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The beneficial effect of a prolyl oligopeptidase inhibitor, KYP-2047, on alpha-synuclein clearance and autophagy in A30P transgenic mouse

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Abstract

The misfolding and aggregation of α -synuclein (aSyn) eventually leads to an accumulation of toxic forms that disturb normal neuronal function and result in cell death. aSyn rich inclusions are seen in Parkinson's disease, dementia with Lewy bodies and other synucleinopathies. Prolyl oligopeptidase (PREP) can accelerate the aggregation process of aSyn and the inhibition of PREP leads to a decreased amount of aggregated aSyn in cell models and in aSyn transgenic mice. In this study, we investigated the effect of 5- and 28-day PREP inhibitor (KYP-2047) treatment on a mouse strain carrying a point mutation in the aSyn coding gene. Following PREP inhibition, we found a decrease in high molecular-weight oligomeric aSyn and a concomitant increase in the amount of the autophagosome marker, LC3BII, suggesting enhanced macroautophagy (autophagy) and aSyn clearance by KYP-2047. Moreover, 28-day treatment with KYP-2047 caused significant increases in striatal dopamine levels. In cell culture, overexpression of PREP reduced the autophagy. Furthermore, the inhibition of PREP normalized the changes on autophagy markers (LC3BII and p62) caused by an autophagy inhibition or aSyn overexpression, and induced the expression of beclin 1, a positive regulator of autophagy. Taken together, our results suggest that PREP inhibition accelerates the clearance of protein aggregates via increased autophagy and thus normalizes the cell functions *in vivo* and *in vitro*. Therefore, PREP inhibition may have future potential in the treatment of synucleinopathies.

Keywords

synucleins; protein misfolding; serine protease; Parkinson's disease; Dementia with Lewy bodies; synucleinopathies; autophagy

Introduction

Synucleinopathies including Lewy body dementia, multiple system atrophy and the most common neurodegenerative movement disorder, Parkinson's disease (PD), all share the hallmark pathologic feature of α -synuclein (aSyn) protein accumulation in the brain (Spillantini and Goedert, 2000). Lewy bodies and Lewy neurites are neuropathological hallmarks of PD and Lewy body dementia and also multiple system atrophy is characterized by filamentous inclusions of insoluble form of aSyn (Baba et al., 1998, Spillantini et al., 1998, Ueda et al., 1993). aSyn consists of 140 amino acids (Jakes et al., 1994) and in its native forms it is a soluble, cytosolic protein that mainly localizes to presynaptic terminals (Iwai et al., 1995). aSyn is suggested to play a role in synaptic transmission, axonal transport and the regulation of dopamine (DA) homeostasis (Surguchov, 2008). This protein interacts with and regulates the function and distribution of the dopamine transporter (DAT), which is responsible for DA recycling (Bellucci et al., 2011, Wersinger and Sidhu, 2003). aSyn also localizes to synaptic vesicles where it can control vesicle recycling by modulating phospholipase D2 function (Jenco et al., 1998). Therefore, aSyn is purported to affect DA packaging and vesicle pools in DAergic nerve terminals (Murphy et al., 2000).

Genetic studies have revealed several forms of early-onset autosomal dominant familial Parkinson's disease that are associated with point mutations in the aSyn coding gene, namely A30P, A53T and E46K (Kruger et al., 1998, Conway et al., 2000, Polymeropoulos et al., 1997, Zarranz et al., 2004). Furthermore, duplications and triplications in the aSyn wildtype gene also increase the incidence of this disease (Chartier-Harlin et al., 2004, Singleton et al., 2003).

Wildtype aSyn (Wt aSyn) and its mutated forms are initially natively unfolded proteins (Conway et al., 1998). In the aSyn aggregation process, protein misfolding leads to the formation of insoluble fibrils and higher order aggregates. Many factors enhance aSyn nucleation including mutations, oxidative stress, low pH, metal ions and impairments in protein trafficking and processing (Uversky et al., 2001, Uversky, 2007, Cuervo et al., 2004, Lee et al., 2013). Interestingly, the aggregation of Wt aSyn is accelerated by the serine protease enzyme, prolyl oligopeptidase (PREP; POP; PO; EC 3.4.21.26), even under cell-free conditions, which implies a direct interaction between these proteins (Brandt et al., 2008, Lambeir, 2011). Furthermore, this action of PREP can be blocked by PREP inhibitors or by mutation of the active site (Brandt et al., 2008).

PREP is widely distributed throughout the brain and other tissues and it normally functions to hydrolyze peptides smaller than 30 amino acids (Mantle et al., 1996). PREP inhibition has been proposed as a therapeutic approach to counteract low levels of some neuropeptides in an effort to enhance memory deficits (Yoshimoto et al., 1987). In fact, PREP inhibitors do mildly improve memory in some animal models of cognitive decline (Marighetto et al., 2000, Morain et al., 2002, Toide et al., 1997, Shinoda et al., 1999, Miyazaki et al., 1998, Kato et al., 1997, Peltonen et al., 2010, Männistö et al., 2007, Jalkanen et al., 2007). PREP's role in protein aggregation, a common denominator in neurodegeneration, remains to be fully explored.

As mentioned above, PREP has been shown *in vitro* and *in vivo* to play a role in aSyn protein aggregation (Brandt et al., 2008, Lambeir, 2011, Myöhänen et al., 2012). We have also shown that the PREP inhibitor, KYP-2047, effectively prevents the formation of aSyn aggregates in aSyn overexpressing cell lines and increases the clearance of aSyn in two mouse strains carrying the pathogenic human A30P aSyn gene (Myöhänen et al., 2012). Our studies suggest that PREP may enhance aSyn aggregation by influencing the nucleation rate and/or possibly by slowing down the degradation of misfolded proteins.

Several mouse models that overexpress Wt aSyn or mutant aSyn (A30P and A53T) have been generated (Kahle et al., 2000, Fleming and Chesselet, 2006, Rockenstein et al., 2002). In this study, we further characterized the role of PREP on the aSyn accumulation process and the effect of PREP inhibition on dopaminergic system *in vivo* using an A30P point-mutated transgenic mouse strain (Plaas et al., 2008). Moreover, since we have previously shown that a PREP inhibitor, KYP-2047, decreases the amount of aSyn in the brain of aged transgenic mice, here we examined the mechanism of this beneficial effect of aSyn clearance by characterizing relevant markers of proteasomal and autophagy-lysosomal protein degradation pathways *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals

Chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Ethanol was purchased from Altia (Helsinki, Finland). The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine), was synthesized in School of Pharmacy, University of Eastern Finland, as previously described (Jarho et al., 2004). KYP-2047 was chosen as a reference compound since the biochemical and pharmacological data indicate that it is potent, selective, enters cells in culture and crosses the blood-brain barrier effectively in the rodents (Jalkanen et al., 2007, Jarho et al., 2004, Jalkanen et al., 2011, Venäläinen et al., 2006).

In vivo experiments

Animals

Transgenic homozygous A30P mice (Snca^{tm(A30P)}) and wildtype non-transgenic littermates (WT) were used. The A30P knock-in mutation was inserted into mouse SNCA gene as previously described (Plaas et al., 2008). In short, recombination positive 129/OlaHSD clones were injected into C57BL/6 blastocysts. The chimeric mice were backcrossed into C57BL/6-strain (Scanbur BK, Sollentuna, Sweden) and the line was maintained by het × het mating. The generation used in these experiments was F13. Mice were obtained from University of Tartu, Estonia. They were 12-16 months old when used for the experiments and maintained at 20-22 °C room temperature with 12:12 h light:dark rhythm and had *ad libitum* access to food and water. The experiments were carried out according to the European Community guidelines for the use of experimental animals and approved by the Finnish National board of animal experiments.

Treatments

5-day treatment—Snca^{tm(A30P)} (n=20) and WT (n=20) mice were divided into groups (n=10 per group) to receive either PREP inhibitor KYP-2047 5 mg/kg or vehicle twice a day intraperitoneally (i.p.) for 5 days (5-d) (subchronic treatment). KYP-2047 was diluted in 0.5 % dimethylsulfoxide (DMSO) in saline. Vehicle treatment groups received 0.5 % DMSO in saline. Mice were 16 months old when the experiments were initiated.

28-day treatment—Snca^{tm(A30P)} (n=13) and WT (n=15) mice were treated for 28 days (28-d) with KYP-2047 (n=7-8) 10 mg/kg per day or vehicle (n=6-7). For this chronic treatment, the drug was delivered using Alzet® osmotic pumps (Durect, Cupertino, Canada). The pumps were filled with KYP-2047 diluted in 50 % DMSO in saline and primed overnight in 37 °C. In a separate experiment KYP-2047 was shown to remain active in this solution for one month (data not shown). Mice were deeply anesthetized with isoflurane and pumps were surgically inserted inside the peritoneum. Buprenorphine was given to relief post-operative pain. Mice were single-housed after the surgery. Mice were 12-13 months old at the start of the experiments.

Tissue dissection

Mice were deeply anaesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) to remove blood. The brains were quickly removed and halves were separated to obtain right hemisphere from which striatal sample was taken for HPLC analysis and the rest of the brain half was stored for Western blot (WB) analysis. The striatal samples were frozen on dry ice and stored in -80 °C until analysis. The samples were collected 32 hours after the last i.p. injection or on the 28th day of i.p. infusion.

Left hemisphere of the brain was saved for immunohistochemistry (IHC). The brain halves were immersed in 4 % paraformaldehyde overnight to fix the tissue. The brains were then immersed first in 10 % sucrose solution in PB for overnight and after that in 30 % sucrose solution, kept in +4°C until they sank, after which the samples were kept in +4 °C until sectioning.

aSyn fractionation and WB

Soluble and insoluble fractions of aSyn were prepared using the method described by (Feng et al., 2010) and in (Myöhänen et al., 2012). Briefly, tissues were mechanically homogenized in 3 volumes of ice cold 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), phenylmethanesulfonyl fluoride (PMSF, Product# P7626) and Halt Phosphatase Inhibitor (Product# 87786, Thermo Fisher Scientific). After centrifuging at 1,000 g for 15 min at 4°C, the supernatants were collected and centrifuged at 18,700 g for 15 min at 4°C, high speed supernatants (soluble fraction) were collected. Pellets were resuspended in denaturing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% beta-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 aSyn, and also SDS-resistant high molecular weight oligomers and

aggregates. Protein amounts were measured by the BCA method (Thermo Scientific, Rockford, IL, USA), and lysates (50 µg) were loaded onto a 12% polyacrylamide-SDS-gel. Standard transfer and blocking techniques were used. Membranes were incubated in mouse anti-aSyn antibody (see details of primary antibodies in Table 1). Horse anti-mouse (tissues; dilution 1:2000 in 2% milk in 0.05 % Tween-20 in tris-buffered saline (TTBS); Product #7076, Cell Signalling Technology) and goat anti-mouse HRP conjugated secondary antibodies (cells; dilution 1:2000 in 2% milk in TTBS; Product #AP130P, Millipore) were used as secondary antibodies. β -actin (tissues) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cells) served as the loading controls with goat anti-mouse secondary antibody (dilution 1:2000 in 2% milk in TTBS; Product #AP130P, Millipore). The images were captured using GeneGnome (Syngene, Cambridge, UK). Three independent WB experiments were performed.

WB of autophagy, glial and PREP markers

The levels of autophagy markers (autophagosome marker microtubule associated protein light chain 3B I-II (LC3BI-II), protein accumulation marker SQSTM1 / p62 and autophagy inducing protein beclin 1), glial cell markers (glial fibrillar acidic protein, GFAP; ion calcium binding adaptor molecule 1, Iba1) and PREP were studied by WB. Soluble fractions of aSyn fractionation were used as the samples. Lysates (20 µg) were loaded onto a 12% polyacrylamide-SDS-gel and standard transfer and blocking techniques were used. β -actin served as the loading control. Primary antibodies and dilutions are shown in Table 1. Appropriate HRP-conjugated secondary antibodies were used for β -actin and SQSTM1/p62 (p62) WB, goat anti-mouse (tissues; dilution 1:5000; Product #7076, Cell Signalling Technology / cells; dilution 1:2000; Product #AP130P, Millipore), for LC3BI-II, beclin 1, GFAP and Iba1, goat anti-rabbit (tissues; dilution 1:2000; Product #31463, Thermo Fisher Scientific, Rockford, IL, USA / cells; dilution 1:2000; Product #AP132P, Millipore), for PREP, rabbit anti-goat (dilution 1:2000; Product #31402, Thermo Fisher Scientific). Images were captured using GeneGnome (Syngene) and three independent WB experiments were performed.

Brain homogenization and PREP activity measurement

The samples were stored at -80°C after microdissection. The brains were homogenized in 10 vol. of assay buffer (0.1 M Na-K-phosphate buffer, Ph 7.0) and centrifuged at 16 000 g at 4°C for 20 min. The supernatants were collected and used for the activity assay as previously described by (Myöhänen et al., 2008b). Briefly, the whole brain homogenate was preincubated with assay buffer for 30 min at 30°C . Substrate (4 mM Suc-Gly-Pro-AMC) was added to initiate the reaction, and the incubation continued for 60 min at 30°C . The reaction was stopped with 1 M sodium acetate buffer (pH 4.2). The formation of 7-amino-4-methylcoumarin (AMC) was measured using The Wallac 1420 Victor fluorescence plate reader (PerkinElmer, Waltham, MA, USA). The excitation and emission wavelengths were 360 and 460 nm, respectively. The protein concentration of the brain homogenate was determined using BCA protein assay kit (Thermo Scientific). To detect pure PREP protein activity, E.coli produced recombinant human PREP (Product #O9515) was used.

Proteasome activity assay

For determining proteasomal activity, the protocol described in (Ebrahimi-Fakhari et al., 2011) was used. Brain homogenates obtained for the PREP activity measurements were used for the proteasomal activity assay (see above). Homogenates were incubated with Suc-Leu-Leu-Val-Tyr-AMC (Product #I-1395, Bachem, Bubendorf, Switzerland) substrate to measure the chymotrypsin-like activity of the 20S proteasome in a fluorescence-based assay (buffer for 20S activity: 250 mM HEPES, pH 7.5, 5 mM EDTA, and 0.01% SDS). A standard curve for AMC was generated to evaluate substrate turnover in the samples, and specificity of the proteasomal assay was ascertained by the ability of additional lactacystin (50 μ M) to inhibit the fluorescence change. After 1 h incubation at 37°C, fluorescence was read with Victor 2 well-plate reader (PerkinElmer). Proteolytic activity was expressed as the amount of free AMC/min * mg protein.

HPLC analysis of DA and its metabolites DOPAC and HVA

The tissue concentrations of DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were analyzed with a high-pressure liquid chromatography (HPLC) equipped with an electrochemical detector as earlier described (Airavaara et al., 2006). Concentrations are calculated as nanograms per milligram of brain tissue.

IHC

aSyn IHC—For the analysis of aSyn in the mouse brain IHC was performed as described in (Myöhänen et al., 2012). In brief, the endogenous peroxidase activity was inactivated with 10% methanol and 3% H₂O₂ in PBS (pH 7.4) for 10 min, and non-specific binding was blocked with 10% normal donkey serum (Product #S30, Millipore, Temecula, CA, USA) in PBS containing 0.5 % Triton-X-100. The sections were incubated overnight at room temperature with a sheep anti-aSyn antibody (see Table 1 for details of primary antibodies), followed by washing with PBS. The sections were then incubated with donkey anti-sheep HRP conjugated secondary antibody (dilution 1:500 in 1% normal serum in PBS; Product #ab6900, AbCam). The antigen-antibody complexes were identified following incubation with 0.05% 3,3'-diaminobenzidine and 0.03% hydrogen peroxide solution. Finally, the sections were transferred to glass slides, dehydrated in alcohol series and mounted with Depex (BDH, Poole, UK).

Tyrosine hydroxylase and dopamine-transporter IHC—Tyrosine hydroxylase (TH) and DAT-immunostainings were modified from Mijatovic et al., (2007). In short, after blocking the endogenous peroxidase activity (as above) the sections were incubated for 30 min in 10% normal goat (TH) or normal rabbit (DAT) serum to block nonspecific binding, after which the sections were incubated overnight in rabbit anti-TH or rat anti-DAT primary antibodies (See Table 1 for details). Subsequently, the sections were placed in either goat anti-rabbit (TH) or rabbit anti-rat (DAT) biotin conjugated secondary antibodies for 2h (1:500, BA1000 for TH and 1:750, BA4000 for DAT, Vector laboratories, Peterborough, UK). The signal was enhanced with the avidin-biotin complex –method (Vectastain® ABC kit standard, Vector Laboratories) and visualized with DAB. All incubations were done at room temperature.

Semiquantitative analysis of optical density

Immunohistochemically processed brain sections were imaged and the optical densities (OD) for the striatum, primary motor cortex and substantia nigra were analyzed using the line analysis tool of Image-Pro Plus software (Media Cybernetics, Inc, Rockville, MD, USA) as described in Lindholm et al., (2007). The background values of each brain area (area without immunostaining) were subtracted from raw data values of the same brain area. Altogether 3-4 samples of each brain area/IHC staining from 3-8 different brains per group were analysed.

OD-values of the WB images were analysed using the free-hand tool of QuantityOne-software (version 4.6.9, Bio-Rad, Hercules, CA, USA) as described previously (Myöhänen et al., 2007, Myöhänen et al., 2012,). Antibody-antigen complexes were quantified by taking into account the OD (intensity) and the area of the band. The OD values were normalized to the loading control OD's.

In vitro experiments

Plasmid construction

Human PREP and green fluorescent protein—The open reading frame for human PREP (hPREP) was amplified from pCMV XL5 plasmid (Origene, Rockville, MD, USA) using PCR with oligos containing linkers for ligation-independent cloning. This insert was recombined into the BamHI and EcoRV sites of pAAV-EF1a- backbone vector (pOTTC374) using In-Fusion HD cloning kit (Clontech, Mountain View, USA). All of the plasmids described herein were transformed into Stbl3 One-Shot competent cells (Invitrogen, Grand Island, USA). An insert-containing clone of each of the generated plasmid was verified by sequencing.

Enhanced green fluorescent protein (eGFP) was used as a control in our studies and it was digested from its original pENTR-1A plasmid (pOTTC256) and ligated into BamHI and EcoRV sites of pAAV EF1a vector (Ligate-IT Rapid Ligation Kit, USB, Cleveland, USA).

S554A mutant of human PREP—A S554A mutation was inserted into the active site of PREP (Fülop et al., 1998) to study the role of inactive enzyme. pAAV EF1a hPREP plasmid was used as a source for mutated site to create pAAV EF1a hPREP (S554A). Two separate PCR amplicons were constructed to contain the desired S554A mutation and linkers for the ligation-independent cloning. Amplicons were inserted into backbone vector using In-Fusion (Clontech) and transformed as above.

Human Wt aSyn—The open reading frame for human alpha-synuclein was amplified from a cDNA originally obtained from Benoit Giasson (Przedborski et al. 2001) using polymerase chain reaction with oligos containing linkers for ligation-independent cloning. A single V5-epitope tag was also appended to the amino terminus at this time. The amplicon was recombined into the BamHI and EcoRI sites of pAAV-EF1a-DIO-hChr2(H143R)-mCherry. (Addgene #20297) using In-Fusion (Clontech), and transformed into Stbl3 One-Shot competent cells (Invitrogen). An insert-containing clone was verified by sequencing and renamed pOTTC293 (pAAV EF1a V5-aSyn(WT)).

Cellular experiments for the role of PREP in autophagy

In order to clarify the role of PREP on autophagy and aSyn clearance, Wt aSyn, hPREP and hPREP (S554A) DNA plasmids were used to transfect HEK-293 cells. HEK-293 cells (200,000 cell/well in 12 wellplate (Nunc, Product #Z688797)) were plated in Dulbecco's modified eagle medium (DMEM; Product #10-013, Cellgro/Mediatech, Manassas, VA, USA) containing 10% fetal bovine serum (FBS, Product #F2442). The next day, the cells were transfected with hPREP (1.0 µg of DNA/well), hPREP (S554A) (1.0 µg of DNA/well), aSyn plasmid (1.5 µg of DNA/well), or hPREP constructs with aSyn (0.8 µg of each DNA/well) using Opti-MEM (Product #31985; Invitrogen/Life Technologies, Grand Island, NY, USA) and lipofectamine 2000 (Product #11668; Invitrogen/Life Technologies) according to manufacturer's instructions. After 5 h, the Opti-MEM was replaced with DMEM containing 10% FBS. KYP-2047 (1 µM) or vehicle (0.001% DMSO) was added as described in the text. After 24 h (hPREP transfections) or 48 h (aSyn and hPREP + aSyn), cells were lysed using cell homogenization buffer for PREP experiments (50 mM KH₂PO₄, 1.5 mM MgCl₂, 10 mM NaCl, 1mM EDTA; pH 7.4) or modified RIPA as described above for aSyn experiments.

To study the effect of PREP inhibition in presence of autophagy inducers/inhibitors, the HEK-293 cells were plated with density of 200,000 cells/well in 12-well plate in DMEM containing 10% FBS. The next day, autophagy inhibitors (3-methyladenine [3-MA; 1 or 5 mM; Product #M9281], bafilomycin A1 [10 nM or 50 nM; Product #B1793]) in the presence or absence of KYP-2047 (1 µM) (vehicle; 0.001% DMSO) were added, and incubated for an additional 24 h. Following treatment, cells were homogenized using cell homogenization buffer.

Proteins were measured using BCA assay (Thermo Scientific), and PREP activity, and WB for aSyn, PREP, p62, LC3B, beclin 1 and GAPDH were run as described above (details of antibodies see Table 1).

Beclin 1 mRNA transcription levels

To detect the levels of beclin 1 mRNA levels after KYP-2047 incubation, a modified qPCR protocol described in Myöhänen et al. (2012) and in Miracco et al. (2007) was used. Cells were grown in 6-well plate (400,000 cells/well) and exposed to 1 µM KYP-2047 or 0.001 % DMSO (vehicle). After 24h, the cells were homogenized, and the total RNA was extracted with Aurum Total RNA Mini Kit (Bio-Rad), including DNase treatment. Reverse transcriptase reaction was done on 10 ng of RNA in standard conditions using iScript™ cDNA Synthesis kit (Bio-Rad). PCR was performed with primers designed to amplify a fragment of beclin 1 cDNA (Miracco et al. 2007) (forward, 5' CAA GAT CCT GGA CCG TGT CA 3'; reverse, 5' TGG CAC TTT CTG TGG ACA TCA 3') or GAPDH cDNA (forward, 5' TGC ACC ACC AAC TGC TTA 3'; reverse, 5'-GAG GGC ATG GAC TGT GGT CAT-3'), using iQ™SYBR® Green Supermix (Bio-Rad). After optimization of PCR conditions, amplification efficiency was evaluated for both beclin 1 and GAPDH primers using four consecutive 10-fold dilutions of the cDNA. Determination was done in triplicate. The fold change of expression of beclin 1, with respect to GAPDH, is reported as $-2^{-C(t)}$.

Statistics

Statistical analysis was completed using the Graphpad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) and PASW Statistics 18 software (SPSS Ltd, Quarry Bay, Hong Kong). Statistical tests that were used were one- and two-way ANOVA with Bonferroni post-test and Student's t-test. $P < 0.05$ was considered as statistically significant.

RESULTS

The effect of KYP-2047 on brain aSyn

Since we have previously demonstrated (Myöhänen et al., 2012) that PREP inhibition can markedly reduce aSyn levels in aSyn overexpressing animals, we studied the effects of a 5-d and 28-d KYP-2047 administration on the level of aSyn conformers in the brain.

aSyn immunoreactivity

Both 5-d and 28-d treatments with KYP-2047 significantly reduced immunoreactive aSyn in the motor cortex, striatum and substantia nigra (Fig. 1A–H; two-way ANOVA, treatment effect: $F_{1,18}=10.46$; $P=0.002$ (5-d) and $F_{1,10}=6.95$; $P=0.0028$ (28-d)) The most robust effect was seen in the substantia nigra ($P < 0.01$ (5-d) and $P < 0.05$ (28-d) Bonferroni post hoc-test).

Soluble and insoluble aSyn protein levels detected by WB analysis

We investigated the levels of soluble and insoluble aSyn protein by WB analysis. High molecular-weight (HMW) oligomers of aSyn were increased in $\text{Snca}^{\text{tm(A30P)}}$ mice, and interestingly 28-d KYP-2047 treatment significantly reduced (two-way ANOVA, treatment effect: $F_{1,12}=7.217$; $P=0.019$; $P < 0.05$ $\text{Snca}^{\text{tm(A30P)}}$ Bonferroni post hoc test) the level of HMW oligomers (molecular weight over 100 kDa) (Fig. 1J,M). Dimeric forms of aSyn (40–75 kDa) appeared to be increased in $\text{Snca}^{\text{tm(A30P)}}$ mice, but this trend never reached statistical significance (Fig. 1K, N). However, KYP-2047 treatment also altered the dimeric forms of aSyn (Fig. 1K, N). No differences in aSyn levels were seen in the soluble aSyn or SDS-soluble monomeric aSyn fractions between WT and $\text{Snca}^{\text{tm(A30P)}}$ animals (Fig. 1L, O).

Effect of KYP-2047 treatment on DA, DOPAC and HVA concentrations and markers of the DAergic pathway

Previously Plaas et al. (2008) reported that $\text{Snca}^{\text{tm(A30P)}}$ mice have decreased striatal DA compared to their WT littermates. Since the amount of aSyn in the brain decreased with the KYP-2047 treatment, we next used HPLC analysis to determine if there were any effects on DA system after PREP inhibition and aSyn clearance.

After a 5-d subchronic treatment with KYP-2047, there was no statistical difference in DA levels after the treatments or between genotypes (Fig. 2A). However, we observed significantly increased HVA and DOPAC levels in both of the genotypes compared to vehicle treatment (Fig. 2B–C; two-way ANOVA, treatment effect: DOPAC: $F_{1,34}=4.356$; $P=0.044$ and HVA: $F_{1,34}=5.329$; $P=0.027$).

A 28-d chronic treatment with KYP-2047 had differential effects on the genotypes tested. There was a significant interaction with genotype effect on DA levels (Fig. 2D; two-way

ANOVA, interaction: $F_{1,17}=15.72$; $P=0.01$ and genotype effect: $F_{1,17}=6.647$; $P=0.02$). Vehicle treated $Snc\alpha^{tm(A30P)}$ mice had lower DA levels than their WT littermates but in the group that received KYP-2047, DA was increased in $Snc\alpha^{tm(A30P)}$ mice but not in WT mice. HVA (Fig. 2E; two-way ANOVA, interaction: $F_{1,17}=5.428$; $P=0.032$) and DOPAC (Fig. 2F; two-way ANOVA, interaction: $F_{1,17}=8.909$; $P=0.008$) concentrations demonstrated a significant interaction effect.

TH and DAT immunoreactivity

In the striatum, OD analysis of TH immunoreactivity did not show any statistical difference between genotypes or treatments (Fig. 3A, C) after 5- or 28-d.

Striatal DAT expression was also measured using IHC and OD analysis. There was no difference in DAT levels between WT and $Snc\alpha^{tm(A30P)}$ mice. However, a 5-d treatment with KYP-2047 showed a trend to increased DAT immunoreactivity in the striatum (Fig. 3B). Interestingly, after 28-d treatment with KYP-2047, striatal DAT immunostaining was significantly decreased, particularly in the $Snc\alpha^{tm(A30P)}$ mice (Fig. 3D; Two-way ANOVA, treatment effect: $F_{1,12}=7.37$; $P=0.02$).

Glial cells *in vivo*

Since PREP has been implicated in neuroinflammation (Penttinen et al., 2011), we also studied PREP inhibition and the expression of glial cell markers. The levels of the astroglial cell marker, GFAP, were increased in $Snc\alpha^{tm(A30P)}$ mice compared to WT (see supplementary material, Fig. S2B, D, E; two-way ANOVA, genotype effect: $F_{1,14}=5.304$; $P=0.037$), pointing to increased astrocyte activation. However, the microglial marker, Iba1, did not show any differences when compared to WT littermates. Treatment with the PREP inhibitor, KYP-2047, had no significant effect on glial cell immunoreactivity (see supplementary material, Fig. S2A–E).

PREP activity *in vivo*

PREP activity was measured at the end of 28-d experiment in order to verify the proper function of the osmotic pumps and that the inhibitor activity of KYP-2047 was maintained during the whole experiment. The PREP activity decreased approximately 50 % as a result of inhibitor administration compared to the vehicle group (data in supplemental material Fig. S3A). PREP activity was not measured after 5-d administration since the mice were sacrificed approximately 32 h after the last injection, and PREP would not be inhibited at that point (for the reference, see Jalkanen 2011). However, as previously reported the dose used in a 5-d administration inhibits brain PREP after i.p. administration (Jalkanen et al. 2011).

Effect of KYP-2047 on proteasomal activity and autophagy markers *in vivo*

Since KYP-2047 showed significant effect on aSyn levels by WB analysis of aged $Snc\alpha^{tm(A30P)}$ mice that already have substantial amounts of HMW aSyn oligomers (Figure 1.), we hypothesized that PREP inhibition may increase the clearance of aSyn via proteasomal or autophagy-lysosomal systems. The rationale for this is that PREP inhibition has previously been shown to decrease the aSyn aggregation process, and the effects seen on

the existing aSyn load might occur via a distinct pathways. Therefore, we next studied the effects of 5- and 28-d KYP-2047 treatments on these protein degradation pathways.

First, we measured the activity of the 20S proteasome and found that it was reduced in the *Snca*^{tm(A30P)} mice compared to WT mice (Fig. 4B, two-way ANOVA, interaction of genotype and KYP-2047 treatment: $F_{1,32} = 3.598$; $P=0.066$ (28-d)). Interestingly, a 5-d treatment with KYP-2047 did not change proteasomal activity (Fig. 4A), but following 28-d of KYP-2047 treatment, the 20S proteasomal activity of the *Snca*^{tm(A30P)} mice returned to WT levels (Fig. 4B). KYP-2047 had no effect on proteasomal activity in the WT mouse (Fig. 4A–B). Therefore, it is likely that the recovery of proteasomal activity occurs because of the reduced amount of HMW aSyn oligomers which are known to inhibit proteasomal function.

Second, we investigated the effects of PREP inhibition on macroautophagy (referred hereafter as autophagy; a pathway of autophagy-lysosome system) an established pathway for the clearance of aSyn aggregates (Watanabe et al., 2012). In order to determine changes in autophagy we analysed treated mice for expression of the autophagosome marker, LC3BII, protein accumulation marker p62 and beclin 1, a marker of autophagy induction.

In *Snca*^{tm(A30P)} animals, LC3BII levels were increased (Fig. 5A; two-way ANOVA, genotype effect: $F_{1,8}=8.427$, $P=0.02$), pointing to an increase in autophagic activity. A 5-d administration of KYP-2047 significantly increased LC3B-II levels, both in WT and *Snca*^{tm(A30P)} mice but more evidently in the mutant mice (Fig. 5A; two-way ANOVA, treatment effect: $F_{1,8}=8.980$; $P=0.017$; Bonferroni post-test $P<0.05$ KYP-2047 vs. vehicle in *Snca*^{tm(A30P)}). However, the 28-d treatment with KYP-2047 did not show a significant effect, perhaps due to the already decreased aSyn levels seen following the 5-d treatment (Fig. 5B). Levels of p62 and beclin 1 were not altered in the *Snca*^{tm(A30P)} animal with or without KYP-2047 treatment (Fig. 5C–F).

Behavioral experiments

We performed several behavioral experiments to study the effect of PREP on *Snca*^{tm(A30P)} mouse motor behavior. However, there were no differences between genotype baselines and no effect by KYP-2047 on animal behavior (see experiments and results in Supplementary Material).

Role of PREP and PREP inhibition on autophagy *in vitro*

Since PREP inhibition in *Snca*^{tm(A30P)} mice caused a significant increase in the autophagy marker LC3BII (Fig. 3), possibly increasing the aSyn clearance, we next studied the role of PREP in autophagy in HEK-293 cells. Plasmids that overexpress hPREP or inactive hPREP (S554A) were prepared and the expression and activity of each protein was confirmed following transfection of HEK-293 cells. A plasmid overexpressing eGFP was used as a control and its expression was confirmed visually in every experiment.

hPREP overexpression in HEK-293 cells resulted in a 4-fold increase in PREP activity whereas the inactive mutant did not increase the activity in the cells (see supplemental data Fig. S3B). 24 h after the hPREP transfection, p62 levels were slightly increased (NS) while

inactive PREP had no effect (Fig. 6A). However, LC3BII levels were significantly decreased with hPREP overexpression (Fig. 6B; $P<0.05$, one-way ANOVA and Tukey post-test, hPREP vs. hPREP (S554A)), suggesting decreased autophagosome formation and suppression of autophagy. However, PREP overexpression did not alter beclin1 levels (Fig. 6C).

We next asked whether the PREP inhibitor could overcome the effects of the autophagy inhibitors. Inhibition of autophagosome formation by 1 mM or 5 mM 3-MA significantly increased p62 levels (two-way ANOVA $F_{2,12}=4.042$; $P=0.045$; 3-MA vs. control), and decreased the amount of the autophagosome marker, LC3BII (Fig. 7A–B), pointing to impaired autophagy. The administration of the PREP inhibitor, KYP-2047 (1 μ M), significantly increased LC3BII levels in all groups (Fig. 7B; two-way ANOVA; $F_{1,12}=19.28$; $P=0.0009$ KYP-2047 vs. vehicle; Bonferroni post-test: $P<0.01$ KYP-2047 vs. vehicle in 3-MA 5 mM group). KYP-2047 returned p62 levels to the normal in the 5 mM 3-MA group (Fig. 7A; $P<0.05$, Bonferroni post-test KYP-2047 vs. vehicle). Both 1 mM and 5 mM 3-MA decreased the levels of beclin 1 (Fig. 7C; two-way ANOVA $F_{2,15}=4.242$; $P=0.035$ 3MA vs. control), suggesting suppressed autophagy. However, KYP-2047 increased the beclin1 levels significantly when incubated with 3-MA and also in the control situation (two-way ANOVA $F_{1,5}=6.42$; $P=0.023$ KYP-2047 vs. vehicle).

To confirm that the observed increase in autophagosome amount was caused by autophagy induction via KYP-2047 and not due a block in their degradation, we used a lysosomal inhibitor, bafilomycin A1. When the degradation of autophagosomes was blocked by 10 or 50 nM bafilomycin A1 (Fig. 7D–E), only a minor increase in accumulation of p62 was seen. As expected, the levels of LC3BII were increased 2-3-fold by bafilomycin A1 compared to control (Fig 7E; two-way ANOVA; $F_{2,22}=15.73$; $P=0.0001$ bafilomycin A1 vs. control). KYP-2047 (1 μ M) further increased the LC3BII levels, pointing to its positive effect on autophagosome formation (Fig. 7E; two-way ANOVA $F_{1,22}=5.762$; $P=0.025$ KYP-2047 vs. vehicle; Bonferroni post hoc test: $P<0.05$ KYP-2047 vs. vehicle Bafilomycin A1 50 nM group). Similar to 3-MA, both bafilomycin A1 doses apparently decreased the levels of beclin 1 dose-dependently although the difference was not significant by two-way ANOVA (Fig. 7F). Nevertheless, KYP-2047 significantly increased beclin 1 levels in all study groups (two-way ANOVA $F_{1,12}=11.00$; $P=0.006$ KYP-2047 vs. vehicle) indicating its role in positive regulation of autophagy.

The role of PREP inhibition on beclin 1 levels on WB led us to study the effect of KYP-2047 on beclin 1 mRNA levels. 24 h incubation of HEK-293 cells with KYP-2047 caused significant ($P=0.025$, Student's t-test) increase in mRNA levels of beclin 1 (Fig. 8).

The effect of aSyn overexpression combined with PREP inhibition on autophagy

aSyn oligomerization and aggregation may disturb the cellular chaperone mediated autophagy and/or proteasomal degradation systems (Martinez-Vicente and Cuervo, 2007). The main pathway for degradation of aggregated aSyn is macroautophagy. We transfected HEK-293 cells for 48 h with a Wt aSyn plasmid to induce aSyn overexpression. Treatment with PREP inhibitor, KYP-2047, reduced the aSyn mediated increase in p62 level (Fig. 9A; two-way ANOVA; $F_{1,9}=7.377$; $P=0.024$ Wt aSyn vs. control, Fig. 9A; two-way ANOVA

$F_{1,9}=7.033$; $P=0.026$ KYP-2047 vs. vehicle; $P<0.05$ KYP-2047 vs. vehicle in Wt aSyn group, Bonferroni post-test). Interestingly, aSyn overexpression did not cause changes on LC3BII levels, but KYP-2047 significantly increased the autophagosomes in treated cells (Fig. 9B; two-way ANOVA $F_{1,7}=5.763$; $P=0.048$ KYP-2047 vs. vehicle).

DISCUSSION

The main results of the present study were that pharmacological inhibition of PREP using KYP-2047, in transgenic *Snca*^{tm(A30P)} mouse with the A30P point mutation in SNCA gene: 1) reduces aSyn immunoreactivity, 2) lowers the amount of HMW aSyn oligomers after chronic treatment, 3) elevates striatal DA metabolites after both short-term and chronic administration and 4) increases the autophagy enhancing the clearance of aSyn aggregates. We further demonstrated using HEK-293 cells that PREP inhibitor enhanced autophagy by augmenting the expression of beclin 1 protein, an important positive regulator of autophagy, and that PREP has a negative regulatory role in autophagosome formation. Importantly, PREP inhibition also normalized the p62 increase mediated by aSyn overexpression by enhancing autophagy.

The A30P point mutation in the SNCA gene caused increased accumulation of aSyn protein in the brain of aged *Snca*^{tm(A30P)} mice compared with aged WT littermates. Fractionation of aSyn conformers and WB analysis revealed the presence and accumulation of HMW aSyn in the brain. Furthermore, following the 5- and 28-d KYP-2047 treatment of these mice, we observed a decrease in aSyn immunoreactivity and the SDS insoluble fraction using IHC and WB analysis, respectively. It has been previously shown that PREP inhibitors effectively reduce aSyn aggregation both *in vitro*, in cell lines and *in vivo* in two different transgenic mouse lines (Brandt et al., 2008, Myöhänen et al., 2012, Van der Veken et al., 2012). Here we demonstrate for the first time that chronic administration of KYP-2047 reduces HMW aSyn aggregates in *Snca*^{tm(A30P)} mice. PREP may interact with aSyn directly to increase and accelerate aggregation (Brandt et al., 2008; Myöhänen et al., 2012). We have earlier hypothesized that the PREP inhibitor may break this interaction by modifying the active site or active conformation of PREP, thus blocking the aggregation process (Myöhänen et al., 2012). However, aSyn oligomers and aggregates are already formed in the brains of aged A30P mice, and blocking aggregation at this timepoint would not have an effect on pre-existing aSyn accumulation. Therefore, the current study did not further characterize the interaction between aSyn and PREP but rather the effect of PREP and its inhibition on aSyn clearance by autophagy was studied.

Since aSyn has a role in synthesis, storage and release of DA (reviewed by Sidhu 2006), we wanted to further characterize the effect of genotype and consequences of PREP inhibition (KYP-2047 treatment) and the subsequent decrease of the oligomeric aSyn load on the DAergic system. This is well justified since PREP is present in the nigrostriatal pathway, mostly in GABAergic neurons, but also in some TH positive cells (Myöhänen 2008; Peltonen et al., 2012). Unfortunately, the results on striatal DA and metabolite levels were not clear and did not allow any easy conclusion. *Snca*^{tm(A30P)} mice showed no basal differences in striatal DA levels compared to their WT littermates in contrast to previous results by Plaas et al. (2008). However, when striatal DA concentrations were measured at

the end of the 28-d treatment, there was a significant genotype and KYP-2047 effect. DA levels increased in *Sncat^{tm(A30P)}* mice after KYP-2047 treatment but not in the WT mice. Striatal DOPAC and HVA concentrations were generally, but not consistently increased by KYP-2047 in transgenic mice but not in WT mice after 28-d treatment. High DA metabolite levels after 5-d treatment may indicate that DA tone had been high earlier during the treatment. In recent studies, a single dose of KYP-2047 had no effect on striatal DA content in rats but an increase of DA and its metabolites was reported in the substantia nigra (Jalkanen et al., 2012, Peltonen et al., 2012). However, the mechanism of how PREP inhibition could affect the dopaminergic system is not known, and since the two genotypes in our study show different responses to a chronic PREP inhibitor administration, it is likely that the clearance of HMW aSyn has some influence on the DAergic tract. In addition to changes in DA and metabolites, we observed a significant decrease in DAT immunostaining in the striatum after a 28-d KYP-2047 treatment in both genotypes. It is known that aSyn negatively regulates DAT activity and expression, and aggregation of aSyn hampers this regulation (Bellucci et al., 2011, Unger et al., 2006). Therefore, we propose that a decreased aSyn load after KYP-2047 treatment may have dampened the regulatory function of aSyn on DAT. These results could explain the absence of robust behavioral changes in *Sncat^{tm(A30P)}* mice as well as to a lack of effect of KYP-2047.

Proteasomal degradation of proteins (ubiquitin-proteasome system) and autophagy are known to be crucial for the clearance of aSyn and most especially for the aggregated forms of this protein (Ebrahimi-Fakhari et al., 2012, Pan et al., 2008). Proteasomal degradation is responsible for the clearance of excessive monomeric aSyn and small oligomers, but the p62-dependent autophagy is needed to clear larger aSyn aggregates (Ebrahimi-Fakhari et al., 2011, Watanabe et al., 2012, Petroi et al., 2012). p62 is a protein that binds to ubiquitinated proteins which are then degraded via autophagy. Since chronic PREP inhibition was able to reduce HMW aSyn oligomers from the brain of the aged mice, it was predicted that PREP inhibition could increase the clearance of aggregated particles from the cells. This led us to study the role of PREP and its inhibition on proteasomal activity and autophagy.

In this current study we observed that in the brain of *Sncat^{tm(A30P)}* mice, the proteasomal activity was significantly decreased, suggesting that accumulated aSyn impairs the 20S proteasomes as previously shown (Ebrahimi-Fakhari et al., 2011, Chen et al., 2006). We also determined that 20S-mediated proteolytic activity was brought back to WT levels after a 28-d KYP-2047 treatment in *Sncat^{tm(A30P)}* mice. Since PREP inhibition by KYP-2047 for 5-d on *Sncat^{tm(A30P)}* mice and for HEK-293 cells did not have a direct effect on proteasomal activity (data not shown), we propose that the decreased HMW aSyn oligomer load following chronic KYP-2047 treatment has a favorable effect on proteasomal function and accounts for the restoration of proteasome activity.

Sncat^{tm(A30P)} animals showed increased autophagosome formation as indicated by the presence of the autophagosomal membrane-protein, LC3BII (Kadowaki and Karim, 2009). It is known that aSyn overexpression and aggregation eventually lead to impairment of autophagy, and compounds increasing autophagy have been studied as potential drugs for protein aggregation disorders (Rubinshtein et al., 2012). Interestingly, both 5-d and 28-d

administrations of KYP-2047 further enhanced the autophagosome formation, as was demonstrated by increased levels of LC3BII.

To further examine the effects of PREP and KYP-2047 on autophagy, we used cell culture models. We found that overexpression of PREP, unlike its inactive mutant (S554A), decreased LC3BII levels, suggesting a reduced formation of autophagosomes and autophagy suppression. When the formation of autophagosomes was blocked by 3-MA, an autophagy inhibitor that works through inhibition of PI3 class III kinase to block the generation of autophagosomes from cell membranes (Blommaert et al., 1997, Seglen and Gordon, 1982, Petiot et al., 2000), we detected an increase of the accumulation marker p62 (Komatsu and Ichimura, 2010) and a decrease in LC3BII levels. KYP-2047 was able to normalize the 3-MA-related changes to p62 and LC3BII. KYP-2047 also further increased elevated LC3BII levels when incubated with bafilomycin A1, an inhibitor of autophagosome-lysosome interaction (Yamamoto et al., 1998), without any effect on p62 levels. We also observed that overexpression and accumulation of aSyn in HEK-293 cells increased the accumulation of p62, whereas PREP inhibition normalized these changes by increasing the autophagosome formation. Taken together, these results suggest that the active PREP protein is apparently a negative regulator of autophagosome formation.

Both 3-MA and bafilomycin A1 decreased the amount of beclin 1 protein, which was reversed with KYP-2047. Moreover, KYP-2047 was shown to increase beclin 1 protein levels in the control cells that can be explained by the fact that KYP-2047 increased the mRNA levels of beclin 1. Beclin 1 is important positive regulator of autophagy and particularly essential for autophagosome formation (for review see Kang et al., 2011). Beclin 1 forms an interaction with Vsp34 on the surface of intracellular membranes thus activating phagophore formation from the membranes. The increase in beclin 1 leads to increased autophagosome formation and autophagy, and this may well be the mechanism behind the positive effect of PREP inhibition on autophagy. How PREP inhibition regulates the expression of beclin 1 is not known. It has been identified that NF κ B, E2F transcription factors and microRNAs can regulate the transcription of beclin 1 (for review see Fu et al., 2013, Kang et al., 2011). However, there are no reports that PREP or PREP inhibition affects these systems. In addition, PREP overexpression in cells did not affect beclin 1 levels although decreased the levels of LC3BII. This indicates that in the normal state, there is already enough PREP to regulate the expression of beclin 1, or then PREP and KYP-2047 regulate the expression levels of beclin 1 indirectly. In fact, it has been earlier proposed that PREP could indirectly regulate gene expression (Harwood, 2011) Another possibility could be an off-target effect of KYP-2047 but in the off-target screening (Jalkanen et al., 2012), no significant off-targets were found. Moreover, PREP is localized to the membranes of the Golgi-endoplasmic reticulum network (Myöhänen et al., 2008a), and these membranes are one of the most important sources of autophagosomes (for review, see Kraft and Martens, 2012). Hence, the subcellular localization of PREP is favorable for a direct interaction with beclin 1. PREP is able to form protein-protein interactions (Di Daniel et al., 2009, Szeltner et al., 2010), and may interact with beclin 1 and other autophagy-related proteins, thus regulating their functions, and this can be countered with PREP inhibition. However, the regulatory effect of KYP-2047 on beclin 1 levels requires further studies to clarify this point.

The mouse strain that was used in this experiment differs from many other aSyn transgenic strains because it has an A30P mutation in the mouse SNCA gene, does not carry normal Wt mouse SNCA gene, or an additional human *SNCA*. Thus it may recapitulate the human condition of Parkinson's disease better than other strains. We have previously characterized the *Snca*^{tm(A30P)} mice behaviour but found only mild hyperactivity after 12 months (Piltanen et al., 2013). The general problem with most of the aSyn transgenic mouse strains is that many of them do not exhibit behavioral deficits until the animals are aged, and even then the motoric changes are mild (Unger et al., 2006, Lee et al., 2002). In this study, we performed several motoric behavioral tests but we noted only mild hyperactivity and no other behavioral changes (see supplementary material). Consequently we did not see significant changes in the motor tests when the KYP-2047 treatment was given (see supplementary material).

aSyn is known to activate both microglia and astrocytes via toll-like receptors (Fellner et al., 2013, Beraud et al., 2011). We observed that the levels of astrocytes, but not in microglia, were significantly increased in the cortex of *Snca*^{tm(A30P)} mice. PREP has been suggested to play a role in neuroinflammation, but it has been localized to glial cells only following toxin administration (Penttinen et al., 2011). Here, we asked whether PREP inhibition had an effect on glial cell activation in the aSyn transgenic mouse brain. However, no changes in microglial or astroglial cell markers were seen following KYP-2047 administration.

CONCLUSIONS

Taken together our findings in cell culture and *in vivo* models suggest that PREP inhibitor induces autophagy, evidently by regulating beclin 1 levels, and that PREP protein has a negative regulatory effect on autophagy. The resulting clearance of accumulated HMW forms of aSyn has a beneficial effect on brain proteasomal activity and even modifies DAergic systems in this aSyn transgenic mouse model. Our studies suggest that the use of PREP inhibitors as a potential treatment of synucleinopathies is worthy of further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AMC	7-amino-4-methylcoumarin
aSyn	α -synuclein

DA	dopamine
DAT	dopamine transporter
DOPAC	dihydroxyphenylacetic acid
DMSO	dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
eGFP	enhanced green fluorescent protein
GFAP	glial fibrillar acidic protein
FBS	glyceraldehyde 3-phosphate dehydrogenase
GAPDH	fetal bovine serum
HMW	high molecular-weight
HVA	homovanillic acid
IHC	immunohistochemistry
Iba1	ion calcium binding adaptor molecule 1
KYP-2047	4-phenylbutanoyl-1-prolyl-2(S)-cyanopyrrolidine
LC3BI-II	microtubule-associated protein light chain 3B I-II
3-MA	3-methyladenine
OD	optical density
PD	Parkinson's disease
PBS	phosphate-buffered saline
PREP	prolyl oligopeptidase
p62	SQSTM1/p62
Snca_{tm}(A30P)	transgenic homozygous A30P mice
TTBS	0.05 % Tween-20 in tris-buffered saline
TH	tyrosine hydroxylase
WB	Western blot

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Figure 1A-O.

Effect of 5-d and 28-d KYP-2047 administrations on brain aSyn forms of wildtype (WT) and *Snca*^{tm(A30P)} (Tm) mice. In immunohistochemistry, both 5-d and 28-d treatments with KYP-2047 significantly reduced aSyn immunoreactivity (A-F) in substantia nigra (SN), striatum (Stri) and primary motor cortex (M1 cortex) when measured with optical density (OD analysis, G-H; two-way ANOVA, treatment effect: $F_{1,18}=10.46$; $P=0.002$ (5-d, G) and $F_{1,10}=6.95$; $P=0.0028$ (28-d, H); WT in panels A and D is vehicle treated). In Western blot (I), high-molecular weight (HMW) oligomers of aSyn were clearly reduced after 28-d of

KYP-2047 (I, M; two-way ANOVA, treatment effect: $F_{1,12}=7.217$; $P=0.019$; *, $P<0.05$ on $\text{Snca}^{\text{tm(A30P)}}$ group KYP-2047 vs. vehicle Bonferroni post-test). Some fluctuation was observed in dimeric form of aSyn (black arrowhead in I; K, N) but no statistical differences were seen. Monomeric forms remained stable (L, O). White arrowheads in I are depicting non-specific bands.

Figure 2A-F.

Effect of 5-day (5d) and 28-day (28d) KYP-2047 administrations on striatal DA and metabolite levels. KYP-2047 increased DOPAC and HVA in both WT and *Sncra*^{tm(A30P)} (Tm) animals after 5-d treatment. After 5 days there is a significant KYP-2047 treatment effect in both genotypes for dopamine metabolites DOPAC (B, two-way ANOVA, treatment effect: $F_{1,34}=4.356$; $P=0.044$) and for HVA (C, two-way ANOVA, treatment effect $F_{1,34}=5.329$; $P=0.027$). After 28 days, the effects were seen only in *Sncra*^{tm(A30P)} mice. For DA, there was a significant interaction (D, two-way ANOVA, interaction: $F_{1,17}=15.72$;

P=0.01) with genotype effect (two-way ANOVA $F_{1,17}=6.647$; $P=0.02$), Bonferroni post-test reveals KYP-2047 vs. vehicle effect of Tm mice ($P<0.01$). For DOPAC there was a significant interaction (E, two-way ANOVA, interaction: $F_{1,17}=5.428$; $P=0.032$) and for HVA, significant interaction (F, two-way ANOVA, interaction: $F_{1,17}=8.909$; $P=0.008$; Bonferroni post-test: $P<0.01$ KYP-2047 vs. vehicle in Tm mice). Data are expressed as mean $\pm$ SEM, $n=4-12$.

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Figure 3A-D.

Optical densities (OD) of striatal tyrosine hydroxylase (TH) and dopamine transporter (DAT) immunoreactivity in vehicle and KYP-2047 treated mice. No significant differences were seen in TH-immunoreactivity in the mouse striatum between *Snca*^{tm(A30P)} (Tm) and wildtype (WT) mouse (A,C). The 28-d KYP-2047 treatment significantly affected TH-immunoreactivity neither in WT nor *Snca*^{tm(A30P)} mouse (C). DAT OD values were not changed due the genotype (B-D) but a 28-d KYP-2047 treatment significantly reduced the DAT-immunoreactivity in the striata of both genotypes (D two-way ANOVA, treatment effect: $F_{1,12}=7.37$; $P=0.02$). Values are calculated as a percentage of WT vehicle treated mice. Data are mean $\pm$ SEM. $n=2-6$, 3 striatal samples per animal.

Figure 4A-B.

Proteasomal activity in $\text{Snca}^{\text{tm(A30P)}} (\text{Tm})$ and WT mouse brains following vehicle and KYP-2047 treatment. 20S proteasomal activity was reduced in the brains of $\text{Snca}^{\text{tm(A30P)}}$ mice (A, 5-day (5d) treatment; B, 28-day (28d) treatment), while 28-day treatment with KYP-2047 returned the proteasomal activity to normal levels in the $\text{Snca}^{\text{tm(A30P)}}$ mouse (B; two-way ANOVA, interaction of treatment and genotype: $F_{1,33}=3.598$; $P=0.066$).

Figure 5A-F.

The effect of KYP-2047 on autophagy markers in *Snca*^{tm(A30P)} (Tm) and WT mouse brains. The protein level of LC3BII, a common marker of autophagosome formation, was increased in *Snca*^{tm(A30P)} mouse brains (A; two-way ANOVA, genotype effect: $F_{1,8}=8.427$; $P=0.02$). A 5-d treatment with KYP-2047 significantly increased the protein levels of LC3B II (two-way ANOVA, treatment effect $F_{1,8}=8.980$; $P=0.017$) both in the brains of *Snca*^{tm(A30P)} and WT mice. After the 28-d treatment, no such effect was seen (B). The autophagy markers,

p62 and beclin 1, showed no differences between genotype or KYP-2047 treatment (C-D for p62 and E-F for beclin 1).

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Figure 6A-C.

The effect of hPREP and inactive hPREP (S554A) overexpression on autophagy markers in HEK-293 cells. 24 h hPREP transfection did not increase the p62 accumulation marker (A), but significantly decreased the autophagosome marker, LC3BII (B; $P < 0.05$, one-way ANOVA and Tukey post-hoc test, hPREP vs. S554A hPREP), pointing to inhibition of autophagosome formation. S554A hPREP did not have similar effect (B). However, neither hPREP nor S554A PREP had an effect on beclin 1 levels (C). Transfection reagent lipofectamine (LFC) was used as a control.

Figure 7A-F.

The effect of autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin A1 (Baf) combined with KYP-2047 or vehicle on autophagy markers LC3BII, p62 and beclin 1. 3-MA significantly increased p62 levels on HEK-293 cells (A, two-way ANOVA $F_{2,12}=4.042$; $P=0.045$ 3-MA vs. vehicle control), and incubation with 1 μ M KYP-2047 significantly decreased the 5 mM 3-MA-dependent increase in p62 levels ($P<0.05$, Bonferroni post-test KYP-2047 vs. vehicle). 3-MA decreased the formation of autophagosomes interpreted from decreased LC3BII levels (B). However, the incubation with KYP-2047 overcame the 3-MA effect, and significantly increased the LC3BII levels (two-way ANOVA $F_{1,12}=19.28$; $P=0.0009$ KYP-2047 vs. vehicle; **, $P<0.01$ KYP-2047 vs. vehicle in 3-MA 5 mM group). Beclin 1 was significantly decreased by 3-MA (C; two-way ANOVA $F_{2,15}=4.242$; $P=0.035$

3MA vs. control) while KYP-2047 significantly increased beclin 1 levels (two-way ANOVA $F_{1,5}=6.42$; $P=0.023$ KYP-2047 vs. vehicle). A 24 h incubation with bafilomycin A1 had no effect on p62 levels (D), but significantly increased autophagosomes (E; LC3BII levels, two-way ANOVA $F_{2,22}=15.73$; $P=0.0001$ bafilomycin A1 vs. vehicle control). Incubation with KYP-2047 did not have effect on p62 levels (C), but further increased the LC3BII levels with bafilomycin A1 (E; two-way ANOVA $F_{1,22}=5.762$; $P=0.025$ KYP-2047 vs. vehicle; *, $P<0.05$ Bonferroni post-test on bafilomycin A1 50 nM group KYP-2047 vs. vehicle). Bafilomycin A1 showed an insignificant trend to decrease dose-dependently the levels of beclin1 but KYP-2047 significantly increased the levels of beclin1 (F; two-way ANOVA $F_{1,12}=11.00$; $P=0.006$ KYP-2047 vs. vehicle). The values were compared to vehicle control that was set as 100%.

Figure 8.

The effect of KYP-2047 on beclin 1 mRNA levels. Incubation of HEK-293 cells for 24 h caused significant increase in mRNA levels of beclin 1 (*, $P=0.025$ Student's t-test). The $-2^{\Delta\Delta C(t)}$ determinations were done in triplicate from 3 cell treatments.

Figure 9A-B.

KYP-2047 reduces p62 accumulation induced by wildtype aSyn (Wt aSyn) overexpression and increases autophagosome formation. When HEK-293 cells were transfected with Wt aSyn plasmid and incubated with KYP-2047 or vehicle for 48 h, KYP-2047 blocked the increase in p62 levels (A; two-way ANOVA $F_{1,9}=7.033$; $P=0.024$ Wt aSyn vs. control; two-way ANOVA $F_{1,9}=7.377$; $P=0.026$ KYP-2047 vs. vehicle; *, $P<0.05$ KYP-2047 vs. vehicle in Wt aSyn group, Bonferroni post-test), and caused significant increase in autophagosome formation (LC3BII, B; two-way ANOVA $F_{1,7}=5.763$; $P=0.048$ KYP-2047 vs. vehicle).

Table 1.

Details of Primary Antibodies

Antigen	PREP	aSyn (WB)	aSyn (IHC)	LC3B	p62	Beclin 1	GFAP	Iba1
Marker for	PREP protein	α -synuclein protein	α -synuclein protein	Micr tubule-associated protein light chain 3 (autophagy marker)	SQSTM1 / p62 protein (autophagy marker)	Beclin 1 protein	Glial fibrillary acidic protein (astroglial cells)	Ionized calcium binding adaptor molecule 1 protein (microglial cells)
Species	Chicken IgY	Mouse monoclonal; clone 4D6	Sheep IgG	Rabbit IgG	Mouse monoclonal	Rabbit IgG	Rabbit IgG	Rabbit IgG
Immunogen	Purified pig PREP	Whole-length recombinant human α -syn	Synthetic peptide MPVDPDNEAYEMPSEE of human synuclein	Synthetic peptide corresponding to amino acids 2-15 of human LC3B	Recombinant full length amino acids 1-441 of Human SQSTM1 / p62	Synthetic peptide sequence within residues 1-100	Full length human recombinant GFAP	Synthetic peptide corresponding to C-terminus of Iba1
Manufacturer	Venäläinen et al. 2006	AbCam; Cambridge, UK	AbCam	Sigma-Aldrich	AbCam	AbCam	AbCam	Wako Chemicals; Neuss, Germany
Product #	-	ab1903	ab6162	L7543	Ab56416	Ab16998	Ab7260	016-20001
Dilution used	1:1000 (WB)	1:1000 (WB) 1:5000 (ELISA)	1:600 (IHC)	1:2000 (WB)	1:2000 (WB)	1:2000 (WB)	1:10000 (WB)	1:500 (WB)
Specificity and reference	WB (human and mouse), (Myöhänen et al 2007 & 2008)	WB; AbCam datasheet, our experiments	WB; AbCam datasheet, our experiments	WB; Sigma-Aldrich datasheet, our experiments	WB; AbCam datasheet, our experiments	WB; AbCam datasheet, our experiments	WB; AbCam datasheet, our experiments	WB; Wako datasheet, our experiments
Antigen	TH	DAT	VMAT2	GAPDH	β-actin			
Marker for	Dopaminergic neurons	Dopamine transporters	Vesicular monoamine transporter 2	Glyceraldehyde-3-Phosphate Dehydrogenase, loading control	Beta-actin protein, loading control (WB)			
Species	Rabbit IgG	Rabbit IgG	Rabbit IgG	Mouse monoclonal, clone 6C5	Mouse monoclonal; clone AC-15			
Immunogen	Denatured tyrosine hydroxylase from rat	Synthetic peptide corresponding to amino acid residues from the intracellular C-terminal region	Synthetic peptide from the intracellular C-terminal region of human vesicular monoamine transporter 2	Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle	Synthetic peptide DDDIAALVIDNGSGL			

Antigen	PREP	aSyn (WB)	aSyn (IHC)	LC3B	p62	Beclin 1	GFAP	Iba1
		conjugated to KLH						
Manufacturer	Millipore; Billerica, MA, USA	Novus Biologicals; Littleton, CO, USA	Millipore	Millipore	AbCam			
Product #	AB152	NB300-254	AB1767	MAB374	Ab6267			
Dilution used	1:1000 (IHC) 1:2000 (WB)	1:1000 (WB)	1:1000 (WB)	1:20,000 (WB)	1:5000 (WB)			
Specificity and reference	WB; Millipore datasheet	WB; Novus Biologicals datasheet	WB; Millipore datasheet	WB; Millipore datasheet, our experiments	WB; AbCam datasheet, our experiments			

IHC, immunohistochemistry; WB, Western blot