



# Prolyl oligopeptidase inhibition by KYP-2407 increases alpha-synuclein fibril degradation in neuron-like cells

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## ABSTRACT

Growing evidence emphasizes insufficient clearance of pathological alpha-synuclein ( $\alpha$ SYN) aggregates in the progression of Parkinson's disease (PD). Consequently, cellular degradation pathways represent a potential therapeutic target. Prolyl oligopeptidase (PREP) is highly expressed in the brain and has been suggested to increase  $\alpha$ SYN aggregation and negatively regulate the autophagy pathway. Inhibition of PREP with a small molecule inhibitor, KYP-2407, stimulates autophagy and reduces the oligomeric species of  $\alpha$ SYN aggregates in PD mouse models. However, whether PREP inhibition has any effects on intracellular  $\alpha$ SYN fibrils has not been studied before. In this study, the effect of KYP2407 on  $\alpha$ SYN preformed fibrils (PFFs) was tested in SH-SY5Y cells and human astrocytes. Immunostaining analysis revealed that both cell types accumulated  $\alpha$ SYN PFFs intracellularly but KYP-2407 decreased intracellular  $\alpha$ SYN deposits only in SH-SY5Y cells, as astrocytes did not show any PREP activity. Western blot analysis confirmed the reduction of high molecular weight  $\alpha$ SYN species in SH-SY5Y cell lysates, and secretion of  $\alpha$ SYN from SH-SY5Y cells also decreased in the presence of KYP-2407. Accumulation of  $\alpha$ SYN inside the SH-SY5Y cells resulted in an increase of the auto-lysosomal proteins p62 and LC3BII, as well as calpain 1 and 2, which have been shown to be associated with PD pathology. Notably, treatment with KYP-2407 significantly reduced p62 and LC3BII levels, indicating an increased autophagic flux, and calpain 1 and 2 levels returned to normal in the presence of KYP-2407. Our findings indicate that PREP inhibition can potentially be used as therapy to reduce the insoluble intracellular  $\alpha$ SYN aggregates.

## 1. Introduction

As one of the most common neurodegenerative disorders, Parkinson's disease (PD) affects almost six million people worldwide, with age as the major risk factor [1]. Due to the loss of dopaminergic neurons in substantia nigra, PD patients suffer severely from motor symptoms. The histopathological feature of PD is the presence of intra-neuronal inclusions called Lewy bodies and Lewy neurites that are mainly comprised of fibrillary alpha-synuclein ( $\alpha$ SYN) [2,3]. Braak and

colleagues proposed that  $\alpha$ SYN pathology spreads throughout the brain in an anatomical manner [4]. Moreover, the presence of  $\alpha$ SYN aggregates in grafted neurons in the brains of PD patients decades after transplantation, gave rise to the idea that  $\alpha$ SYN may act as a prion-like protein [5,6]. The suggested mechanisms for this propagation are: release of  $\alpha$ SYN aggregates from dying neurons to the surroundings, cell-to-cell contact, and secretion.

Reactive gliosis is another hallmark of PD where the glial cells of the brain enter an inflammatory state. Astrocytes, which are the most

**Abbreviations:** KYP-2047, 4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine;  $\alpha$ SYN, alpha-synuclein; ALP, autophagy-lysosomal pathway; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle medium; FBS, Fetal Bovine Serum; IGF-1, insulin-like growth factor; NEAA, non-essential amino acids; OD, optical density; PD, Parkinson's disease; PFF, preformed fibrils; PREP, prolyl oligopeptidase; ThT, Thioflavin T.

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numerous glial cells of the CNS, are crucial for brain homeostasis and neuronal health [7]. In addition to neurons,  $\alpha$ SYN fibrils are also found in reactive astrocytes during PD [8,9]. Previously, *in vitro* studies have demonstrated that astrocytes engulf aggregated  $\alpha$ SYN that accumulates inside the cell and causes cellular stress [10,11]. The stressed astrocytes respond by spreading  $\alpha$ SYN to healthy surrounding astrocytes through direct contact [10]. Consequently, astrocytes may be indirectly involved in progression of PD pathology by not being able to perform their physiological function and directly by spreading  $\alpha$ SYN aggregates.

As a disordered protein,  $\alpha$ SYN is believed to undergo conformational changes upon certain circumstances and become more aggregation-prone. Several identified mutations in the  $\alpha$ SYN encoding gene, *SNCA*, result in  $\alpha$ SYN aggregation and thereby initiate the pathology [12]. In the sporadic cases, however, the underlying cause for the disease is not yet fully understood. Nevertheless, accumulating evidence emphasizes that direct interaction of  $\alpha$ SYN with certain proteins [13,14], oxidative stress [15,16] as well as impaired degradation pathways [17,18] can initiate  $\alpha$ SYN aggregation.

Prolyl oligopeptidase (PREP) is a highly conserved serine peptidase that is widely expressed in the body with the highest activity in the brain [19–21]. Previous studies have shown that direct interaction between PREP and  $\alpha$ SYN increases the aggregation propensities of  $\alpha$ SYN [14,22,23]. Moreover, PREP levels increase with age in the brain, and in the PD post-mortem brain it co-localizes with  $\alpha$ SYN aggregates [24,25]. Notably, increased PREP activity has been indicated to negatively regulate the autophagy–lysosomal pathway (ALP) both *in vitro* and *in vivo* [26,27]. In addition to PREP, calpains are enzymes identified to have increased proteolytic activity in PD [28]; they increase  $\alpha$ SYN aggregation by cleaving the c-terminal part of the protein [13,29]. In accordance, activated calpains have been shown to co-localize with truncated  $\alpha$ SYN in the brains of PD patients. Similar to PREP, enhanced calpain activity has been suggested to negatively regulate the autophagy pathway [30–32]. Furthermore, previous reports have illustrated that intracellular  $\alpha$ SYN aggregates also diminish the ALP by either directly interacting with key enzymes or affecting other molecules required for functionality [33–36]. Therefore, it is of great importance to improve the cellular degradation pathways using pharmacological agents.

Small molecule inhibitors of PREP, such as KYP-2407, have been shown to stimulate the autophagy pathway in both *in vitro* and *in vivo* studies [26,37]. Thus, PREP inhibition was shown to reduce the levels of  $\alpha$ SYN aggregates, particularly soluble oligomers, in the brains of different PD mouse models and improve the motor symptoms [26,38,39]. However, the impact of PREP inhibition on  $\alpha$ SYN fibrils that are suggested to be responsible for  $\alpha$ SYN propagation in PD has not been studied. Here, we show that KYP-2407 reduces intracellular as well as extracellular  $\alpha$ SYN in SH-SY5Y cells through increased auto-lysosomal turnover. Furthermore, PREP inhibition reduced the levels of calpain 1 and 2 in SH-SY5Y cells. However, KYP-2407 had no effect on the intracellular astrocytic  $\alpha$ SYN aggregates. Altogether, these data imply that KYP-2407 prevents  $\alpha$ SYN accumulation and further aggregation in neuron-like cells.

## 2. Materials and methods

### 2.1. PREP inhibitor

The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine), was synthesized for us in Division of Pharmaceutical Chemistry, University of Helsinki [40], and it was diluted to cells from 100 mM stock in DMSO before use.

### 2.2. Culture of SH-SY5Y cells

The human neuroblastoma cell line (SH-SY5Y) was cultured in Dulbecco's modified Eagle medium (DMEM-Glutamax, 31966021, ThermoFisher Scientific) containing 15 % Fetal Bovine Serum (FBS;

ThermoFisher), 1 % non-essential amino acids (NEAA; ThermoFisher) and 50  $\mu$ g/ml Gentamycin (ThermoFisher). Cells were used between passages 3–15.

### 2.3. Culture of human astrocytes

Human astrocytes were generated from neuroepithelial-like stem (NES) cells [41,42]. The cells were cultured in Advanced DMEM/F12 (Thermo Fischer 12634-010) supplemented with 1 % Penicillin–Streptomycin (ThermoFischer 15140-122), 1 % B27 supplement (ThermoFischer), 1 % non-essential amino acids (Merc Millipore) and 1 % L-Glutamine (ThermoFischer 25030-024). The following factors were added to the medium before addition to the cells: Basic fibroblast growth factor (bFGF) 10 ng/ml (ThermoFischer, 13256029), heregulin 10 ng/ml (Sigma–Aldrich, SRP3055), activin A 10 ng/ml (Peprotech, 120-14E) and insulin-like growth factor (IGF-1) 200 ng/ml (Sigma–Aldrich, SRP3069). Additionally, 20 ng/ml ciliary neurotropic factor (CNTF; ThermoFischer, PHC7015) was added to the medium the last two weeks of culture. Cells were passaged using Trypsin-EDTA (Life Technologies) and were used for experiments directly after the differentiation protocol was completed.

### 2.4. PREP activity measurement

For the PREP activity assay, the following protocol [39] was used. Briefly, SH-SY5Y cells were plated on 12-well plates at a density of 200,000 cells/well and allowed to attach overnight. Thereafter, the cells were treated with  $\alpha$ SYN PFFs  $\pm$  10  $\mu$ M KYP-2047 for 4 and 24 h. The astrocytes were cultured on 60 mm plates with a density of 200,000 cells/plate, and treated  $\pm$  10  $\mu$ M KYP-2407 for 24 h before lysis. The cells were then lysed by incubating them in the lysis buffer (50 mM  $\text{KH}_2\text{PO}_4$ , 1.5 mM  $\text{MgCl}_2$ , 10 mM NaCl, 1 mM EDTA; pH 7.4) on ice for 20 min. Cell homogenates were centrifuged at 16,000g for 10 min at +4 °C, and the supernatant was recovered and stored at –80 °C until use. PREP activity was measured from supernatants using Suc-Gly-Pro-aminomethylcoumarin substrate (AMC; #4005321, Bachem,) as described earlier. Total protein amounts were measured using the bicinchoninic acid method (BCA; Pierce BCA Protein Assay Kit, #23225, ThermoFisher), and released AMC was correlated to that.

### 2.5. Generation of $\alpha$ SYN preformed fibrils

Endotoxin-free recombinant human  $\alpha$ SYN (Anaspec A5555-1000) was incubated at 37 °C on a shaker (IKA MS3 Basic, 1000 rpm) at a concentration of 5 mg/ml. Thereafter, the preformed  $\alpha$ SYN fibrils (PFFs) were diluted to a working concentration of 2 mg/ml (140  $\mu$ M) and stored at –70 °C until use.

#### 2.5.1. Characterization

The  $\alpha$ SYN PFFs were characterized using Thioflavin T (ThT) assay and electron microscopy (EM) to confirm the formation of  $\beta$ -sheet structure after the incubation time. The ThT solution (100  $\mu$ M) was added to recombinant monomers (diluted 1:100 from 2 mg/ml in PBS) and PFFs (diluted 1:100 from 2 mg/ml in PBS) and the absorbance was measured instantly at 420 nm. Moreover, the PFFs as well as monomers (diluted 1:5 in PBS) were placed on carbon-coated 300-mesh copper grids, negatively stained with 1 % uranyl acetate for 5 min and then air-dried. The samples were analyzed using a Hitachi H-7100 transmission electron microscope.

#### 2.5.2. Cy3-labeling

The Cy3 Antibody Labelling Kit (GE Healthcare, PA33000) was used to label  $\alpha$ SYN fibrils according to the manufacturer's protocol. Unbound Cy3 was removed by centrifugation at 23,000  $\times$  g for 30 min, the supernatant was removed, and the pellet was resuspended in sterile PBS. The washing procedure was repeated three times.

### 2.5.3. Sonication

The  $\alpha$ SYN PFFs were diluted 1:2 in sterile PBS and sonicated at 20 % amplitude, 1 s off and 1 s on for 30 s using a Vibra Cell sonicator (Sonics&Materials Inc), immediately prior to the experiment.

### 2.6. Alpha-synuclein exposure

SH-SY5Y cells were treated with 0.5  $\mu$ M sonicated Cy3-labeled or unlabeled  $\alpha$ SYN PFFs for 24 h and washed twice with the culture medium. Following the washes, the SH-SY5Y cultures were treated with either 1  $\mu$ M or 10  $\mu$ M KYP-2407 for 48 h, or 10  $\mu$ M DMSO (vehicle control). Astrocytes were exposed to Cy3-labeled  $\alpha$ SYN PFFs for 24 h and washed twice with the culture medium. The cells were then treated with 10  $\mu$ M KYP-2407 for 48 h or left untreated for the same amount of time.

### 2.7. Western blot analysis

SH-SY5Y cells were lysed in modified RIPA buffer (50 mM Tris HCl pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl) containing a phosphatase inhibitor (#87786, ThermoFisher) and protease inhibitor cocktail (#78430, ThermoFisher) unless otherwise specified. The lysates were centrifuged at 16,000g for 15 min, and the supernatant was removed as soluble fraction. The pellets were diluted to 1 % SDS treatment and sonicated for 3  $\times$  1 s (SDS-soluble and insoluble fraction). Protein concentration was measured from soluble fraction with BCA (bicinchoninic acid; #23225, ThermoFisher Scientific). Standard SDS-PAGE techniques were used and approximately 30  $\mu$ g of sample was loaded to 12 % Mini-Protean TGX Stain-Free gels (#4568044, Bio-Rad, Hercules, CA). Gels were transferred by Trans-Blot Turbo Transfer System (#1704150, Bio-Rad) onto Trans-Blot Turbo Midi PVDF (#1704157, Bio-Rad) membranes. Soluble  $\alpha$ SYN species were measured in the lysates. The insoluble  $\alpha$ SYN fractions were measured in the pellets from the cell lysates. Total protein amount was visualized from Stain-Free gels by using the ChemiDoc XRS+ (Bio-Rad) and was used to normalize the insoluble  $\alpha$ SYN fraction. Membranes were incubated at +4  $^{\circ}$ C overnight in 5 % skim milk in Tris-buffered saline with 0.05 % Tween-20 (TBS-T). The following antibodies and dilutions were used:  $\alpha$ SYN (sheep anti- $\alpha$ SYN 1:2000; ab6162, Abcam); p62 (mouse anti-p62 1:5000; ab56416, Abcam); LC3B (rabbit anti-LC3B 1:1000; L7543, Sigma-Aldrich); Calpain 1 (rabbit anti-calpain 1 1:1000; ab28258, Abcam); Calpain 2 (rabbit-anti Calpain 2 1:1000; ab39165, Abcam); beta-actin (loading control; rabbit anti-beta-actin 1:2000; ab8227; Abcam). After overnight incubation, the membranes were washed and incubated with appropriate HRP-conjugated secondary antibodies for 2 h in room temperature (dilution 1:2000), donkey anti-sheep HRP (ab6900; Abcam); goat anti-mouse HRP (#31430, ThermoFisher); goat-anti rabbit (#31463, ThermoFisher). The images were captured using the ChemiDoc XRS+ (Bio-Rad). To verify that bands are in the linear range of detection, increasing exposure time and automatic detection of saturated pixels in ImageLab software (version 6.01; Bio-Rad) was used. Thereafter, images were converted to 8-bit grayscale format, and the optical densities (OD) of the bands were measured by ImageJ (histogram area analysis; version 1.48; National Institute of Health, Bethesda, MD). The quantified OD value obtained for each band was normalized against the corresponding beta-actin band; in the insoluble fraction of  $\alpha$ SYN, total protein amount was used for normalizing the signal. The control group for each studied protein was set as 100 % and treatments were compared to the control group of the current protein.

### 2.8. ELISA analysis

The  $\alpha$ SYN concentration in conditioned media was measured using the human  $\alpha$ SYN ELISA kit (Abcam, ab210973). The sandwich ELISA was carried out according to the manufacturer's protocol, and the signal was read at 450 nm using Victor 2 well-plate reader (PerkinElmer).

### 2.9. Immunocytochemistry

#### 2.9.1. SH-SY5Y cells

SH-SY5Y were plated over glass coverslips in a 12-well plate with a density of 100,000 cells/well. After the various treatments, the cells were fixed with 4 % paraformaldehyde at room temperature for 15 min and permeabilized with 0.5 % Triton X-100 in PBS for 5 min. Unspecific binding was blocked with 10 % normal goat serum (NGS; S-1000, Vector Laboratories) for 30 min. Thereafter, cells were incubated with primary antibodies against cadherin isoforms (mouse pan-anti-cadherin 1:400; ab6528, Abcam) or LC3B (rabbit anti-LC3B 1:400; L7543) overnight at +4  $^{\circ}$ C. After 3  $\times$  5 min PBS washes, the cells were incubated for 1 h in room temperature with the following secondary antibodies: for cadherin, anti-mouse AlexaFluor488 (dilution 1:200; ab150113, Abcam); for LC3B, anti-rabbit AlexaFluor 488 (dilution 1:400; ab150077, Abcam). Cells were mounted with Vectashield containing DAPI to stain nuclei (H-1200, Vector Laboratories). Imaging was performed using Leica TCS SP5 confocal microscope (Leica Microsystems).

#### 2.9.2. Astrocytes

Astrocytes were plated on glass coverslips in 12 well plates with a density of 25 000 cells/well. Cells were fixed in 4 % paraformaldehyde in PBS at room temperature for 15 min and washed twice with PBS. Blocking and permeabilization was performed with 5 % normal goat serum (NGS) and 0.1 % Triton-X 100 in PBS for 30 min at room temperature. The cells were incubated for 30 min with Alexa Fluor 488-labeled phalloidin (Sigma Aldrich, P5282) to stain F-actin, and washed in PBS before mounting with EverBrite Hardset Mounting medium (Bionordika). Images were captured using Zen Observer.1 fluorescence microscope (Zeiss).

### 2.10. Image analysis

Cy3-labeled  $\alpha$ SYN aggregates were measured in immunocytochemistry experiments from SH-SY5Y cells and astrocytes. Furthermore, image analysis was also performed on LC3B immunostainings from SH-SY5Y cells. These analyses were carried out to determine if PREP inhibition had any effect on the  $\alpha$ SYN and LC3B levels inside the cells.

#### 2.10.1. SH-SY5Y cells

For the  $\alpha$ SYN measurements, three independent experiments were performed. From each experiment, six to fourteen Z-stack images were captured. For the LC3B measurements, two independent experiments were performed where six to nine Z-stacks were captured. Integrated density (mean intensity  $\times$  area) was measured from the merged Z-layers of the LC3B and  $\alpha$ SYN channels in ImageJ and normalized to the number of cells, which were counted manually.

#### 2.10.2. Astrocytes

To analyze  $\alpha$ SYN in the astrocyte cultures, three independent experiments were performed. Twelve Z-stacks were taken from each experiment. Integrated density (mean intensity  $\times$  area) was measured from the merged Z-layers of the  $\alpha$ SYN channel in ImageJ and normalized to the number of cells.

### 2.11. Measurements of basal $Ca^{2+}$ concentration

80 000–130 000 SH-SY5Y cells were plated on 25 mm  $\varnothing$ /0.15 mm thick glass coverslips (Warner Instruments; Hamden, CT, USA). The cells were cultured for 24 h before treating with 1 or 10  $\mu$ M KYP-2407. After a 48 h treatment, the coverslips were loaded for 20 min at +37  $^{\circ}$ C with 4  $\mu$ M fura-2 acetoxymethyl ester (Invitrogen; Carlsbad, CA, USA) in HEPES-buffered medium (HBM; 137 mM NaCl, 5 mM KCl, 1 mM  $CaCl_2$ , 1.2 mM  $MgCl_2$ , 0.44 mM  $KH_2PO_4$ , 4.2 mM  $NaHCO_3$ , 10 mM glucose, and 20 mM HEPES, adjusted to pH 7.4 with NaOH), rinsed twice with HBM, and used immediately; KYP-2407 was also included in the loading and

experimental medium.  $\text{Ca}^{2+}$  measurements were performed at  $+37^\circ\text{C}$  with a Nikon TE2000 fluorescence microscope ( $20\times/0.75$  air objective) and an Andor iXon 885 electron-multiplying charge-coupled device camera under the control of Nikon NIS Elements AR software with 6D extension (Nikon, Tokyo, Japan). The cells were excited with alternating 340 and 380 nm light (Sutter DG4 Plus wavelength switcher; Sutter Instrument Company, Novato, CA, USA), and the emitted light was collected through a 420 nm dichroic mirror and a 420–570 nm band-pass filter. After the acquisition of the images, regions of interest were defined with the NIS software, and the data were extracted to Microsoft Excel (Microsoft, Redmond, WA) for observation and quantitation. 166–382 cells were measured per coverslip, and the experiment was repeated 4–5 times for each condition. The values are given in 340/380 ratio.

## 2.12. Statistical analysis

### 2.12.1. Western blot analysis

All immunoblotting analysis were performed at least three times. The data are shown as mean  $\pm$  SEM and analyzed using ANOVA followed by Tukey's post-hoc comparison in Prism statistical software (version 6.07; GraphPad Software, Inc., San Diego, CA). Significance was defined by  $p < 0.05$ .

### 2.12.2. ELISA analysis

Media from SH-SY5Y cells were collected from triplicates of three independent experiments. The control group was set as 100 % and the other treatments were normalized to the control group. The data are expressed as mean  $\pm$  SEM. ANOVA analysis followed by Tukey's post-hoc comparison was performed in Prism to compare all the groups. Significance was defined by  $p < 0.05$ .

Astrocyte media were collected from duplicates of two independent experiments. The data are expressed as mean  $\pm$  SD and was analyzed using Student's *t*-test. Significance was defined by  $p < 0.05$ .

### 2.12.3. Immunocytochemistry

For the  $\alpha\text{SYN}$  and LC3B measurements in SH-SY5Y cells, ANOVA analysis followed by Tukey's post-hoc comparison was performed in Prism to compare all the groups. The data are presented as mean  $\pm$  SD. Significance was defined by  $p < 0.05$ .

For the  $\alpha\text{SYN}$  measurements in human astrocytes, Student's *t*-test analysis was performed. The data are presented as mean  $\pm$  SD. Significance was defined by  $p < 0.05$ .

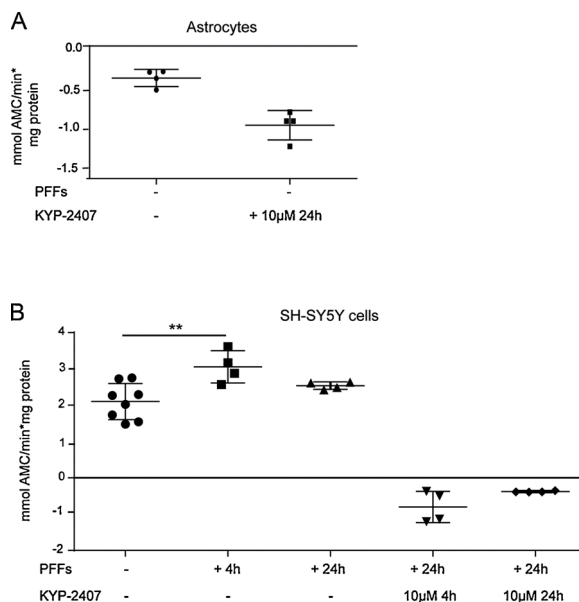
## 3. Results

### 3.1. $\alpha\text{SYN}$ PFFs elevate PREP activity in SH-SY5Y cells

To investigate whether PREP is active in the cell types used in this study, PREP activity in SH-SY5Y cells and human astrocytes was measured. Interestingly, PREP activity was only detected in SH-SY5Y cells and not in human astrocytes (Fig. 1A, B). Next, we investigated the effect of  $\alpha\text{SYN}$  PFFs on the PREP activity in SH-SY5Y cells. The cells were exposed to  $\alpha\text{SYN}$  PFFs for 4 h and 24 h. The PREP activity seemed to have increased at the 4 h time point; however, this effect was no longer evident at 24 h (Fig. 1B). Importantly, KYP-2407 eliminated the PREP activity in SH-SY5Y cells that were exposed to  $\alpha\text{SYN}$  PFFs at both 4 h and 24 h (Fig. 1B). Henceforward, both SH-SY5Y cells and astrocytes were exposed to  $\alpha\text{SYN}$  PFFs for 24 h, as this time point allowed more engulfment of  $\alpha\text{SYN}$  PFFs by the cells.

### 3.2. Inhibition of PREP by KYP-2407 decreases intracellular and extracellular $\alpha\text{SYN}$ in SH-SY5Y cells

Previous studies have shown that PREP increases  $\alpha\text{SYN}$  aggregation and acts as a negative regulator to autophagy [14,22,27,43]. Moreover,



**Fig. 1. KYP-2407 completely eliminates PREP activity in SH-SY5Y cells in the presence of  $\alpha\text{SYN}$  PFFs.** PREP activity was not detectable in astrocytes (A). PREP activity was measurable in SH-SY5Y cells. Addition of  $\alpha\text{SYN}$  PFFs to SH-SY5Y cells increased PREP activity at the 4 h time point. This, however, returned to control levels at 24 h. KYP-2407 abolishes PREP activity in the presence of  $\alpha\text{SYN}$  PFFs at both 4 h and 24 h (B). \*\*:  $p < 0.01$  in the comparison between control SH-SY5Y cells and SH-SY5Y + PFF 4 h.

PREP inhibition has been able to reduce  $\alpha\text{SYN}$  oligomers in transgenic and  $\alpha\text{SYN}$ -virus vector based mouse models [38,43]. To study whether PREP inhibition affects  $\alpha\text{SYN}$  PFF degradation, we treated SH-SY5Y with  $\alpha\text{SYN}$  PFFs and exposed them to KYP-2407. Immunostaining analysis revealed that SH-SY5Y cells engulfed and accumulated  $\alpha\text{SYN}$  PFFs (Fig. 2A). Importantly, quantification of the intracellular  $\alpha\text{SYN}$  aggregates illustrated a reduction of  $\alpha\text{SYN}$  PFFs in the SH-SY5Y cells in the presence of 1  $\mu\text{M}$  and 10  $\mu\text{M}$  KYP-2407 (Fig. 2B). Decreased levels of the intracellular  $\alpha\text{SYN}$  could also be due to increased secretion of the aggregates from the cells to the medium. Hence, we analyzed the  $\alpha\text{SYN}$  concentration in the medium and found that extracellular  $\alpha\text{SYN}$  was also reduced, suggesting an increased degradation of the protein (Fig. 2C).

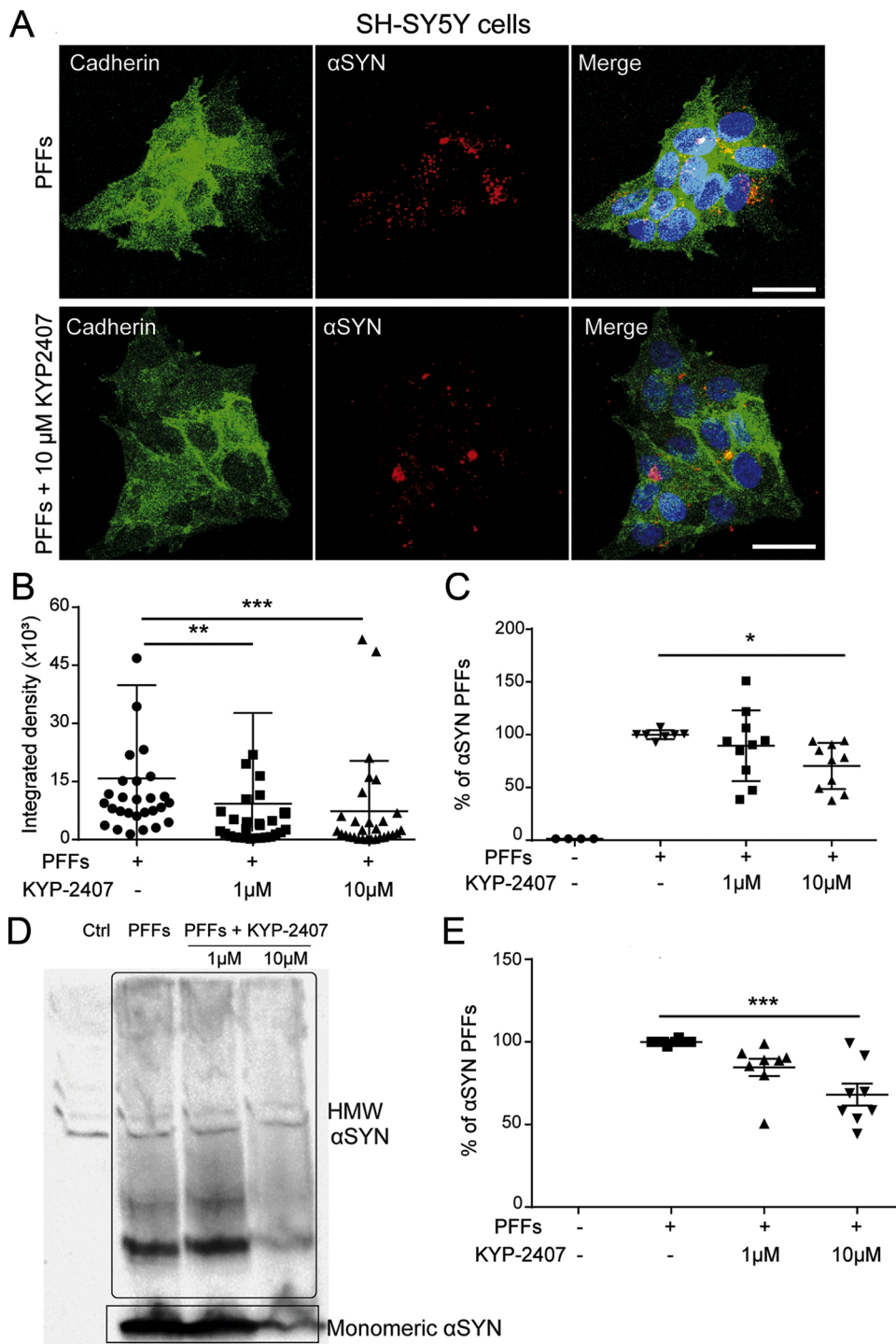
Next, we performed Western blot analysis on the SDS-fraction of the lysates to analyze whether KYP-2407 had any effect on high molecular weight (HMW)  $\alpha\text{SYN}$ . Our results showed that KYP-2407 reduced the levels of HMW  $\alpha\text{SYN}$  (Fig. 2D, E; normalized to the total insoluble proteins, Fig. S1). However, we did not observe any significant changes in the monomeric  $\alpha\text{SYN}$  from either the SDS- fraction or the soluble fraction in the presence of KYP-2407 (Figs. S2 and S3).

### 3.3. KYP-2407 has no effect on astrocytic $\alpha\text{SYN}$ inclusions

To further investigate if KYP-2407 has the same effect in astrocytes, human iPSC-derived (see characterization in Fig. S4) astrocytes were treated with  $\alpha\text{SYN}$  PFFs and KYP-2407. Similarly to SH-SY5Y cells, the astrocytes engulfed and accumulated  $\alpha\text{SYN}$  PFFs. However, neither in the immunostaining experiments nor in the medium could we find an effect of the KYP-2407 on astrocytic  $\alpha\text{SYN}$  aggregates (Fig. 3A–C). This could be explained by the fact that no PREP activity was detected in astrocytes. These data indicate that PREP inhibition decreases intracellular  $\alpha\text{SYN}$  load in neuron-like cells, but not in astrocytes.

### 3.4. PREP inhibition enhances the lysosomal pathway

The ALP is one of the major degradation pathways within the cell and has been shown to be impaired in PD [44,45]. Unlike monomeric  $\alpha\text{SYN}$ ,



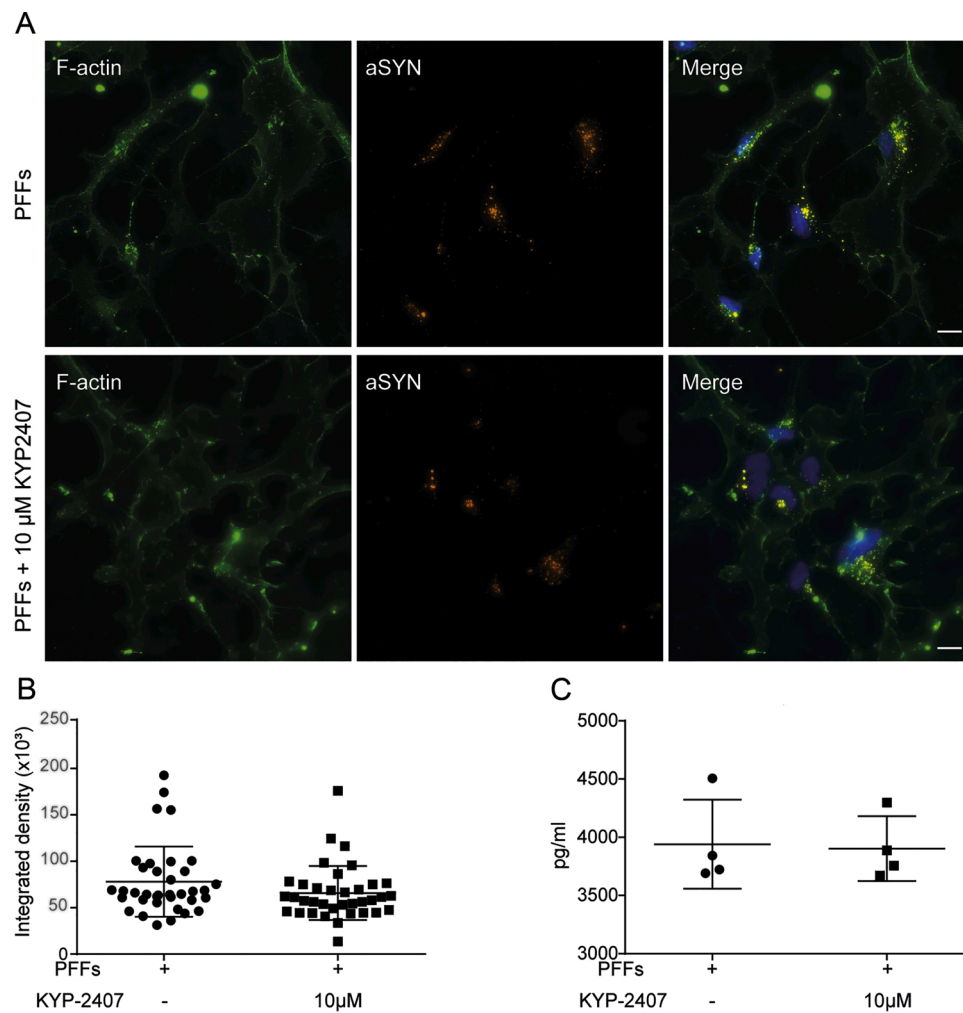
**Fig. 2. KYP-2407 decreases intracellular and extracellular  $\alpha$ SYN PFFs in SH-SY5Y cells.** SH-SY5Y cells engulf and accumulate  $\alpha$ SYN PFFs (A). Quantification of Cy3-labeled  $\alpha$ SYN PFFs using ImageJ confirmed that KYP-2407 decreases  $\alpha$ SYN PFFs in SH-SY5Y cells (B). ELISA analysis revealed that the levels of extracellular  $\alpha$ SYN also decrease in conditioned media from SH-SY5Y cells (C). Western blot analysis from the insoluble fraction of SH-SY5Y cell lysates showed a reduction in HMW and monomeric  $\alpha$ SYN with 10  $\mu$ M KYP-2407 (D–E). \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Scale bar is 10  $\mu$ m.

aggregated species of  $\alpha$ SYN are directed towards the ALP [36]. Therefore, we next studied if PREP inhibition increased lysosomal degradation in the presence of  $\alpha$ SYN PFFs. Immunostaining for total LC3B, an autophagosome marker, illustrated no significant difference between the treatment groups of SH-SY5Y cells (Fig. 4A, B). This suggests that KYP-2407 does not affect the total LC3B levels. Next, we examined the LC3BII and p62 levels using Western blot analysis to measure the autolysosomal turnover. Both p62 and LC3BII protein levels were increased in the PFF-treated cells (Fig. 4C, D), indicating an accumulation of the autophagosomal proteins. This could be possible due to inefficient degradation in the lysosomes. Subsequently, treatment with 10  $\mu$ M KYP-2407 significantly reduced both p62 and LC3BII almost to

the control levels (Fig. 4C, D; see the effect of KYP-2407 on p62 and LC3BII in absence of PFFs in Fig. S5). Hence, the lysosomal pathway could be partially responsible for the increased degradation of  $\alpha$ SYN.

### 3.5. KYP-2407 reduces calpain levels in the presence of $\alpha$ SYN PFFs

Calpains are proteolytic enzymes that are sensitive to intracellular calcium levels [46]. These proteins are associated with synucleinopathies as they cleave the C-terminus of  $\alpha$ SYN and thereby increase its aggregation properties. Furthermore, calpains, similar to PREP, negatively regulate autophagy [30,31]. Therefore, we examined whether exposure to  $\alpha$ SYN PFFs resulted in any changes in calpain 1 and 2 levels



**Fig. 3.** KYP-2407 has no effect on accumulated  $\alpha$ SYN PFFs in astrocytes. Immunostainings showed that 10  $\mu$ M KYP-2407 had no effect on the accumulated  $\alpha$ SYN PFFs inside the astrocytes (A). This was also confirmed with image analysis (B). ELISA analysis of extracellular  $\alpha$ SYN in astrocytic conditioned media showed no changes in  $\alpha$ SYN levels in the presence of 10  $\mu$ M KYP-2407 (C).

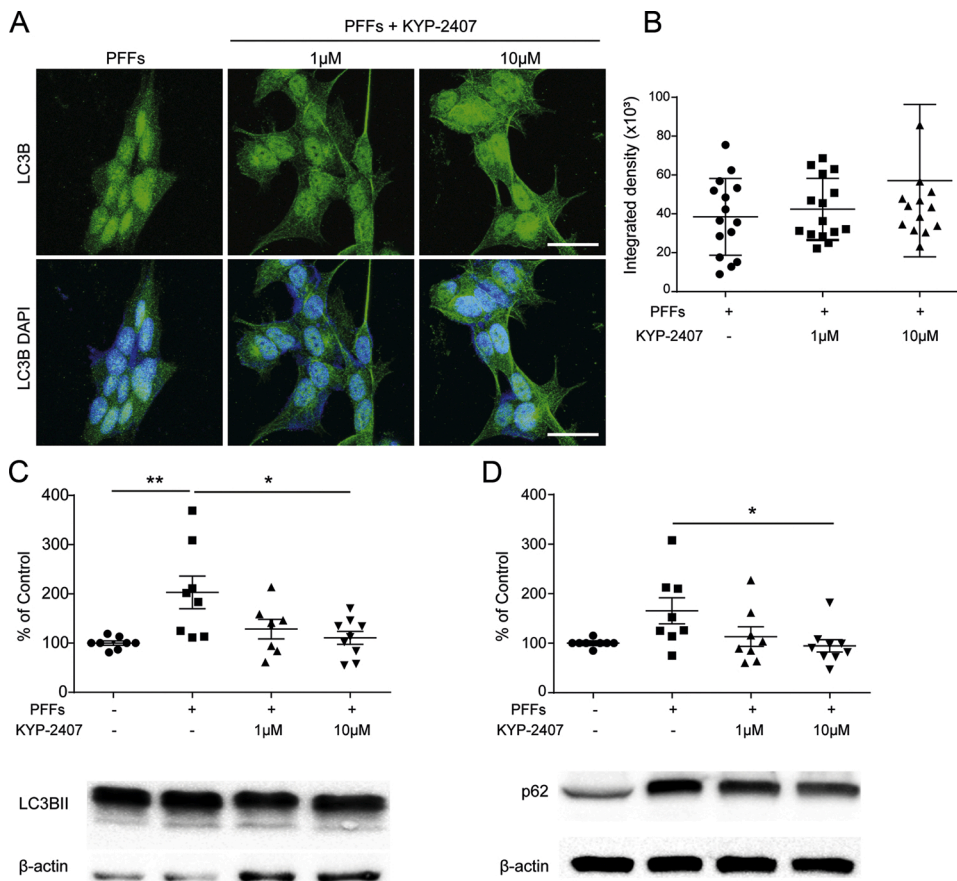
in SH-SY5Y cells. Western blot analysis revealed that SH-SY5Y cells, which were incubated with the PFFs, had significantly higher intracellular calpain 1 and 2 expression (Fig. 5A, B). Notably, PREP inhibition restored normal calpain 1 and 2 levels (Fig. 5A, B). In order to further investigate if PREP inhibition had any effect on calpain levels in untreated SH-SY5Y cells, the cells were only treated with 1  $\mu$ M and 10  $\mu$ M KYP-2407 for 4, 24, and 48 h. Treatment with 1  $\mu$ M KYP-2407 caused a reduction in calpain 1 and 2 levels at the earliest time point, but the levels gradually increased at the later time points (Fig. 5C, D). At 10  $\mu$ M KYP-2407, calpain 1 levels remained low even at the 48 h time point (Fig. 5C). However, calpain 2 behaved similarly in the presence of 10  $\mu$ M KYP-2407 as in the presence 1  $\mu$ M KYP-2407 (Fig. 5D). These results suggest that PREP inhibition has an effect on calpain levels, in particular calpain 1, with or without the presence of  $\alpha$ SYN PFFs.

While calpain is activated by calcium, regulation of its expression levels is largely unknown. As KYP-2407 affected calpain expression levels, we nevertheless tested whether this might occur *via* calcium, *i.e.* impact on the resting intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). Thus, resting  $[\text{Ca}^{2+}]_i$  was measured in control cells as well as cells treated with 1 and 10  $\mu$ M KYP-2407 for 48 h. No significant difference between the values for different conditions was observed:  $0.360 \pm 0.011$  (control),  $0.358 \pm 0.010$  (1  $\mu$ M KYP-2407) and 10  $\mu$ M  $0.350 \pm 0.008$  (10  $\mu$ M KYP-2407) ( $n = 4-5$ ).

#### 4. Discussion

Enhanced degradation of aggregated proteins which are involved in several neurodegenerative disorders, such as fibrillary  $\alpha$ SYN, has been of great interest from the therapeutic perspective. Degradation and spreading are two processes that are directly connected to each other as reduced degradation may lead to increased secretion. Hence, treatments that enhance the degradation of protein aggregates can probably prevent or at least reduce spreading of the pathology through the brain. In this study, we have shown that PREP inhibition by KYP-2407 enhanced degradation of preformed intracellular  $\alpha$ SYN fibrils in SH-SY5Y cells by stimulating the auto-lysosomal turnover as well as lysosomal degradation. Furthermore, KYP-2407 reduced the extracellular  $\alpha$ SYN released by the SH-SY5Y cells. Interestingly, these effects were seen only in neuronal SH-SY5Y cells but not in astrocytes that lack PREP activity, which indicates that the impact of the PREP inhibitor is indeed PREP-dependent.

PREP is a peptidase that has been implicated in the degradation of peptides shorter than 30 amino acids with proline as the cleavage site [47]. However, recent findings suggest that regulation of other proteins *via* direct protein-protein interactions is the main physiological function for PREP, and PREP also induces  $\alpha$ SYN aggregation *via* direct interaction [22,23,27]. In human and rodent brain sections, PREP expression and activity has mainly been detected in neurons [48]. However, in a mouse model with experimental neuroinflammation, PREP activity also



**Fig. 4. KYP-2407 induces lysosomal degradation.** LC3B immunostaining was performed to analyze the effect of KYP-2407 on autophagy (A). Quantification of LC3B immunoreactivity showed that there was no significant difference between  $\alpha$ SYN PFF-treated SH-SY5Y cells and  $\alpha$ SYN PFF + KYP-2407-treated cells (B). Western blot analysis revealed increased levels of LC3BII and p62 in SH-SY5Y cells that were treated with  $\alpha$ SYN PFFs. Treatment with KYP-2407 at 1  $\mu$ M and 10  $\mu$ M reversed this effect to the control levels (C–D). Scale bar =10  $\mu$ m.

appeared in activated microglial cells [49]. Interestingly, secretion of PREP from reactive microglia cells *in vitro* gave rise to neuronal death, suggesting a role of PREP in apoptosis [50]. Compelling evidence strengthens the involvement of astrocytes, the major glial cells in the brain, in the progression of PD. *Ex vivo* studies of human PD brain tissue as well as *in vitro* studies have demonstrated that astrocytes phagocytose and accumulate  $\alpha$ SYN aggregates instead of digesting it [8,10,11,51]. Therefore, we raised the question of whether PREP inhibition might be beneficial against the accumulation of  $\alpha$ SYN degradation in astrocytes. This could be explained by the fact that the human astrocytes used in this study did not express any PREP.

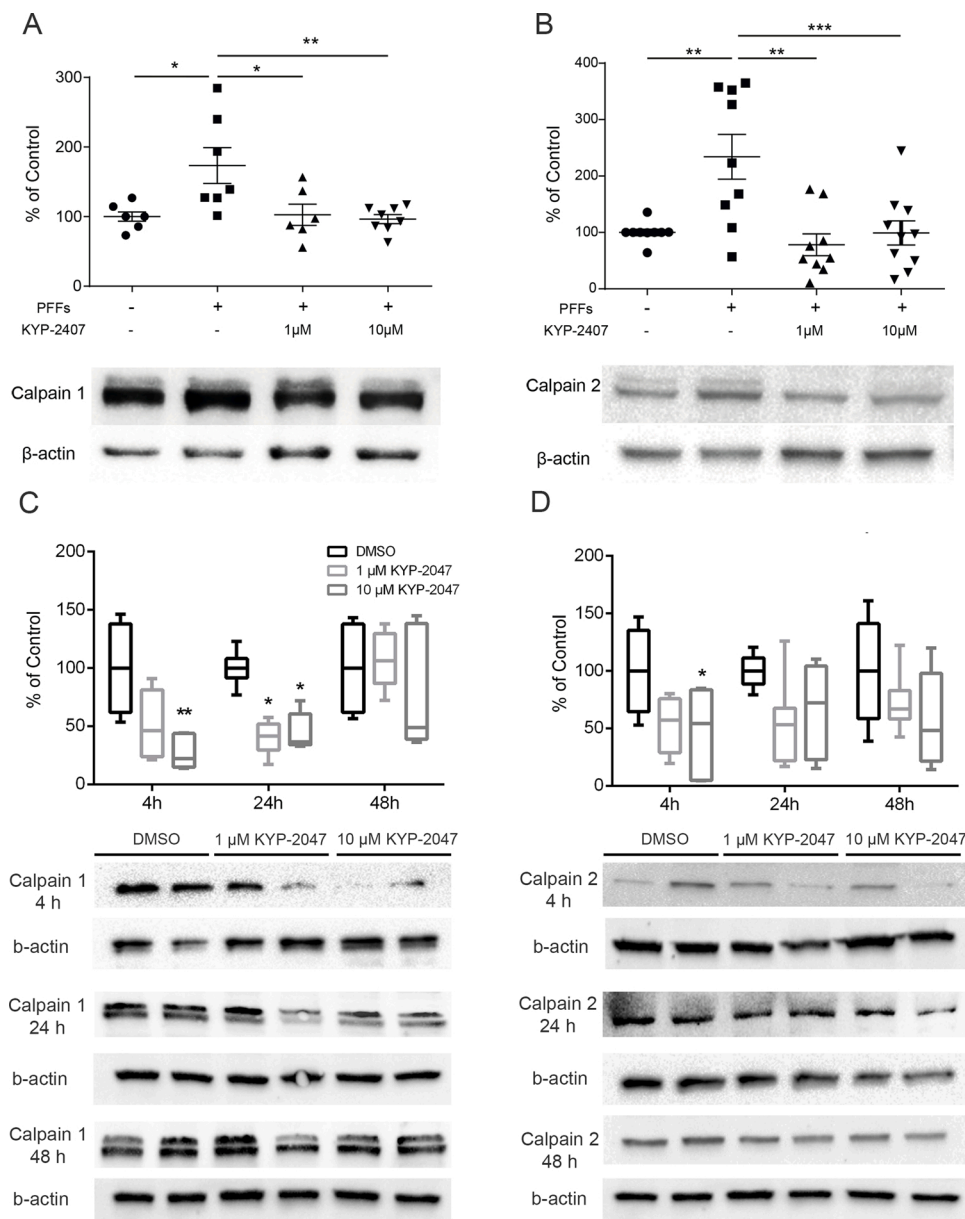
The autophagy pathway is divided into three parts: microautophagy, macroautophagy and chaperone-mediated autophagy. Macroautophagy, which the name implies, is responsible for elimination of bigger components such as dysfunctional organelles as well as aggregated proteins. The pathway is initiated by the formation of autophagosomes where LC3BII and p62 proteins play a crucial role. In order to digest the encapsulated materials, the autophagosomes need to fuse with lysosomes where low pH and enzymes degrade the material. During this turnover, p62 and LC3BII are also degraded in the lysosomes. Subsequently, these proteins can be used as markers for the autolysosomal pathway [52].

Soluble  $\alpha$ SYN is normally degraded through the ubiquitin–proteasome pathway as well as by chaperone-mediated autophagy, whereas bigger  $\alpha$ SYN aggregates and fibrils are directed to the ALP. However, all three pathways have been demonstrated to be diminished in PD [18,34,36]. Recently, we have shown that PREP overexpression inhibits autophagy [26] by negatively regulating protein phosphatase 2A (PP2A) [27], and that removal of PREP or PREP inhibition by KYP-2047 induces autophagic flux [27,37]. Furthermore, *in vivo* experiments in various Parkinsonian mouse models, including the

A30 P and the adeno-associated virus (AAV)-based mouse models, have revealed that KYP-2407 increases degradation of high molecular weight  $\alpha$ SYN in the brain [38,43]. Here we show that exposure of SH-SY5Y cells to  $\alpha$ SYN PFFs gives rise to an accumulation of LC3BII and p62, indicating an impaired auto-lysosomal turnover. Importantly, treatment of these cells with KYP-2407 caused a downregulation of LC3BII and p62, which may indicate an improved turnover as well as lysosomal degradation. In addition, KYP-2407 treatment also increased degradation of high molecular weight  $\alpha$ SYN in SH-SY5Y cells.

Increasing evidence suggests that  $\alpha$ SYN aggregates can spread throughout the brain through cell–cell contact as well as secretion [53]. Uptake of released  $\alpha$ SYN aggregates by the surrounding neurons can lead to increased seeding and pathology in the brain. Seeding is a process in which the fibrillary  $\alpha$ SYN binds to the monomeric  $\alpha$ SYN as a template and enables its aggregation as well as elongation. Therefore, it would be of enormous interest to reduce  $\alpha$ SYN spreading and seeding. We showed that  $\alpha$ SYN secretion significantly decreased in SH-SY5Y cultures upon inhibition of PREP. We also showed that KYP-2407 reduced the soluble  $\alpha$ SYN monomers in the cell lysates derived from SH-SY5Y cultures. This suggests that PREP inhibition can reduce seeding of  $\alpha$ SYN by decreasing the template required for the process.

In addition to PREP,  $\alpha$ SYN has been shown to interact with calpain 1 and 2. These proteins are proteolytic enzymes that are activated by increased intracellular calcium levels. Calpain 1, which is also called  $\mu$ -calpain, reacts to micromolar concentrations of calcium, whereas calpain 2 or m-calpain, reacts to millimolar concentrations [46]. Calpain 1 has been associated with the cleavage of the C-terminal part of  $\alpha$ SYN, thereby increasing its aggregation. The C-terminal part of  $\alpha$ SYN is considered to act as a chaperone and prevent aggregation [29]. Calcium dysregulation in neurons and glial cells has been reported in several studies related to PD. Consequently, increased intracellular calcium levels induce  $\alpha$ SYN aggregation, and  $\alpha$ SYN aggregates have also been



**Fig. 5. Calpain levels are reduced inside the cell in the presence of KYP-2407.** Exposure to  $\alpha$ SYN PFFs caused an increase in intracellular calpain 1 and 2 levels in SH-SY5Y cells. Similar to the auto-lysosomal proteins, calpain 1 and 2 levels were reduced to the control levels in the presence of KYP-2407 (A). In control cells, 1  $\mu$ M KYP-2407 caused a reduction in calpain 1 levels at 4 h and 24 h, whereas at 48 h the levels increased to normal conditions. However, at 10  $\mu$ M KYP-2407, calpain 1 levels remained reduced even at 48 h (B). Calpain 2 levels were also reduced in control cells in the presence of 1  $\mu$ M KYP-2407 at 4 h but gradually increased at the later time points. Similarly, 10  $\mu$ M KYP-2407 had no additional effect at the later time points (C).

shown to disrupt calcium homeostasis inside cells [54]. Our results indicate that KYP-2407 decreases the expression levels of calpain 1 and 2 in the presence of  $\alpha$ SYN PFFs. Previously, it has been reported that PREP regulates the generation of inositol 1,4,5-trisphosphate ( $IP_3$ ) [55], an important signaling molecule in the calcium pathway. We hypothesized that intracellular calcium might also play a part here. However, we did not see any difference in the measured resting calcium levels in cells treated with KYP-2407 and control cells. This suggests that KYP-2407 does not elicit its effect on calpain levels by regulating the resting  $[Ca^{2+}]_i$  and the regulatory effect of PREP inhibition on calpains should be studied further. However, we should note that the measurement of gross  $[Ca^{2+}]_i$  is not sensitive to local differences in  $[Ca^{2+}]_i$  and fura-2 is altogether not very sensitive to small changes in the low end of the fluorescence. The resting  $[Ca^{2+}]_i$  is quite low in SH-SY5Y cells – e.g. on average 59 nM [56] and here probably even lower, i.e. quite much lower than the  $K_d$  of fura-2, 224 nM.

**5. Conclusion**

Altogether, our data present a beneficial effect of PREP inhibition

using KYP-2407 on degradation and secretion of  $\alpha$ SYN aggregates potentially alleviating PD pathology.

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**Declaration of Competing Interest**

The authors report no declarations of interest.

**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.110788>.



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