BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.01846.x
www.brjpharmacol.org

RESEARCH PAPER

A prolyl oligopeptidase inhibitor, KYP-2047, reduces α-synuclein protein levels and aggregates in cellular and animal models of Parkinson's disease

TT Myöhänen[1,2], MJ Hannula[1], R Van Elzen[2], M Gerard[3], P Van Der Veken[4], JA García-Horsman[1], V Baekelandt[5], PT Männistö[1] and AM Lambeir[2]

[1]*Division of Pharmacology and Toxicology, University of Helsinki, Finland,* [2]*Laboratory of Medical Biochemistry, University of Antwerp, Wilrijk (Antwerp), Belgium,* [3]*Laboratory of Biochemistry, Katholieke Universiteit Leuven-Kortrijk, Belgium,* [4]*Laboratory of Medicinal Chemistry, University of Antwerp, Wilrijk (Antwerp), Belgium, and* [5]*Laboratory for Neurobiology and Gene Therapy, Division of Molecular Medicine, Katholieke Universiteit Leuven, Leuven, Belgium*

Correspondence
Timo T Myöhänen, Division of Pharmacology and Toxicology, Faculty of Pharmacy, University of Helsinki, P.O. Box 56 (Viikinkaari 5E), FI-00014 University of Helsinki, Finland. E-mail: timo.myöhänen@helsinki.fi

Keywords
serine protease; prolyl oligopeptidase inhibitor; α-synuclein; Parkinson's disease; protein aggregation

Received
7 July 2011
Revised
7 December 2011
Accepted
18 December 2011

BACKGROUND AND PURPOSE
The aggregation of α-synuclein is connected to the pathology of Parkinson's disease and prolyl oligopeptidase (PREP) accelerates the aggregation of α-synuclein *in vitro*. The aim of this study was to investigate the effects of a PREP inhibitor, KYP-2047, on α-synuclein aggregation in cell lines overexpressing wild-type or A30P/A53T mutant human α-syn and in the brains of two A30P α-synuclein transgenic mouse strains.

EXPERIMENTAL APPROACH
Cells were exposed to oxidative stress and then incubated with the PREP inhibitor during or after the stress. Wild-type or transgenic mice were treated for 5 days with KYP-2047 (2×3 mg·kg^{-1} a day). Besides immunohistochemistry and thioflavin S staining, soluble and insoluble α-synuclein protein levels were measured by Western blot. α-synuclein mRNA levels were quantified by PCR. The colocalization of PREP and α-synuclein,and the effect of KYP-2047 on cell viability were also investigated.

KEY RESULTS
In cell lines, oxidative stress induced a robust aggregation of α-synuclein,and low concentrations of KYP-2047 significantly reduced the number of cells with α-synuclein inclusions while abolishing the colocalization of α-synuclein and PREP. KYP-2047 significantly reduced the amount of aggregated α-synuclein,and it had beneficial effects on cell viability. In the transgenic mice, a 5-day treatment with the PREP inhibitor reduced the amount of α-synuclein immunoreactivity and soluble α-synuclein protein in the brain.

CONCLUSIONS AND IMPLICATIONS
The results suggest that the PREP may play a role in brain accumulation and aggregation of α-synuclein, while KYP-2047 seems to effectively prevent these processes.

Abbreviations
AMC, amino methyl coumarin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; KYP-2047, 4-phenylbutanoyl-*L*-prolyl-2(*S*)-cyanopyrrolidine; LDH, lactate dehydrogenase assay; NEAA, non-essential amino acids; PREP, prolyl oligopeptidase; WT, wild-type

Introduction

α-Synuclein is a cytosolic brain protein of 140 aminoacids and a member of the synuclein family. It interacts with membranes and vesicles, and is found also in the nucleus (McLean *et al.*, 2000; Yu *et al.*, 2007; Surguchov, 2008). Several physiological functions have been suggested for α-synuclein,including synaptic transmission, axonal transport and the regulation of dopamine release (see Surguchov, 2008; Bisaglia *et al.*, 2009).

Insoluble fibrillar inclusions of α-synuclein have been identified as the main component of Lewy bodies in Parkinson's disease and Lewy body dementia, concomitant with neuronal death and clinical symptoms (Spillantini *et al.*, 1997; Arima *et al.*, 1998; Surguchov, 2008). Further clinical evidence for the importance of α-synuclein in Parkinson's disease is derived from mutations in the α-synuclein gene. Duplication or triplication, or three missense mutations in the α-synuclein gene, producing the changes A30P, A53T and E46K in the protein, have been identified as the cause of early-onset autosomal-dominant familial Parkinson's disease (Polymeropoulos *et al.*, 1997; Kruger *et al.*, 1998; Singleton *et al.*, 2003; 2004; Ibanez *et al.*, 2004; Zarranz *et al.*, 2004).

The aggregation of α-synuclein is a nucleation-dependent polymerization process (Wood *et al.*, 1999). Several factors increasing the nucleation and aggregation of α-synuclein have been identified, including oxidative stress, low pH, increased ionic strength and defects in protein trafficking and processing (Uversky *et al.*, 2001; Cooper *et al.*, 2006; Kim *et al.*, 2008). Recently, it was shown that enzymes of the FK506 binding protein family increase the aggregation of α-synuclein *in vitro* and *in vivo* (Gerard *et al.*, 2008; 2010; Deleersnijder *et al.*, 2011). Moreover, a serine protease, prolyl oligopeptidase (PREP; POP; PO; EC 3.4.21.26), accelerates and increases the aggregation of wild-type (WT) α-synuclein under cell-free conditions, possibly by increasing the nucleation rate (Brandt *et al.*, 2008; Lambeir, 2011). Interestingly, this action can be blocked by PREP inhibitors or by PREP active site mutation.

PREP is present in different species, and, in mammals, it is widely distributed in the body, hydrolysing bioactive peptides of less than 30 amino acids at the carboxyl side of proline residue (see Garcia-Horsman *et al.*, 2007). Alterations in PREP enzyme activity have been measured in several diseases, including Parkinson's disease and Lewy body dementia (Mantle *et al.*, 1996). These findings combined with earlier reports of antiamnesic effects (Yoshimoto *et al.*, 1987) have served as the rationale for developing PREP inhibitors to combat neurodegenerative diseases via restoring low neuropeptide levels. Several potent substrate-like PREP inhibitors have been developed and extensively characterized. Although some beneficial effects in animal memory models (Yoshimoto *et al.*, 1987; Toide *et al.*, 1997; Shishido *et al.*, 1998) and in senescence accelerated mice (Kato *et al.*, 1997) have been reported, the *in vivo* results of PREP inhibitors are rather controversial (see Männistö *et al.*, 2007). Recent studies have suggested that PREP has other actions beyond its peptidase activity, relying on protein–protein interactions (Brandt *et al.*, 2008; Di Daniel *et al.*, 2009; Myöhänen *et al.*, 2010a).

To further clarify the role of PREP in α-synuclein aggregation, we tested the effect of a PREP inhibitor on α-synuclein aggregation *in vitro*, in several cell lines overexpressing different forms of α-synuclein,and *in vivo*, in two mouse strains overexpressing α-synuclein with the pathogenic A30P mutation. As a result, in this study we have found that a PREP inhibitor, KYP-2047 [4-phenylbutanoyl-*L*-prolyl-2(*S*)-cyanopyrrolidine], prevented the formation of α-synuclein aggregates in α-synuclein-overexpressing cell lines, possibly via abolishing the colocalization between PREP and α-synuclein. Moreover, KYP-2047 accelerated the clearance of α-synuclein aggregates after oxidative stress in cells and reduced the soluble and insoluble α-synuclein protein levels in overexpressing cellular and *in vivo* models. Furthermore, based on these results, we suggest that, besides nucleation, PREP may influence the α-synuclein aggregation process through some other mechanism, for example, by affecting the aggresomal processing of misfolding proteins.

Methods

Antibodies

Commercial antibodies were chosen carefully, taking into account that the manufacturer provided a proof of quality based on publications in which the production and specificity of the antibodies have been properly validated. See details and dilutions of primary antibodies in Table 1.

Cell lines

WT SH-SY5Y human neuroblastoma cell line was purchased from ATCC (LGC Standards; product # CRL-2266, Middlesex, UK). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM-Glutamax, product # 31966-021; Invitrogen/Gibco, Paisley, UK) containing 15% fetal bovine serum (FBS; product # 16000-044; Invitrogen/Gibco), 1% non-essential amino acids (NEAA; product # 11140; Invitrogen/Gibco) and 50 $\mu g \cdot mL^{-1}$ Gentamycin (product # 15750-094; Invitrogen/Gibco).

Stable cell lines expressing WT, A30P and A53T α-synuclein were generated using a lentiviral vector as described in Gerard *et al.* (2010), and transfected cells were selected by puromycin resistance. α-Synuclein-overexpressing cells were cultured with DMEM-Glutamax (Invitrogen/Gibco) containing 15% FBS (Invitrogen/Gibco), 1% NEAA (Invitrogen/Gibco), 50 $\mu g \cdot mL^{-1}$ gentamycin (Invitrogen/Gibco) and 1 $\mu g \cdot mL^{-1}$ puromycin (Invitrogen/Gibco). Cell lines were used at passages 3 to 15 and grown at 37°C and 5% CO_2 in a humidified atmosphere.

Induction of α-synuclein aggregation by oxidative stress and the effect of KYP-2047

Cells were seeded in 12-well-plates or T-25 flasks and allowed to grow overnight. The number of cells used depended on the assay (see below). Thereafter, the aggregation process of α-synuclein was induced by adding 100 μM H_2O_2 and 10 mM $FeCl_2$ in cell culturing medium for 3 days, as described (Gerard *et al.*, 2010). The following groups were formed to test the effects of KYP-2047 on α-synuclein aggregation in cells: (1) oxidative stress for 3 days with 0.001% DMSO (stress 3d with veh); (2) oxidative stress for 3 days with 1 μM KYP-2047 in 0.001% DMSO (stress 3d with KYP); (3) medium for

Table 1
Details of primary antibodies

Antigen	PREP	Human α-synuclein	Human and mouse α-synuclein	β-Actin
Marker for	PREP protein	Human α-synuclein protein	Human and mouse α-synuclein protein	Loading control (WB)
Species	Chicken IgY	Mouse monoclonal; clone 4B12	Mouse monoclonal; clone 3H9	Mouse monoclonal; clone AC-15
Immunogen	Purified pig PREP	Whole-length recombinant human α-syn	Whole-length recombinant human α-syn	synthetic β-cytoplasmic N-terminal peptide
Manufacturer	Venäläinen *et al.* 2006	AbCam, Cambridge, UK; Thermo Fisher Scientific, Waltham, MA, USA	AbCam, Cambridge, UK	Sigma-Aldrich
Product #	–	ab1904; MA1-90346	ab78155	A1978
Dilution used	1:500 (IHC)	1:1000 (IFL)	1:1000 (IHC)	1:3000 (WB)
	1:5000 (WB)	1:600 (IHC)	1:2000 (WB in α-synuclein fractions)	
		1:10000 (WB)	1:10000 (WB)	
Specificity and reference	WB (human and mouse), (Myöhänen *et al.*, 2007; 2008b)	WB; AbCam and Thermo Fisher datasheets, our experiments	WB; AbCam datasheet, our experiments	WB; our experiments

IHC, immunohistochemistry; IFL, cell immunofluorescence; WB, Western blotting.

3 days (negative control); (4) 1 μM KYP-2047 in 0.001% DMSO for 3 days (KYP 3d); (5) 0.001% DMSO for 3 days (veh 3d); (6) oxidative stress for 3 days (stress 3d). The concentration–response of KYP-2047 was tested with various concentrations (1 nM, 1 μM, 10 μM and 100 μM) in stress 3d experiments for A30P and A53T cell lines.

To test if the outcome is dependent on antioxidant effects of KYP-2047 during the induction of oxidative stress, an experiment was performed where KYP-2047 and vehicle/medium control were added for 1 day following the 3 days of oxidative stress with the same concentrations as in the stress 3d experiment described above.

Cell immunofluorescence

The effect of oxidative stress and KYP-2047 on α-synuclein aggregation in cells was studied using immunofluorescence by a modification of the protocol by Gerard *et al.* (2010). Briefly, cells were seeded in 12 well-plates with coverslips (Nunc, Roskilde, Denmark) at a density of 5 x 10^5 cells·mL^{-1} and allowed to grow in medium for 24 h. Thereafter, the aggregation of α-synuclein was induced by oxidative stress as described above. After treatment and fixation of the cells with 4% paraformaldehyde solution, non-specific binding was blocked by incubation of 30 min with 10% normal goat serum for α-synuclein (NGS, product # S-1000; Vector Laboratories, Burlingame, CA) and with 10% normal rabbit serum for PREP (NRS, product # S-5000; Vector Laboratories) in PBS, pH 7.4. Primary antibodies were added (see Table 1) and incubated overnight at room temperature. After washing with PBS, 2 h incubation with secondary antibody followed (1:500 goat anti-mouse fluorescein-conjugated for α-synuclein, product # 31966, Thermo Fisher Scientific, Rockford, IL, USA; 1:500 rabbit anti-chicken Texas Red-conjugated for PREP, product # ab6751, AbCam, Cambridge, UK) Wavelengths for fluorescein were 494 nm (excitation) and 512 (emission), and for Texas Red, 596 nm and 620 nm respectively. The coverslips were mounted on slides using Vectashield with 4′,6-diamidino-2-phenylindole (DAPI) (product # H-1200, Vector Laboratories) as a nuclear marker [wavelengths 358 nm (excitation) and 461 nm (emission)]. Control stainings for the immunofluorescence protocol were carried out by omission of primary antibody. No evidence of any staining was observed in these negative controls (data not shown).

Immunofluorescence photomicrographs were captured by a digital camera connected to a microscope (Olympus BX61 microscope and DP40 Digital Camera, Olympus Corporation, Tokyo, Japan). Part of the sections were photographed using Leica TCS SP2 AOBS (Leica Microsystems, Inc., Wetzlar, Germany) equipped with an argon–He/Ne laser mounted on an inverted Leica DM IRE2 microscope (Leica Microsystems Inc.). Minor corrections to brightness and contrast were made with Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated, Mountain View, CA).

α-Synuclein fractionation and Western blotting

To separate soluble and insoluble fractions of α-synuclein, the method described in Feng *et al.* (2010) was used. Briefly, 1 × 10^6 cells were seeded to T-25 flasks, and oxidative stress and study groups were prepared as described above. Cells were first lysed and mechanically homogenized on ice in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl) with protease inhibitor cocktail (product # P8340), PMSF (product # P7626) and Halt Phosphatase Inhibitor (product # 87786, Thermo Fisher Scientific). After centrifuging at 11 865× *g* for 15 min at 4°C,

supernatants (soluble fraction) were collected. Pellets were resuspended in denaturing sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue), boiled for 5 min and centrifuged as above for 1 min. This fraction contained SDS-soluble monomers and oligomers of α-synuclein and also SDS-resistant high molecular weight oligomers and aggregates. Protein levels were measured by using the method of Bradford (1976), and lysates were loaded on SDS-gel (12%) with equal protein amounts. Standard transfer and blocking techniques were used. For details of primary antibodies for α-synuclein, see Table 1. Horse anti-mouse HRP [dilution 1:2000 in 2% milk in TTBS (0.05% Tween20-TBS); product #7076, Cell Signalling Technology, Danvers, MA] was used as secondary antibody. The images were captured using GeneGnome (Syngene, Cambridge, UK). Three independent Western blots were performed.

The levels of PREP in cell lines and brains of A30P transgenic mice were quantified by Western blotting, modified from Myöhänen *et al*. (2008b). The cells were homogenized as described above, tissues were homogenized in five volumes of assay buffer (0.1 M Na–K-phosphate buffer, pH 7.0) and the tissue homogenates were centrifuged at 16,000× *g*, 4°C, for 20 min discarding the pellet. Standard SDS-PAGE (12%), transfer and blocking techniques were used, and β-actin served as the loading control. For details of primary antibodies for PREP and β-actin, see Table 1. For the α-synuclein and β-actin Western blots, goat anti-mouse secondary antibody with HRP conjugate (product #31430, Thermo Fisher Scientific; dilution 1:5000 in 1% milk in TTBS) was used. To detect PREP from cells and mice brain, rabbit anti-chicken HRP secondary antibody (product #31401, Thermo Fisher Scientific; dilution 1:5000 in 1% milk in TTBS) was used. The images were captured using OptiGo (Isogen Life Sciences, De Meern, The Netherlands). Three independent Western blotting experiments were performed.

Thioflavin S staining

Thioflavin S staining of amyloid-type fibrils with β structure was used as described earlier (Gerard *et al*., 2010). Thioflavin S is a commonly used self-fluorescent marker of amyloid-type protein structure that binds also to α-synuclein aggregates (Conway *et al*., 2000). Briefly, cells were fixed for 15 min in 4% paraformaldehyde. After two PBS washes, cells were incubated with 0.05% thioflavin S (product # T1892) for 20 min, washed 2 × 5 min in 80% ethanol and 1 × 5 min in PBS, and covered with mounting medium (Vectashield; product # H-1000, Vector Laboratories). Wavelengths for thioflavin S were 430 nm (excitation) and 550 nm (emission). Thioflavin S-stained cells were photographed using a fluorescence microscope as described above.

Double-label immunofluorescence

Double-label immunofluorescence for α-synuclein and PREP markers was performed by modifying the protocol described earlier (Myöhänen *et al*., 2008a). Briefly, after staining for α-synuclein, the slides were washed with PBS or TBS 3 × 5 min, incubated with 10% RBS, and thereafter, PREP staining was done as described above (see antibodies in Table 1). Fluorescent double-labelled sections were photographed using an inverted microscope with laser scanning confocal device as described above, and the red channel was converted to magenta using Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated).

Cell calculations

The percentages of cells with cytosolic α-synuclein inclusions from immunofluorescence and thioflavin S stainings were calculated with Stereo Investigator software attached to Olympus BX61 microscope and DP40 Digital Camera (MBF Bioscience, Williston, VT) by using the optical fractionator method (Lindholm *et al*., 2007) and comparing the number of cells with clear and intense cytosolic α-synuclein expression with the total number of cells (see examples in Figures 1 and 2). The percentage of α-synuclein inclusion-positive cells for each condition was determined from three different experiments, each with two or three wells per condition and with 450–800 cells for each coverslip.

Colocalization percentages between PREP and α-synuclein were calculated as described (Myöhänen *et al*., 2008a) without Abercrombie's correction since the cells grew in monolayers. The colocalization percentage for each condition was calculated from three different experiments, each with two or three wells per condition and with 60–150 cells for each coverslip.

α-Synuclein mRNA transcription levels

Cells were grown in flasks and exposed to oxidative stress as described above. After 3 days, the cells were homogenized, and the total RNA was extracted with Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA), which includes DNase treatment. Reverse transcriptase reaction was done on 100 ng of RNA in standard conditions using iScrpt™ cDNA Synthesis kit (Bio-Rad). PCR was performed with primers designed to amplify a fragment of α-synuclein cDNA (forward, AGGACTTTCAAA GGCCAAGG; reverse, TCCTCCAACATTTGTCACTTGC) or β-actin cDNA (forward, TCACCATGGATGATGATATCGCC; reverse, CCACACGCAGCTCATTGTAGAAGG), using iQ™ SYBR® Green Supermix (Bio-Rad).

After optimization of PCR conditions, amplification efficiency was evaluated for both α-synuclein and β-actin primers using four consecutive 10-fold dilutions of the cDNA. Determination was done in triplicate. The fold change of expression of α-synuclein,with respect to β-actin, is reported as $-2^{\Delta\Delta C(t)}$.

PREP enzyme activity measurements

In the cellular activity assays, the cells were seeded in T-25 flasks at a density of 10^6 cells per flask and, after 1 day of incubation, were exposed to oxidative stress following the experimental setup described above (six groups). After 3 days, the cells were homogenized with lysis buffer (50 mM KH_2PO_4, 1.5 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA; pH 7.4). Cell homogenates were centrifuged 16 000× *g* for 10 min in +4°C. The supernatant was recovered and stored at –80°C until use. PREP activity was measured from supernatants using Z-Gly-Pro-aminomethylcoumarin (AMC) substrate as described earlier (Moreno-Baylach *et al*., 2008; Myöhänen *et al*., 2008b). Total protein amounts were measured using the method of Bradford (1976). All activity measurements were made in triplicate. The cell homogenates were also used in Western

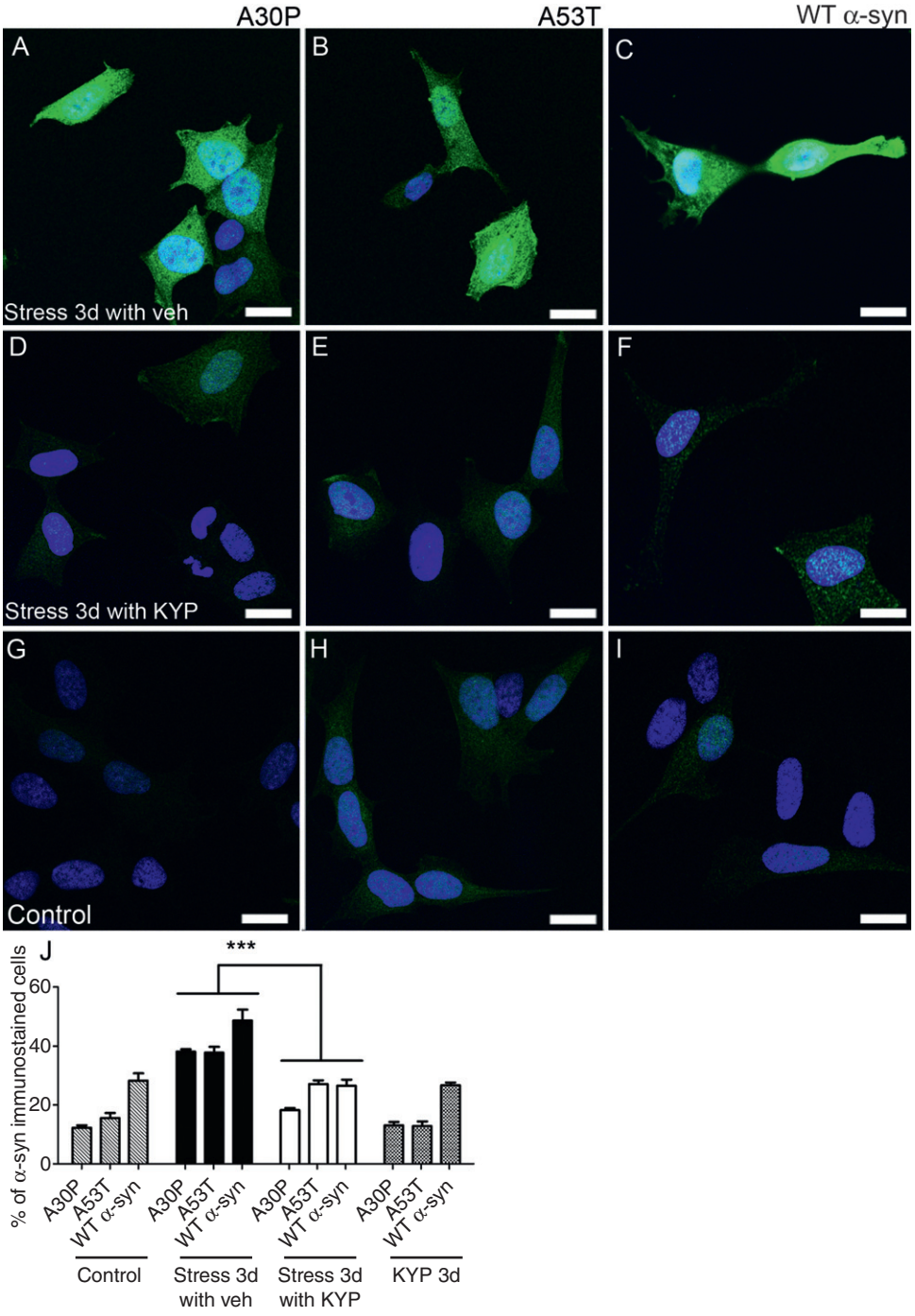

Figure 1

The effect of KYP-2047 on immunoreactive α-synuclein in three different α-synuclein-overexpressing cell lines. Representative confocal photomicrographs show that oxidative stress of 3 days with vehicle (0.001% DMSO; stress 3d with veh) clearly increased the amount of cells with highly immunoreactive α-synuclein in all cell lines as seen in panels A–C and J (α-syn is visualized with green, nuclear marker DAPI is visualized with blue). Incubation of 1 μM KYP-2047 during the oxidative stress (D–G; stress 3d with KYP) significantly reduced the cells with immunoreactive α-synuclein in all cell lines ($P < 0.001$; J). Incubation of cells with KYP-2047 without oxidative stress did not affect the α-synuclein immunoreactivity (J, KYP 3d). In non-stressed control cells (G–I, Control), only low amount of α-synuclein immunoreactivity was seen. Bars are mean ± SEM in G–H (n = 3 individual experiments). Scale bars are 10 μm in all the images. For detailed information of study groups, see Methods. ***$P < 0.001$, stress 3d with veh, significantly different from stress 3d with KYP; Cell line codes: A30P cells, SH-SY5Y cells expressing human A30P mutated α-syn; A53T cells, SH-SY5Y cells expressing human A53T mutated α-syn; WT α-syn cells, SH-SY5Y cells expressing human wild-type α-synuclein.

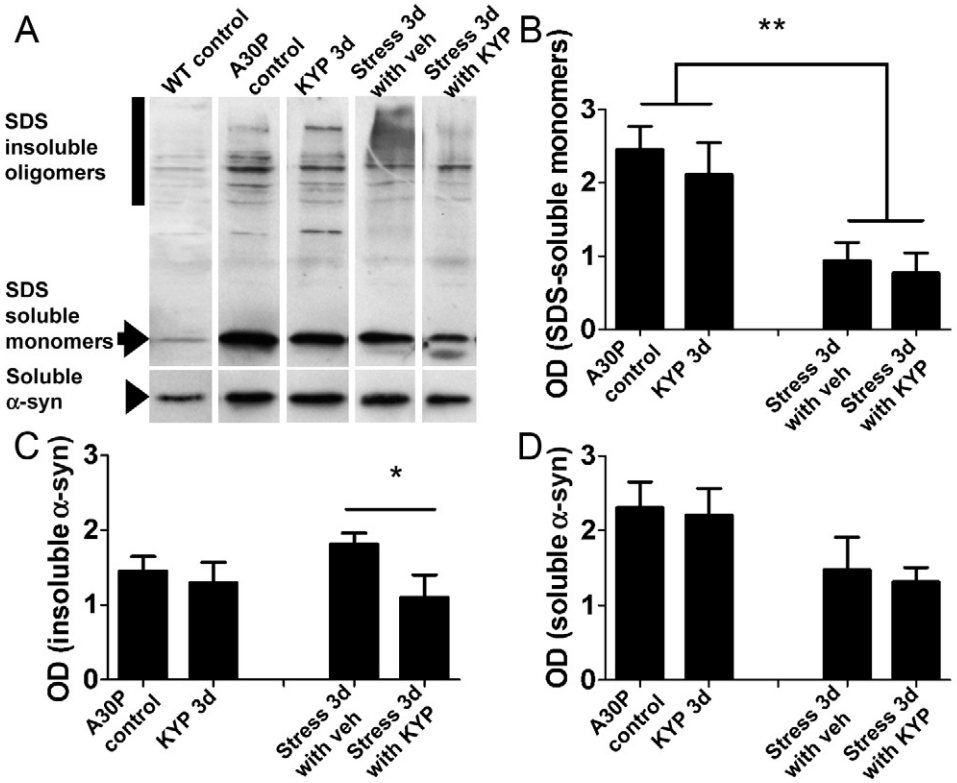

Figure 2

The effect of oxidative stress and KYP-2047 on SDS-insoluble α-synuclein (α-syn) on Western blot. In non-stressed A30P control cells (A30P control) and in non-stressed A30P cells incubated with KYP-2047 (KYP 3d), some high MW, SDS-insoluble oligomers were seen due to their A30P[α-synuclein] overexpression (A). In non-stressed and non-overexpressing WT cells (WT control;), hardly any SDS-soluble monomers or high MW, SDS-insoluble oligomers were seen. OD value of WT control was used as a background value in Western blotting band OD analysis of A30P cells. A 3-day oxidative stress with vehicle (0.001% DMSO; stress 3d with veh) increased the amount (not significantly compared to A30P control, $P = 0.094$) and changed the pattern of high MW, SDS-insoluble α-synuclein (A and C). Oxidative stress caused reduction of SDS-soluble monomers (A, B; $P < 0.01$) and also some decrease in soluble α-synuclein (A and D). Incubation with KYP-2047 during stress reduced high MW, SDS-insoluble α-synuclein in Western blotting compared with vehicle incubation ($P < 0.05$, Student's *t*-test; stress 3d with veh, significantly different from stress 3d with KYP; A–B). Only slight reduction by KYP-2047 was seen in SDS-soluble α-synuclein monomers or soluble α-synuclein (B, D). Representative α-synuclein Western blots are from A30P cells. For detailed information of study groups, see Methods. Bars are mean ± SEM in panels B–D (n = 3 independent experiments). *$P < 0.05$ in stress 3d with veh, significantly different from stress 3d with KYP. **$P < 0.01$ in stressed, significantly different from non-stressed cells.

blot experiments. The same PREP activity assay was used also for mouse brain homogenates (see preparation below).

Cell viability

To assess cell viability under oxidative stress and the effects of KYP-2047, the LDH release assay was used, as described by Talman *et al*. (2011). The effects of different dilutions of oxidative stress medium (see above; 100%, 75% and 50%) on cell viability and the effects of KYP-2047 were also studied. In case of negative values in LDH assay, these values were set as 0%, and other values were proportioned to this.

Animals and tissue preparation

All animal care and experimental procedures were conducted according to the Council of Europe (directive 86/609) guidelines and approved by the Animal Ethics Committee of the University of Antwerp. As the α-synuclein mouse model, we used Thy1-A30P-α-syn transgenic mice, where human A30P α-synuclein overexpression is driven by the Thy1 promoter as reported before (Kahle *et al*., 2000). This transgenic mouse strain develops α-synucleinopathy in several brain regions, age-dependent cognitive decline after 12 months and progressive deterioration of locomotor function (Neumann *et al*., 2002; Freichel *et al*., 2007). Thy1-A30P-α-syn transgenic mice (16 males, weight 32–38 g, aged 59–72 weeks) and 13 WT control mice (C57BL/6JRccHsd, weigh 30–45 g, aged 62–68 weeks) were maintained under a 12:12 h light/dark cycle at temperature of 20–22°C. Food and water were available *ad libitum*. The second A30P-α-syn transgenic mouse and the experiments performed on this strain are described in supplementary material.

Tissue processing for immunohistochemistry and Western blotting were performed by protocols described earlier (Myöhänen *et al*., 2008a; 2008b). Briefly, animals were anaesthetized with an overdose of pentobarbital (100 mg·kg^{-1}; Orion Corporation, Espoo, Finland) and then perfused transcardially with PBS. The brains were then removed and dissected in two hemispheres; one hemisphere

was fixed for immunohistochemistry (4 + 18 h in 4% PFA), and the other one was frozen for Western blotting analysis. Fixation, sectioning and homogenization were done as described earlier (Myöhänen *et al.*, 2008b).

Treatment of A30P transgenic mice with a PREP inhibitor

A30P transgenic mice were separated in two groups, a group treated with KYP-2047 (n = 7) and a vehicle group (n = 6). KYP-2047 (1 mg·mL^{-1}) in saline containing 0.5% DMSO was injected i.p. (3 mg·kg^{-1}) twice a day (12/12 h) for 5 days. The vehicle group received the same treatment but with omission of KYP-2047 (same volume of 0.5% DMSO in saline). After 5 days of injections, the animals were killed, and tissues were removed as described above.

Immunohistochemistry

Immunohistochemistry for the detection of α-synuclein in the mouse brain was performed by modifying the protocol described in Myöhänen *et al.* (2010b). Briefly, the endogenous peroxidase activity was inactivated with 10% methanol and 3% H_2O_2 in Tris-buffered saline (TBS; pH 7.4) for 10 min, and non-specific binding was blocked with MOM Basic kit (product BMK-2202, Vector Laboratories) in TBS. The sections were incubated overnight at room temperature with α-synuclein antibody (Table 1), followed by washing with PBS. The slides were then incubated with goat anti-mouse HRP conjugated secondary antibody (product # 31430, Thermo Fisher Scientific; dilution 1:500 in TBS). The brown colour was developed with 0.05% 3,3′-diaminobenzidine and 0.03% hydrogen peroxide in TBS. Finally, the sections were transferred to objective glasses, dehydrated in alcohol series and mounted with Depex (BDH, Poole, UK). Slides were photographed and processed as described above.

In double-label immunofluorescence of PREP and α-synuclein, the protocol described in Myöhänen *et al.* (2008a) was used. Briefly, free-floating sections were washed with PBS, and thereafter the double-label staining was carried out similarly as cells (see above).

Semiquantitative analysis of optical density (OD)

Immunohistochemically processed sections were photographed and the ODs of the striatum, primary motor cortex and substantia nigra were analysed using the line analysis tool of AnalySISpro software (version 5.0, Olympus Soft Imaging Solutions GmBH, Münster, Germany) as described in Lindholm *et al.* (2007). The background values of each brain area (background obtained by control staining without primary antibody) were subtracted from raw data values of the same brain area. Altogether, 15–20 samples of each brain area/staining from three different experiments were analysed, and then the average and SEM of the values were calculated. OD values of the Western blot images were analysed using the free-hand tool of QuantityOne-software (version 4.6.9, Bio-Rad) as described earlier (Myöhänen *et al.*, 2007). The bands in Western blotting were quantified taking into account the OD (intensity) and the area of the band. When measuring the SDS-insoluble fractions of α-synuclein Western blots, the value of non-stressed wild-type cells was used as background that was subtracted from the value. In other Western blotting analysis, after subtracting the background of the gel, the OD values were proportioned to the values of the loading control, and this value was considered indicative of the protein amounts. The averages and SEM of optical densities from each area were calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5.0, GraphPad Software, Inc., San Diego, CA). To detect difference between the groups in α-synuclein immunofluorescence, thioflavin S staining and cellular PREP enzyme activities, two-way ANOVA with Bonferroni post-test was used. Differences in α-synuclein OD values from animal immunohistochemical experiments were calculated with two-way ANOVA and Bonferroni post-test. Two-tailed Student's t-test was used to assess the differences in cellular and animal Western blotting results. Statistically significant differences were considered at $P < 0.05$.

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified in the text. The PREP inhibitor, KYP-2047, was synthesized as previously described (Jarho *et al.*, 2004). KYP-2047 was chosen as a reference compound since the biochemical and pharmacological data indicate it is potent and selective, that it enters SH-SY5Y cells in culture and crosses the blood–brain barrier effectively in rodents (Jarho *et al.*, 2004; Venäläinen *et al.*, 2006; Jalkanen *et al.*, 2007; 2011). The drug and molecular target nomenclature follows Alexander *et al.*, (2011).

Results

Cell culture studies: KYP-2047 reduces α-synuclein immunoreactivity and aggregation in cells exposed to oxidative stress

α-Synuclein immunofluorescence. The aggregation of α-synuclein was induced by oxidative stress (stress 3d) in three stable SH-SY5Y cell lines overexpressing human WT, mutated A30P or A53T α-synuclein. The oxidative stress treatment with or without vehicle increased the percentage of cells with clearly elevated immunoreactive α-synuclein from control level [12% (A30P cells) – 26 % (WT α-synuclein) up to 38% (A30P cells) – 48% (WT α-synuclein)] (Figure 1A–C, J). Vehicle (0.001% DMSO) or KYP-2047 incubation alone did not affect the number of cells with α-synuclein immunoreactivity (Figure 1J). KYP-2047 significantly reduced the number of cells with immunoreactive α-synuclein, both when present together with the oxidative stress (stress 3 days with KYP; $P < 0.001$ in all cell lines; Figure 1D–F,J) or when incubated for 1 day after the oxidative stress treatment (stress 3 days + 1 day KYP; $P < 0.001$ in all cell lines; Figure S1A) when compared with vehicle (stress 3d with veh; Figure 1A–C,J) in all α-synuclein overexpressing cell lines. Only minor α-synuclein immunoreactivity was seen in non-stressed cells with the antibody used in this study (Figure 1G–I).

The effect of increasing concentrations of KYP-2047 (1 nM–100 μM) on α-synuclein immunoreactivity was studied during the stress 3d treatment of A30P and A53T

Table 2

Transcription changes of the α-synuclein gene, relative to the β-actin control gene, assayed by quantitative reverse transcriptase PCR in A30P overexpressing cells after 3-day oxidative stress and controls (value ± SD)

	$2^{-\Delta\Delta C(t)} \pm$ SD*
3 d Stress with VEH	1.33 ± 0.4
3 d Stress with 1 μM KYP-2047	1.37 ± 0.8
3 d 1 μM KYP-2047	1.13 ± 0.1
Control	1.33 ± 0.1

*Average of three measurements.

cells. 1 nM of KYP-2047 in the culture medium did not cause any significant change in the number of cells with high α-synuclein immunoreactivity (Figure S1B). At higher KYP-2047 concentrations, the number of cells with α-synuclein immunoreactivity was significantly reduced compared to stress 3d with vehicle group ($P < 0.001$; Figure S1B).

Soluble and insoluble α-synuclein protein levels detected by Western blotting. Western blots of soluble and insoluble fractions of α-synuclein were performed to correlate the results obtained from the immunocytochemical analysis of all experimental conditions. A30P cells were used as a reference in Western blotting experiments. Stress 3d treatment with vehicle reduced the levels of soluble α-synuclein and the amount of SDS-soluble monomers, but increased the amount of high-molecular-weight (high MW) SDS-insoluble oligomers (Figure 2A–D). The increase was not significant compared with non-stressed overexpressing cells, and the overexpression of A30P[α-synuclein] itself gave rise to some high MW, SDS-insoluble oligomers (Figure 2A,C). However, the oxidative stress changed the pattern of high MW bands that were increased (Figure 2A). This was also seen as changes in the staining patterns of cell immunohistochemistry (Figure 1). Importantly, KYP-2047 incubation during the stress significantly reduced the levels of high MW, SDS-insoluble α-synuclein oligomers, when administered with the oxidative stress (Figure 2A,C; $P < 0.05$ compared with stress 3d with vehicle). KYP-2047 incubation without the stress decreased slightly (not significantly) SDS-soluble monomers (Figure 2) but had no effect on soluble α-synuclein levels (Figure 2A,B,D). Moreover, oxidative stress itself clearly decreased the levels of SDS-soluble α-synuclein monomers and slightly the levels of soluble α-synuclein (Figure 2A,B,D). In non-stressed and non-overexpressing WT cells, hardly any SDS-soluble monomers or high MW, SDS-insoluble oligomers were seen (Figure 2A).

Thioflavin S staining as a marker of α-synuclein aggregation. The results obtained by immunohistochemistry and Western blots were confirmed by the appearance of thioflavin S (a specific marker for β-sheet structures) positive cells in the same conditions (Figure 3A–C). In all cell lines, thioflavin S staining was also significantly reduced by addition of KYP-2047 (Figure 3D–G), supporting the immunohistochemistry and Western blotting results.

α-Synuclein mRNA transcription levels. To clarify whether oxidative stress or PREP inhibitor has an effect on α-synuclein mRNA levels and protein transcription, a quantitative rtPCR experiment was performed for A30P cells. No changes in α-synuclein mRNA levels were detected in any condition tested (Table 2).

Colocalization of α-synuclein with PREP. We investigated potential colocalization of α-synuclein with PREP in all α-synuclein-overexpressing cells by double-label immunofluorescence and confocal microscopy. PREP and α-synuclein partially colocalized when α-synuclein aggregation was induced by the stress 3d treatment with vehicle (Figure 4A–C). However, after incubation with KYP-2047, the observed colocalization was lost (Figure 4D,E). KYP-2047 treatment did not affect the localization or amount of immunoreactive PREP (Figure S2B), although some colocalization may have been lost due to the reduced amount of high MW,SDS-insoluble oligomers and SDS-soluble monomers as detected by Western blotting (Figure 2A–C). However, there was also a decrease of the amounts of PREP protein after the oxidative stress, but not following the KYP-2047 incubation (Figure S2B). In non-stressed cells, only minor colocalization was seen (Figures 2B and 4G–I; Table 3). Since (1) the levels of soluble α-synuclein and SDS-soluble monomers were higher in non-stressed than stressed cells (Figure 2C), and we detected (2) increased amounts of high MW, SDS-insoluble α-synuclein and (3) increased colocalization between PREP and α-synuclein as a result of the oxidative stress (Figure 4A–C), our interpretation is that PREP colocalized with α-synuclein aggregates (Figures 1 and 4). The colocalization percentage in stress 3d with vehicle varied between 24% (A53T) and 42% (WT α-synuclein). After KYP-2047 treatment, the percentages were 18–22% (Table 3). The colocalization percentages correlated with the amount of cells with α-synuclein inclusions (Figures 1 and 3).

PREP enzyme activity. There was no significant difference in the PREP enzyme activities between the A30P, A53T or WT α-synuclein overexpressing cells and normal SH-SY5Y cells (Figure S2A). 1 μM KYP-2047 drastically reduced the PREP activity in the cells during exposure to stress 3d (Figure S2A). The oxidative agents used slightly reduced PREP activity, but this reduction was not statistically significant (Figure S2A). This minor inhibition may be due to the fact that residual Fe^{2+} ions from the culture medium inhibit PREP (Cunningham and O'Connor, 1998).

Cell viability. To detect the effect of oxidative stress and PREP inhibition on cell viability of the A30P and A53T overexpressing cell lines and wild-type cells, the standard lactate dehydrogenase (LDH) release assay was used. Moreover, to study the effect of oxidative stress on cell viability, we induced the aggregation of α-synuclein using 50%, 75% and 100% concentrations of oxidative stress medium. In the LDH cell viability assay, measuring the membrane integrity of the cells, KYP-2047 treatment reduced cell death in α-synuclein-overexpressing cell lines compared with vehicle in all stress groups (Figure 5A–C). The most significant differences were seen at 50% stress medium, but there was a significant

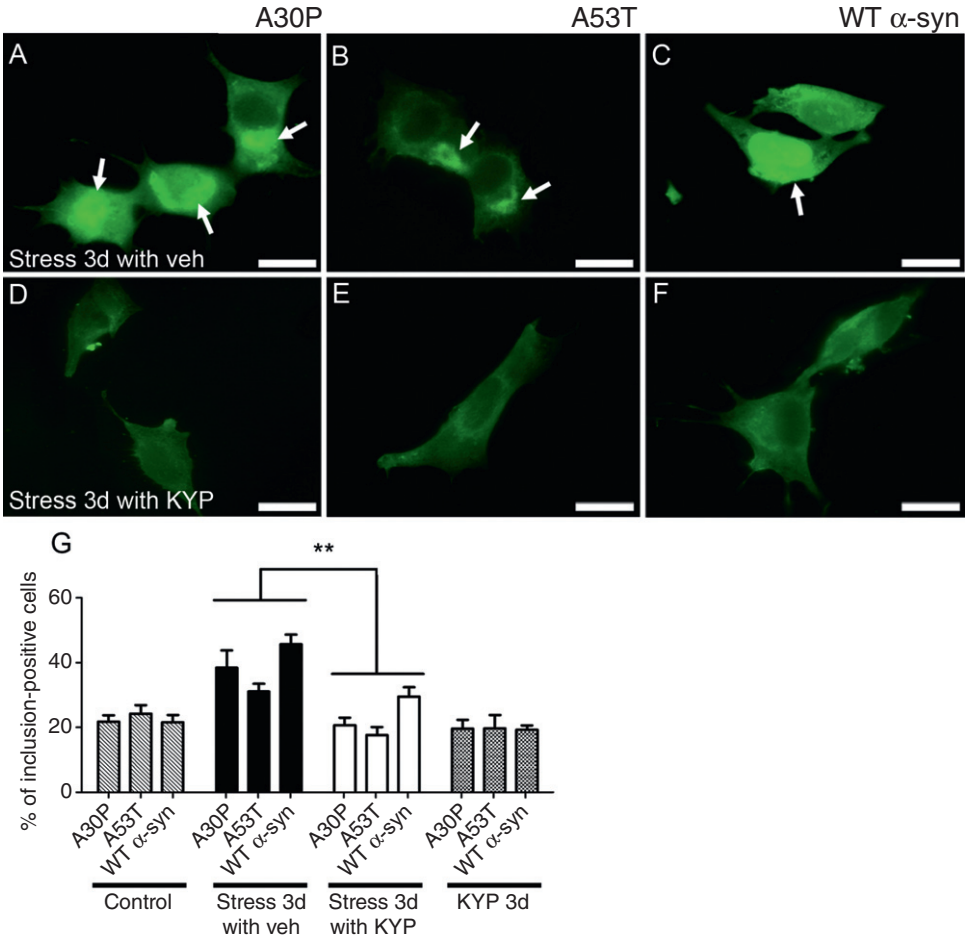

Figure 3

Thioflavin S staining of protein aggregates in three different α-synuclein-overexpressing cells. Thioflavin S-stained fluorescence photomicrographs are very similar to the results of α-synuclein (α-syn) immunofluorescence in Figure 1. Incubation of 1 μM KYP-2047 during the 3-day oxidative stress (stress 3d with KYP; D–F) reduced significantly the amount of cells with cytosolic aggregates compared with vehicle incubation (stress 3d with veh; A–C) in all cell lines (G; $P < 0.01$). As shown by immunofluorescence staining, treatment with the PREP inhibitor without the oxidative stress did not cause any difference in aggregates compared with the control (G; KYP 3d). Bars are mean ± SEM in panel I ($n = 3$ individual experiments); **$P < 0.01$, stress 3d with veh, significantly different from stress 3d with KYP. Cell line codes are similar to those for Figure 1. Scale bars are 10 μm in all the images.

Table 3

Colocalization percentages between PREP and α-synuclein in overexpressing cell lines (% of all cells ± SEM). All values are averages of three individual experiments

	A30P	A53T	WT α-syn
3 d Stress with vehicle	31 ± 1.6	24 ± 2.2	42 ± 3.8
3 d Stress with 1 μM KYP-2047	18 ± 2.3**	11 ± 2.2**	20 ± 1.7***
3 d 1 μM KYP-2047	10 ± 0.3	12 ± 1.9	17 ± 2.2
Control	12 ± 2.1	11 ± 1.3	19 ± 1.3

**$P < 0.01$; ***$P < 0.001$ compared with 3d Stress with vehicle.

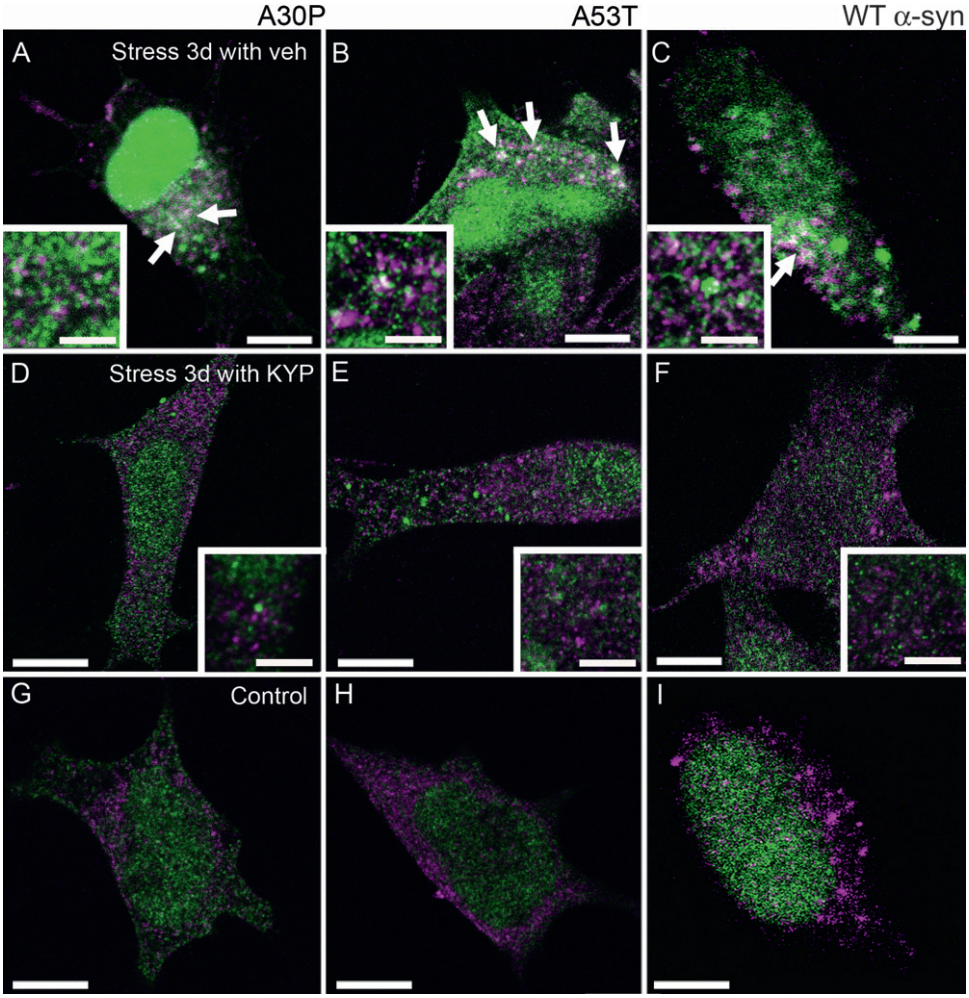

Figure 4

Changes in colocalization between PREP (magenta) and α-synuclein (α-syn) (green) during oxidative stress vehicle (stress 3d with vehicle) and PREP inhibitor treatment (stress 3d with KYP) visualized by immunohistochemistry and confocal microscopy. When α-synuclein-overexpressing cells are exposed to stress for 3 days with vehicle (A–C), partial intracellular colocalization between α-synuclein and PREP is formed (white colour and white arrows). However, when 1 μM KYP-2047 is present (stress 3d with KYP, D–E), the colocalization disappears. Small picture panels in panels A–E are magnifications from the larger picture, showing the colocalization and its disappearance after KYP-2047 incubation. In non-stressed cells, only sparse colocalization between PREP and α-synuclein is seen (Control, G–I). Cell line codes are as in Figure 1. Scale bars are 10 μm in large images and 1 μm in small picture panels.

difference also in the 100% group (A53T cells; Figure 5B). In non-overexpressing WT cells, there was no difference between the effects of vehicle and KYP-2047 treatments (Figure 5C), suggesting that the protective effect of KYP-2047 was related to α-synuclein overexpression. In 3-day KYP or vehicle incubations without oxidative stress, no clear differences in cell viability were observed (Figure S3).

Animal studies: the effects of KYP-2047 in the brain of A30P α-synuclein transgenic mice

Immunohistochemistry and Western blotting for α-synuclein. To confirm the *in vitro* and cellular effects of KYP-2047 on α-synuclein aggregation, we tested the effect of a 5-day treatment on the amount of α-synuclein immunoreactive and soluble protein levels in the brain of old Thy1-A30P-α-syn transgenic mice (Freichel *et al.*, 2007). Even this short exposure of KYP-2047 (3 $mg \cdot kg^{-1}$ i.p. twice a day) significantly reduced the amount of α-synuclein protein in the A30P-α-syn mice in all the brain areas analysed (immunohistochemistry) and in the whole-brain homogenates (Western blotting) compared with vehicle treatment [Figure 6A–M; Figure S6A,B; Western blotting, $P < 0.01$ (mouse and human α-synuclein) and $P < 0.05$ (human α-synuclein); immunohistochemistry, $0.05 < P < 0.001$)]. Antibodies recognizing both human and mouse α-synuclein or only human α-synuclein showed similar effect (Figure 6J–M), but the effect of the latter was more pronounced in the immunohistochemistry analysis (Figure 6K,M).

We also tested if the effect of KYP-2047 on the amount of the α-synuclein immunoreactivity/protein was dependent on

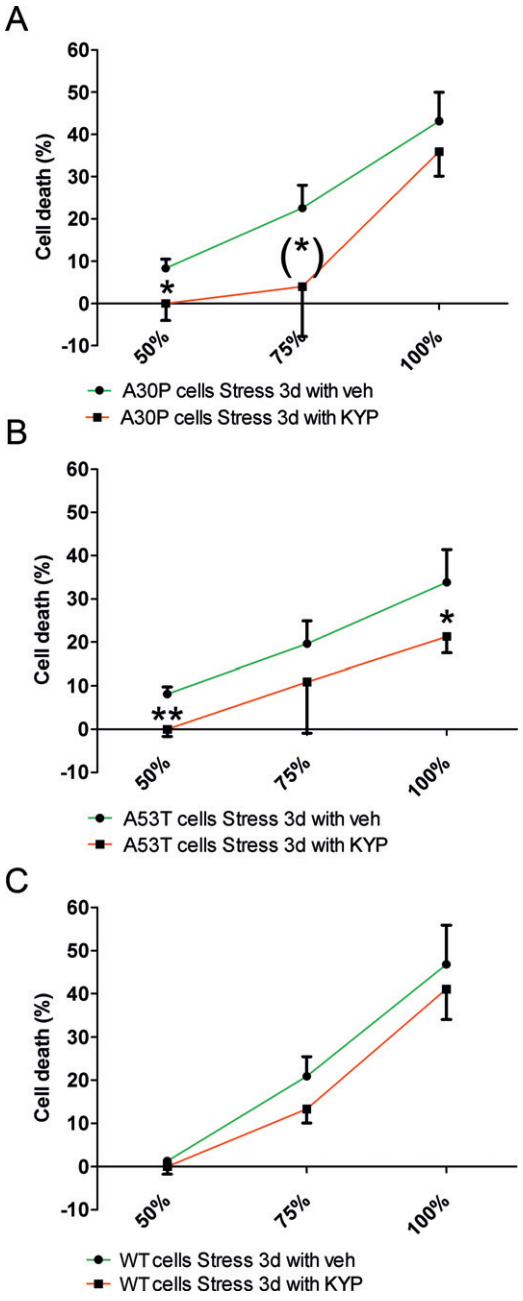

Figure 5

The effect of 3-day oxidative stress with vehicle or KYP-2047 on cell viability studied by lactate dehydrogenase (LDH) assay. Cell viability was measured using different concentrations of oxidative stress medium (100%, 75% and 50%), and A30P (A) and A53T (B) cells. WT cells (without α-synuclein overexpression) were used as a control (C). In α-synuclein-overexpressing cells, KYP-2047 (red line) clearly reduced the cell death compared with vehicle, and the difference was significant even in 100% stress medium (A53T cells; B). The most significant effects were seen in 50% stress medium (A–B). In WT cells, no statistical differences were seen in any stress concentration (C). $n = 5$ individual experiments. $^{**}P < 0.01$, stress 3d with veh, significantly different from stress 3d with KYP; $^{*}P < 0.05$, stress 3d with veh, significantly different from stress 3d with KYP; (*)$P = 0.054$, stress 3d with veh, significantly different from stress 3d with KYP in A30P cells and 75% stress medium.

the specific type of the α-synuclein mouse model and performed the same experiment with a different A30P transgenic mouse strain where A30P α-synuclein is under the control of a prion promoter (see details in Supplementary material and in Yavich *et al.*, 2005). The results in both mice strains were similar; a 5-day exposure of KYP-2047 (3 mg·kg^{-1}) reduced the amount of α-synuclein to the levels found in the substantia nigra, striatum and motor cortex of the WT animals when analysed with immunohistochemistry (see details and Figure S4).

Colocalization of PREP with α-synuclein. Similar to the results of cellular studies, colocalization between α-synuclein and PREP was seen in the vehicle-treated transgenic mouse striatum (Figure 7A) and substantia nigra pars compacta (Figure 7C). After a 5-day KYP-2047 treatment, the colocalization clearly reduced (Figure 7B,D), pointing to the ability of the PREP inhibitor to interfere with the interaction between α-synuclein and PREP also *in vivo*. However, some colocalization may have lost due to the reduced α-synuclein protein levels (Figure 6A–M). Practically no colocalization was seen in the substantia nigra of a wild-type animal (Figure 7E).

PREP protein levels and PREP activity. A 5-day KYP-2047 treatment (3 mg·kg^{-1} i.p., twice a day) did not alter the amount of PREP protein amount assayed by Western blotting in whole brains of A30P transgenic mice (Figure S5A). Moreover, KYP-2047 treatment did not significantly reduce the overall protein amounts in brain assayed by β-actin in whole brain homogenate, as shown by Western blotting (Figure S5B). There were also no statistical differences in PREP activity between A30P transgenic and WT mice measured from various brain areas (Figure S5C).

Discussion and conclusions

To our knowledge, and based on this study, it is the first time that a PREP inhibitor, KYP-2047, has been shown to reduce α-synuclein aggregation in cellular and animal models of Parkinson's disease. In the A30P α-synuclein-overexpressing cells in culture, the PREP inhibitor reduced high MW, SDS-insoluble forms of α-synuclein induced by oxidative stress. In the A30P transgenic mouse models, this reduction was also accompanied by a decrease in the amount of soluble α-synuclein in the brain.

Studies with cells in culture suggest that, when the α-synuclein aggregation process has been triggered by oxidative stress, the active site of PREP needs to be free to promote the aggregation, although PREP does not act as a trigger itself – this would cause a drop in the baseline of cells with overexpression upon inhibitor treatment. We have shown reliably that, under oxidative stress, a 3-day KYP-2047 incubation can reduce the amount of α-synuclein immunoreactivity and high-molecular-weight SDS-insoluble α-synuclein protein levels in α-syn-overexpressing cells. Moreover, KYP-2047 treatment reduced β-sheet structure-containing cells revealed by staining with thioflavin S, demonstrating that KYP-2047 also affected α-synuclein aggregates. At least in the cell

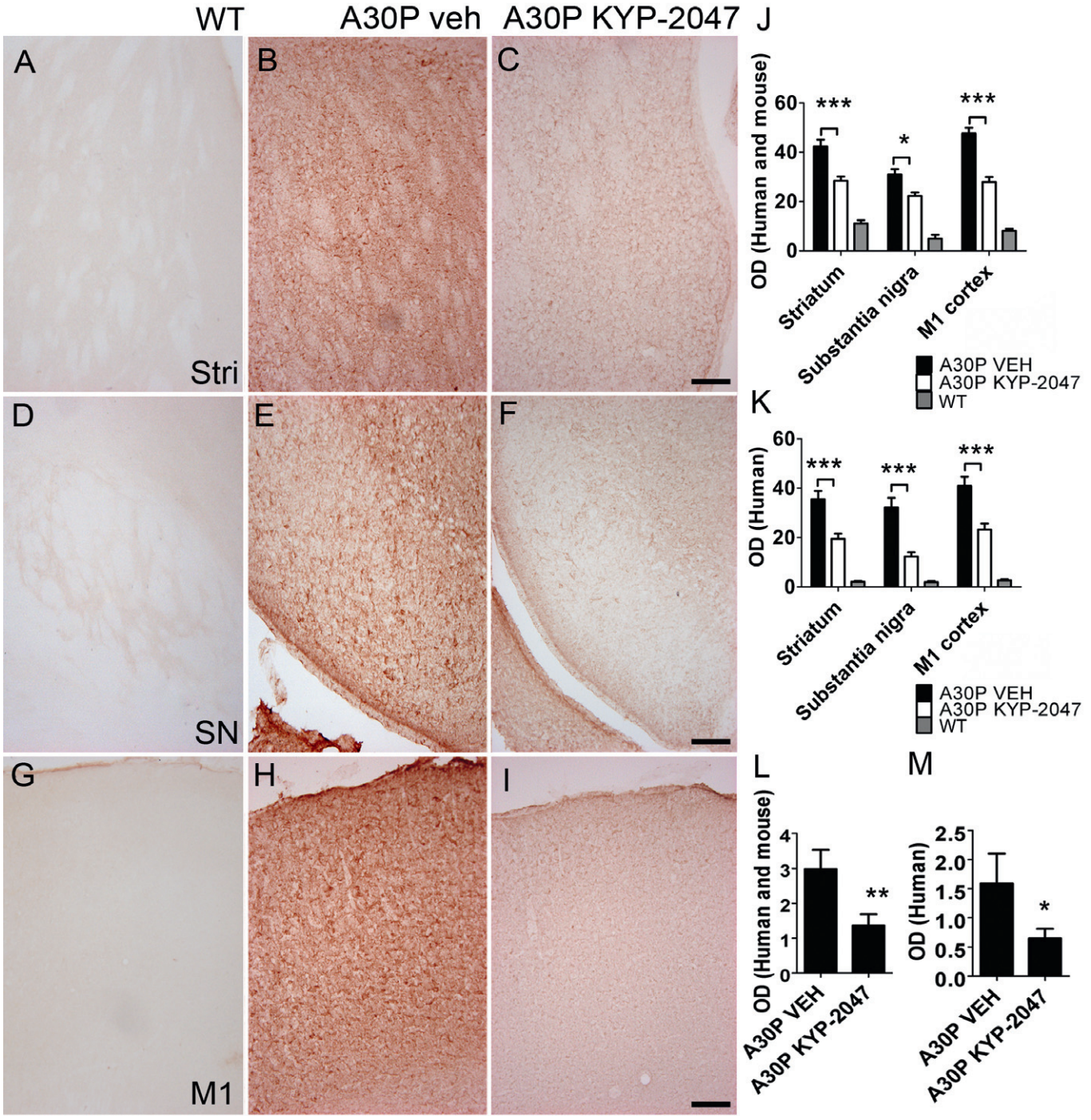

Figure 6

The effect of a 5-day KYP-2047 exposure on α-synuclein protein expression in A30P-α-syn transgenic mice. A clear effect of PREP inhibition (A30P KYP-2047) was seen on immunoreactive α-synuclein in various brain areas of A30P-α-syn transgenic mice (A–K; $0.05 < P < 0.001$) and also in the whole-brain α-synuclein determined with Western blotting (L–M; $0.05 < P < 0.01$). The effect is accentuated in A30P mutated human α-synuclein by the immunohistochemical analysis (J–K). Panels L–M depict α-synuclein detected by Western blotting from KYP-2047 and vehicle (veh) exposed A30P-α-syn transgenic mouse brain. Representative Western blots are presented in Figure S6A-B. Bars are mean ± SEM in panels J–M ($n = 3$ individual experiments). Scale bars are 100 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, significantly different from A30P vehicle (A30P veh). Codes: A30P KYP-2047, PREP inhibitor treated A30P-α-syn transgenic mouse; A30P veh, vehicle-treated A30P α-synuclein transgenic mouse; M1, primary motor cortex; SN, substantia nigra; Stri, striatum; WT, wild-type animal.

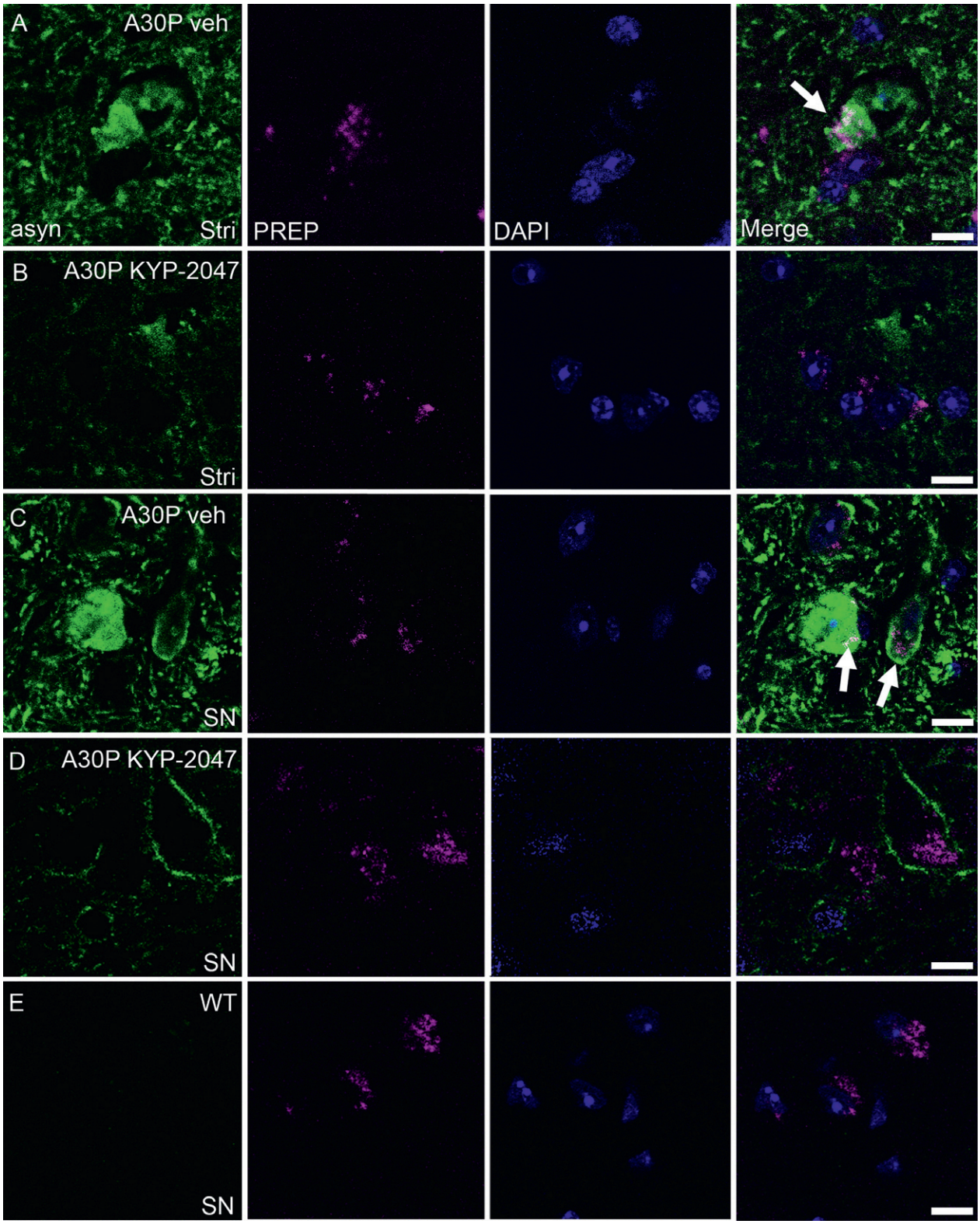

Figure 7

Confocal immunofluorescence pictures presenting the *in vivo* colocalization between α-synuclein (green) and PREP (magenta) in the nigrostriatal pathway. Clear colocalization is seen both in striatum (A, Stri) and substantia nigra (C, SN) of vehicle (A30P veh) treated A30P α-synuclein transgenic mice while in KYP-2047-exposed animals the colocalization was not present (B, D). In the substantia nigra of wild-type (WT) mice, very minor amounts of α-synuclein were seen (E). α-Synuclein is visualized with fluorescein, PREP with Texas red and nuclei with DAPI. Pictures are merged, and colocalization between α-synuclein and PREP is shown in white colour. Scale bars are 10 μm in all figures. Codes are similar to Figure 6.

cultures, the reduction of α-synuclein level is not the result of the changes in transcription, and in α-synuclein overexpressing cells, PREP inhibition seems also to accelerate recovery of cells exposed to oxidative stress. Importantly, KYP-2047 also reduced the toxicity of oxidative stress on α-synuclein overexpressing cells assayed by the LDH cell viability assay. This was not observed in non-overexpressing cells, suggesting that KYP-2047 reduced the α-synuclein-based cell toxicity. As observed in cells, a 5-day treatment with KYP-2047 significantly reduced the α-synuclein immunoreactivity and soluble protein levels *in vivo*.

Both in cells and *in vivo*, these proteins were partially colocalized when aggregation had occurred and the colocalization was abolished by the PREP inhibitor. This reduced colocalization may also be caused by the decreased amount of α-synuclein or PREP proteins, especially in the mouse brain. However, in the cellular experiments, although the oxidative stress reduced also the amount of PREP protein, KYP-2047 did not have an additional effect. Moreover, at least in cells, the reduction in colocalization was more pronounced than the reduction of high MW, SDS-insoluble α-synuclein protein detected by Western blots. Therefore, it seems probable that colocalization disappears as a result of treatment with KYP-2047 rather than reflecting the reduction in protein amount. Moreover, in the non-stressed control cells, only minor colocalization of PREP and α-synuclein was seen, and oxidative stress even reduced the amounts of soluble α-synuclein and SDS-soluble monomers of α-synuclein below the non-stressed cells. This further supports the hypothesis that the interaction of PREP and α-synuclein occurs only after oxidative stress has triggered the aggregation process and that PREP colocalizes with aggregated forms of α-syn. These results support previous *in vitro* observations by Brandt *et al*. (2008) where, under cell-free conditions, α-synuclein aggregation was accelerated by PREP, an effect that was blocked by two different PREP inhibitors (Z-pro-prolinal and UAMC-21) or by mutation of the active site of PREP.

There are several phases in the process of α-synuclein aggregation. It has been suggested earlier, based on cell-free *in vitro* experiments, that PREP acts in early phases of α-synuclein aggregation, possibly in the nucleation phase (Lambeir, 2011), but the mechanism remained unknown. It may be proposed that PREP acts as a nucleation center and therefore assists the aggregation process. Moreover, we hypothesize that when KYP-2047 binds to PREP, the aggregation process is slowed down, giving time for cellular housekeeping mechanisms, such as proteasomes and lysosomes (Chu *et al*., 2009; Lorenc-Koci *et al*., 2011), to clear α-synuclein from the cells under the critical concentrations for aggregation (Wood *et al*., 1999).

Because a PREP inhibitor accelerated the clearance of α-synuclein from the cells after the oxidative stress and had potent effects on α-synuclein protein levels in old transgenic mouse, there are very probably some other mechanisms for PREP in α-synuclein aggregation besides acting as a nucleation center. One possibility is that the PREP interaction with α-synuclein can interfere with the functions of aggresomes that are known to be the most effective in clearing the α-synuclein aggregates (Opazo *et al*., 2008). PREP inhibition could then abolish the interaction and thus increase the activity of aggresomes. Aggresomal processing of misfolding proteins is also a microtubule-dependent process and localization of PREP along the microtubules supports this function (Schulz *et al*., 2005). The effect of KYP-2047 on a number of stressed α-synuclein -overexpressing cells, as seen with immunohistochemical assays, is very similar to aggresomal clearance reported earlier (Opazo *et al*., 2008).

Eventually, the action of the PREP inhibitor leads also to a reduced number of aggregates, as shown by both Western blotting and thioflavin S staining. PREP might also directly regulate the levels of α-synuclein protein by regulating gene expression, as Moreno-Baylach *et al*. (2011) recently reported that PREP and PREP inhibition can affect gene expression of various intracellular proteins. However, we did not see any changes in α-synuclein mRNA levels after PREP inhibition. According to our results, it is clear that the active site or a specific active conformation of PREP was needed for the meaningful interaction of the two proteins. Further studies of this protein–protein interaction are warranted.

In summary, we conclude as follows. Firstly, incubation with KYP-2047 reduced the amount of high MW, SDS-insoluble α-synuclein oligomers in the α-synuclein-overexpressing cells and increased cell survival in the LDH assay. Secondly, even a short-term treatment with KYP-2047 reduced the soluble α-synuclein *in vivo*. Finally, PREP inhibitors were able to break the spatial interaction between PREP and α-synuclein in three different α-synuclein-overexpressing cell lines and also *in vivo*. This is proposed to lead to a block of the α-synuclein aggregation process or to an enhancement of the α-synuclein clearance. Although these findings suggest that PREP may increase the α-synuclein aggregation via a protein–protein interaction, the molecular mechanism remains unclear. The rapid and significant effect of a short-term exposure to a PREP inhibitor on levels of α-synuclein and its aggregation may open new ways to develop a drug therapy for α-synuclein-based Parkinson's disease.

Acknowledgements

The authors thank the Department of Veterinary Sciences and the Core Facility Biomedical Microscopic Imaging of the University of Antwerp for providing excellent facilities for tissue processing and staining, Prof PP De Deyn, Dr D Van Dam and members of the Laboratory of Neurochemistry and Behaviour of the University of Antwerp for housing mice and their assistance studying mice behaviour, and Dr P Jäkälä (University of Eastern Finland) and Dr P Kahle (University of Tübingen) for providing the A30P transgenic mice. We also acknowledge MSc G Duran-Torres for his excellent technical assistance. These studies were supported by grants from FWO-Vlaanderen, UA Research Council, Finnish Cultural Foundation, Orion-Pharmos Foundation, Academy of Finland (No. 138127/2010) and Jane and Aatos Erkko Foundation to Timo T Myöhänen, by the grants of the Academy of Finland (No. 1131915/2008 and no. 117881/2006) to Pekka T Männistö and by the grant (G.0114.08) of the FWO-Vlaanderen to Anne-Marie Lambeir and Veerle Baekelandt. This research was supported by the nEuroPro project, EU FP7-HEALTH-2007-B-223077.

Conflict of interest

The authors state that they have no conflicts of interests.

References

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Arima K, Ueda K, Sunohara N, Hirai S, Izumiyama Y, Tonozuka-Uehara H *et al.* (1998). Immunoelectron-microscopic demonstration of NACP/alpha-synuclein-epitopes on the filamentous component of Lewy bodies in Parkinson's disease and in dementia with Lewy bodies. Brain Res 808: 93–100.

Bisaglia M, Mammi S, Bubacco L (2009). Structural insights on physiological functions and pathological effects of alpha-synuclein. FASEB J 23: 329–340.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brandt I, Gerard M, Sergeant K, Devreese B, Baekelandt V, Augustyns K *et al.* (2008). Prolyl oligopeptidase stimulates the aggregation of alpha-synuclein. Peptides 29: 1472–1478.

Chu Y, Dodiya H, Aebischer P, Olanow CW, Kordower JH (2009). Alterations in lysosomal and proteasomal markers in Parkinson's disease: relationship to alpha-synuclein inclusions. Neurobiol Dis 35: 385–398.

Conway KA, Harper JT, Landsbury PT Jr (2000). Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. Biochemistry 39: 2552–2563.

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B *et al.* (2006). Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science 313: 324–328.

Cunningham DF, O'Connor B (1998). A study of prolyl endopeptidase in bovine serum and its relevance to the tissue enzyme. Int J Biochem Cell Biol 30: 99–114.

Deleersnijder A, Van Rompuy AS, Desender L, Pottel H, Buee L, Debyser Z *et al.* (2011). Comparative analysis of different peptidyl-prolyl isomerases reveals FK506-binding protein 12 as the most potent enhancer of {alpha}-synuclein aggregation. J Biol Chem 286: 26687–26701.

Di Daniel E, Glover CP, Grot E, Chan MK, Sanderson TH, White JH *et al.* (2009). Prolyl oligopeptidase binds to GAP-43 and functions without its peptidase activity. Mol Cell Neurosci 41: 373–382.

Feng LR, Federoff HJ, Vicini S, Maguire-Zeiss KA (2010). α-Synuclein mediates alterations in membrane conductance: a potential role for α-synuclein oligomers in cell vulnerability. Eur J Neurosci 32: 10–17.

Freichel C, Neumann M, Ballard T, Muller V, Woolley M, Ozmen L *et al.* (2007). Age-dependent cognitive decline and amygdala pathology in alpha-synuclein transgenic mice. Neurobiol Aging 28: 1421–1435.

Garcia-Horsman JA, Männistö PT, Venäläinen JI (2007). On the role of prolyl oligopeptidase in health and disease. Neuropeptides 41: 1–24.

Gerard M, Debyser Z, Desender L, Baert J, Brandt I, Baekelandt V *et al.* (2008). FK506 binding protein 12 differentially accelerates fibril formation of wild type alpha-synuclein and its clinical mutants A30P or A53T. J Neurochem 106: 121–133.

Gerard M, Deleersnijder A, Daniels V, Schreurs S, Munck S, Reumers V *et al.* (2010). Inhibition of FK506 binding proteins reduces alpha-synuclein aggregation and Parkinson's disease-like pathology. J Neurosci 30: 2454–2463.

Ibanez P, Bonnet AM, Debarges B, Lohmann E, Tison F, Pollak P *et al.* (2004). Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. Lancet 364: 1169–1171.

Jalkanen AJ, Puttonen KA, Venäläinen JI, Sinervä V, Mannila A, Ruotsalainen S *et al.* (2007). Beneficial effect of prolyl oligopeptidase inhibition on spatial memory in young but not in old scopolamine-treated rats. Basic Clin Pharmacol Toxicol 100: 132–138.

Jalkanen AJ, Hakkarainen JJ, Lehtonen M, Venäläinen T, Kääriäinen TM, Jarho E *et al.* (2011). Brain pharmacokinetics of two prolyl oligopeptidase inhibitors, JTP-4819 and KYP-2047, in the rat. Basic Clin Pharmacol Toxicol 109: 443–451.

Jarho EM, Venäläinen JI, Huuskonen J, Christiaans JA, Garcia-Horsman JA, Forsberg MM *et al.* (2004). A cyclopent-2-enecarbonyl group mimics proline at the P2 position of prolyl oligopeptidase inhibitors. J Med Chem 47: 5605–5607.

Kahle PJ, Neumann M, Ozmen L, Muller V, Jacobsen H, Schindzielorz A *et al.* (2000). Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. J Neurosci 20: 6365–6373.

Kato A, Fukunari A, Sakai Y, Nakajima T (1997). Prevention of amyloid-like deposition by a selective prolyl endopeptidase inhibitor, Y-29794, in senescence-accelerated mouse. J Pharmacol Exp Ther 283: 328–335.

Kim M, Jung W, Lee IH, Bhak G, Paik SR, Hahn JS (2008). Impairment of microtubule system increases alpha-synuclein aggregation and toxicity. Biochem Biophys Res Commun 365: 628–635.

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S *et al.* (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 18: 106–108.

Lambeir AM (2011). Interaction of prolyl oligopeptidase with α-synuclein. CNS Neurol Disord Drug Targets 10: 349–354.

Lindholm P, Voutilainen MH, Lauren J, Peränen J, Leppänen VM, Andressoo JO *et al.* (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. Nature 448: 73–77.

Lorenc-Koci E, Lenda T, Antkiewicz-Michaluk L, Wardas J, Domin H, Smialowska M *et al.* (2011). Different effects of intranigral and intrastriatal administration of the proteasome inhibitor lactacystin on typical neurochemical and histological markers of Parkinson's disease in rats. Neurochem Int 58: 839–849.

Männistö PT, Venäläinen JI, Jalkanen AJ, Garcia-Horsman JA (2007). Prolyl oligopeptidase: a potential target for the treatment of cognitive disorders. Drug News Perspect 20: 293–305.

Mantle D, Falkous G, Ishiura S, Blanchard PJ, Perry EK (1996). Comparison of proline endopeptidase activity in brain tissue from normal cases and cases with Alzheimer's disease, Lewy body dementia, Parkinson's disease and Huntington's disease. Clin Chim Acta 249: 129–139.

McLean PJ, Kawamata H, Ribich S, Hyman BT (2000). Membrane association and protein conformation of alpha-synuclein in intact neurons. Effect of Parkinson's disease-linked mutations. J Biol Chem 275: 8812–8816.

Moreno-Baylach MJ, Felipo V, Männistö PT, Garcia-Horsman JA (2008). Expression and traffic of cellular prolyl oligopeptidase are regulated during cerebellar granule cell differentiation, maturation, and aging. Neuroscience 156: 580–585.

Moreno-Baylach MJ, Puttonen KA, Tenorio-Laranga J, Venäläinen JI, Storvik M, Forsberg MM *et al.* (2011). Prolyl endopeptidase is involved in cellular signalling in human neuroblastoma SH-SY5Y cells. Neurosignals 19: 97–109.

Myöhänen TT, Venäläinen JI, Tupala E, Garcia-Horsman JA, Miettinen R, Männistö PT (2007). Distribution of immunoreactive prolyl oligopeptidase in human and rat brain. Neurochem Res 32: 1365–1374.

Myöhänen TT, Venäläinen JI, Garcia-Horsman JA, Piltonen M, Männistö PT (2008a). Cellular and subcellular distribution of rat brain prolyl oligopeptidase and its association with specific neuronal neurotransmitters. J Comp Neurol 507: 1694–1708.

Myöhänen TT, Venäläinen JI, Garcia-Horsman JA, Piltonen M, Männistö PT (2008b). Distribution of prolyl oligopeptidase in the mouse whole-body sections and peripheral tissues. Histochem Cell Biol 130: 993–1003.

Myöhänen TT, Schendzielorz N, Männistö PT (2010a). Distribution of catechol-O-methyltransferase (COMT) proteins and enzymatic activities in wild-type and S-COMT deficient mice. J Neurochem 113: 1632–1643.

Myöhänen T, Tenorio-Laranga J, Jokinen B, Vazquez-Sanchez R, Moreno-Baylach M, Garcia-Horsman J *et al.* (2010b). Prolyl oligopeptidase induces angiogenesis both in vitro and in vivo in a novel regulatory manner. Br J Pharmacol 163: 1666–1678.

Neumann M, Kahle PJ, Giasson BI, Ozmen L, Borroni E, Spooren W *et al.* (2002). Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies. J Clin Invest 110: 1429–1439.

Opazo F, Krenz A, Heermann S, Schulz JB, Falkenburger BJ (2008). Accumulation and clearance of α-synuclein aggregates demonstrated by time-lapse imaging. J Neurochem 106: 529–540.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A *et al.* (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276: 2045–2047.

Schulz I, Zeitschel U, Rudolph T, Ruiz-Carrillo D, Rahfeld JU, Gerhartz B *et al.* (2005). Subcellular localization suggests novel functions for prolyl endopeptidase in protein secretion. J Neurochem 94: 970–979.

Shishido Y, Furushiro M, Tanabe S, Taniguchi A, Hashimoto S, Yokokura T *et al.* (1998). Effect of ZTTA, a prolyl endopeptidase inhibitor, on memory impairment in a passive avoidance test of rats with basal forebrain lesions. Pharm Res 15: 1907–1910.

Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J *et al.* (2003). Alpha-synuclein locus triplication causes Parkinson's disease. Science 302: 841.

Singleton A, Gwinn-Hardy K, Sharabi Y, Li ST, Holmes C, Dendi R *et al.* (2004). Association between cardiac denervation and parkinsonism caused by alpha-synuclein gene triplication. Brain 127: 768–772.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997). Alpha-synuclein in Lewy bodies. Nature 388: 839–840.

Surguchov A (2008). Molecular and cellular biology of synucleins. Int Rev Cell Mol Biol 270: 225–317.

Talman V, Tuominen RK, Boije af Gennäs G, Yli-Kauhaluoma J, Ekokoski E (2011). C1 Domain-targeted isophthalate derivatives induce cell elongation and cell cycle arrest in HeLa cells. PLoS ONE 6: e20053.

Toide K, Shinoda M, Fujiwara T, Iwamoto Y (1997). Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on spatial memory and central cholinergic neurons in aged rats. Pharmacol Biochem Behav 56: 427–434.

Uversky VN, Li J, Fink AL (2001). Evidence for a partially folded intermediate in alpha-synuclein fibril formation. J Biol Chem 276: 10737–10744.

Venäläinen JI, Garcia-Horsman JA, Forsberg MM, Jalkanen A, Wallen EA, Jarho EM *et al.* (2006). Binding kinetics and duration of in vivo action of novel prolyl oligopeptidase inhibitors. Biochem Pharmacol 71: 683–692.

Wood SJ, Wypych J, Steavenson S, Louis JC, Citron M, Biere AL (1999). alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. J Biol Chem 274: 19509–19512.

Yavich L, Oksman M, Tanila H, Kerokoski P, Hiltunen M, van Groen T *et al.* (2005). Locomotor activity and evoked dopamine release are reduced in mice overexpressing A30P-mutated human alpha-synuclein. Neurobiol Dis 20: 303–313.

Yoshimoto T, Kado K, Matsubara F, Koriyama N, Kaneto H, Tsura D (1987). Specific inhibitors for prolyl endopeptidase and their anti-amnesic effect. J Pharmacobiodyn 10: 730–735.

Yu S, Li X, Liu G, Han J, Zhang C, Li Y *et al.* (2007). Extensive nuclear localization of alpha-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody. Neuroscience 145: 539–555.

Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros R, Ampuero I *et al.* (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 55: 164–173.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The effect of a 1-day KYP-2047 incubation after a 3-day oxidative stress in α-synuclein-overexpressing cells (A) and dose–response test of KYP-2047 in A30P and A53T cells (B). When 1 μM KYP-2047 was administered after 3 days of oxidative stress (stress 3d + 1d KYP), it significantly reduced the amount of cells with clear α-synuclein immunoreactivity compared with vehicle incubation (stress 3d + 1d veh) in all α-syn-overexpressing cell lines (A; ***P < 0.001). In concentration–response studies (B), at 1 nM, KYP-2047 was not effective in reducing the number of cells with α-synuclein inclusions when administered during oxidative stress, while 10-100 μM were fully effective (***P < 0.001; **P < 0.01, compared with control). Error bars are ±SEM (n = 3 individual

experiments). Cell line codes: A30P cells, SH-SY5Y cells with human A30P mutated α-synuclein gene addition; A53T cells, SH-SY5Y cells with human A53T mutated α-synuclein gene addition; WT α-syn cells, SH-SY5Y cells with human wild-type α-synuclein gene addition.

Figure S2 PREP enzyme activities and protein amounts in 3-day oxidative stress experiment. Incubation of 1 μM KYP-2047 significantly reduced PREP activity of α-synuclein-overexpressing cells when incubated for 3 days of oxidative stress (stress 3d with KYP; A). Vehicle together with oxidative stress (stress 3d with veh) or alone (veh 3d) did not have effect on PREP activity. There was no difference in PREP activity between WT cells (WT) and α-synuclein-overexpressing cells (A). In A30P cells, a 3-day oxidative stress reduced PREP protein levels (stress 3d with veh; B) and KYP-2047 had no effect on this (stress 3d with KYP; B). Moreover, a 3-day KYP-2047 (KYP 3d) or vehicle (veh 3d) incubation did not affect PREP protein amounts when assayed by Western blotting (B). Western blotting band of A30P control cells is shown in control band (B). Bars in panel A are mean ± SEM (n = 3 individual experiments). Cell line codes are similar to Figure S1.

Figure S3 The effect of a 3-day KYP-2047 or vehicle incubation on cell viability in LDH assay of α-synuclein-overexpressing and non-overexpressing WT cells. When the cells were incubated with 1 μM KYP-2047 or vehicle (0.001% DMSO) without oxidative stress, practically no change in cell viability was seen in the LDH assay.

Figure S4 The effect of a 5-day KYP-2047 exposure on α-synuclein protein expression in A30P-α-syn transgenic mice of Yavich *et al*. (2005). A clear overexpression of α-synuclein is seen in the striatum (Stri) and substantia nigra (SN) of vehicle-treated animals (A30P veh; B, E). A 5-day exposure to the PREP inhibitor reduced the amount of immunoreactive α-synuclein protein (A30P KYP-2047; C, F) to the levels in the WT animals (WT; A, D). Similarly, there was a significant reduction in cells containing a cytosolic α-synuclein overexpression (G–H), when determined by OD measurements and cell counting. Bars are mean ± SEM (n = 3 individual experiments). Scale bar is 150 μm in all figures. ***P < 0.001; **P < 0.01; A30P KYP-2047, PREP inhibitor-treated animal; A30P veh, vehicle-treated animal; WT, wild-type.

Figure S5 A 5-day exposure to KYP-2047 did not affect the PREP or β-actin protein amounts in A30P transgenic mouse, and there was no difference in PREP activity between WT and A30P mice. No statistical difference was seen in whole-brain homogenates between vehicle-treated (A30P veh) and KYP-2047 (A30P KYP-2047) exposed animals (A). Moreover, a 5-day exposure to KYP-2047 did not significantly affect the protein amounts of β-actin when assayed by Western blotting (B). There were no differences in the PREP activity in various brain areas between A30P and WT mice. Bars are mean ± SEM (n = 3 individual measurements). A30P KYP-2047, PREP inhibitor-treated animal; A30P veh, vehicle-treated animal; A30P, A30P transgenic mouse; WT, wild-type mouse.

Figure S6 Representative Western blots for soluble α-synuclein protein levels from A30P transgenic mouse brain after a 5-day exposure to vehicle or KYP-2047. A, Western blots for human and mouse α-synuclein. B, Western blots for antibody detecting only human α-syn. Veh, a 5-day treatment with 0.5% DMSO (twice a day); KYP, a 5-day treatment with KYP-2047 (3 mg·kg^{-1}, twice a day). The quantification is shown in Figure 6.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.