Accumulation of oligomer-prone α-synuclein exacerbates synaptic and neuronal degeneration in vivo

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In Parkinson’s disease and dementia with Lewy bodies, α-synuclein aggregates to form oligomers and fibrils; however, the precise nature of the toxic α-synuclein species remains unclear. A number of synthetic α-synuclein mutations were recently created (E57K and E35K) that produce species of α-synuclein that preferentially form oligomers and increase α-synuclein-mediated toxicity. We have shown that acute lentiviral expression of α-synuclein E57K leads to the degeneration of dopaminergic neurons; however, the effects of chronic expression of oligomer-prone α-synuclein in synapses throughout the brain have not been investigated. Such a study could provide insight into the possible mechanism(s) through which accumulation of α-synuclein oligomers in the synapse leads to neurodegeneration. For this purpose, we compared the patterns of neurodegeneration and synaptic damage between a newly generated mThy-1 α-synuclein E57K transgenic mouse model that is prone to forming oligomers and the mThy-1 α-synuclein wild-type mouse model (Line 61), which accumulates various forms of α-synuclein. Three lines of α-synuclein E57K (Lines 9, 16 and 54) were generated and compared with the wild-type. The α-synuclein E57K Lines 9 and 16 were higher expressings of α-synuclein, similar to α-synuclein wild-type Line 61, and Line 54 was a lower expressing of α-synuclein compared to Line 61. By immunoblot analysis, the higher-expressing α-synuclein E57K transgenic mice showed abundant oligomeric, but not fibrillar, α-synuclein whereas lower-expressing mice accumulated monomeric α-synuclein. Monomers, oligomers, and fibrils were present in α-synuclein wild-type Line 61. Immunohistochemical and ultrastructural analyses demonstrated that α-synuclein accumulated in the synapses but not in the neuronal cells bodies, which was different from the α-synuclein wild-type Line 61, which accumulates α-synuclein in the soma. Compared to non-transgenic and lower-expressing mice, the higher-expressing α-synuclein E57K mice displayed synaptic and dendritic loss, reduced levels of synapsin 1 and synaptic vesicles, and behavioural deficits. Similar alterations, but to a lesser extent, were seen in the...
Introduction

α-Synucleinopathies are a heterogeneous group of neurodegenerative disorders characterized by widespread neurodegeneration (Hardy, 2010) and by abnormal accumulation of α-synuclein. α-Synuclein is a 14 kDa (Weinreb et al., 1996) synaptic protein (Iwai et al., 1995) involved in vesicular release (Burre et al., 2010). Pathological accumulation of α-synuclein occurs predominantly in neuronal cells and synapses, as observed in Parkinson’s disease and dementia with Lewy bodies (Spillantini et al., 1997; Takeda et al., 1998), or in glial cells, as observed in multiple system atrophy (Papp and Lantos, 1994; Wakabayashi et al., 1998). There is great interest in what causes α-synuclein to undergo conformational changes to form oligomers, protofibrils and fibrils, which accumulate in cells (Uversky et al., 2001; Tsigeln, 2012). The relative toxic contribution of the different α-synuclein arrays, namely monomers, oligomers and fibrils, has been scrutinized. Although the precise identification of the toxic α-synuclein species remains unclear, clues to its identity come from familial forms of α-synucleinopathy where mutated forms of α-synuclein (A53T, E46K) (Polymeropoulos et al., 1997) form oligomers faster than wild-type α-synuclein (Conway et al., 1998). Likewise, in sporadic forms of α-synucleinopathies, post-translational modifications, including C-terminus cleavage (Sung et al., 2005), oxidation, nitration (Hodara et al., 2004) and phosphorylation (Fujiwara et al., 2002) might contribute to oligomerization, polymerization and toxicity (Paleologou et al., 2010).

In facilitating our ability to study the oligomeric forms of α-synuclein and to assess its toxic characteristics, we previously generated variants of α-synuclein bearing synthetic mutations (E35K and E57K) designed to disrupt α-synuclein-specific salt bridges and thereby alter the structure of α-synuclein. These mutations resulted in α-synuclein species that specifically formed oligomers but not fibrils (Winner et al., 2011) and when delivered into the substantia nigra by way of lentiviral vector injection, led to dopaminergic cell loss. The viral vector injection system provides information about the acute local effects of α-synuclein oligomers; however, the effects of chronic and more widespread α-synuclein E57K expression in other areas of the brain associated with dementia with Lewy bodies and the other α-synucleinopathies remain unexamined. A model with these characteristics could provide insight into the possible mechanism(s) through which accumulation of α-synuclein oligomers in the synapse leads to neurodegeneration.

In this context, we generated oligomer-prone novel transgenic mouse lines expressing higher- and lower-levels of human α-synuclein bearing the synthetic α-synuclein E57K mutation under the control of the neuronal mThy-1 promoter, and then we compared them to α-synuclein wild-type mice. α-Synuclein E57K mice displayed synaptic and dendritic loss, reduced levels of synapsin 1 and synaptic vesicles, and behavioural deficits. Similar alterations were seen in α-synuclein wild-type mice; however, although oligomer-prone α-synuclein mice displayed neurodegeneration in the frontal cortex and hippocampus, α-synuclein wild-type mice only displayed neuronal loss in the hippocampus. These results support the hypothesis that accumulating oligomeric α-synuclein may mediate early neurodegenerative changes by disrupting synaptic vesicles. This oligomer-prone model might be useful for evaluating therapies directed at oligomer reduction.

Materials and methods

Generation of mThy-1 human α-synuclein E57K transgenic mice

The University of California at San Diego’s animal subjects committee approved all experiments. Mice expressing human (h)α-synuclein bearing the oligomer-prone E57K mutation under the neuronal mThy-1 promoter cassette (provided by Dr H. van der Putten, Ciba-Geigy, Basel, Switzerland) were generated. The α-synuclein E57K variant was constructed by site-directed mutagenesis (QuikChange kit; Stratagene) and confirmed by DNA sequencing (Winner et al., 2011). The α-synuclein E57K complementary DNA fragment was ligated into pCRII (Invitrogen), sequenced for accuracy, inserted into the mThy-1 expression cassette between exons 2 and 4, purified, and microinjected into single-cell embryos (C57BL/6 × DBA/2F1) (Rockenstein et al., 2002). Genomic DNA, extracted from tail biopsies, was analysed by PCR amplification (Rockenstein et al., 1995; Masliah et al., 2000). Heterozygous α-synuclein E57K mice were crossed with wild-type (DBA) mice to generate transgenic and non-transgenic littermates. Lines expressing lower (Line 54) and higher (Lines 9 and 16) levels of α-synuclein were selected for analysis compared to our previously described mThy-1 α-synuclein wild-type Line 61 (Rockenstein et al., 2002), which accumulates various species of α-synuclein accompanied by axonal pathology, dopaminergic and behaviour deficits (Games et al., 2013).

Behavioural analysis

Context-dependent learning in an open field data were collected using a Kinder SmartFrame Cage Rack Station activity monitor system (Kinder Scientific), in 3D space using a 7 x 15 beam configuration.
Data collection began when an animal was placed in the test chamber. Animals were evaluated for 10 min for three consecutive days, given a 2-day dishabituation period, followed by a fourth trial (Chen et al., 2012; Sanchez et al., 2012).

Tissue preparation

After behavioural analysis, mice were sacrificed following NIH guidelines. The right hemi-brain was post-fixed for 48 h in 4% phosphate-buffered paraformaldehyde (pH 7.4) at 4°C and sagittal sectioned Vibratome 2000 (40 μm; Leica). The left hemi-brain was snap-frozen and stored at −70°C.

Real-time polymerase chain reaction

RNA was extracted from mice in triplicate using the RNeasy kit (QIAGEN) (Rockenstein et al., 2002), and quantified by spectrophotometer readings. For complementary DNA synthesis, 1 μg total RNA was reverse transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad). Real-time PCR experiments were performed using the iQ™SYBR® Green Detection System (Bio-Rad). Amplification was performed on complementary DNA equivalent to 25 ng total RNA with 1 × iQ™SYBR® Green Supermix (Bio-Rad). Template PCR reactions were performed in triplicate and run in duplicate using the following PCR cycling parameters: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 15 s, 60°C for 1 min before a dissociation protocol to verify the presence of a single product for each amplicon. The amount of complementary DNA was calculated by the comparative threshold cycle method and expressed using mouse actin as an internal control.

Immunohistochemical analysis

Analysis of α-synuclein accumulation was performed using free-floating, blind-coded sections (Ubhi et al., 2010; Masliah et al., 2011). Sections were incubated overnight at 4°C with antibodies against total α-synuclein (1:500, affinity purified rabbit polyclonal, Millipore) (Masliah et al., 2000), human α-synuclein (LB509, mouse monoclonal), and pSer129 α-synuclein (mouse monoclonal, Wako laboratories), followed by biotin-tagged anti-rabbit or anti-mouse IgG (1:100, Vector Laboratories, Inc.) secondary antibodies, Avidin D-HRP (1:200, ABC Elite, Vector), and visualized with diaminobenzidine. Sections were scanned with a digital Olympus bright field digital microscope (BX41).

Neurodegenerative pathology was observed using sections immunolabelled overnight with antibodies against the dendritic marker microtubule-associated protein 2 (MAP2; 1:500, Millipore), neuronal marker NeuN (1:500, Millipore), astroglial marker glial fibrillary acidic protein (GFAP, 1:1000, Millipore), and synaptic marker synaptophysin (SY38, 1:500, Millipore) (Ubhi et al., 2010; Masliah et al., 2011). Sections reacted with antibodies against NeuN or GFAP were incubated with secondary antibodies, Avidin D-HRP, and visualized with diaminobenzidine. Sections reacted with antibodies against MAP2 and synaptophysin were visualized with FITC-tagged secondary antibody or the Tyramide Signal Amplification™ Direct (Red) system (1:100, NEN Life Sciences), respectively, mounted under glass coverslips with anti-fading media (Vector Laboratories), and imaged with the laser scanning confocal microscope (MRC1024, Bio-Rad).

The numbers of NeuN-immunoreactive neurons were estimated using unbiased stereological methods (Owerk et al., 2009). Hemisections containing the neocortex, hippocampus and striatum were outlined using an Olympus BX51 microscope running StereoInvestigator 8.21.1 software (Micro-BrightField). Grid sizes for the striatum, frontal cortex, and hippocampal CA3 pyramidal layer were: 900 × 900, 800 × 800, and 300 × 300 μm, respectively, and the counting frames were 40 × 40, 30 × 30, and 50 × 50 μm, respectively. The average coefficient of error for each region was 0.9.

Sections were analysed using a 100 × 1.4 PlanApo oil-immersion objective. A 5-μm high disector, allowed for 2 μm top and bottom guard-zones.

Double immunolabelling and fluorescence co-labelling

To determine the co-localization between α-synuclein and synaptic markers, double-labelling experiments were performed (Masliah et al., 2011). Sections were immunolabelled with antibodies against human α-synuclein (SY211) and synaptophysin (SY38, Millipore). To compare the proportion of synapses containing synapsin 1 in non-transgenic versus α-synuclein E57K mice and α-synuclein wild-type mice, double-labelling experiments were performed with antibodies against synapsin 1 and synaptophysin (SY38, Millipore). All sections were processed simultaneously, and experiments were performed in triplicate. The synaptophysin-immunoreactive terminals were detected with Tyramide Red (NEN Life Sciences), whereas SYN211 and synapsin 1 were detected with FITC-tagged antibodies (1:75, Vector). Sections were imaged with a Zeiss × 63 1.4 objective on an Axiovert 35 microscope (Zeiss) with an attached MRC1024 laser scanning confocal microscope system (Bio-Rad) (Masliah et al., 2011). Paired optical sections were analysed with ImageJ co-localization colour map software to determine the per cent of synaptophysin-positive terminals containing α-synuclein- or synapsin-1-immunoreactivity. For each mouse, ~20 digital images, each containing 700 synapses on average, were analysed. Per cent neuropil was calculated based on area of intensity above threshold divided by the geometric area.

Tissue fractionation, immunoblot analysis and size exclusion chromatography

Conventional immunoblot

The distribution and levels of α-synuclein in mouse brains were analysed using lysate that were extracted and fractioned into soluble and insoluble fractions by ultracentrifugation (Masliah et al., 2011). Protein (20 μg/lane) was loaded onto 4–12% SDS/PAGE gels and blotted onto polyvinylidene difluoride membranes, incubated with rabbit polyclonal anti-α-synuclein (1:1000; Millipore) or mouse monoclonal α-synuclein antibody (1:1000; BD), followed by HRP-tagged secondary antibodies (1:5000; Santa Cruz Biotechnology). Bands were visualized by enhanced chemiluminescence (PerkinElmer) and analysed with a quantitative Versadoc XL imaging apparatus (Bio-Rad). β-Actin (1:3000) was the loading control.

Sequential extraction

Oligomers were evaluated using additional immunoblot analysis of protein following three sequential extractions to generate Tris-buffered saline (TBS)-, TBST-, and urea-soluble fractions (Tofaris et al., 2006). Protein extracts were run on 4–12% Bis-Tris gels (Invitrogen) and electroblotted onto nitrocellulose membranes (Millipore). Membranes were fixed in 0.4% paraformaldehyde for 30 min (Lee and Kamitani, 2011), washed in PBS, and blocked for 30 min in PBS Tween (PBS with 0.2% Tween-20, and 5% bovine serum albumin), followed by
incubation with either SYN211 (1:600; Sigma) or human- and rodent-specific antibody SYN1 (1:1000; BD Bioscience in PBS Tween containing 5% bovine serum albumin) overnight. After washing with PBS Tween, membranes were probed with anti-mouse secondary antibodies (1:5000, American Qualex), normalized as above and quantified (Nuber et al., 2008).

Native gel electrophoresis was conducted on 12% Tris-glycine gels with native (SDS-free) running and sample loading buffer (Invitrogen) (Fauvet et al., 2012).

Sequential homogenates were further fractionated by size exclusion chromatography using a 2500 10/300 GL column (GE Healthcare), previously equilibrated in TBS + buffer with 0.05% (w/v) sodium azide. Elution was performed at 0.5 ml/min monitoring UV signals at 280 nm and 260 nm. Fractions of 0.5 ml were collected during elution, starting at 6 ml (just before the void volume) and ending at 22 ml (close to the end of the column). Aliquots of the eluted fractions were then used for SDS-PAGE and western blot analyses. LB509 from Abcam was used as primary antibody.

Cases with dementia with Lewy bodies
For comparison with the sequential immunoblot assay in the α-synuclein mice, frozen samples from the right hemisphere from six dementia with Lewy bodies cases were obtained from patients evaluated neurologically and psychometrically at the Alzheimer Disease Research Centre, University of California, San Diego (Hansen, 1997). Control cases were obtained from the German Brain Bank. The diagnosis was based on the clinical presentation of dementia and the pathological findings of Lewy bodies were detected with antibodies against α-synuclein, as recommended (McKeith et al., 1996). Tissue was sequentially extracted and 25 µg of TBS and urea extracts used for immunoblot studies, as described above (Nuber et al., 2013).

Fibril assembly
For generation of a dot blot standard, fibrils were prepared using 1 mg/ml α-synuclein monomer in assembly buffer (50 mM Tris, 100 mM NaCl, pH 7.0) and agitated (37°C; 1000 rpm). Reaction was stopped after 3 days, diluted with PBS (10 µM final concentration) and stored at −20°C. A short sonication with a tip sonicator (Fisher Scientific) increased the dissociation of small oligomers to generate ‘seeds’.

Dot blot analysis
Formation of α-synuclein oligomers was determined using dot blot measurements with the A11 antibody (1:4000; BioSource) (Kayed et al., 2003). Duplicate cytosolic or hippocampal Triton™ X-100 membrane fractions (8 µg) were spotted on a polyvinylidene difluoride membrane, blocked for 30 min with 5% bovine serum albumin in PBS Tween, incubated with A11 for 1.5 h at room temperature, and treated with HRP-conjugated anti-rabbit IgG for 45 min. Bands were visualized with enhanced chemiluminescence, and analysed using Image Quant Software (Amersham Bioscience).

Electron microscopy and immunogold analysis
Vibratome sections from the non-transgenic and α-synuclein E57K mice (Line 16) were post-fixed in 1% glutaraldehyde, treated with osmium tetroxide, embedded in Epon® araldite and sectioned with the ultramicrotome (Leica) (Masliah et al., 2011). Grids were analysed with a Zeiss OM10 electron microscope (Rockenstein et al., 2001). These same serial sections were used for analysis of synaptic vesicle counts (Applegate and Landfield, 1988). Approximately 100 terminals and 2500 vesicles were analysed per animal. Immunogold-labelled sections were mounted on nickel grids, etched and incubated with the LB509 antibody (1:50) and labelled with 10 nm Aurion ImmunoGold particles (1:50, Electron Microscopy Sciences) with silver enhancement. Electron micrographs (∗×25 000 magnification) were obtained.

Statistical analysis
All analyses used GraphPad Prism (version 5.0). Differences among means were assessed by one-way ANOVA with Dunnett’s post hoc test when compared to non-transgenic and by Tukey-Kramer when comparing transgenic groups. Two-way ANOVA with repeated measures followed by a Bonferroni multiple comparisons post hoc test was used for analysing the interactions between groups and time. The null hypothesis was rejected at the 0.05 level.

Results
Characterization and comparison of messenger RNA and protein expression in transgenic mouse lines expressing the α-synuclein E57K mutation versus wild-type α-synuclein
To investigate the effects of chronic widespread expression of oligomer-prone α-synuclein, we generated novel transgenic mouse lines (Lines 9, 16 and 54) expressing the α-synuclein E57K mutation under the control of the neuronal mThy-1 promoter (Fig. 1A), and analysed them with mThy-1 α-synuclein wild-type Line 61 for expression of α-synuclein at the messenger RNA (Fig. 1B) and protein levels (Fig. 1C). Quantitative PCR analysis of messenger RNA isolated from brain tissue demonstrated that α-synuclein E57K Lines 9 and 16 were higher-expressing lines, similar to α-synuclein wild-type Line 61 (Fig. 1B) (Rockenstein et al., 2002), whereas α-synuclein E57K Line 54 was a low-expressing line producing a fifth of the Line 61 expression (Fig. 1B). Consistent with the quantitative PCR results, immunoblot analysis demonstrated that α-synuclein E57K Lines 9 and 16 and α-synuclein wild-type Line 61 expressed significantly higher levels of α-synuclein compared to non-transgenic mice (Fig. 1C–F). α-Synuclein was primarily found in the cytosolic fraction, and the most abundant band was detected at 14 kDa, corresponding to monomeric α-synuclein (Fig. 1C and D). Other bands between 28 and 62 kDa were found in the Lines 9 and 16 and, to a lesser extent, in Line 61. In the membrane fraction, the 14 kDa band was detected to a significantly greater extent in α-synuclein E57K Lines 9 and 16 and α-synuclein wild-type Line 61, but not in Line 54 (Fig. 1E and F). Compared to the α-synuclein E57K lines, the α-synuclein wild-type mice displayed a prominent band at 28 kDa in the membrane fraction (Fig. 1E). Levels of α-synuclein protein in α-synuclein E57K Lines 9 and 16 were comparable to those observed in α-synuclein wild-type mice (Line 61; Fig. 1E and F).
Characterization of α-synuclein aggregates in the α-synuclein E57K transgenic mouse lines

To investigate the formation of α-synuclein aggregates in the α-synuclein E57K mice, we performed sequential extraction procedures with detergent-containing buffers yielding three main extracts. The first extract was enriched in TBS-soluble cytosolic proteins, the second extract contained Triton™ X-100-soluble membrane and organelle proteins, and the third extract contained SDS/urea-soluble proteins, including integral membrane proteins and cytoskeletal filaments (Schindler et al., 2006). In urea-treated extracts, complex α-synuclein aggregates typically solubilize as a high-molecular weight band and its monomer (Culvenor et al., 1999; Campbell et al., 2001; Kahle et al., 2001; Liu et al., 2005; Tong et al., 2010) (Fig. 2A). Western blot analyses of the TBS-soluble extracts of hippocampi showed a similar band at ~14 kDa in Lines 9, 16 and 61 (Fig. 2A). Incubation with the human-specific anti-α-synuclein with the urea-soluble fraction showed a specific band running at ~70 kDa molecular weight.

Figure 1 Generation of transgenic mice expressing the synthetic α-synuclein mutation E57K under the mThy-1 promoter. (A) Diagram of the mThy-1 construct. (B) Levels of α-synuclein messenger RNA expressed as a ratio to the housekeeping gene Gadph in non-transgenic and α-synuclein E57K Lines 9, 16, and 54, showing that Line 54 had 0.2 of the expression compared to α-synuclein E57K Line 9 and 16 and α-synuclein wild-type Line 61 transgenic mice. (C) Representative western blot (SDS) and (D) analysis of the levels of α-synuclein in the cytosolic fractions showing that Lines 9 and 16 were higher expressers of α-synuclein monomers and dimers, which was to a similar extent as Line 61. (E) Representative western blot (SDS) and (F) analysis of levels of monomer, dimer, and oligomer α-synuclein immunoreactivity in the membrane-bound fractions. Across all lines monomeric α-synuclein (14 kDa) was present to a greater extent in the cytosolic fraction compared to the membrane fraction. For analysis, six non-transgenic and six mThy-1 α-synuclein E57K transgenic mice (3–4 months old) from each line were used. *P < 0.05 when compared to non-transgenic control using one way ANOVA with Dunnet’s post hoc test. Tg = transgenic; wt = wild-type.
marker in α-synuclein E57K Lines 9 and 16 mice, consistent with pentamer α-synuclein conformer (Fig. 2B), which was also present in cases with dementia with Lewy bodies (Fig. 2C). Although urea-soluble fraction from α-synuclein wild-type Line 61 mice had a strong band at ~14 kDa (Fig. 2B), it was faint in the α-synuclein E57K, non-transgenic lines, and dementia with Lewy body cases (Fig. 2C). Immunoblot analysis of α-synuclein in native gels was consistent with previous studies (Fauvet et al., 2012), which showed a faint band at ~55 kDa for Lines 9, 16 and 54, and a dense smear was most prominent in Lines 9 and 16 between 70–200 kDa. In the α-synuclein wild-type transgenic line, there was a prominent band at ~55 kDa and less smearing between 70 and 200 kDa. (E) Representative image of the dot blot analysis with the A11 and FILA-1 antibodies using Triton™ X-100 membrane-extracted fraction indicated that α-synuclein oligomers (F) were present in all α-synuclein E57K transgenic lines, and Line 61, albeit to a lesser extent than in Line 16. (G) Only Line 61 had significant levels of fibrils above non-transgenic background. Seeded and fibrillized dose responses of recombinant α-synuclein protein were generated for qualitative concentration analysis. For analysis, four non-transgenic and six transgenic mice (3-4 months old) from each line were used. Tg = transgenic; wt = wild-type * = p-value < 0.05 compared to non-tg; # = p-value < 0.05 compared to Line 61.

Patterns of α-synuclein immunoreactivity and distribution in α-synuclein E57K transgenic compared with α-synuclein wild-type transgenic mice

To analyse the anatomical distribution of α-synuclein in the α-synuclein E57K mice, sections were immunolabelled with antibodies against total α-synuclein. As expected, in the non-transgenic mice the antibody against total α-synuclein reacted mildly with fine granular structures in the neuropil throughout the CNS,
Figure 3 Separation of oligomeric and monomeric species in extracts of wild-type and E57K transgenic mice using size exclusion chromatography-profiles (top) before western blot (bottom). (A) α-Synuclein extracted from α-synuclein wild-type mice with TBS+ buffer was found as monomers and small oligomers (lanes 4 and 5). (B) α-Synuclein E57K-transgenic mice brain extracts contained monomeric α-synuclein (lane 5 bottom) and oligomers with a molecular weight >200 kDa, which elute near the void volume and are retained in the stacking gel of standard SDS-PAGE (lanes 1–2). (C) Eluted fractions from urea-soluble extracts of α-synuclein wild-type transgenic mice reveal a monomeric α-synuclein band (lane 4) in addition to an intense absorbance peak near the void volume. (D) Eluted fractions from urea-soluble extracts of α-synuclein E57K transgenic mice also had an intense absorbance peak near the void volume although the oligomeric species were bigger in size than those extracted from wild-type transgenic mice (compare lanes 1–4 of C with lanes 1–4 of D). There was no evidence of a band corresponding to monomeric α-synuclein in the α-synuclein E57K transgenic urea-soluble homogenates. EV = elution volume; HMW = high molecular weight; tg = transgenic; WB = western blot; wt = wild-type.
consistent with the distribution of the endogenous murine α-synuclein (Fig. 4A–D). Transgenic α-synuclein E57K mice from Lines 9 and 16 displayed intense immunoreactivity within the neuropil in the neocortex (Fig. 4A), limbic system (Fig. 4A and C), basal ganglia (Fig. 4A and C), thalamus and substantia nigra pars compacta (Fig. 4A and D), whereas α-synuclein was not observed in most neuronal cell bodies in any of the α-synuclein E57K lines (Fig. 4B and C), although occasionally there were α-synuclein-positive neuronal cell bodies in the substantia nigra (Fig. 4C). Consistent with the immunoblot analysis, mice from Line 54 showed a similar distribution of α-synuclein, but to a lesser extent than Lines 9 and 16 (Fig. 4). In contrast, tissue from Line 61 overexpressing α-synuclein wild-type mice (Rockenstein et al., 2002) showed intense immunoreactivity that was detected in the neuropil and in neuronal cell bodies, in some instances mimicking intracellular inclusions (Fig. 4B–D arrows).

To further investigate the presence and distribution of α-synuclein aggregates in the brains of the α-synuclein E57K mice, immunocytochemical analysis was performed in sections pretreated with proteinase K. Previous studies have shown that α-synuclein aggregates are proteinase K resistant (Takeda et al., 1998; Kahle et al., 2001). As expected, in untreated (proteinase K/C0) sections from the α-synuclein E57K mice, most α-synuclein immunoreactivity was present in the neuropil and neuronal cell bodies (arrow). The inset box illustrates a detailed area at higher magnification. (C) Immunoreactive α-synuclein punctae were also present in the neuropil of the hippocampus and striatum in Lines 9 and 16, and to a lesser extent in Line 54, whereas in the α-synuclein wild-type transgenic Line 61 mice, α-synuclein immunostaining was also present in neuronal cell bodies (N) and dystrophic neurites (arrows). (D) α-Synuclein was present in the neuropil of the thalamus in Lines 9 and 16, and to a lesser extent in Line 54, and occasionally in the cell bodies in the substantia nigra pars compacta (insets) whereas in the α-synuclein wild-type transgenic Line 61 tissue, α-synuclein immunostaining was present in the neuropil and dystrophic neurites. In the substantia nigra pars compacta, α-synuclein accumulated in the neuronal cell bodies (arrows). For analysis, six non-transgenic, n = 6 α-synuclein E57K transgenic mice from each line and six α-synuclein wild-type transgenic Line 61 mice (8–10 months old) were used. Scale bars: A = 150 μm; B = 25 μm; C = 50; D = 25 μm.

Figure 4 Comparison of the patterns of α-synuclein distribution in the brains of the α-synuclein E57K and α-synuclein wild-type transgenic mice. Vibratome sections were immunostained with the rabbit polyclonal antibody (Millipore) against full-length total α-synuclein. (A) Low-magnification photomicrographs of regions of interest, including the neocortex (1), hippocampus (2), striatum (3) and (4) thalamus in the non-transgenic α-synuclein E57K and α-synuclein wild-type transgenic mice. (B) Staining against α-synuclein was present in the neuropil of the neocortex in Lines 9 and 16, and to a lesser extent in Line 54, whereas in the α-synuclein wild-type transgenic Line 61 mouse model, α-synuclein immunoreactivity was present in the neuropil and neuronal cell bodies (arrow). The inset box illustrates a detailed area at higher magnification. (C) Immunoreactive α-synuclein punctae were also present in the neuropil of the hippocampus and striatum in Lines 9 and 16, and to a lesser extent in Line 54, whereas in the α-synuclein wild-type transgenic Line 61 mice, α-synuclein immunostaining was also present in neuronal cell bodies (N) and dystrophic neurites (arrows). (D) α-Synuclein was present in the neuropil of the thalamus in Lines 9 and 16, and to a lesser extent in Line 54, and occasionally in the cell bodies in the substantia nigra pars compacta (insets) whereas in the α-synuclein wild-type transgenic Line 61 tissue, α-synuclein immunostaining was present in the neuropil and dystrophic neurites. In the substantia nigra pars compacta, α-synuclein accumulated in the neuronal cell bodies (arrows). For analysis, six non-transgenic, n = 6 α-synuclein E57K transgenic mice from each line and six α-synuclein wild-type transgenic Line 61 mice (8–10 months old) were used. Scale bars: A = 150 μm; B = 25 μm; C = 50; D = 25 μm.
wild-type mice, proteinase K pretreatment resulted in the appearance of \( \alpha \)-synuclein-positive granular structures in the neuropil, whereas the neuronal and synaptic \( \alpha \)-synuclein labelling was reduced (Fig. 5A). The proteinase K-resistant \( \alpha \)-synuclein neurites were most abundant in the neocortex (Fig. 5A), in the hippocampal molecular layer and lacunosum, and in the basal ganglia and substantia nigra (not shown). In agreement with these findings, analysis with the LB509 antibody, which could detect pathological as well as other types of human \( \alpha \)-synuclein (Jakes et al., 1999), showed the presence of abundant immunoreactive granular structures in the neuropil of the \( \alpha \)-synuclein E57K mice (Fig. 5B). In the \( \alpha \)-synuclein wild-type mice, LB509 reacted with the neurites in the neuropil, as well as with the neuronal cell bodies (Fig. 5B). Similarly, immunostaining with an antibody against phosphorylated \( \alpha \)-synuclein (Ser129) showed the presence of abnormal immunoreactive neurites in the higher-expressing \( \alpha \)-synuclein

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**Figure 5** Accumulation of proteinase K-resistant \( \alpha \)-synuclein in the \( \alpha \)-synuclein E57K transgenic mice. (A) Representative photomicrographs (frontal cortex) of brain sections from non-transgenic and \( \alpha \)-synuclein transgenic mice immunostained with the \( \alpha \)-synuclein polyclonal antibody (Millipore) without proteinase K (PK−, top) and with proteinase K (PK+, bottom) treatment. In the proteinase K-treated tissue from non-transgenic and \( \alpha \)-synuclein transgenic mice, the antibody stained the neuropil as well as the neurons (n) in the Line 61 mice. The proteinase K + treatment eliminated most of the neuropil immunostaining in the non-transgenic mice, while at the same time revealing \( \alpha \)-synuclein in axons (arrowheads), as well as in dystrophic neurites in the neuropil of the \( \alpha \)-synuclein E57K transgenic Lines 9 and 16 and the \( \alpha \)-synuclein wild-type transgenic Line 61 mice. (B) Patterns of LB509 (pathological \( \alpha \)-synuclein, top) immunostaining in the neocortex of the non-transgenic and \( \alpha \)-synuclein transgenic mice. The non-transgenic mice did not immunoreact with the LB509 antibody, whereas in the \( \alpha \)-synuclein E57K transgenic mice, \( \alpha \)-synuclein immunostaining was present in the neuropil. The \( \alpha \)-synuclein wild-type transgenic Line 61 mice displayed \( \alpha \)-synuclein accumulation in the neuron cell body (n) and neuropil. Patterns of p129S \( \alpha \)-synuclein immunostaining in the neocortex of the non-transgenic and \( \alpha \)-synuclein transgenic (bottom) indicated that the non-transgenic mice showed no labelling with the pSer129 antibody whereas the \( \alpha \)-synuclein E57K transgenic mice showed \( \alpha \)-synuclein immunostaining in the neuropil (arrowheads). The \( \alpha \)-synuclein wild-type transgenic Line 61 mice presented \( \alpha \)-synuclein accumulation in the neuron cell body (n) and neuropil. Scale bar = 10 \( \mu \)m. Four non-transgenic and four \( \alpha \)-synuclein E57K transgenic mice (8–10 months old) from each line were used. Tg = transgenic; wt = wild-type.
E57K mice (Fig. 5B). In the α-synuclein wild-type mice, the pSer129 antibody reacted with the neurites as well as with the neuronal cell bodies (Fig. 5B).

To analyse the distribution of the transgenic human α-syn, immunofluorescent and confocal analysis was performed with the SYN211 antibody. Using confocal microscopy we found close co-localization between human α-synuclein (SYN211) and synaptophysin in the neocortex (Fig. 6A), hippocampus (Fig. 6B) and other brain regions (not shown) in all three lines of α-synuclein E57K mice, as well as in the α-synuclein wild-type expressing Line 61, indicating the accumulation of α-synuclein in the synapse. However in the latter mouse line, α-synuclein also accumulated in the neuronal cell bodies (Fig. 6).

**Accumulation of oligomer-prone α-synuclein results in synaptic pathology and reduced vesicles**

To determine if the accumulation of oligomerization prone α-synuclein E57K in the synapses led to neurodegenerative pathology, immunohistochemical analysis was performed (Fig. 7). As we were interested in the chronic effects of a more global accumulation of α-synuclein accumulation in brain regions relevant to dementia with Lewy bodies and Parkinson’s disease with dementia, analyses hereafter were performed in the frontal cortex and hippocampus. Confocal analysis of synaptic terminals with an antibody against synaptophysin showed a significant 20–40% loss in the frontal cortex in the higher-expressing Lines 9 and 16, as well as Line 61, but not in Line 54 (Fig. 7A and C). In the hippocampus there was a similar loss in Lines 16 and 61, but not Lines 9 or 54 (Fig. 7C). Likewise, analysis of dendrites with an antibody against MAP2 displayed a significant 20–40% loss in the frontal cortex of the higher-expressing Lines 9 and 16, as well as Line 61, but not Line 54 (Fig. 7C). In the CA1-CA3 regions of the hippocampus, there was a significant reduction across all lines (Fig. 7B and C).

As the higher-expressing α-synuclein E57K mice displayed synaptic accumulation of α-synuclein accompanied by decreased synaptophysin and MAP2 immunoreactivity, and as we have previously shown that accumulation of α-synuclein oligomers impairs the transport of synaptic proteins (Scott et al., 2010), immunoblot analysis was performed using fronto-temporal cortex. Levels of synaptophysin showed a trend toward a reduction in Line 9 as well as Line 61 and were significantly reduced by 20% in Line 16 (Fig. 8A and B). In Lines 9 and 16, as well as Line 61, levels of synapsin 1 were significantly reduced.
reduced by 30–50%, and levels of RIMS3 were significantly reduced by 55% in Line 16 (Fig. 8A and B). In contrast, levels of SNAP25 were not altered when compared to non-transgenic controls (Fig. 8A and B).

Further analysis of the frontal cortex using double-labelling and confocal microscopy confirmed that the proportion of synaptophysin (SY38) immunoreactive terminals containing synapsin 1 was significantly decreased by 50–60% in the higher-expressing Lines 9 and 16, as well as α-synuclein wild-type Line 61, displayed a significant reduction in synaptophysin in the frontal cortex, whereas line 54 (lower-expressing) was comparable non-transgenic littermates. In the hippocampus (hippo), synaptophysin was reduced in α-synuclein E57K Line 16 and the α-synuclein wild-type Line 61. MAP2 was reduced in the frontal cortex in α-synuclein E57K Lines 9 and 16, as well as α-synuclein wild-type Line 61, but not in α-synuclein E57K Line 54. In the hippocampus, MAP2 was reduced across all transgenic lines. *P < 0.05 when compared with non-transgenic using one-way ANOVA with Dunnet post hoc test. Scale bar = 10 μm for top row, 40 μm for the second row. Tg = transgenic; wt = wild-type.

Neuronal loss pathology in α-synuclein E57K mice

To determine if the alternations in the synapses (Figs 7–9) had an effect on neuron cell bodies, stereological assessment of NeuN-
positive cells was determined in the frontal cortex, hippocampus, and basal ganglia (Fig. 10A and B). In the frontal cortex, only the α-synuclein E57K Line 16 had a significant reduction in the number of neurons compared with non-transgenic controls, whereas in the hippocampus, there was a significant loss across α-synuclein E57K and α-synuclein wild-type mice (Fig. 10B). There were no changes in the neuronal population in the striatum (Fig. 10B). These neuronal alterations were accompanied by a significant increase in GFAP immunoreactivity in the neocortex and hippocampus in all mutant α-synuclein lines, as well as in Line 61 (Fig. 10C).

**Behavioural deficits are exacerbated in α-synuclein E57K compared to α-synuclein wild-type mice**

To evaluate the functional effects of the accumulation of the oligomer-prone α-synuclein E57K, analysis was performed using a context-dependent open field test in young and older α-synuclein E57K mice and compared with non-transgenic and α-synuclein wild-type mice. As most of the neuropathological analysis was performed in the frontal cortex and hippocampus, behavioural assessment of memory...
acquisition was performed. At the younger age, there was a significant interaction between time and genotype \( (P < 0.0001) \), in addition to a significant effect as a result of time \( (P = 0.0003) \), as evaluated using a two-way ANOVA with repeated measures. Moreover, the young (3–4 months old) non-transgenic and lower-expressing \( \alpha \)-synuclein E57K Line 54 mice showed habituation to the novel environment, as evidenced by decreased activity on the last day of testing (Fig. 11A). In contrast, \( \alpha \)-synuclein E57K Lines 9 and 16 mice displayed trends toward continued high activity and failure to habituate (no learning; Fig. 11A). These trends were reiterated between genotypes when compared to the non-transgenic mice during the fourth trial (Fig. 11B). At the older age, there was a significant interaction between time and genotype \( (P = 0.0011) \), in addition to a significant effect because of time \( (P = 0.0003) \) and genotype \( (P = 0.0135) \), as evaluated using a two-way ANOVA with repeated measures (Fig. 11C). Both the older, 8–10 month non-transgenic and

Figure 9  Ultrastructural and immunogold analyses of the synaptic terminals in the \( \alpha \)-synuclein E57K and \( \alpha \)-synuclein wild-type transgenic mice. Vibratome sections were post-fixed with glutaraldehyde and embedded in Epon\textsuperscript{a} araldite, and ultra-thin sections from the neocortex were prepared for transmitted electron microscopy (TEM) and immunogold analysis. (A) Representative electron micrographs from the neuropil of non-transgenic mice displaying normal characteristics for presynaptic terminals (PST), post-synaptic densities, and dendrites (d). In the neuropil of the higher expressing \( \alpha \)-synuclein E57K transgenic Lines 9 and 16, and in the \( \alpha \)-synuclein wild-type Line 61, the synapses showed depletion of vesicles in the nerve terminals. Electrodense bodies were seen in Line 16 (arrow). (B) Image analysis of serial micrographs to estimate the numbers of synaptic vesicles per terminal. Compared with non-transgenic controls, the higher-expressing \( \alpha \)-synuclein E57K transgenic mouse Lines 9 and 16, and \( \alpha \)-synuclein wild-type Line 61, showed a significant reduction in the number of vesicles. No alterations were observed in mice from Line 54 (lower expressing). (C) Representative electron micrographs from the neuropil of non-transgenic mice immunostained with the LB509 antibody against pathological \( \alpha \)-synuclein. Only a few scattered gold particles were detected in the neuropil. In the neuropil of the \( \alpha \)-synuclein E57K transgenic and \( \alpha \)-synuclein wild-type lines, the synapses showed abundant gold particles clustering at the nerve terminals and mitochondria. (D) Image analysis of serial micrographs to estimate the numbers of gold particles (LB509 reactive) per terminal. Compared with non-transgenic controls the higher-expressing \( \alpha \)-synuclein E57K transgenic mouse Lines 9 and 16 showed a significant increase, as did \( \alpha \)-synuclein wild-type Line 61. To a lesser extent, mice from Line 54 (lower expressing) also displayed accumulation of gold particles in synapses. \( n = 4 \) mice per group, age 8–10 months. * \( P < 0.05 \) when compared with non-transgenic using one-way ANOVA with Dunnet post hoc test. Scale bar = 1 \( \mu \)m for A and B; 0.5 \( \mu \)m for D and E. Tg = transgenic; wt = wild-type.
lower expressing α-synuclein E57K Line 54 mice showed habituation and thus learning, whereas in the α-synuclein E57K Line 9 there was a significant difference in activity (P < 0.0001; two-way ANOVA followed by Bonferroni multiple comparisons post hoc test). When comparing genotypes to the non-transgenic controls during the fourth trial, α-synuclein E57K Lines 9 and 16 and α-synuclein wild-type Line 61 were significantly different and failed to habituate to the environment (forgetting) (Fig. 11D).

**Discussion**

In the present study, we investigated the contribution of long-term accumulation of α-synuclein oligomers to early synaptic toxicity in mice expressing low and high levels of oligomerization-prone α-synuclein E57K compared to α-synuclein wild-type (Line 61) and non-transgenic mice. We found that, with this artificial mutation, α-synuclein accumulates primarily in the synapses, leading to early reduction in synaptophysin- and MAP2-immunoreactivity and alterations associated with impaired localization of synapsin 1 to the terminals. Loss of synaptic markers could indicate synaptic degeneration or reduced localization or expression of the protein as a result of α-synuclein accumulation in the synapse. We previously showed that the α-synuclein E57K mutant tended toward oligomerization rather than fibril formation, and direct lentiviral vector injection of α-synuclein E57K resulted in greater dopaminergic neuronal loss compared with other mutants and to wild-type α-synuclein (Winner et al., 2011). The tyrosine hydroxylase deficits within the E57K viral vector model were similar to those observed with naturally occurring mutations such as E46K. The present study is different from this earlier study in that we investigated the effects of more widespread and chronic expression of oligomer-prone α-synuclein on early synaptic pathology rather than the local acute effects on a restricted circuitry, as in the experiments involving the lentiviral injections.
A recent study used AAV2 to investigate the effects of aggregation of \( \alpha \)-synuclein (Taschenberger et al., 2012) on the progression of neurodegeneration by expressing proline substitution variants with different fibrillation propensities in the rat substantia nigra. The conformational changes induced by proline substitutions slowed fibril formation, most likely by impairing \( \alpha \)-helix formation. Although these proline substitutions (A30P/A56P/A76P) had a pathologic effect when combined in rat cortical neurons and Caenorhabditis elegans, formation of proteinase K-resistant \( \alpha \)-synuclein aggregates was associated with loss of nigral dopaminergic neurons and striatal fibres. Expression of two prefibrillar, structure-based design mutants of \( \alpha \)-synuclein (i.e. A56P and A30P/A56P/A76P) resulted in some neuronal loss in nigral dopaminergic neurons. However, only the \( \alpha \)-synuclein variants capable of forming fibrils (wild-type/A30P) induced a sustained progressive loss of adult nigral dopaminergic neurons. These AAV-based systems had slower onset of tyrosine hydroxylase loss, depending on the promoter, between 4 and 24 weeks (St Martin et al., 2007; Taschenberger et al., 2012). The differences seen between models might differ in the extent and duration of prefibrillar toxicity. Burre et al. (2012) also introduced different proline substitutions by injecting a lentivirus construct into mouse substantia nigra. Depending on the localization of the mutation within \( \alpha \)-synuclein, the introduction of these mutational changes by proline substitution were able to induce dopaminergic loss within the NAC region (residues 61–95) and in the familial

![Figure 11](http://brain.oxfordjournals.org/)

**Figure 11** Memory alterations in the \( \alpha \)-synuclein E57K and \( \alpha \)-synuclein wild-type transgenic mice. Mice were evaluated for context-dependent learning in an open field area first at 3–4 months of age and then at 8–10 months in a Kinder SmartFrame Cage Rack Station activity monitor system. (A) At 3–4 months of age, non-transgenic controls and \( \alpha \)-synuclein E57K transgenic Line 54 (lower-expressing) mice had reduced levels of activity over time as they became more familiar with the environment in the cage. The \( \alpha \)-synuclein E57K transgenic mice from Lines 9 and 16 and the \( \alpha \)-synuclein wild-type Line 61 transgenic mice showed a similar level of activity over time. (B) Evaluation of the fourth day of assessment compared to non-transgenic mice suggested trends toward dishabituation in \( \alpha \)-synuclein E57K Line 9 and \( \alpha \)-synuclein wild-type Line 61 (one-way ANOVA with Dunnett’s multiple comparisons post hoc test). (C) Both the older 8–10 month non-transgenic and lower-expressing \( \alpha \)-synuclein E57K Line 54 mice showed habituation and thus learning, whereas in the \( \alpha \)-synuclein E57K Line 9 there was a significant difference in activity (\( P < 0.0001 \); two-way ANOVA followed by Bonferroni multiple comparisons post hoc test). (D) When comparing genotypes to the non-transgenic controls during the fourth trial, \( \alpha \)-synuclein E57K Lines 9 and 16 and \( \alpha \)-synuclein wild-type Line 61 were significantly different and failed to habituate to the environment (forgetting). \( n = 12 \) non-tg, \( n = 7 \) \( \alpha \)-synuclein E57K Line 9, \( n = 8 \) \( \alpha \)-synuclein E57K Line 16, \( n = 14 \) \( \alpha \)-synuclein E57K Line 54, \( n = 14 \) \( \alpha \)-synuclein wild-type Line 61 mice per age group. *\( P < 0.05 \); **\( P < 0.001 \) when compared with non-transgenic by one-way ANOVA with Dunnet post hoc analysis. Tg = transgenic; wt = wild-type; m/o = month old.
A30P mutation, but not in mutations between residues 31 and 59. In addition, these mutations do not have an impact on the physiological role of α-synuclein in synaptic vesicles. In contrast, in this system the familial A53T, E46K mutations also had a major increase in pathology (Burre et al., 2012). We therefore concluded that proline substitutions might induce more selective toxicity. The difference between our results and those of the study by Taschenberger et al., (2012) can be explained by differences in α-synuclein structure and synaptic function induced by E-K mutations (negative charge to positive charge) that either led to different forms of prefibrillar structures or had differential impact on nucleation of aggregation of α-synuclein wild-type.

As behavioural and neurodegenerative pathology was present primarily in the higher-expressing mice as compared with the lower-expressing line, this implies a dose-dependent effect. Although the mutation we used is artificial, the α-synuclein oligomers are biochemically and ultrastructurally similar to those described with α-synuclein wild-type and with familial mutants such as A53T (Lashuel et al., 2002). Although the identity of toxic α-synuclein conformers and species in vivo are intensely investigated, it is clear from this and other studies that oligomerization plays a central role in neurodegeneration and the damage begins at the synaptic site and spreads back to the axons and neuronal cell bodies. For example, in the brains of patients with Parkinson’s disease and dementia with Lewy bodies, proteinase K-resistant α-synuclein accumulates primarily at the nerve terminals, where it might affect synaptic function and axonal transport (Kahle et al., 2001; Scott et al., 2010). Similar proteinase K-resistant α-synuclein aggregates were detected in synapses and axons of α-synuclein E57K mice. These proteinase K-resistant α-synuclein deposits correlate with synaptic and dendritic damage in dementia with Lewy bodies/Parkinson’s disease (Schulz-Schaeffer, 2010). The precise mechanisms through which α-synuclein oligomers might damage the synapse are not completely understood; however, interference with synaptic vesicle proteins (Burre et al., 2010; Fortin et al., 2010; Choi et al., 2013), axonal transport (Scott et al., 2010), calcium homeostasis (Dänzer et al., 2007; Reznichenko et al., 2012) and mitochondrial function (Nakamura et al., 2008) are involved. Consistent with the possibility that α-synuclein oligomers might impair synaptic function by interfering with the vesicles, the present study showed that the oligomer-prone α-synuclein E57K accumulated at the presynaptic site, where they might disrupt the presynaptic localization of synapsin 1, which is essential for vesicle clustering in the active zone (Shupliakov et al., 2011). In addition, the synapses had fewer synaptic vesicles, which is in agreement with a previous study which showed that in the brains of patients with dementia with Lewy bodies/Parkinson’s disease and in primary neuronal cultures from α-syn-GFP transgenic mice, synapsin 1 is mislocalized, which impairs overall synaptic function and plasticity (Scott et al., 2010) and results in deficient long term potentiation (Diogenes et al., 2012). The mechanisms through which synaptic oligomers disrupt synaptic vesicle function are under intense investigation. A recent study showed that dopamine-induced, large α-synuclein oligomers effectively inhibit neuronal Soluble NSF Attachment Protein Receptor (SNARE)-mediated vesicle lipid mixing by preferentially binding to the SNARE protein, synaptotubrin 2 (Choi et al., 2013). In addition, large α-synuclein oligomers reduced vesicular exocytosis in PC12 cells (Choi et al., 2013). Another interesting synaptic protein affected by the mutant α-synuclein was RIMS3. RIMs 1-3 are effector proteins for Rab3s, synaptic GTP-binding proteins. RIMs are localized close to the active zone at the synapse, where they interact in a GTP-dependent manner with Rab3s located on synaptic vesicles (Wang et al., 2000). We currently do not have direct evidence for the interaction of the α-synuclein oligomers with RIMS3 or Rab3s proteins; however, this is a potential mechanism through which α-synuclein could disrupt synaptic vesicle localization. Further investigation will be needed in the future.

α-Synuclein can associate with cell membranes, including synaptic vesicles, retrograde axonal transport vesicles, and detergent-resistant membrane-microdomains (Maroteaux et al., 1988; Jensen et al., 1998; Cole et al., 2002; Outeiro and Lindquist, 2003; Fortin et al., 2004). We therefore used different detergents (Triton™ X-100, SDS) and a chaotropic reagent (urea) to study the solubility and equilibrium of α-synuclein and its multimers at distinct neuronal structures. Consistent with our previous finding that α-synuclein oligomers are prone to form membrane-assembling multimeric aggregates (Tsigelny et al., 2007), which is further increased by the ‘conformation-trapped’ α-synuclein E57K mutation (Winner et al., 2011), we detected a specific SDS/urea-stable α-synuclein multimer in E57K transgenic mice, and in homogenates from dementia with Lewy bodies cases. Unlike α-synuclein E57K, α-synuclein wild-type oligomers dissolve mainly as an unspecific higher-molecular smear and its monomer (Kahle et al., 2002; Tofaris et al., 2006). The stability of the multimer suggests that the protein may be rigid, similar to other multimeric membrane proteins (Le Coutre et al., 1998; Hibino and Kurachi, 2007). It is possible that tight association with detergent-resistant membrane fractions such as lipid-rafts (Fortin et al., 2004) or conformational changes induced by integration into the membrane stabilize the α-synuclein E57K multimer, whereas α-synuclein wild-type aggregates are less membrane-associated and therefore dissociate into multiple higher-oligomeric and monomeric species in the presence of SDS/urea mixture. This observation is supported by new evidence that amyloid and oligomeric structures display a higher tendency to disrupt lipid formations of plasma membranes (Reynolds et al., 2011; Barrett et al., 2012). Therefore, the aggregates formed by the α-synuclein E57K (pentameric) and α-synuclein wild-type (monomeric) lines suggest structural similarities with urea-insoluble multimeric and monomeric α-synuclein detected in dementia with Lewy body cases.

Although higher-expressing α-synuclein E57K mice displayed similar levels of synaptic degeneration and neuronal loss in the hippocampus when compared to the α-synuclein wild-type mice, they differed in that the oligomer-prone mice displayed more severe synaptic pathology, behavioural differences and neuronal loss in the frontal cortex. This finding is consistent with the concept that oligomers drive the neurodegenerative process in dementia with Lewy bodies/Parkinson’s disease in a significant manner. Failure to form fibrils, as was the case in the α-synuclein E57K lines, was associated with exacerbating pathology. In the case of the α-synuclein wild-type line 61 mice, both oligomers and fibrillar (FILA-1 immunoactivity) α-synuclein were present, suggesting that the conversion of α-synuclein toward fibrillar
structures may attenuate the extent of the neurodegeneration and behavioural deficits.

In summary, this study supports the hypothesis that synaptic pathology, which is common to dementia with Lewy bodies and Parkinson’s disease with dementia and is an early pathological consequence of α-synuclein accumulation, may be mediated by oligomeric α-synuclein interfering with the synaptic vesicles, leading to neuronal loss. Moreover, this oligomer-prone model might be useful for evaluating therapies directed at oligomer reduction.

Acknowledgements

Human control cases were supplied by the German Brain Bank and analyses done in accordance with the Ethics Committee guidelines.

Funding

This work was funded by NIH grants AG18440, AG022074, and NS044233, as well as CIRM, the Michael J. Fox Foundation, The JPB Medical Foundation, Interdisciplinary Center of Clinical Research (IZFK), and The Lookout Fund. The authors declare no competing financial interests.

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