α-Synuclein is phosphorylated in synucleinopathy lesions

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The deposition of the abundant presynaptic brain protein α-synuclein as fibrillary aggregates in neurons or glial cells is a hallmark lesion in a subset of neurodegenerative disorders. These disorders include Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy, collectively referred to as synucleinopathies[1,2]. Importantly, the identification of missense mutations in the α-synuclein gene in some pedigrees of familial PD has strongly implicated α-synuclein in the pathogenesis of PD and other synucleinopathies[3]. However, specific post-translational modifications that underlie the aggregation of α-synuclein in affected brains have not, as yet, been identified. Here, we show by mass spectrometry analysis and studies with an antibody that specifically recognizes phospho-Ser 129 of α-synuclein, that this residue is selectively and extensively phosphorylated in synucleinopathy lesions. Furthermore, phosphorylation of α-synuclein at Ser 129 promoted fibril formation *in vitro*. These results highlight the importance of phosphorylation of filamentous proteins in the pathogenesis of neurodegenerative disorders.

We have previously identified α-synuclein as one of the major components of Lewy Bodies (LBs), by raising antibodies against whole LBs isolated from DLB brains[2]. LBs are pathognomonic for PD and DLB. To identify the changes in α-synuclein that are key to its conversion from a molecule that is normally soluble into insoluble aggregates deposited in synucleinopathy lesions, we sequentially extracted cerebral cortices of DLB brains with Tris-HCl, 1% Triton-X100 (TX), 1% Sarkosyl and 8 M urea (Fig. 1a). A polypeptide with a relative molecular mass of ~15,000 (M_r ~15K) was detected with a human-specific anti α-synuclein antibody, LB509 (refs 2,4), in Tris-HCl- or TX-soluble fractions of both DLB and normal brains. This represents normal α-synuclein, the amounts of which were similar between DLB and control brains. A major band of ~15K and minor additional higher molecular weight polypeptides were specifically detected by the LB509 antibody in Sarkosyl-insoluble–urea-soluble fractions from DLB brains (Fig. 1a) and may correspond to aggregated forms of α-synuclein[5]. By contrast, no immunoreactive bands were detected in the same fractions from control brains (Fig. 1a). The urea-soluble fractions from DLB brains were further purified by Q-sepharose column chromatography and then separated by reverse-phase high-performance liquid chromatography (RP–HPLC). In this way, purified α-synuclein from insoluble fractions of DLB brains, as well as recombinant α-synuclein and normal α-synuclein recovered from Tris-HCl-soluble brain fractions, were subjected to chemical cleavage by cyanogen bromide (CNBr) and separation by RP–HPLC (Fig. 1b). Analyses of the RP–HPLC fractions of CNBr digests from recombinant and normal α-synuclein by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) demonstrated that peak A (acetonitrile concentration, 21.5%) and peak B (acetonitrile concentration, 23.0%) correspond to carboxy-terminal fragments of α-synuclein. Peak A corresponds to amino acid residues 117–127 of α-synuclein (m/z: 1231.5) and peak B to residues 128–140 (m/z: 1513.6), (Fig. 1b, top and middle). RP–HPLC separation of urea-soluble α-synuclein from DLB cortices generated two peaks that eluted at the same positions as peaks A and B. We designated these peaks A′ and B′. In contrast to the peaks generated from recombinant or normal α-synuclein, the height of peak A′ was consistently greater than that of B′ (Fig. 1b, bottom). MALDI-TOF MS analysis revealed that like peak B, B′ corresponds to residues 128–140 of α-synuclein, whereas peak A′ generated two mass signals, that is, m/z 1232.3 corresponding to residues 117–127 of α-synuclein (as for peak A), and another signal of m/z 1593.4. As the latter mass number is equivalent to the 128–140 C-terminal fragment of α-synuclein (1513.6) plus one phosphate (80), it is likely that one of the three amino acid residues that can serve as a phosphate acceptor within this fragment (that is, Ser 129, Tyr 133 or Tyr 136) is phosphorylated. To determine which of these amino acid residues is phosphorylated in α-synuclein aggregates, we analysed the peptide fragment of m/z 1593.4 by nanoelectrospray tandem mass spectrometry (nano ES-MS/MS). The product ion spectra derived from fragmentation of the doubly charged ion at m/z 797.5 resulted in series of b and y sequence ions. Thus, we identified that the phosphopeptide corresponded to residues 128–140 and was specifically phosphorylated on Ser 129 (Fig. 2).

To examine further the phosphorylation of Ser 129 of α-synuclein in normal and disease conditions, we raised antibodies against a synthetic peptide that corresponds to amino acid residues 124–134 of human α-synuclein with a phosphorylated Ser 129 residue. We termed this antibody anti-PSer129. Anti-PSer129 specifically reacted with the phosphopeptide immunogen, but never reacted with recombinant human α-synuclein by ELISA (Fig. 3a). Immunoblot analysis of differentially solubilized α-synuclein from human brains demonstrated that anti-PSer129 specifically recognized a ~15K polypeptide, as well as some minor bands in the urea-soluble and

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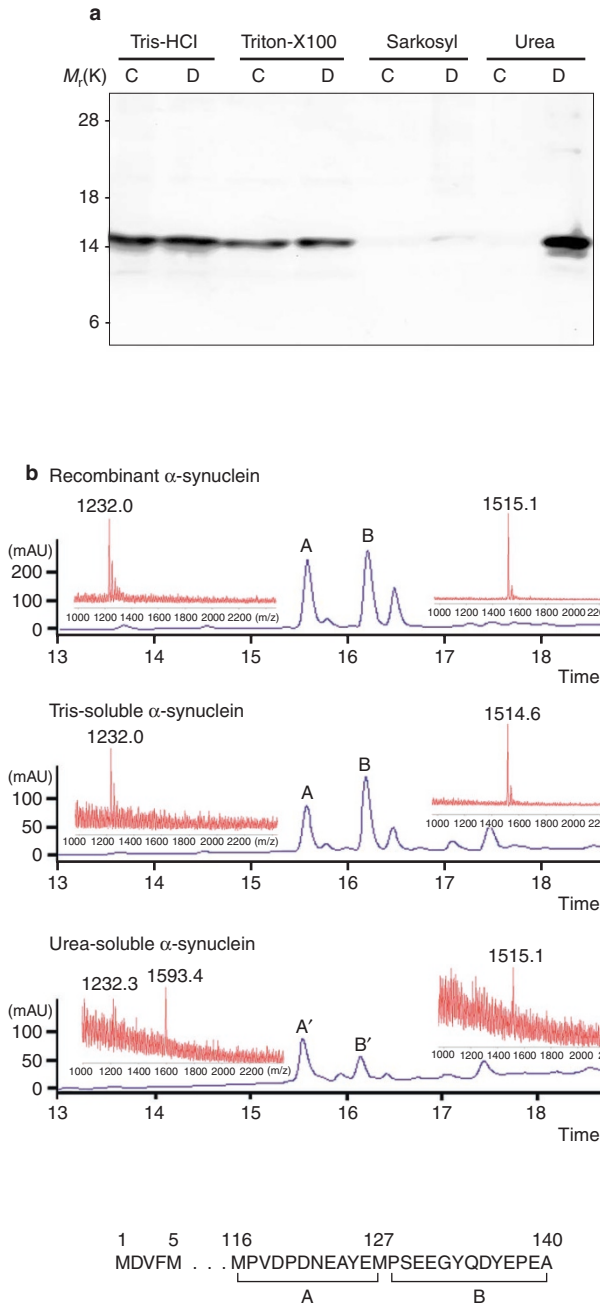

Figure 1 **Purification and characterization of α-synuclein from human brains. a**, Western blot analysis of α-synuclein differentially extracted with Tris-HCl, Triton-X100, Sarkosyl or urea, from the cerebral cortices of a patient with DLB (D) and a normal control individual (C), with the antibody LB509. Molecular weight markers are shown at the left side of the panel. **b**, Separation profiles of CNBr digests (purple lines) of recombinant human α-synuclein (top), Tris-HCl-soluble normal α-synuclein (middle) and urea-soluble α-synuclein (bottom) purified from DLB brains by RP–HPLC. Peaks A and B in the upper two panels were assigned to amino acid residues 117–127 and 128–140 of human α-synuclein (as shown at the bottom of the panels), respectively, by MALDI-TOF MS analysis (insets in the left and right sides of the top and middle panels correspond to peaks A and B, respectively). Peak A′ from the insoluble fraction of DLB brains contained a signal of m/z 1593.4 (insets in the left and right sides of the bottom panel correspond to peaks A′ and B′, respectively), equivalent to residues 128–140 plus one phosphate. mAU, milli-absorbance units at 215 nm.

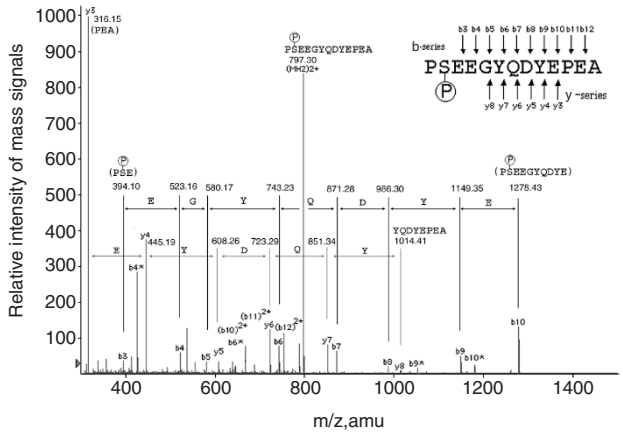

Figure 2 **Identification of a phosphorylated amino acid residue by nanoelectrospray tandem mass spectrometry.** Product ion spectrum of a mass signal $(M+2H)^{2+}$ of m/z 797.5 from urea-soluble α-synuclein from DLB brains, showing the b and y ion series (fragmentation sites shown in the upper right), identifying the site of phosphorylation as Ser 129. Vertical bars denote consecutive mass signals in b and y series (m/z shown at the top). Asterisks (that is, b4*, b6*, b9* and b10*) indicate mass signals from peptide fragments containing dephosphorylated Ser 129 with a loss of 98 relative molecular mass units (H_3PO_4).

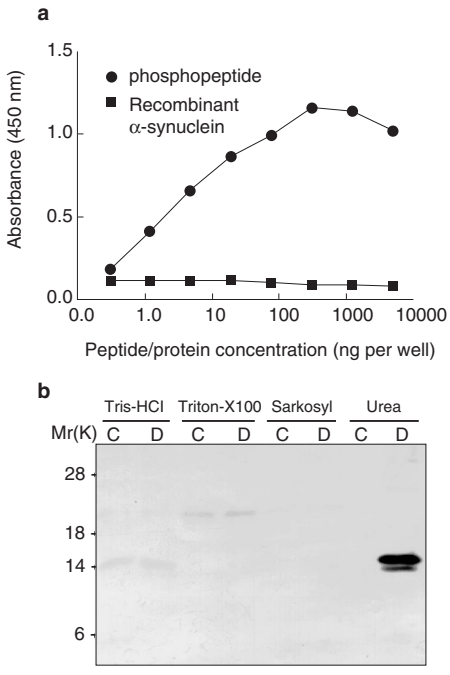

Figure 3 **Characterization of the anti-PSer129 antibody. a**, A synthetic phosphopeptide corresponding to residues 124-134 (plus a cysteine residue at the amino terminus) of human α-synuclein with phospho-Ser 129 (circle) or recombinant human α-synuclein (square) were probed with the anti-PSer129 antibody. The abscissa and ordinate represent the amount of peptide/protein and absorbances at 450 nm, respectively. **b**, Western blot analysis of α-synuclein differentially extracted with Tris-HCl, Triton-X100, Sarkosyl or urea from cerebral cortices of a patient with DLB (D) and a normal control individual (C) probed with the anti-PSer129 antibody. The same samples examined in Fig. 1a were analysed.

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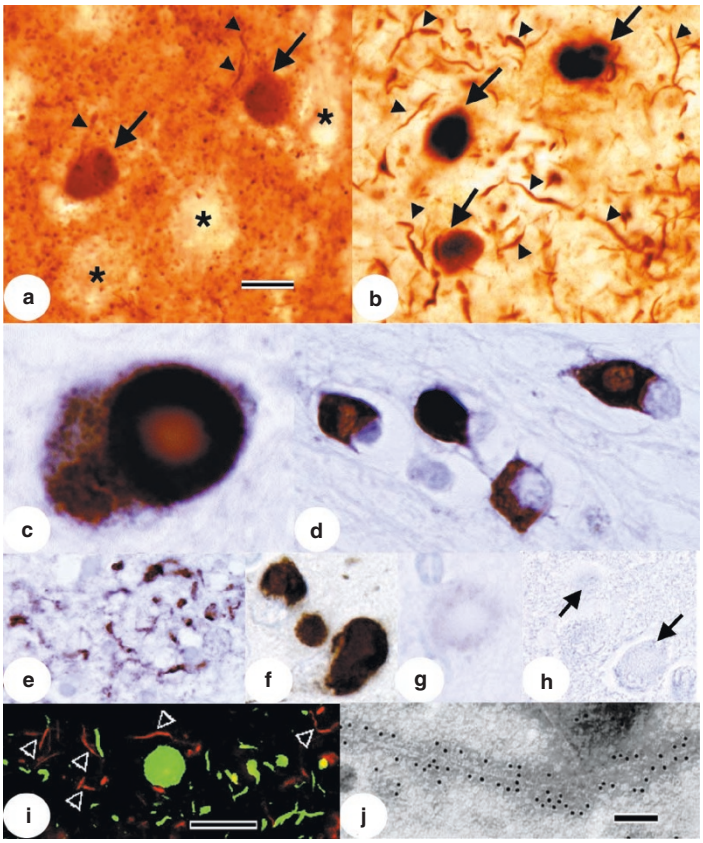

Figure 4 **Immunohistochemistry of synucleinopathy lesions.** 50-μm-thick floating sections from the temporal neocortex of DLB brains were immunostained with LB509 (**a**) or anti-PSer129 (**b**). Arrows and arrowheads indicate LBs and Lewy neurites, respectively, and asterisks in (**a**) show unstained neuronal cell bodies. Brainstem LBs in pigmented neurons of the substantia nigra in PD (**c**), glial cytoplasmic inclusions in the pontine basis of multiple system atrophy (**d**), dystrophic neurites in the temporal neocortex of Hallervorden-Spatz disease (**e**) in 6-μm-thick, formalin-fixed paraffin-embedded sections also were immunolabelled by anti-PSer129. Immunolabelling of neocortical LBs in DLB brains (**f**) was diminished by pretreating the sections with 20U ml^{-1} alkaline phosphatase for 12 h (**g**) and totally abolished after adsorption with the phosphopeptide immunogen (**h**). Arrows in **h** denote unstained LBs. **i**, Confocal image of a floating section from the temporal neocortex of DLB doubly immunolabelled with anti-PSer129 and a phosphorylated-tau specific antibody AT8, showing an LB (large round green fluorescent profile) and Lewy neurites (green) that are distinct from tau-positive neuropil threads (red, arrowheads). **j**, Immunoelectron micrograph of dispersed ~10 nm filaments extracted from DLB neocortex, immunolabelled by anti-PSer129 and a secondary antibody conjugated to 10 nm colloidal gold particles. **c**–**h**, Samples counterstained with haematoxylin. Scale bar in **a** is equivalent to 10 μm in **a** and **b**, 5 μm in **c**, 8 μm in **d** and **g**, 14 μm in **e**, 12 μm in **f** and 16 μm in **h**. Scale bars in **i** and **j** are equivalent to 20 μm and 10 nm, respectively.

urea-insoluble fractions from DLB brains, whereas normal α-synuclein in Tris-HCl-soluble or TX-soluble fractions was not labelled (Fig. 3b). Alkaline phosphatase (ALP) treatment of urea-soluble α-synuclein totally abolished the positive reaction with anti-PSer129 (data not shown). Next, we analysed the levels of α-synuclein in the urea-soluble fraction from DLB brains by quantitative immunoblotting with the anti-PSer129 antibody and the phosphorylation-independent antibody LB509, with purified recombinant human α-synuclein phosphorylated *in vitro* by casein kinase 2 as a control. By this method we demonstrated that in the urea-soluble fraction, ~90% of α-synuclein is phosphorylated at Ser 129 (Supplementary information, Fig. S1). Furthermore, similar analysis of α-synuclein that was freshly prepared from normal adult rat brains revealed that ~4% of total brain α-synuclein is phosphorylated at Ser 129, but it is readily dephosphorylated *post mortem* (Supplementary information, Fig. S2). Immunohistochemical analysis of human postmortem brains with the LB509 antibody identified fine granular labelling of presynaptic termini within the neuropil, together with LBs and Lewy neurites in DLB brains (Fig. 4a). Neuronal cell bodies were unstained (Fig. 4a). By contrast, the anti-PSer129 antibody generated specific and intense labelling of synucleinopathy lesions, that is, cortical LBs and Lewy neurites in DLB brains without neuropil staining (Fig. 4b), brainstem LBs in the substantia nigra of PD brains (Fig. 4c), glial cytoplasmic inclusions in multiple system atrophy (Fig. 4d) and dystrophic neurites in Hallervorden-Spatz disease (Fig. 4e). Positive labelling of LBs in DLB cortices with the anti-PSer129 antibody (Fig. 4f) was diminished by ALP treatment (Fig. 4g). Furthermore, pre-absorption of the anti-PSer129 antibody with immunogen phosphopeptide abolished the specific labelling of LBs (Fig. 4h). Double immunolabelling of DLB cortices with the anti-PSer129 antibody and a phosphorylated tau-specific antibody (AT8) demonstrated that phosphorylated α-synuclein-positive LBs or Lewy neurites and tau-positive neurofibrillary lesions were differentially immunolabelled (Fig. 4i). This suggests that these two types of phosphoprotein aggregates are neither codeposited nor cross-recognized by the anti-PSer129 antibody. Immunoelectron microscopic analysis of dispersed filaments from DLB cortices (Fig. 4j) with the anti-PSer129 antibody confirmed that the filaments which comprise LBs or Lewy neurites are associated with α-synuclein that is phosphorylated at Ser 129. In addition, we examined two independently generated lines of transgenic mice that overexpress human wild-type α-synuclein, under the control

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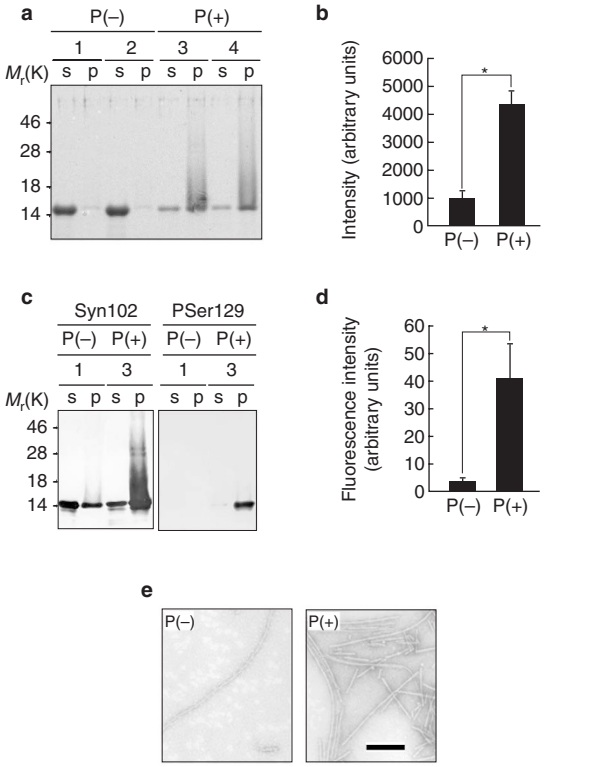

Figure 5 **Phosphorylation of Ser 129 of recombinant human α-synuclein and its effect on fibrillization. a**, SDS–PAGE analysis of recombinant human α-synuclein with (P(+)) or without (P(-)) phosphorylation at Ser 129 by casein kinase 2, after 5 days of incubation. Coomassie brilliant blue stained SDS–PAGE gel of samples in two independent, representative experiments each for P(-) (experiments 1, 2) and for P(+) (experiments 3,4) are shown. s and p denote supernatants and pellets after ultracentrifugation, respectively. Supernatants (4 μl) were resuspended in 4 μl of SDS sample buffer and 12 μl of pellets resuspended in 30 μl of SDS sample buffer were loaded into each lane. Note that the intensities of ~15K α-synuclein bands decreased in s, whereas they increased in p, with some high molecular weight smearing substances in P(+), compared with those in P(–). **b**, Densitometric quantification of sedimented α-synuclein in insoluble pellets. Intensities of ~15K α-synuclein bands were quantified in five independent experiments, and mean values ±S.E. are shown in arbitrary units. *$p<0.0005$ by Student's *t* test. **c**, Immunoblot analysis of supernatants (s) and pellets (p) of α-synuclein with a phosphorylation-independent monoclonal antibody Syn102 (ref.19; left) or anti-PSer129 (right). Loaded samples (equivalent to 2% of the loading in **a**) and abbreviations are identical to those in **a**, and the data of experiment 1 and 3 are shown. **d**, Thioflavin-T fluorescence analysis of aliquots of non-phosphorylated (P(–)) or phosphorylated (P(+)) α-synuclein in experiments shown in **b**. Mean fluorescence intensities ±S.E. are shown. *$p<0.05$ by Student's *t* test. **e**, Negative electron microscopic analysis of filaments formed by non-phosphorylated (P(–), left panel) or phosphorylated (P(+), right) α-synuclein. Note that abundant filaments are present in P(+). Scale bar = 200 nm.

of a PDGF-β promotor[6,7] (Supplementary information, Fig. S3). The anti-PSer129 antibody positively immunostained inclusion-like structures in a subset of neuronal somata in the cerebral neocortex (Supplementary information, Fig. S3b), or the hippocampus (Supplementary information, Fig. S3f) in one of the two lines that formed intraneuronal inclusions and exhibited motor disturbances[6] (Supplementary information, Fig. S3a,b,e and f). By contrast, the anti-PSer129 antibody did not label any structures (Fig.S3d and h) in the other line, which was asymptomatic and did not form inclusions[7] (Supplementary information, Fig. S3c,d,g and h), despite comparable levels of normal α-synuclein protein expression in the neuropil (compare Supplementary information, Fig. S3a and e with c and g).

To examine whether phosphorylation of α-synuclein at Ser 129 has a causative function in fibrillization, we incubated recombinant human α-synuclein with or without prior phosphorylation by casein kinase 2, which has been shown to selectively phosphorylate Ser 129 (refs 8,9), and compared the formation of insoluble filaments *in vitro*. SDS–polyacrylamide gel electrophoresis (PAGE) analysis of insoluble pellets from recombinant α-synuclein (2 mg ml^{-1}), after five days of incubation with shaking at 37 °C, showed that α-synuclein phosphorylated at Ser 129 yielded ~4 fold more insoluble sediments (Fig. 5a–c). A thioflavin-T fluorescence assay showed increased thioflavin-T signals in aliquots of α-synuclein phosphorylated at Ser 129 (Fig. 5d), suggesting the formation of β-sheeted fibrils. In addition, electron microscopic analysis confirmed the formation of abundant filaments from phosphorylated α-synuclein (Fig. 5e) that were positively immunolabelled by the anti-PSer129 antibody (Supplementary information, Fig. S4a). Furthermore, incubation with a higher concentration of α-synuclein phosphorylated at Ser 129 (~5.3 mg ml^{-1}) induced the accumulation of more oligomers than non-phosphorylated α-synuclein (Supplementary information, Fig. S4d).

It has been shown that α-synuclein can be phosphorylated by a subset of protein kinases *in vitro*, including casein kinase 1 (CK1), CK2 or G-protein coupled receptor kinases, or in culture cells at a few serine residues, including Ser129 (refs 8,9). However, most of the α-synuclein is not phosphorylated under physiological conditions *in vivo* (ref. 8 and Supplementary Information, Fig. S2), suggesting that the extensive phosphorylation of α-synuclein at Ser 129 in synucleinopathy brains is a highly pathological event. The phosphorylation at Ser 129 seems to be relatively specific, because we have not detected any incorporation of phosphates in other possible phosphate acceptor sites in our preliminary MALDI-TOF MS analysis of whole-molecule and lysylendopeptidase fragments covering the entire length of α-synuclein from DLB brains (N.D., M.H., H.F., K.T. and T.I., unpublished observation). Although the function of α-synuclein is unknown, recent studies have shown that phosphorylation at Ser 129 inhibited the interaction of α-synuclein with membrane phospholipids or phospholipase D2 (ref. 9). In Alzheimer's disease and other tauopathies, excessively phosphorylated tau is thought to dissociate from microtubules, causing destabilization of the microtubular network and an increase in the concentration of free tau molecules that are in turn assembled into abnormal filaments[10,11]. A similar increase in the concentration of free phosphorylated α-synuclein may also contribute to the formation of abnormal filaments in the cytoplasm. Furthermore, our *in vitro* studies suggested that phosphorylation of Ser 129 promotes formation of α-synuclein filaments as well as oligomers. The latter are thought to be pathogenic and to function as nuclei for fibril propagation[12,13]. Further studies should examine what kind of conformational changes are induced by the addition of a phosphate to the negatively charged C terminus of α-synuclein. Although, the possibility that hyperphosphorylation at Ser 129 is partially a secondary phenomenon resulting from the aggregation of α-synuclein cannot be completely excluded at present. However, our observations strongly suggest that excessive phosphorylation of α-synuclein in synucleinopathy brains (including those in transgenic animals), as well as the underlying alterations in the activities of various kinases and phosphatases, and in the conformation of α-synuclein, may contribute to abnormalities of α-synuclein. In conjunction with other post-translational modifications of α-synuclein, these changes would cause aggregation, eventually leading to neuronal cell death[14,15]. □

Methods

Purification of α-synuclein from human brains.

Cerebral cortices from patients with DLB (4 cases, mean age 72.3 y, mean postmortem interval 8.9 h), or normal control individuals (2 cases, mean age: 69 y), were homogenized in buffer A (50 mM Tris-HCl

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at pH 7.5, 1 mM EGTA, 0.5 mM PMSF, 1 μg ml^{-1} antipain, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} leupeptin, 50 mM imidazole, 25 mM β-glycerophosphate, 20 mM sodium fluoride, 10 mM $Na_4P_2O_7$) and centrifuged at 1000*g*. Resulting pellets were sequentially extracted by homogenization in Triton-X100 (buffer A containing 1% Triton-X100, 10% sucrose and 0.5 M sodium chloride), Sarkosyl (50 mM Tris-HCl at pH 7.5, 1% Sarkosyl, 1 mM EGTA) and urea (50 mM Tris-HCl at pH 7.5, 8 M urea, 1 mM EGTA) followed by centrifugation at 350,000*g*. 1000*g* supernatants were centrifuged at 350,000*g* and the supernatant was used as a Tris-HCl-soluble fraction. Each fraction was separated by SDS–PAGE and analysed by immunoblotting, as described[16]. Precipitates in 50% ammonium sulfate of the Tris-soluble fraction were boiled, separated by a DEAE-cellulose column, and the 0.1 M sodium chloride eluates were fractionated by size exclusion chromatography on a TSKgel SuperSW3000 column (Tosoh, Tokyo, Japan), and then separated by RP–HPLC on an Aquapore RP300 column (PerkinElmer, Norwalk, CT). Normal α-synuclein was recovered at an acetonitrile concentration of 40%. Recombinant α-synuclein was prepared as previously described[4]. The urea-soluble fraction was adsorbed by a Q-sepharose column and the 0.3 M sodium chloride eluates were separated by RP–HPLC on an Aquapore RP300 column. α-synuclein was recovered at an acetonitrile concentration of 40%.

Cyanogen bromide digestion and mass spectrometry.

Purified α-synucleins were cleaved by overnight digestion with 0.2% CNBr in 70% formic acid. Digests were resuspended in 6 M guanidine-HCl and then separated by RP–HPLC on a Superspher Select B column (Merck, Darmstadt, Germany). Peptide fragments were analysed by MALDI-TOF MS (PerSeptive Biosystems, Voyager-DE, Applied Biosystems, Foster City, CA) and the phosphopeptide was further analysed by nano ES-MS/MS (API QSTAR, Applied Biosystems) according to previously described methods (reviewed in ref. 17).

Antibody production and immunohistochemistry.

Rabbits were immunized with a synthetic phosphopeptide based on residues 124–134 of human α-synuclein, with phospho-Ser 129 conjugated at the amino terminus by an additional cysteine to keyhole limpet haemocyanine (KLH-CAYEMPS(PO_3H_2)EEGYQ), together with adjuvants as described[18]. Antisera were purified by obtaining flow-through fractions of an Affi-Gel 10 column conjugated with recombinant α-synuclein (BioRad, Richmond, CA). PSer129 was characterized by ELISA through plating immunogen peptide or recombinant α-synuclein on multiwell plates, as described[18]. Immunohistochemistry was performed on human postmortem brain samples that were clinically and pathologically diagnosed as PD, DLB[2], multiple system atrophy[19] or Hallervorden-Spatz disease[20]. Sections were fixed in 10% neutral buffered formalin for 24 h (50-μm-thick floating sections) or 1 week (6-μm-thick paraffin-embedded sections) by the avidin–biotin complex method, as previously described[2]. For immunohistochemistry of transgenic mice, the brains of 10-month-old mice from two independently generated lines overexpressing human wild-type α-synuclein under the control of PDGF-β promoter (refs 6,7; the latter will be described elsewhere by M.S.G.) were fixed in 10% formalin, embedded in paraffin, and sections were similarly immunostained. Confocal images of double immunofluorescence labelling of floating sections[2] and immunoelectron micrographs of dissociated filaments[4] were obtained, as described.

In vitro phosphorylation and fibrillization of α-synuclein.

Recombinant human α-synuclein (0.4 mg) was incubated in 200 μl of 30 mM Tris-HCl at pH 7.5 containing 4 mM magnesium chloride, 2 mM ATP, with or without 1000U of purified casein kinase 2 (New England Biolabs, Beverly, MA) for 14 h at 37 °C. After boiling, phosphorylated or non-phosphorylated α-synuclein proteins were separated by RP–HPLC. To study fibrillization, lyophilized α-synuclein was resuspended in 30 mM Tris-HCl at pH 7.5 and then ultracentrifuged at 350,000*g* for 20 min to remove aggregates. The supernatants were adjusted to a final protein concentration of 2 mg ml^{-1}, and incubated in 50-μl aliquots at 37 °C for five days with continuous vigorous shaking (250 strokes min^{-1}), according to the previously described methods[21–23]. After incubation, aliquots were diluted 2-fold in 30 mM Tris-HCl at pH 7.5 and centrifuged at 350,000*g* for 20 min. α-synuclein filaments that sedimented in pellets were separated by SDS–PAGE, stained with Coomassie brilliant blue, and the intensity of the ~15K bands corresponding to monomerized α-synuclein were quantified by a densitometer with an NIH-image program. The thioflavin-T fluorescence assay was performed according to the previously described methods[13].

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Supplementary Information is available on *Nature Cell Biology*'s website (http://cellbio.nature.com).

This PDF replaces the one originally posted on 28 January 2002. The contact e-mail address has been corrected to iwatsubo@mol.f.u-tokyo.ac.jp and in Fig. 3a a-synuclein has been replaced by α-synuclein.

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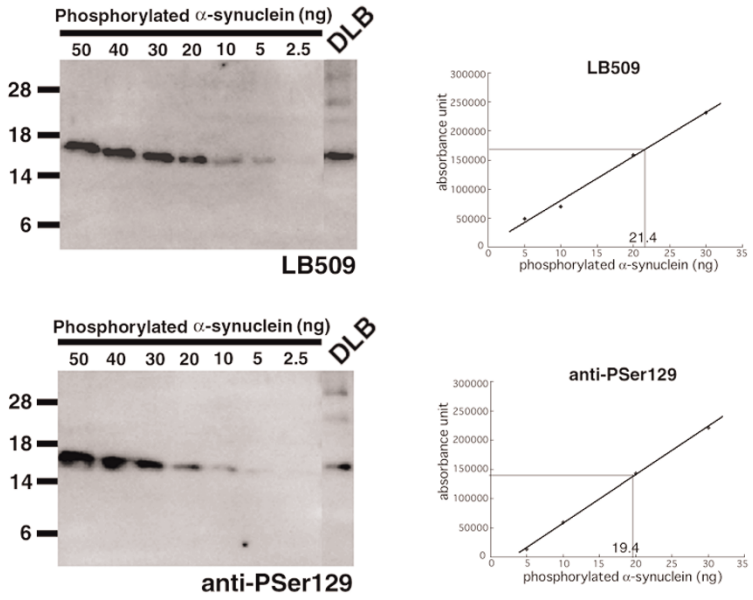

Figure S1. **Quantitative analysis of the extent of a-synuclein phosphorylation in the urea-soluble fractions of DLB brains.** Recombinant human α-synuclein was phosphorylated *in vitro* by casein kinase 2, and α-synuclein phosphorylated at serine 129 was separeted by a Q-sepharose column according to a previously described method[7]. Phosphorylated α-synuclein was further purified and protein concentration was estimated by RP-HPLC. Urea-soluble fractions of DLB brains (10 μlof urea-soluble fraction) were separated by SDS-PAGE together with known amounts of phosphorylated α-synuclein (2.5-50 ng/lane) purified as above, subjected to immunoblot analysis by LB509 (phosphorylation-independent; upper left panel) or anti-PSer129 (lower left panel) using HRP-labeled secondary antibody and development by an ECL system. Intensities of the bands of recombinant phosphorylated α-synuclein were quantitated by a densitometer using a NIH-image program, and standard curves were drawn within the linear ranges (upper right diagram for LB509 and lower right for anti-PSer129). The percentage of phosphorylated α-synuclein as a fraction of total α-synuclein in the urea-soluble fractions of DLB brains was calculated by extrapolating the intensities of bands of DLB samples. 89.1 ± 5.6% (mean ± S.E. of four independent experiments) of total a-synuclein was phosphorylated at serine 129. (data in a single experiment, showing 90.7% of phosphorylation, are presented).

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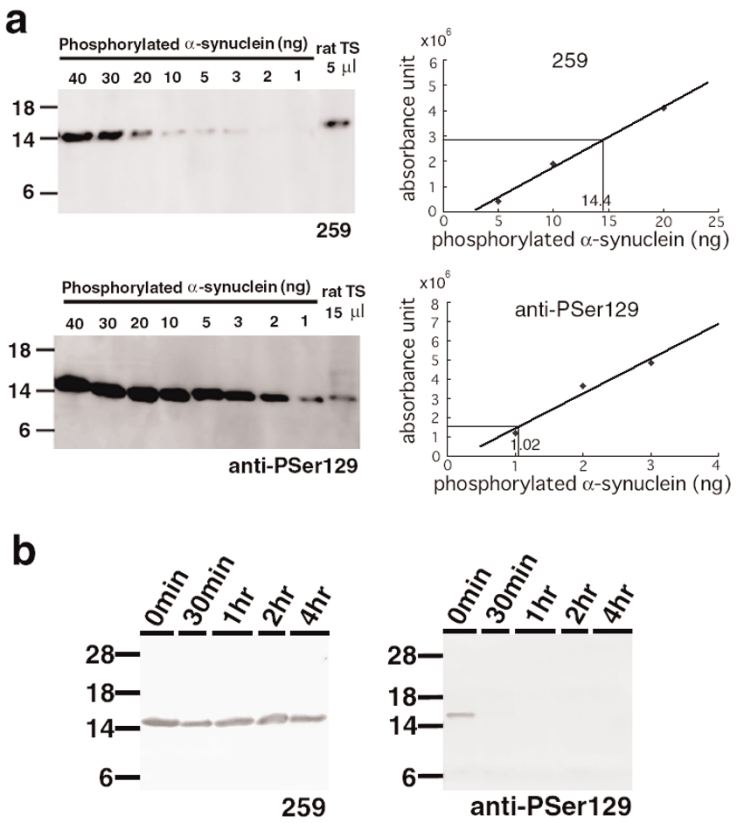

Figure S2. **Quantitative analysis of the extent of α-synuclein phosphorylation in freshly prepared normal α-synuclein from adult rat brains (**a**) and rapid dephosphorylation of normal α-synuclein *post mortem* (**b**).** TS-soluble fractions of adult rat brains (8 weeks old, rat TS) were prepared immediately after decapitation by an identical method to that used for human soluble α-synuclein. Extent of α-synuclein phosphorylation was quantitated as in Fig.S1, except that a polyclonal antibody 259[2], which was raised against a synthetic peptide corresponding to amino acid residues 104-119 of rat α-synuclein (identical to human α-synuclein except for residue 107) and crossreacts with human and rat α-synuclein, was used for the detection of total rat α-synuclein instead of LB509, that is specific for human α-synuclein[4]. 4.3 ± 1.2% (mean ± S.E. of three independent experiments) of normal rat α-synuclein was phosphorylated at serine 129 (data in a single experiment, showing 2.4% of phosphorylation, are presented). b, To examine the stability of phosphorylation of serine 129 *post mortem*, rat brains were left at room temperature after decapitation for 30 min to 4 hr prior to extraction, and soluble fractions were subjected to immunoblot analysis by 259 (left) and anti-PSer129 (right). Reactivities of α-synuclein with anti-PSer129 rapidly decreased at 30 min and were abolished after 1 hr, suggesting that normal α-synuclein, that is phosphorylated at serine 129 to a very low extent, undergoes rapid dephosphorylation *post mortem*.

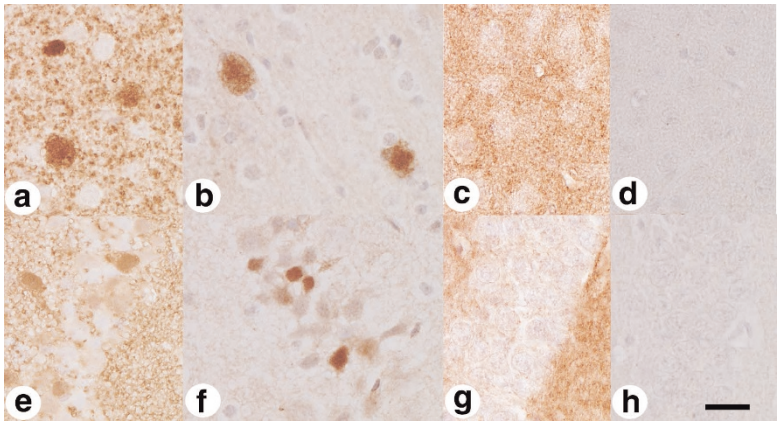

Figure S3. **Immunohistochemistry of brains of transgenic mice overexpressing wild-type human α-synuclein under the control of PDGF-β promotor.** Frontal neocortices (**a**-**d**) or hippocampus (**e**-**h**) of 10-month-old animals from two independently generated lines of transgenic mice with[6] (**a**,**b**,**e** and **f**) or without[7] (**c**,**d**,**g** and **h**) α-synuclein-positive intraneuronal inclusions were immunostained with LB509 (**a**,**c**,**e** and **g**) or anti-PSer129 (**b**,**d**,**f** and **h**). Note that PSer129-positive structures (that is, inclusions) are exclusively seen in **b** and **f**. Scale bar= 20 μm.

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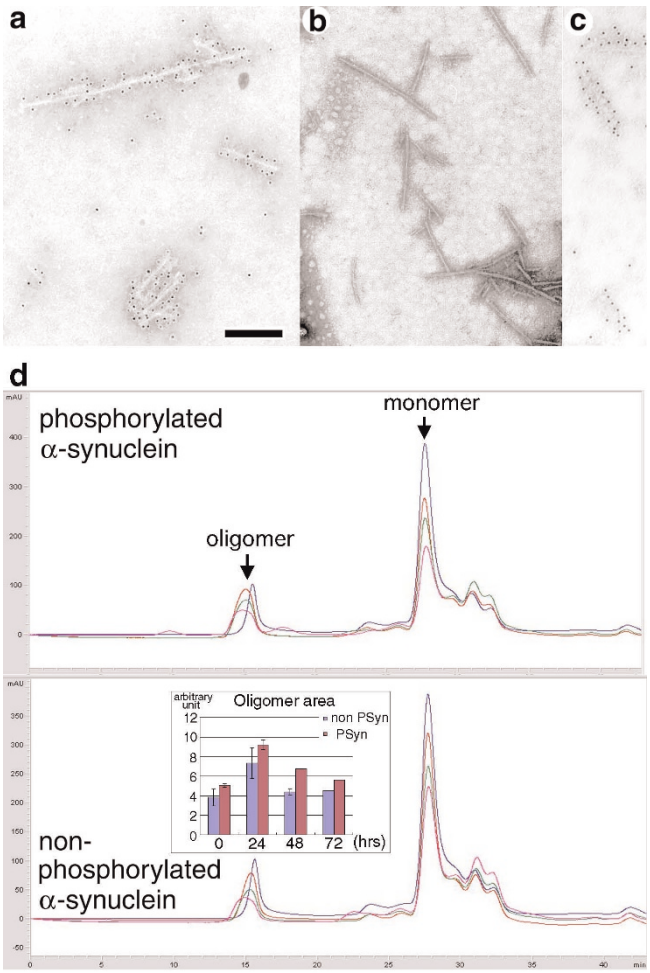

Figure S4. **Supplementary data on polymerization of recombinant α–synuclein. a**, positive immunolabeling by anti-PSer129 of filaments assembled from recombinant α-synuclein phosphorylated by CK2 (10 nm colloidal gold). **b**, lack of immunodecoration with anti-PSer129 of filaments assembled from recombinant α-synuclein without prior phosphorylation. **c**, immunolabeling of Sarkosyl-insoluble filaments isolated from DLB cortices with anti-PSer129. Scale bar=200 nm (**a**-**c**) **d**, oligomer formation of phosphorylated (upper panel) and non-phosphorylated (lower panel) α–synuclein incubated for 0 (blue), 24 (red), 48 (green) and 72 (purple) hrs, respectively, was monitored (at 215 nm) by gel filtration separation on a Superdex 200 column. Monomeric α-synuclein was eluted at ~28 min, whereas peaks at ~15 min represent α-synuclein oligomers[24,25]. Note that phosphorylated α-synuclein yielded more oligomers at each time point, whereas levels of monomeric phosphorylated α-synuclein decreased more rapidly compared to non-phosphorylated α-synuclein. Inset in the lower panel shows quantitative analysis of oligomer accumulation by calculation of areas under the peaks corresponding to the oligomer (purple, phosphorylated α-synuclein; blue, non-phosphorylated α-synuclein). Method. Oligomer formation and gel filtration assays were performed according to the previously described method[23,24] with some modifications. Briefly, recombinant a-synuclein purified without boiling was concentrated by precipitation in 50% ammonium sulfate, purified by size exclusion chromatography on a TSKgel SuperSW3000 column, and phosphorylated by CK2 as described in Methods. Phosphorylated α-synuclein and non-phosphorylated α-synuclein as a control were again fractionated by TSK column, lyophilized, and then reconstituted in 10 mM phosphate buffer containing 2.7 mM KCl, 137 mM NaCl and 0.02% NaN_3. After passing through a 0.22 μm filter, the solutions were adjusted to a final protein concentration of 5.3 mg/ml, and incubated at 37 ˚ with gentle rotation (60 cycles/ min). 20 μl aliquots were taken at every 24 hr, diluted with 30 μl phosphate buffer, centrifuged at 16,000 x g for 5 min to remove fibrils and then separated on a Superdex 200 column.

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