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

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A B S T R A C T

The misfolding and aggregation of α-synuclein (αSyn) eventually lead to an accumulation of toxic forms that disturb normal neuronal function and result in cell death. αSyn rich inclusions are seen in Parkinson’s disease, dementia with Lewy bodies and other synucleinopathies. Prolyl oligopeptidase (PREP) can accelerate the aggregation process of αSyn and the inhibition of PREP leads to a decreased amount of aggregated αSyn in cell models and in αSyn transgenic mice. In this study, we investigated the effect of 5- and 28-day PREP inhibitor (KYP-2047) treatments on a mouse strain carrying a point mutation in the αSyn coding gene. Following PREP inhibition, we found a decrease in high molecular-weight oligomeric αSyn and a concomitant increase in the amount of the autophagosome marker, LC3BII, suggesting enhanced macroautophagy (autophagy) and αSyn 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 αSyn over-expression, 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.

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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 (αSyn) 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 αSyn (Baba et al., 1998; Spillantini et al., 1998; Ueda et al., 1993). αSyn 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). αSyn 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). αSyn also localizes to synaptic vesicles where it can control vesicle recycling by modulating phospholipase D2 function (Jenco et al., 1998). αSyn 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). αSyn also localizes to synaptic vesicles where it can control vesicle recycling by modulating phospholipase D2 function (Jenco et al., 1998). Therefore, αSyn 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 αSyn coding gene, namely A30P, A53T and E46K (Conway et al., 2000; Kruger et al., 1998; Polymeropoulos et al., 1997). Synucleinopathies include Parkinson’s disease, multiple system atrophy and the most common neurodegenerative movement disorder, Parkinson’s disease (PD), all share the hallmark pathologic feature of α-

synuclein (αSyn) 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 αSyn (Baba et al., 1998; Spillantini et al., 1998; Ueda et al., 1993). αSyn 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). αSyn 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). αSyn also localizes to synaptic vesicles where it can control vesicle recycling by modulating phospholipase D2 function (Jenco et al., 1998). Therefore, αSyn is purported to affect DA packaging and vesicle pools in DAergic nerve terminals (Murphy et al., 2000).

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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 (Cuervo et al., 2004; Lee et al., 2013; Uversky, 2007; Uversky et al., 2001). 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 (Jalkanen et al., 2007; Kato et al., 1997; Männistö et al., 2007; Marighetto et al., 2000; Miyazaki et al., 1998; Morain et al., 2002; Peltonen et al., 2010; Shinoda et al., 1999; Toide et al., 1997). 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; Myöhänien 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 (Myöhänien 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 (Fleming and Chesselet, 2006; Kahle et al., 2000; 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 form 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)-cyano-pyrdrolidine), was synthesized in the 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\textsuperscript{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 a 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\textsuperscript{tm(A30P)} (n = 20) and WT (n = 20) mice were divided into groups (n = 10 per group) to receive either the 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\textsuperscript{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\textsuperscript{®} osmotic pumps (Durect, Cupertino, Canada). The pumps were filled with KYP-2047 diluted in 50% DMSO in saline and primed overnight at 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 relieve 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 anesthetized 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 the 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 at −80 °C until analysis. The samples were collected 32 h after the last i.p. injection or on the 28th day of i.p. infusion.

The 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 at +4 °C until they sank, after which the samples were kept at +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 Myöhänien 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# 77786, Thermo Fisher Scientific). After centrifuging at 1000 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 TTBS; Product #7076, Cell Signaling 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) and three independent WB experiments were performed.

**Table 1**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>PREP</th>
<th>aSyn (WB)</th>
<th>aSyn (IHC)</th>
<th>LC3B</th>
<th>p62</th>
<th>Beclin 1</th>
<th>GFAP</th>
<th>lba1</th>
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</thead>
<tbody>
<tr>
<td>Marker for</td>
<td>PREP</td>
<td>α-Synuclein protein</td>
<td>α-Synuclein protein</td>
<td>Microtubule associated protein light chain 3 (autophagy marker)</td>
<td>SQSTM1/p62 protein (autophagy marker)</td>
<td>Beclin 1 protein</td>
<td>Gial fibrillary acidic protein (astroglial cells)</td>
<td>Ionized calcium binding adaptor molecule 1 protein (microglial cells)</td>
</tr>
<tr>
<td>Species</td>
<td>Chicken IgY</td>
<td>Mouse monoclonal; clone 4G6</td>
<td>Sheep IgG</td>
<td>Mouse monoclonal</td>
<td>Rabbit IgG</td>
<td>Mouse monoclonal</td>
<td>Rabbit IgG</td>
<td>Rabbit IgG</td>
</tr>
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<td>Immunogen</td>
<td>Purified pig PREP</td>
<td>Recombinant full length human α-syn</td>
<td>Synthetic peptide corresponding to amino acids 1–441 of human LC3B</td>
<td>Recombinant full length protein, corresponding to amino acids 1–441 of Human SQSTM1/p62</td>
<td>AbCam</td>
<td>AbCam</td>
<td>AbCam</td>
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<td>AbCam</td>
<td>AbCam</td>
<td>Sigma-Aldrich</td>
<td>Full length human recombinant GFAP</td>
<td>Wako Chemicals; Neuss, Germany</td>
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<td></td>
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<tr>
<td>Specificity and reference</td>
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<td>WB; AbCam datasheet, our experiments</td>
<td>WB; AbCam datasheet, our experiments</td>
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**Table 2**

<table>
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<th>DAT</th>
<th>VMAT2</th>
<th>GAPDH</th>
<th>β-actin</th>
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</thead>
<tbody>
<tr>
<td>Marker for</td>
<td>Dopaminergic neurons</td>
<td>Dopamine transporters</td>
<td>Vesicular monoamine transporter 2</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase, loading control</td>
<td>Beta-actin protein, loading control (WB)</td>
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<tr>
<td>Species</td>
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<td>Rabbit IgG</td>
<td>Rabbit IgG</td>
<td>Mouse monoclonal, clone 6C5</td>
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<td>Immunogen</td>
<td>Denatured tyrosine hydroxylase from rat brain</td>
<td>Synthetic peptide corresponding to amino acid residues from the intracellular C-terminal region</td>
<td>Synthetic peptide from the intracellular C-terminal region of human vesicular monoamine transporter 2</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle</td>
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<td>Novus Biologicals; Littleton, CO, USA</td>
<td>Millipore</td>
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<tr>
<td>Product #</td>
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<td>NB300-254</td>
<td>AB1767</td>
<td>MAB374</td>
<td>Ab6207</td>
</tr>
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<td>Dilution used</td>
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<td>1:2000 (WB)</td>
<td>1:1000 (WB)</td>
<td>1:2000 (WB)</td>
<td>1:5000 (WB)</td>
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<tr>
<td>Specificity and reference</td>
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<td>WB; Novus Biologicals datasheet</td>
<td>WB; Millipore datasheet</td>
<td>WB; Millipore datasheet</td>
<td>WB; AbCam datasheet, our experiments</td>
</tr>
</tbody>
</table>

IHC, immunohistochemistry; WB, Western blot.
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 a BCA protein assay kit (Thermo Scientific). To detect pure PREP protein activity, *Escherichia coli* produced recombinant human PREP (Product #09515) was used.

**Proteasome activity assay**

For determining proteasomal activity, the protocol described in Eбраhimi-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 #L-1395, Bachem, Bubendorf, Switzerland) substrate to measure the chymotrypsin-like activity of the 20S proteasome in a fluorescence-based assay (buffer for 205 assay: 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 the specificity of the proteasomal assay was ascertained by the addition of additional lactacytin (50 μM) to inhibit the fluorescence change. After 1 h incubation at 37 °C, fluorescence was read with a 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 chromatograph (HPLC) equipped with an electrochemical detector as earlier described (Airavaara et al., 2006). Concentrations are calculated as nanograms per milligram of brain tissue.

**IHC**

αSyn IHC. For the analysis of αSyn 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 #530, Millipore, Temecula, CA, USA) in PBS containing 0.5% Triton-X-100. The sections were incubated overnight at room temperature with a sheep anti-αSyn 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 2 h (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 (ODs) 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 to 8 different brains per group were analyzed.

OD-values of the WB images were analyzed using the free-hand tool of QuantityOne-software (version 4.6.9, Bio-Rad, Hercules, CA, USA) as described previously (Myöhänen et al., 2007, 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 ODs.

**In vitro experiments**

**Plasmid construction**

**Human PREP and green fluorescent protein.** The open reading frame for human PREP (hPREP) was amplified from pCMV XLS plasmid (Origene, Rockville, MD, USA) using PCR with oligos containing linkers for ligation-independent cloning. This insert was recombinated into the BamHI and EcoRV sites of pAAV-EF1α-backbone vector (pOTTTC347) using an 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 (pOTTTC256) and ligated into the BamHI and EcoRV sites of pAAV EF1α 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 (Fulop et al., 1998) to study the role of inactive enzyme. pAAV EF1α hPREP plasmid was used as a source for mutated site to create pAAV EF1α 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 αSyn.** 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-EF1α-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 pOTTTC293 (pAAV EF1α V5-αSyn(WT)).

**Cellular experiments for the role of PREP in autophagy**

In order to clarify the role of PREP on autophagy and αSyn clearance, Wt αSyn, hPREP and hPREP (S554A) DNA plasmids were used to transfect HEK-293 cells. HEK-293 cells (200,000 cells/well in a 12 well plate (Nunc, Product #2088797)) were plated in Dulbecco’s modified Eagle’s medium (DMEM; Product #10-013, Cellgro/Mediatech, Manassas, VA, USA) containing 10% fetal bovine serum (PBS, 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 the 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, 1 mM EDTA; pH 7.4) or modified RIPA as described above for aSyn experiments.

To study the effect of PREP inhibition in the presence of autophagy inducers/inhibitors, the HEK-293 cells were plated with a density of
200,000 cells/well in a 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 a 6-well plate (400,000 cells/well) and exposed to 1 μM KYP-2047 or 0.001% DMSO (vehicle). After 24 h, the cells were homogenized, and the total RNA was extracted with an Aurum Total RNA Mini Kit (Bio-Rad), including DNase treatment. Reverse transcriptase reaction was done on 10 ng of RNA in standard conditions using an 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′ TGG CAC TTT CTG TGG ACA TCA 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 $-2^{ΔΔC(t)}$.

**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 Sncatm(A30P) mice, and interestingly 28-d KYP-2047 treatment significantly reduced (two-way ANOVA, treatment effect: \( F_{1,12} = 7.217 \); two-way ANOVA, interaction of treatment and genotype: \( F_{1,13} = 3.598; P = 0.086 \)).

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**Fig. 3.** A–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 Sncatm(A30P) (Tm) and wildtype (WT) mouse (A,C). The 28-d KYP-2047 treatment significantly affected TH-immunoreactivity neither in WT nor Sncatm(A30P) mouse (C). DAT OD values were not changed due to 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 ± SEM. \( n = 2–6 \), 3 striatal samples per animal.

**Fig. 4.** A–B. Proteasomal activity in Sncatm(A30P) (Tm) and WT mouse brains following vehicle and KYP-2047 treatment. 20S proteasomal activity was reduced in the brains of Sncatm(A30P) mice (A, 5-day (5 d) treatment; B, 28-day (28 d) treatment), while 28-day treatment with KYP-2047 returned the proteasomal activity to normal levels in the Sncatm(A30P) mouse (B; two-way ANOVA, interaction of treatment and genotype: \( F_{1,33} = 3.598; P = 0.086 \)).
P = 0.019; P < 0.05 Snc\textsuperscript{tm(A30P)} Bonferroni post hoc test) the level of HMW oligomers (molecular weight over 100 kDa) (Figs. 1J, M). Dimeric forms of aSyn (40–75 kDa) appeared to be increased in Snc\textsuperscript{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

**Fig. 5.** A–F. The effect of KYP-2047 on autophagy markers in Snc\textsuperscript{tm(A30P)} (Tm) and WT mouse brains. The protein level of LC3BII, a common marker of autophagosome formation, was increased in Snc\textsuperscript{tm(A30P)} mouse brains (A; two-way ANOVA, genotype effect: F\textsubscript{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\textsubscript{1,8} = 8.980; P = 0.017) both in the brains of Snc\textsuperscript{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).
or SDS-soluble monomeric αSyn fractions between WT and Snca<sup>tm(A30P)</sup> animals (Figs. 1L, O).

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

Previously Plaas et al. (2008) reported that Snca<sup>tm(A30P)</sup> mice have decreased striatal DA compared to their WT littermates. Since the amount of αSyn in the brain decreased with the KYP-2047 treatment, we next used HPLC analysis to determine if there were any effects on the DA system after PREP inhibition and αSyn 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 (Figs. 2B–C; two-way ANOVA, treatment effect: DOPAC: F<sub>1,34</sub> = 4.356; P = 0.044 and HVA: F<sub>1,34</sub> = 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, genotype effect: F<sub>1,17</sub> = 15.72; P = 0.01 and genotype effect: F<sub>1,17</sub> = 6.647; P = 0.02). Vehicle treated Snca<sup>tm(A30P)</sup> mice had lower DA levels than their WT littermates but in the group that received KYP-2047, DA was increased in Snca<sup>tm(A30P)</sup> mice but not in WT mice. HVA (Fig. 2E; two-way ANOVA, interaction: F<sub>1,17</sub> = 5.428; P = 0.032) and DOPAC (Fig. 2F; two-way ANOVA, interaction: F<sub>1,17</sub> = 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 Snca<sup>tm(A30P)</sup> mice. However, a 5-d treatment with KYP-2047 showed a trend to increased DAT immunoreactivity in the striatum (Fig. 3B). Interestingly, after the 28-d treatment with KYP-2047, striatal DAT immunostaining was significantly decreased, particularly in the Snca<sup>tm(A30P)</sup> mice (Fig. 3D; two-way ANOVA, treatment effect: F<sub>1,12</sub> = 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 astrogial cell marker, GFAP, were increased in Snca<sup>tm(A30P)</sup> mice compared to WT (see supplementary material, Figs. S2B, D, E; two-way ANOVA, genotype effect: F<sub>1,14</sub> = 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, Figs. S2A–E).

**PREP activity in vivo**

PREP activity was measured at the end of the 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 the 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 et al., 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 αSyn levels by WB analysis of aged Snca<sup>tm(A30P)</sup> mice that already have substantial amounts of HMW αSyn oligomers (Fig. 1.), we hypothesized that PREP inhibition may increase the clearance of αSyn via proteasomal or autophagy-lysosomal systems. The rationale for this is that PREP inhibition has previously been shown to decrease the αSyn aggregation process, and the effects seen on the existing αSyn load might occur via a distinct pathway. 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<sup>tm(A30P)</sup> mice compared to the WT mice (Fig. 4B, two-way ANOVA, interaction of genotype and KYP-2047
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 Sncatm(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 the autophagy-lysosome system) an established pathway for the clearance of aSyn aggregates (Watanabe et al., 2012). In order to determine changes in autophagy we analyzed treated mice for...
expression of the autophagosome marker, LC3BII, protein accumulation marker p62 and beclin 1, a marker of autophagy induction.

In Sncatm(A30P) animals, LC3BII levels were increased (Fig. 5A; two-way ANOVA, genotype effect: F1,8 = 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 Sncatm(A30P) mice but more evidently in the mutant mice (Fig. 5A; two-way ANOVA, treatment effect: F1,8 = 9.890; P = 0.017; Bonferroni post-test P < 0.05 KYP-2047 vs. vehicle in Sncatm(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 Sncatm(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 Sncatm(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 Sncatm(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 (SS54A) were prepared and the expression and activity of each protein were 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 (SS54A)), suggesting decreased autophagosome formation and suppression of autophagy. However, PREP overexpression did not alter beclin 1 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 F2,12 = 4.042; P = 0.045; 3-MA signiﬁcantly decreased with hPREP overexpression (Fig. 6B; P < 0.05, one-way ANOVA and Tukey post-test, hPREP vs. hPREP (SS54A)), suggesting decreased autophagosome formation and suppression of autophagy.

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–F), only a minor increase in the 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; F2,15 = 15.73; P = 0.0001 bafilomycin A1 vs. control). KYP-2047 (1 μM) further increased the LC3BII levels, pointing to its positive effect on autophagosome formation (Fig. 7E; two-way ANOVA F1,22 = 5.762; P = 0.025 KYP-2047 vs. vehicle; Bonferroni post hoc test: P

Fig. 9. A–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 F1,9 = 7.033; P = 0.024 Wt aSyn vs. control; two-way ANOVA F1,9 = 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 F1,9 = 5.762; P = 0.048 KYP-2047 vs. vehicle).

Expression of p62 in Wt aSyn group (two-way ANOVA F1,9 = 7.033; P = 0.024 Wt aSyn vs. control; two-way ANOVA F1,9 = 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 F1,9 = 5.762; P = 0.048 KYP-2047 vs. vehicle).
< 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 F1,12 = 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 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 main results of the present study were that pharmacological inhibition of PREP using KYP-2047, in transgenic Snc\textsuperscript{A\textsubscript{30P}} mouse with the A30P point mutation in the 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 the 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 Snc\textsuperscript{A\textsubscript{30P}} 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 treatments 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 Snc\textsuperscript{A\textsubscript{30P}} 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 the synthesis, storage and release of DA (reviewed by Sidhu et al., 2004), 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, and also in some TH positive cells (Myöhänen et al., 2008a; Peltonen et al., 2012). Unfortunately, the results on striatal DA and metabolite levels were not clear and did not allow any easy conclusion. Snc\textsuperscript{A\textsubscript{30P}} 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 Snc\textsuperscript{A\textsubscript{30P}} 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 the 28-d treatment. High DA metabolite levels after the 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 Snc\textsuperscript{A\textsubscript{30P}} mice as well as the 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; Petroi et al., 2012; Watanabe et al., 2012). p62 is a protein that binds to ubiquitinated proteins that 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 Snc\textsuperscript{A\textsubscript{30P}} mice, the proteasomal activity was significantly decreased, suggesting that accumulated aSyn impairs the 20S proteasomes as previously shown (Chen et al., 2006; Ebrahimi-Fakhari et al., 2011). We also determined that 20S-mediated proteolytic activity was brought back to WT levels after a 28-d KYP-2047 treatment in Snc\textsuperscript{A\textsubscript{30P}} mice. Since PREP inhibition by KYP-2047 for 5-d on Snc\textsuperscript{A\textsubscript{30P}} 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.

Snc\textsuperscript{A\textsubscript{30P}} 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 (Rubinsztein 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 (SS54A), 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 (Blommaart et al., 1997; Petiot et al., 2000; Seglen and Gordon, 1982), 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 αSyn 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 an important positive regulator of autophagy and particularly essential for autophagosome formation (for review see Kang et al., 2011). Beclin 1 forms an interaction with Vps34 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 NrfB, 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 others αSyn transgenic strains because it has an A30P mutation in the mouse snca gene, and 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(A30P/−) mice behavior but found only mild hyperactivity after 12 months (Pitonen et al., 2013). The general problem with most of the αSyn 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 (Lee et al., 2002; Unger et al., 2006). 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).

αSyn is known to activate both microglia and astrocytes via toll-like receptors (Beraud et al., 2011; Fellner et al., 2013). We observed that the levels of astrocytes, but not in microglia, were significantly increased in the cortex of Snca(A30P/−) mice. KYP-2047 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 αSyn 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 αSyn has a beneficial effect on brain proteasomal activity and even modifies DAergic systems in this αSyn transgenic mouse model. Our studies suggest that the use of PREP inhibitors as a potential treatment of synucleinopathies is worthy of further investigation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.04.003.

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