fyn (blue) and MC1-Tau (green) revealed an aberrant accumulation and differential missorting of soluble tau species in apical dendrites of 6-month-old APP mice. Scale bar = 20 μm. $n = 6$ sections per animal; $N = 6$ animals/age/genotype

Fig. 6. Bidirectional regulation of cell cycle re-entry by αSyn in APP mice and cultured neurons (a, b) Representative confocal images (a) and quantitation (b) of cyclin D1 (green), NeuN (magenta) and MAP2 (blue) from 6-month-old APP/αSyn, APP and APP/αSyn-KO mice. Images were captured in the prefrontal cortex. Histograms show mean ± S.D.; One-way ANOVA [$F_{(5,30)} = 210$, $P < 0.0001$] followed by Student's t test, $\star P < 0.05$ vs. age-matched APP mice; $n = 6-9$ mice/group. (c, d) Representative confocal images (c) and quantitation (d) of cyclin D1 (green), NeuN (magenta) and MAP2 (blue) from cultured primary cortical neurons exposed to 1.5 μM AβOs or vehicle for 24 hours. Neurons were also transfected with scrambled (Scr.) or SNCA shRNAs (two separate shRNAs targeting αSyn transcripts were used). Histograms show mean ± S.D.; One-way ANOVA [$F_{(5,37)} = 11.75$, $P < 0.0001$] followed by Student's t test, $\star P < 0.05$ vs. neurons exposed to vehicle and Scr. shRNA; $n = 8-9$ dishes/group. (e-h) Representative Western blots (e) and quantitation (F-H) of cell cycle markers detected in primary cortical neurons. Histograms show mean ± S.D.; One-way ANOVA [$F_{(2,30)} = 8.336$, $P < 0.0013$, $F_{(2,49)} = 4.84$, $P < 0.0121$, $F_{(2,35)} = 18.64$, $P < 0.0001$] followed by Student's t test, $\star P < 0.05$ vs. age-matched APP mice; $n = 8-9$ dishes/group. αSyn shRNA 1 (TRCN0000003736) and αSyn shRNA 2 (TRCN0000366590) were used for (c, d), αSyn shRNA 1 was used for (e-h)

Fig. 7. α Syn is required to cause A β O-induced tau pathology in cultured neurons

(a) Representative confocal images of conformationally-altered tau molecules labeled with antibodies to a tau conformational variant (MC1; green), α Syn (magenta) and MAP2 (blue) from wild type primary cortical neurons exposed to 1.5 μ M A β O or vehicle for 24 hours. Neurons were also transfected with scrambled (Scr.) or *SNCA* shRNAs. (b) Representative confocal images of eGFP (green), Cyclin D1 (magenta) and MAP2 (blue) from *MAPT*-null primary cortical neurons exposed to 1.5 μ M A β O or vehicle for 24 hours. Neurons were also transfected with lentiviruses expressing eGFP or human α Syn^{WT}. (c, d) Quantitation of Cyclin D1-positive neurons in wild-type (c) or *MAPT*-null (d) cortical neurons. Histograms show mean $\pm$ S.D.; One-way ANOVA [$F_{(7,40)} = 431.2$, $P < 0.0001$] followed by Student's *t* test, $\star P < 0.05$ vs. untransfected neurons exposed to vehicle; $\star P < 0.05$ vs. h- α Syn^{WT} expressing neurons exposed to A β O; $n = 6$ dishes/group