Removal of proteinase K resistant αSyn species does not correlate with cell survival in a virus vector-based Parkinson’s disease mouse model

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ABSTRACT

Parkinson’s disease (PD) is characterized by degeneration of nigrostriatal dopaminergic neurons and accumulation of α-synuclein (αSyn) as Lewy bodies. Currently, there is no disease-modifying therapy available for PD. We have shown that a small molecular inhibitor for prolyl oligopeptidase (PREP), KYP-2047, relieves αSyn-induced toxicity in various PD models by inducing autophagy and preventing αSyn aggregation. In this study, we wanted to study the effects of PREP inhibition on different αSyn species by using cell culture and in vivo models.

We used Neuro2A cells with transient αSyn overexpression and oxidative stress or proteasomal inhibition-induced αSyn aggregation to assess the effect of KYP-2047 on soluble αSyn oligomers and on cell viability. Here, the levels of soluble αSyn were measured by using ELISA, and the impact of KYP-2047 was compared to anle138b, nilotinib and deferiprone. To evaluate the effect of KYP-2047 on αSyn fibrillization in vivo, we used unilateral nigral AAV1/2-A53T-αSyn mouse model, where the KYP-2047 treatment was initiated two- or four-weeks post injection.

KYP-2047 and anle138b protected cells from αSyn toxicity but interestingly, KYP-2047 did not reduce soluble αSyn oligomers. In AAV-A53T-αSyn mouse model, KYP-2047 reduced significantly proteinase K-resistant αSyn oligomers and oxidative damage related to αSyn aggregation. However, the KYP-2047 treatment that was initiated at the time of symptom onset, failed to protect the nigrostriatal dopaminergic neurons. Our results emphasize the importance of whole αSyn aggregation process in the pathology of PD and raise an important question about the forms of αSyn that are reasonable targets for PD drug therapy.

1. Introduction

Parkinson’s disease (PD) is the second most common progressive neurodegenerative disease after Alzheimer’s disease, with a mean age of onset at 55 (Dauer and Przedborski, 2003; Rizek et al., 2016). The incidence of PD increases significantly with age, from 20/100,000 overall to 120/100,000 at the age of 70 (Dauer and Przedborski, 2003). Typically, PD is characterized by motor deficits (e.g., resting tremors, rigidity, bradykinesia) that emerge when the striatal dopamine levels have decreased by 60–80% (Dauer and Przedborski, 2003). Neuronal degeneration occurs also in other brain nuclei (Prakash et al., 2016), and this may play a role in non-motor symptoms such as olfactory impairment, autonomic dysfunctions, and sleep disturbances. Certain non-motor symptoms are present even before the motor symptoms (Gómez-Benito et al., 2020).

Protein aggregation is a pathological process common to several neurodegenerative diseases (Tan et al., 2009). Lewy bodies found in affected brain areas are the key pathological findings in PD, and although alpha-synuclein (αSyn) is the most abundant protein in Lewy bodies, they contain high amounts of cellular membranes and damaged cellular organelles to which αSyn is attached to (Dauer and Przedborski, 2003; Gómez-Benito et al., 2020; Rizek et al., 2016; Shahmoradian et al., 2019; Tan et al., 2009). Another link between PD and αSyn arises from GWAS studies that have identified the αSyn coding SNCA gene as a risk factor for PD (Simón-Sánchez et al., 2009). Known point mutations in the SNCA, such as A30P (Krüger et al., 1998), A53T (Polymeropoulos

Abbreviations: αSyn, alpha-synuclein; PD, Parkinson’s disease; PREP, prolyl oligopeptidase.

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Increase the risk for familial PD. AαSyn is a small and natively unfolded protein that is most abundantly expressed in presynaptic terminals in the central nervous system (Gómez-Benito et al., 2020). The exact endogenous function of AαSyn remains unclear but it is speculated to participate in synaptic plasticity, neurotransmitter release and uptake (Burr et al., 2010; Julku et al., 2018; Venda et al., 2010).

Conformation of the AαSyn protein alters easily and is sensitive to environmental changes and post-translational modifications, which predisposes it to misfolding and consequent aggregation (Uversky, 2007). Certain fibrillar forms of AαSyn can propagate toxicity at least in preclinical models, following to certain extent the theory by Braak et al. (2003) but the nature of AαSyn spreading in clinical PD is still unclear (Steiner et al., 2018). Recently, Garcia et al. (2022) showed that glial activation does not follow the pattern of AαSyn inclusions induced by pre-formed fibrils, but the glial changes were related to smaller AαSyn oligomers that were induced by pre-formed fibrils. Additionally, an ultrastructural study did not find considerable amounts of fibrillar AαSyn in Lewy bodies (Shahmoradian et al., 2019). This supports the view that early stage accumulation of smaller oligomeric AαSyn forms, rather than the more aggregated forms and inclusions of AαSyn, mediate AαSyn neurotoxic effects (Carjia et al., 2017; Villar-Piqué et al., 2016), such as mitochondrial dysfunction, endoplasmic reticulum stress, impairment of protein clearance pathways, disruption of biological membranes and synaptic dysfunction, which eventually lead to neuronal cell death (Gómez-Benito et al., 2020).

Reduced activity of protein degradation systems, such as proteasomes, autophagic systems and lysosomes, have been detected in PD patient samples (Fowler et al., 2021; McNaught and Jenner, 2001) and in patient-derived cells (Laperle et al., 2020). This suggests that deficits in protein degradation have a role in PD pathophysiology, exposing cells also to elevated AαSyn levels and aggregation (Webb et al., 2003; Xilouri et al., 2013). This is a two-way road as accumulation of AαSyn, particularly its aggregated or mutated forms, can damage proteasomes and eventually the autophagic recycling systems (Erustes et al., 2018; Winslow et al., 2010). Another factor that exposes dopaminergic neurons in the substantia nigra pars compacta (SN) for degeneration is their long axonal tracts and wide dendritic tree that have high energy demand. High dopamine, iron and neuromelanin levels in dopaminergic cells of SN combined with high amount of energy producing mitochondria increase the risk for excessive production of reactive oxygen species (ROS) (Jiang et al., 2016). Therefore, it is not surprising that deficits in mitochondrial complex proteins relate to PD (Haque et al., 2022), and increased oxidative damage is seen in PD post-mortem tissue (Yoritaka et al., 1996) and patient-derived cells (Chang et al., 2016). Moreover, studies have presented that increased ROS levels trigger also AαSyn aggregation and vice versa (Dias et al., 2013; Haque et al., 2022). It can be concluded that the neuronal degeneration in PD, as in other neurodegenerative diseases, is a vicious circle that has several connected pathophysiological features.

Current drug therapies for PD are still based on dopamine replacement (de Bie et al., 2020). However, these cannot stop or delay the neuronal degeneration (de Bie et al., 2020), and therefore, there is a huge need for a disease-modifying therapy. Recently, several therapies have been developed to target AαSyn aggregation in PD but so far no AαSyn targeting or other disease-modifying treatment have been approved for patient use (McFarthing et al., 2020). We have studied the effects of small molecules of prolyl oligopeptidase (PREP), a serine protease, on AαSyn-based cellular and animal models as a potential disease-modifying therapy for PD. PREP enhances AαSyn aggregation via direct protein-protein interaction (Savolainen et al., 2015) and high levels of PREP and co-localization with AαSyn is seen in the SN of post-mortem PD patient brains (Hannula et al., 2013; Jiang et al., 2001; Rožner et al., 2005). Small molecular PREP inhibitors can modify this interaction to reduce AαSyn aggregation (Savolainen et al., 2015), and they also induce AαSyn degradation via chaperone-mediated autophagy (Cui et al., 2022) and beclin-1-dependent macroautophagy (Svarcbahs et al., 2020). Macroautophagy activation by PREP inhibitors is based on modifying the interaction network between PREP and protein phosphatase 2A (PP2A), which also reduces oxidative stress (Eteläinen et al., 2021; Svarcbahs et al., 2020). PREP inhibition by KYP-2047 has already shown disease-modifying effects in animal models of PD, even when initiated after symptom onset (Myöhänen et al., 2012; Savolainen et al., 2014; Svarcbahs et al., 2016). In earlier studies, KYP-2047 has reduced particularly SDS-insoluble forms of AαSyn in cell culture models expressing wild type AαSyn, and A30P and A53T-mutated AαSyn (Dokleja et al., 2014; Myöhänen et al., 2012, 2017). In animal models, the reducing effect of KYP-2047 is seen in total AαSyn (Myöhänen et al., 2012), and both in AαSyn oligomers and proteinase-K resistant AαSyn species (Svarcbahs et al., 2016). However, the effect of KYP-2047 on oligomeric AαSyn species has not been completely verified. Although KYP-2047 has effectively reduced AαSyn levels in vivo in various transgenic mice strains and in an AαSyn virus vector model (Svarcbahs et al., 2019), the main weakness of abovementioned models is that AαSyn transgene or AAV-AαSyn did not cause significant toxicity in dopaminergic cells. Therefore, the protective effect of KYP-2047 on brain dopaminergic neurons in vivo AαSyn models remains unclear.

Therefore, in this study, we wanted to assess the effect of KYP-2047 on soluble AαSyn oligomers by using a cell culture assay and AαSyn oligomer specific ELISA. For comparison, we selected compounds that target different AαSyn-connected mechanisms in PD and that are currently in clinical trials for PD; anle138b, an AαSyn aggregation inhibitor (Wagner et al., 2013); nilotinib, an Abelos (c-Abl) tyrosine kinase inhibitor that decreases AαSyn levels via autophagy (Hebron et al., 2013); deferiprone, an iron chelator that aims to reduce oxidative stress that is evident in dopaminergic neurons of PD patients (Martin-Bastida et al., 2017). KYP-2047 was used in further assays where we evaluated the effects of two and four-week chronic KYP-2047 treatment in a virus vector-based unilateral human mutant AαSyn (A53T) overexpression mouse model of PD. A53T-AαSyn was selected since earlier studies have shown that AAV-A53T-AαSyn causes more prominent cellular toxicity than wild-type AαSyn virus vector that we have used in our earlier study (Giasson et al., 2002; Ip et al., 2017). In both in vivo experimental setups, the treatment was initiated at the time of symptom onset as we wanted to reflect the typical situation in PD therapy.

2. Materials and methods

2.1. Reagents

Chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. PREP inhibitor, KYP-2047 (4-phenylbutanoyl-1-prolyl-2(S)-cyano pyrrolidine) was synthesized in the University of Helsinki as described earlier (Jarho et al., 2004) Stock solution of KYP-2047 was 10 mM in dimethyl sulfoxide (DMSO), which were diluted to the final concentration in the cell medium. Anle138b (#SML1515) and Nilotinib (#CD023093) were dissolved in DMSO with stock solutions of 100 mM and 50 mM, respectively. Deferiprone (#Y0001976) was dissolved in distilled water at 100 mM. KYP-2047 readily crosses the blood-brain barrier in rodents (Jalkanen et al., 2011, 2014).

Adeno-associated viruses (AAVs), driven by the chicken β-actin promoter (CBA) hybridized with the cytomegalovirus (CMV) immediate early enhancer sequence, were acquired via Michael J. Fox Foundation for Parkinson’s disease from Vigene Biosciences (Rockville, MD, USA). The AAVs used were AAV1/2-CMV/CBA-human-A53T-alpha-synuclein-WPRE-BGH-polyA (AAV-A53T-a-syn, 5.1 x 10^{12} vg/ml) (GD1001-RV, Vigene Biosciences) and AAV1/2-CMV/CBA-Null/Empty-WPRE-BGH-polyA (AAV-empty, 5.1 x 10^{12} vg/ml) (GD1004-RV, Vigene Biosciences). These chimeric vectors were designed to utilize advantages of both AAV serotype 1 (good ability to penetrate brain tissue) and AAV serotype 2 (high neuronal tropism and ability to produce high titers)
The AAV1/2 construct has been shown to be highly effective in both rats and mice when looking at the coverage of the SN dopaminergic neurons and its axonal transport along the nigrostriatal tract (Ip et al., 2017; Koprich et al., 2010, 2011).

2.2. Cell culture

Mouse neuronal Neuro2A (N2A) cell line was used in this study, due to its neuronal background and easy transfect ability. The cells were obtained from ATCC (VA, USA) and were cultured as described in (Koprich et al., 2010). The AAV1/2 construct has been shown to be stably released the treatment compounds for two weeks, new minipump.

2.3. αSyn oligomer enzyme-linked immunosorbent assay (ELISA) and cell viability assay

N2A cells were seeded on a 12-well-plate with the density of 50,000 cells/well, and the cells were transfected with 1 μg AAV1-EF1α-V5-synuclein (Addgene #60057) by using lipofectamine (LFC) 3000 as earlier (Svarcbahs et al., 2018). Empty AAV1-EF1α plasmid served as a control. Thereafter, the αSyn aggregation was induced by using oxidative stress (100 μM hydrogen peroxide (H₂O₂) and 10 mM ferrous chloride (FeCl₂)) (Eteläinen et al., 2021; Svarcbahs et al., 2018) or 10 μM lactacystin (Möyhänen et al., 2017) for 24 h with the following study compounds: 10 μM KYP-2047, 10 μM Anle138b, 10 μM Nilotinib and 1 mM deferiprone. 0.1% DMSO served as a negative control. After the incubation, the cells were lysed by incubating cells in RIPA buffer on ice for 30 min followed by centrifugation at 20,000 g, 20 min, 4°C. Supernatant was collected and the protein concentration was measured by using the BCA method (#23225, ThermoFisher Scientific).

After the incubation period, the αSyn oligomers were analyzed by using the ELISA protocol described and validated in the study by Lassen et al. (2018). Briefly, MJF-14-6-4-2 antibody (625 ng/ml; rabbit aggregated αSyn specific; ab209538, AbCam) was the capturing antibody and mouse monoclonal anti-αSyn antibody (0.5 µg/ml; #610787, BD Transduction Laboratories, NJ, USA) served as the detecting antibody. 4 µg of protein was added to the wells, and mouse secondary antibody with HRP (#31430; Thermo Fisher Scientific) with 3,3′,5′, 5′-Tetramethybenzidine (TMB; Merck-Millipore) were used to visualize the proteins. The absorbance was measured at 450 nm by using a BioTek ELX800 microplate reader (BioTek, VT, USA). Since we wanted only to compare the impact between the compounds and vehicle, we did not determine absolute αSyn oligomer amounts with standard curve.

The impact of compounds on cell viability was assayed by the end of the incubation period by using standard lactate hydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assays as in (Svarcbahs et al., 2018). The results were correlated to empty AAV1-EF1α plasmid transfection with LFC.

2.4. Animals

Male C57BL/6JRccHsd mice (2-week: n = 41; 4-week: n = 46) obtained from Envigo (The Netherlands) were used in this experiment. The mice were 10–11 weeks old at the onset of the experiments, single housed in individually ventilated cages (Mouse IVC Green Line, Techniplast, Italy), kept under standard laboratory conditions (room temperature 23 ± 2°C, 12 h light/dark cycle), and had ad libitum access to food (Teklad, 2016, Envigo, UK) and irradiated tap water.

Animal experiments were conducted according to the 3R principles of the EU directive 2010/63/EU regarding the care and use of experimental animals and following the local laws and regulations (Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013), Government Degree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)). The experiment protocols were authorized by the national Animal Experiment Board of Finland (ESAVI/42235/2019).

2.5. Treatments and experimental setup

Mice that received AAV-A53T-αSyn injections got treated either with PREP inhibitor (KYP-2047, 10 mg/kg/day in propylene glycol) (2-week: n = 13; 4-week: n = 15) or vehicle (100% propylene glycol) (2-week: n = 14; 4-week: n = 15). The treatments were started two or four weeks after the virus vector surgeries (Fig. 1). The dose of KYP-2047 was based on our earlier studies with the AAV-αSyn virus vector (Svarcbahs et al., 2016) and αSyn transgenic mice (Savolainen et al., 2014), and on a brain pharmacokinetic study with KYP-2047 (Jalkanen et al., 2014). The treatment lasted for 14 days in the 2-week treatment setup and 28 days in the 4-week treatment setup. All AAV-empty injected mice (2-week: n = 14; 4-week: n = 16) were given KYP-2047 10 mg/kg/day.

We have earlier shown that the AAV-empty injection + vehicle does not differ from AAV-empty + KYP-2047 group (Kilpeläinen, Eteläinen, Myöhänen et al., manuscript), hence, this group was not included in order to reduce number of animals in the experiments, as encouraged by the 3R principles. Osmotic minipumps (Micro-osmotic pump, model 1002, lot no. 10400-19, Alzet) implanted in the abdominal cavity were used to provide chronic administration. Priming doses dissolved in 5% tween 80 in saline (intra peritoneal (i.p.), 10 mg/kg) were given on the first day of the treatment to ensure immediate onset of the drug effect. Within the treatment groups, the mice were further divided into groups intended for a microdialysis experiment (2-week: n = 22; 4-week: n = 25) and for immunohistochemistry (IHC, 2-week: n = 19; 4-week: n = 21).

2.6. Stereotactic AAV virus vector microinjections

The mice were injected with AAV-A53T-αSyn (2-week: n = 27; 4-week: n = 30) or AAV-empty (2-week: n = 14; 4-week: n = 16) under isoflurane anesthesia (4% induction, 2% maintenance). The injections were given above the left substantia nigra (SN) in the 2-week treatment setup, and above the right SN (A/P: –3.1, L/M –1.2, D/V –4.2 from bregma) in the 4-week treatment setup according to Paxinos and Franklin and Paxinos (1997), as in studies of Svarcbahs et al. (2016), Svarcbahs et al. (2018) and Julku et al. (2018). The side was chosen according to the base line cylinder test where the natural forepaw preference among the whole group of mice was determined. The used injection volume was 1 μl and it was administered with the rate of 0.2 μl/min. Before the needle was lifted from the brain, it was kept in place for 5 min to prevent leakage up the needle tract. Topical lidocaine (10 mg/ml), Sub-cutaneous (s.c.) buprenorphine (0.1 mg/kg) and s.c. carprofen (5 mg/kg) injections were provided as pre- and postoperative pain management.

2.7. Minipump surgeries

The minipumps were filled with treatment compounds and primed in 0.9% saline at +37°C overnight before implantation. The minipumps were inserted in the abdominal cavity of the mice under isoflurane anesthesia (4% induction, 2% maintenance). A midline skin incision (approx. 1 cm) was made in the lower abdomen under the rib cage, after which the musculoperitoneal layer was carefully tented up and another incision was made beneath the cutaneous incision. The prefilled minipump was then inserted into the cavity. The musculoperitoneal and cutaneous layers were then closed with sutures (Dafilon blue 6/0, C0932060, B.Braun) and the mice were transferred to a heating recovery chamber set at +37°C to recover. Topical lidocaine (10 mg/ml), s.c. buprenorphine (0.1 mg/kg) and s.c. carprofen (5 mg/kg) injections were provided as pre- and postoperative pain management. As the minipumps stably released the treatment compounds for two weeks, new minipump surgeries were repeated two weeks after implantation for the mice who were treated for 28 days.
2.8. Guide cannula surgeries

Animals intended for microdialysis (2-week: n = 22; 4-week: n = 25) had guide canulas inserted above their left or right striatum (left: A/P − 0.6; L/M + 1.8; D/V − 2.7; right: A/P − 0.6; L/M − 1.8; D/V − 2.7) in a stereotactic surgery under isoflurane anesthesia either 3 weeks (2-week treatment setup) or 7 weeks (4-week treatment setup) after viral vector injections (Fig. 1). The mice had a 1-week recovery time after the minipump surgeries before guide canulas were implanted. Dental cement (Aqualox, Voco) and two stainless-steel screws (1.3 × 3 mm, DIN84, Helsingin Ruuvihankinta, Finland) were used to fasten the cannula to the skull. Topical lidocaine (10 mg/ml), s.c. buprenorphine (0.1 mg/kg) and s.c. carprofen (5 mg/kg) injections were provided as pre- and postoperative pain management.

2.9. Cylinder test

Asymmetry in spontaneous forepaw use was studied with the cylinder test as described in (Svarcbahs et al., 2016, 2018). Briefly, the mice were video recorded in transparent plastic cylinders for 5 min or until they had touched the cylinder wall at least 20 times. The cylinder test was first done before the viral vector injections and then repeated with two-week intervals. In addition, the side of the brain for the injection was selected according to the natural paw preference of the whole group determined by the baseline test (2-week: left; 4-week: right). The data is presented as percentage of the ipsilateral forepaw use from the overall forepaw use: [(ipsilateral paw)/(ipsilateral paw + contralateral paw)] × 100%.

2.10. Locomotor activity

Spontaneous locomotor activity was measured in clear walled plastic cages (24 × 24 × 15 cm) attached to activity monitors (open-field activity monitor, MED Associates, GA, USA) from the mice belonging to the IHC group (2-week: n = 19; 4-week: n = 21) before the viral vector injection surgeries (base line) and at the end point of the experiment, i.e. 4 weeks (2-week treatment setup) or 8 weeks (4-week treatment setup) after the viral injections (Fig. 1). The monitor recorded infrared photo-beam interruptions for 21 h starting at 11 a.m. Mice were not habituated to the open-field cages prior to the experiment and had ad libitum access to food and water during the experiment.

2.11. Microdialysis

Microdialysis was performed after 14 days (2-week: n = 22) or 28 days (4-week: n = 25) of KYP-2047 or vehicle treatment as described in (Svarcbahs et al., 2016) and (Julku et al., 2018). Briefly, four baseline samples were collected (20 min/40 μL/sample) after the 2 h stabilization period from microdialysis probe insertion. After collecting the baseline samples, the probe was perfused 2 × 20 min with 10 μM and 30 μM α-amphetamine sulphate with 2 × 20 min recovery time between the concentrations. Mice were sacrificed right after the microdialysis experiment, and the brains frozen in isopentane on dry ice and preserved at −80 °C. The concentration of extracellular dopamine was measured by high-performance liquid chromatography (HPLC) as described previously (Svarcbahs et al., 2016), with slight modifications.

Due to technical issues, slightly different HPLC systems were used in the 4-week treatment setup and 2-week treatment setup. 4-week treatment setup: solvent delivery pump (model PU-2080 Plus, Jasco, Tokyo, Japan), pulse damper (SSI LP-21, Scientific Systems, MA, USA), autosampler (SIL-20AC Autosampler, Shimadzu, Japan), analytical column (Kinetex C-18, 5 m, 4.60.50 mm, Phenomenex, CA, USA) thermostated by a column heater (CROCO CIL, Cluzeau Info-Labo; LaChrom L-7350, Merck), electrochemical detector (Coulochem II detector, ESA Biosciences, MA, USA), model 5014B microdialysis cell (ESA Biosciences). The mobile phase: 0.1 M NaH2PO4 buffer (Merck), 8% (v/v) methanol (Merck), 0.2 M ethylenediaminetetraacetic acid, and 100 mg/L octanesulfonic acid, pH 4.0, flow rate 4.0 l/min. DA was reduced with an amperometric detector (potential −120 mV against an Ag/AgCl reference electrode) after being oxidized with a coulometric detector (300 mV). The sample injection volume 25 μL, column temperature 40 °C. The chromatograms were processed by AZUR chromatography data system software (Cromatek, Essex, UK). 2-week treatment setup: solvent delivery pump (Prominence LC-20A liquid chromatograph, Shimadzu), autosampler (Prominence SIL-20AC autosampler, Shimadzu), analytical column (EICOMPAX CAX, 5 μm, 2.0 × 200 mm, Eicom, Dublin, Ireland), electrochemical detector with temperature control (Decade II LC-EC Workstation, Antec Leyden, The Netherlands). Mobile phase: 70% (v/v) 0.1 M ammonium acetate, 50 mM sodium sulfate, 50 mg/ml EDTA-2Na, 30% (v/v) methanol (CAX mobile phase, 900.800.00, Amuza, CA, US), flow rate 0.250 ml/min. DA was reduced with an amperometric detector (potential 600 mV against an Ag/AgCl reference electrode). Injection volume 10 μL, column temperature 35 °C. The chromatograms were processed as above.

2.12. Tissue processing

At the end of the experiment, mice in the IHC group (2-week: n = 36; 4-week: n = 21) were transcardially perfused (first with PBS followed by 4% paraformaldehyde, PFA) under deep sodium pentobarbital anesthesia (i.p. 200 mg/kg) and their brains were collected. The brains were postfixed for 24 h in 4% PFA at 4 °C, after which they were transferred into 10% sucrose in PBS and kept there overnight at 4 °C. On the following day, brains were transferred further into 30% sucrose in PBS
and kept at 4 °C another 24 h. After this, the brains were frozen on dry ice and kept at –80 °C until sectioning. The brains were cut to 30 μm free-floating sections on a cryostat (Leica CM3050) and kept in cryopreservation solution (30% ethylene glycol and 30% glycerol in 0.5 M phosphate buffer) in -20 °C until staining.

Additionally, striatal (0.2 mm) punches were collected from the frozen brains harvested from mice of the 4-week treatment setup who underwent microdialysis. These samples were used in HPLC tissue analysis to determine the tissue concentration of dopamine, serotonin, and their metabolites. The HPLC tissue analysis was done as in the study of Svarcbahs et al. (2018) and the concentrations were calculated as nanograms per milligram of brain tissue and normalized to the intact side. In addition, nigral punches (0.1 mm) were collected from brains of the 2-week treatment setup microdialysis group, and these were used in Western blot.

2.13. Immunohistochemistry (IHC)

IHC staining from 30 μm striatal and nigral sections was performed for tyrosine hydroxylase (TH) and oligomer-specific αSyn (αSynO5) as in (Svarcbahs et al., 2016) and (Kilpeläinen et al., 2019c). Briefly, after quenching, the nonspecific binding was blocked with 10% normal goat serum (TH; S-1000-20, Vector Laboratories) or by Basic Vector Mouse on Mouse (M.O.M.) Immunodetection kit (αSynO5; BMK-2202, Vector Laboratories) for 30 min. The sections were incubated overnight at room temperature with rabbit anti-TH primary antibody (1:2000 in 1% normal goat serum in 0.5% Triton-X in PBS, AB152, Sigma-Aldrich) or by mouse anti-human αSynO5 primary antibody (1:200 in M.O.M. diluent, AS132718, Agrisera). αSynO5 primary antibody is oligomer-specific (Braunstroth et al., 2014) and does not react with mouse endogenous αSyn in tissue IHC (Svarcbahs et al., 2016). After washes, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (TH; 1:500 in 1% normal goat serum in 0.5% Triton-X in PBS, BA1000, Vector Laboratories) or by biotinylated anti-mouse IgG secondary antibody (αSynO5; 1:300 in M.O.M. diluent, MKB-2225, Vector Laboratories) at room temperature for 2 h. The signal was enhanced with the avidin-biotin complex method (Vectastain ABC standard kit, PK-6100, Vector laboratories) according to instructions provided by the manufacturer and the immunoreactivity was visualized with 0.05% diaminobenzidine solution (0.05% 3,3'-diaminobenzidine and 0.03% H2O2 in PBS). The sections were then moved on gelatin-coated glass slides, air-dried overnight at room temperature, dehydrated in an alcohol series, and coverslipped using Pertex mounting medium (HistoLab).

2.14. Proteinase K treatment

To analyze larger aggregates of αSyn in SN, proteinase K (PK) treatment was applied as in Svarcbahs et al. (2016). Briefly described, the striatal and nigral sections were first mounted on gelatin-coated glass slides and dried overnight at 55 °C. The sections were then wetted with tris-buffered saline with 0.05% Tween20 (TTBS) and digested with 10 μg/ml PK (#V3021, Promega) in TTBS for 10 min at 55 °C. The sections were postfixed with 4% PFA for 10 min. After this, they went through oligomer-specific αSyn (αSynO5) IHC with the same primary and secondary antibodies and, same concentrations, as above.

2.15. Microscopy and stereological count of dopaminergic neurons

The optical densities (OD) of TH and αSynO5 from striatum and SN were determined as described in (Kilpeläinen et al., 2019c). Digital images were single layer scanned at 20x magnification with Pannoramic Flash II Scanner (version 1.15.4, 3DHISTECH). Four sections of both striatum and SN from each mouse were processed for further analyses with Pannoramic Viewer (version 1.15.4., 3DHISTECH) and images were converted to greyscale and inverted in ImageJ (version 1.53c, NIH). Line analysis tool (for αSynO5 in striatum) and freehand tool (for αSynO5 in SN and TH in both striatum and SN) in ImageJ were used to measure the ODs of immunoreactivity. To correct the effect of background staining, correction values were obtained from the corpus callosum (for striatum) and cerebral peduncle (for SN). For the αSynO5 following PK treatment, the threshold analysis method was applied to measure the αSyn aggregates immunoreactive area. The data was presented as percentages of the intact side. Four coronal sections were selected for analysis from each mouse and the person performing the analysis was blinded for the treatment groups.

In addition, the number of tyrosine hydroxylase-positive (TH+) cells in SN were estimated using stereological counting algorithm based on convolutional neural networks in the Aiforia Cloud (version RELEAS Ef4.9.HOTFIX 4, Aiforia Technologies). The counting algorithm for TH+ neurons in substantia nigra has been developed and characterized earlier in the study of Pentinen et al. (2019). For this, the digital images were obtained with extended focus at 20x magnification with Pannoramic Flash II Scanner (3DHISTECH). Four coronal sections were selected for analysis from each mouse and the data was presented as mentioned above.

2.16. Western blot (WB)

Nigral punches from the 2-week treatment setups microdialysis group (n = 22) were in mRIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl) containing 1:100 HALT protease inhibitor cocktail (Product #78429, ThermoFisher Scientific) and 1:100 HALT phosphatase inhibitor cocktail (Product # 87786, ThermoFisher Scientific). The lysates were sonicated and centrifuged at 14,000 rpm at +4 °C for 1h. The supernatant, containing the soluble fraction was collected in separate tubes. The pellet (insoluble fraction) was resuspended in 10 μl of 1% SDS in TBS and sonicated further. The lysates were stored at −80 °C until WB experiment.

Markers of protein aggregation (αSynO5, p62), autophagy (LC3B) and oxidative stress (4 hydroxynonenal (4-HNE) modified proteins) were analyzed by WB as in (Svarcbahs et al., 2020). Standard SDS-PAGE protocol was followed and the entire nigral sample was loaded in the wells of stain-free 4–20% Mini PROTEAN TGX precast gels (product #4568094, Bio-Rad, CA, USA) to ensure sufficient protein loading. Blotting was done on PVDF membranes (Trans-blot Turbo Midi 0.2 μm, Product # 1704157, Bio-Rad) with Trans-blot Turbo Transfer System (Bio-Rad). The membranes were blocked with 5% skim milk in TTBS and incubated with primary antibody in 5% skim milk overnight at +4 °C: mouse anti-αSynO5 (1:500, AS132718, Agrisera, Vännäs, Sweden), mouse anti-p62 (1:5000, ab56416, Abcam, Cambridge, UK), mouse anti-4-HNE (1:1000 in 5% skim milk, ab48506, Abcam), rabbit anti-LC3B (1:1000, L7543, Sigma-Aldrich), rabbit anti-vinculin (1:10,000, Loading control, ab129002, Abcam). The secondary antibodies used were goat anti-rabbit, HRP (1:2000, #31460, Thermo Fisher Scientific) and goat anti-mouse, HRP (1:2000 #31430, Thermo Fisher Scientific). As several different proteins were detected from the same membrane, the membranes were stripped (WesternSure ECL stripping buffer 5x, part No 926–9200, Li-Cor). To verify that bands were 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 greyscale format, and the OD of the bands were measured by ImageJ (histogram area analysis; version 1.53c; NIH). The OD obtained from each band was normalized against the corresponding vinculin band, which was used as loading control. The data was represented as ratio of OD from the injected side (left) to the OD of the intact side (right) and analysis on the different markers was done with at least 2 to 3 technical replicates.
2.17. Statistical analysis

For the cellular assay, 3–4 replicate wells were used in each experiment and at least 3 independent experiments were performed. Data are expressed as mean values ± standard error of the mean (mean ± SEM), and negative control average was set as 100% to reduce variability between repeats. Statistical analysis of the data was performed using SPSS statistics (version 27, IBM Corporation) and the figures were made with GraphPad Prism (version 7.04, GraphPad Software Inc.). Mixed two-way ANOVA was used for datasets comprising of repeated measures with several different groups, to assess significant time × treatment interactions, and one-way ANOVA with Tukey’s HSD post hoc test was used for locating the differences between the treatment groups at separate time points. The rest of the data was analyzed using one-way ANOVA with Tukey’s HSD post hoc or Welch ANOVA with Games-Howell post hoc if the assumption of homogeneity of variances was violated (p < 0.05 in the Levene’s test). Data from the tissue HPLC, IHC and WB experiments is represented as % of the intact side. All results are expressed as mean values ± standard error of the mean (mean ± SEM) and were considered statistically significant at p < 0.05.

3. Results

3.1. αSyn oligomer enzyme-linked immunosorbent assay (ELISA) and cell viability

The effect of PREP inhibition in reducing soluble αSyn oligomers was assessed in a cellular model (N2A) utilizing αSyn overexpression and stress-induced aggregation by either oxidative stress by FeCl₃ and H₂O₂ or lactacystin, a proteasome inhibitor. Compounds that currently are in viability by the MTT assay. The amount of soluble αSyn-related mechanisms in PD, were assessed with an ELISA assay and the effect of the treatments on cell viability. αSyn overexpression and αSyn aggregation was induced by oxidative stress (p < 0.05).

In experiments where αSyn aggregation was induced by oxidative stress, the amount of the soluble αSyn oligomers was significantly increased due to the stress (p = 0.031) compared to the non-stressed group (Fig. 2A) (F(5,37) = 9.791, p = 0.00005, one-way ANOVA, Tukey’s HSD post hoc). Moreover, compared to the stressed and vehicle-treated group 10 μM anle138b (p = 0.022), and 5 μM nilotinib (p = 0.000001) significantly decreased the soluble αSyn oligomers, while the 10 μM KYP-2047 nor 1 mM deferiprone did not have an effect.

The lactacystin-induction caused even more prominent significant increase in the soluble αSyn oligomers (p = 0.000004) compared to the non-stressed cells (Fig. 2B) (F(5,32) = 15.905, p = 7.0931 E⁻³, one-way ANOVA, Tukey’s HSD post hoc). Here the 5 μM nilotinib (p = 0.000001) and 1 mM deferiprone (p = 0.014) reduced the oligomers significantly. Neither the 10 μM KYP-2047 nor the 10 μM anle138b had any effect.

The MTT cell viability assay revealed that αSyn aggregation induced by both stressors significantly increased the toxicity compared to non-stressed cells (oxidative stress: p = 0.000159, F(5,36) = 8.203, p = 0.000003, one-way ANOVA, Tukey’s HSD post hoc; Lactacystin: p = 0.000022, F(5,29) = 18.282, 3.4759 E⁻⁴, respectively) (Fig. 2C and D). In the oxidative stress-induced cells, the 10 μM KYP-2047 (p = 0.047) and 10 μM anle138b (p = 0.039) were able to reduce the toxicity compared to vehicle-treated cells. The 1 mM deferiprone reduced the toxicity only non-significantly. Furthermore, in lactacystin-stressed cells, 10 μM anle138b (p = 0.014) was the only compound that was able to significantly reduce the toxicity, while the 5 μM nilotinib promoted the toxicity (p = 0.013) compared to the vehicle-treatment. The 10 μM KYP-2047 and the 1 mM deferiprone non-significantly improved the viability to some extent. The changes in cell viability did not reach significance in the LDH assay (Additional Fig. S1).

3.2. AAV-A53T-αSyn mouse model

3.2.1. Behavioral data and αSyn oligomer levels

In the 2-week KYP-2047 treatment setup, AAV-A53T-αSyn caused only non-significant elevation in the ipsilateral forepaw use 4-weeks after the mutant αSyn injections compared to the AAV-empty group (Fig. 3A). However, in the 4-week treatment setup, AAV-A53T-αSyn injections caused significant asymmetry in spontaneous forepaw use, which was seen as an increase after the AAV-A53T-αSyn injections compared to the AAV-empty group before PREP inhibitor treatment was administrated (Fig. 2C).
Fig. 3. The effect of nigral AAV-A53T-αSyn on mouse behavior and αSyn levels. Nigral AAV-A53T-αSyn injection did not cause asymmetry in spontaneous forepaw use before PREP inhibition treatment was initiated at two weeks (A) but significant impact was seen at four weeks (B) post viral vector injection. PREP inhibition treatment was not able to revert the effects of AAV-A53T-αSyn in the cylinder test in neither of the setups. Immunoreactivity of oligomer-specific αSyn (αSynO5) increases in substantia nigra (SN) and striatum (STR) after nigral AAV-A53T-αSyn injections (C–E), and longer-lasting PREP inhibition was able to prevent progression of the αSyn pathology (E). Representative images of αSynO5 immunostaining are presented in F (2-week treatment) and G (4-week treatment). In addition, nigral AAV-A53T-αSyn injections result in significant formation of proteinase K resistant αSyn aggregates in a time-dependent manner (H–I), and longer-lasting treatment with PREP inhibitor was able to prevent the aggregation process (I). Representative images of proteinase K resistant αSynO5 immunostaining are presented in J–K. (A–B) Lines represent group means ± SEM at different timepoints. The AAV-A53T-αSyn injection is evident on the left (F and J) or right (G and K) side of the brains in the images. (C–E, H–I) Bars represent group means ± SEM, *=p<0.05, **=p<0.01, ***=p<0.001, Welch ANOVA with Games-Howell post hoc or one-way ANOVA with Tukey’s HSD post hoc. *=p<0.05, two-way mixed ANOVA and one-way ANOVA with Tukey HSD post hoc for separate timepoints.
initiated (Fig. 3B). Mixed two-way ANOVA analysis revealed a significant interaction (time*treatment) in the 4-week treatment setup (F(8,168) = 2.831, p = 0.013). Following a significant one-way ANOVA (week 4: F(2,42) = 4.542, p = 0.016; week 8: F(2,42) = 4.506, p = 0.017), the Tukey HSD post hoc pointed at differences between AAV-empty + KYP-2047 vs. AAV-A53T-dSyn + VEH and AAV-A53T-dSyn + KYP-2047 groups at week 4 (p = 0.025 and p = 0.044, respectively) and at week 8 (p = 0.028 and p = 0.041, respectively). PREP inhibition by KYP-2047 was not able to revert the occurrence of the behavioral change in neither of the treatment setups, as we did not find any difference between the vehicle and treatment groups. Even though it has been reported that A53T-dSyn transgenic mice present a hyperactive phenotype (Graham and Sidhu, 2010; Unger et al., 2006), we could not see changes in the endpoint 21 h locomotor activity in the AAV-A53T-dSyn injected mice compared to the empty vector + KYP-2047 control neither in the 2-week nor in the 4-week treatment setups (Additional Fig. S2).

KYP-2047 control neither in the 2-week nor in the 4-week treatment setup, the AAV-A53T-dSyn injections resulted in decreased extracellular dopamine levels after α-amphetamine-sulphate-induced release in both the 2-week and 4-week treatment setups (Fig. 5A and B). In the 2-week treatment setup, the AAV-A53T-dSyn injections caused significant decrease in α-amphetamine-induced extracellular dopamine levels, which could be seen especially during the 30 μM induction (Fig. 5A, time points: 160, 180, 200 min) and were not prevented by PREP inhibition (F(4,32) = 4.7, p = 0.004, mixed two-way ANOVA with one-way ANOVA and Tukey’s HSD post hoc). 180 min: AAV-A53T-dSyn + VEH (p = 0.004), AAV-A53T-dSyn + KYP-2047 (p = 0.007); 200 min: AAV-A53T-dSyn + VEH (p = 0.023). The decrease in extracellular dopamine levels after the AAV-A53T-dSyn injections were present also during the 10 μM α-amphetamine induction (Fig. 5A, time points: 80, 100, 120 min), even though the mixed two-way ANOVA found only a significant main effect of treatment in this dataset (F(2,18) = 6.013, p = 0.01).

In the 4-week treatment setup microdialysis experiments, the extracellular dopamine levels were even more clearly decreased in the AAV-A53T-dSyn groups compared to the AAV-empty group, while PREP inhibition did not have preventive effect here either (Fig. 5B). The differences were significant in all time points during the 10 μM α-amphetamine perfusion (Fig. 5B; time points: 80, 100, 120 min; F(4,34) = 2.852, p = 0.039, mixed two-way ANOVA with one-way ANOVA and Tukey’s HSD post hoc) and 30 μM perfusion (Fig. 5B; time points 160, 180, 200 min; F(4,34) = 4.574, p = 0.005). After significant one-way ANOVA, the Tukey’s HSD post hoc revealed following statistical differences compared to AAV-empty group: 80 min: AAV-A53T-dSyn + VEH (p = 0.015), AAV-A53T-dSyn + VEH + KYP-2047 (p = 0.024); 100 min: p = 0.009 and p = 0.008, respectively; 120 min: p = 0.012 and p = 0.012, respectively; 160 min: p = 0.004 and p = 0.003, respectively; 180 min: p = 0.002 and p = 0.000475, respectively; 200 min: p = 0.003 and p = 0.001, respectively.

3.2.4. Tissue HPLC for dopamine and metabolites

Since TH⁻ immunoreactivity was significantly decreased in the 4-week treatment setup, HPLC tissue analysis was done to study the tissue levels of neurotransmitters in striatum in this time point. One-way ANOVA (F(2,20) = 13.543, p = 0.000191) with Tukey’s HSD revealed a significant decrease in striatal tissue concentrations of dopamine in AAV-A53T-dSyn groups (+VEH: p = 0.002; +KYP-2047: p = 0.000246) compared to the AAV-empty group (Fig. 5C). Despite the more significant decrease in the AAV-dSyn + KYP-2047 compared to the AAV-empty + VEH group, there was not a significant difference between VEH and KYP-2047 groups among the mice who received AAV-A53T-dSyn injections. Significant decreases were also seen in dopamine metabolites (Fig. 5D and E): 3,4-Dihydroxyphenylacetic acid (DOPAC): AAV-empty vs. KYP-2047 + AAV-A53T-dSyn + VEH and AAV-A53T-dSyn + KYP-2047, p = 0.003 and p = 0.015, respectively, with Tukey’s HSD post hoc after a significant one-way ANOVA (F(2,20) = 8.478, p = 0.002); homovanillic acid (HVA): AAV-empty + KYP-2047 vs. AAV-A53T-dSyn + VEH, p = 0.032 with Games-Howell post hoc following significant Welch ANOVA (F(2,20) = 5.052, p = 0.034). Analysis of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) did not show any significant changes (Fig. 5F and G). The tissue HPLC data complements the findings from the microdialysis experiment.
Fig. 4. The effect of nigral AAV-A53T-αSyn on tyrosine hydroxylase positive (TH⁺) cells in nigrostriatal pathway. TH⁺ immunoreactivity decreased time-dependently after nigral (SN) AAV-A53T-αSyn injections, as after 2 weeks only a non-significant reduction is present (A–D) but significant decrease in nigral TH⁺ staining and cell count can be seen 4 weeks after the injections (E–H). No significant differences were evident in TH⁺ staining in the striatum (STR) (C, G) in either treatment setups. KYP-2047 also had no effect on TH⁺ immunoreactivity in either setup. The AAV-A53T-αSyn injection is evident on the left (A) or right (E) side of the brains in the images. Bars represent group means ± SEM. * = p < 0.05, Welch ANOVA with Games-Howell post hoc.
3.2.5. Western blot analysis

Since AAV-A53T-αSyn injections did not show any significant changes in animal behavior at 2-week treatment setup, and KYP-2047 had no effect on behavior or αSyn levels, we wanted to assess other markers for neuronal degeneration by WB. SN samples were collected from the animals of the 2-week treatment setup, and these were used in WB experiment to assess the protein levels of markers of protein aggregation (αSynO5, p62), autophagy (LC3B) and oxidative stress (4-HNE modified proteins) (Fig. 6). In line with the IHC results, the AAV-A53T-αSyn injection resulted in significantly elevated αSynO5 oligomer protein levels in the vehicle treated group (p = 0.000388) compared to the empty vector injected mice. In contrast to 2-week IHC results, PREP inhibition reduced αSynO5 levels (p = 0.035) (Fig. 6A) (F(2,8) = 22.57, p = 0.000513, one-way ANOVA with Tukey’s HSD post hoc). In support of these findings, a similar effect was seen in the levels of p62 in the soluble fraction (Fig. 6B) (AAV-empty + KYP-2047 vs. AAV-A53T-αSyn + VEH, p = 0.011; AAV-A53T-αSyn + VEH vs. AAV-A53T-αSyn + KYP-2047, p = 0.044; F(2,11) = 7.114, p = 0.01, one-way ANOVA with Tukey’s HSD post hoc). A significant difference could also be detected in the oxidative stress marker, 4-HNE modified proteins, as their levels were significantly elevated after the AAV-A53T-αSyn injections in the vehicle treated group compared to the AAV-empty group (p = 0.000279). Furthermore, PREP inhibition led to a significant reduction of 4-HNE levels (AAV-A53T-αSyn + VEH vs. AAV-A53T-αSyn + KYP-2047, p = 0.014, F(2,10) = 19.45, p = 0.000358, one-way ANOVA with Tukey’s HSD post hoc) (Fig. 6C). A similar trend was observed in the levels of LC3BII, but these differences were not significant (Fig. 6D).

4. Discussion

Here, our goal was to assess the effects of a PREP inhibitor, KYP-2047, on the toxic soluble αSyn oligomers and assess its protective effect on dopaminergic neurons in vivo by using a PD mouse model based on unilateral AAV-A53T-αSyn virus vector injection. Our results show that KYP-2047 did not remove soluble αSyn oligomers but protected cells in αSyn overexpression model, and then again the removal of insoluble αSyn forms did not protect TH+ neurons in SN in vivo. This raises important questions concerning the αSyn targeting therapies for PD.

In our cell culture assay that was based on wild type αSyn overexpression and induction of αSyn aggregation by using oxidative stress or proteasomal inhibition, we observed that removal of soluble αSyn oligomers detected by ELISA did not correlate with cell survival. C-abl kinase inhibitor, nilotinib, removed effectively αSyn oligomers in both setups. However, this did not correlate with improved cell survival, and in lactacystin stressed cells, nilotinib even reduced cell viability. This suggests that removal of αSyn species detected by MJF-14-6-4-2 antibody (Lassen et al., 2018) are not particularly toxic and then again nilotinib may mediate toxicity via autophagy-induced apoptosis as has been suggested earlier (Shaker et al., 2013). In contrast, KYP-2047 had no effects on αSyn oligomers on either of the setups but it improved cell viability on oxidative stress stressed cells. Anle138b reduced significantly αSyn oligomers when aggregation was induced by oxidative stress but had no effect on lactacystin-induced aggregation. However, anle138b was protective for cells in both assays. Dferiprone was close to significance in the cellular protection after oxidative stress but similarly to KYP-2047, it did not have effect on αSyn oligomers. However, deferiprone is an iron chelator and it is obvious that it can reduce the toxicity of oxidative stress where FeCl2 is involved. As the compounds were added together with the stressor, it seems plausible that anle138b blocked aggregation early enough to also block the formation of all later toxic species. However, this suggests that anle138b may be effective only when added at the beginning of the αSyn aggregation process, which may not be optimal in the clinical PD where the αSyn aggregation process has already started.
process has been ongoing for decades. The protective effect of KYP-2047 in the oxidative stress model may be partially explained by its impact on ROS production under oxidative stress (Eteläinen et al., 2021). Earlier studies by KYP-2047 have shown that PREP inhibition reduces particularly αSyn species in SDS-fraction after oxidative stress and lactacystin exposures in αSyn overexpressing cells (Svarcbahs et al., 2019). However, in HEK-293 and PREP knock-out HEK-293 cells that were transiently transfected with αSyn, overexpression nor restoration of PREP did not increase αSyn in SDS-fraction when aggregation was induced by oxidative stress with FeCl₃–H₂O₂ treatment (Svarcbahs et al., 2018). Based on this, we hypothesized that PREP and its inhibition would have effect on soluble αSyn oligomers. However, here our results suggest that PREP inhibition has an effect on other oligomeric species than detected by current ELISA, and that these forms are more toxic for the cells.

To further assess the effect of PREP inhibition on different αSyn forms in vivo, we tested KYP-2047 on an AAV-A53T-αSyn virus vector model. The threonine substitution of alanine at 53rd amino acid disrupts the α-helix and extends the β-sheet structure in the corresponding location on the protein structure, promoting self-aggregation and formation of further pathological aggregates (Li et al., 2002; Polymeropoulos et al., 1997; Teravskis et al., 2018; Völles et al., 2001). Therefore, the use of AAV-A53T-αSyn would allow us to characterize the effect of KYP-2047 on rapidly aggregating fibrillar forms of αSyn. Additionally, AAV-A53T-αSyn significantly decreased TH+ neurons in SN in an earlier study (Ip et al., 2017), and we wanted to assess if KYP-2047 has protective effect on dopaminergic neurons of SN. Although we have already shown the efficacy of KYP-2047 on wild-type AAV-αSyn model, wild-type AAV-αSyn did not cause significant dopaminergic cell toxicity (Svarcbahs et al., 2016). We showed that two weeks exposure time after nigral AAV-A53T-αSyn injections was not enough to produce a significant asymmetry in the spontaneous forepaw use compared to the empty vector injected mice, but again, four weeks after the injections, the asymmetry was significant. This finding suggests a progressive impairment in the pathological behavior in this model. Similarly, Ip et al. (2017) have also described manifestation of significant asymmetry in spontaneous forepaw use at five and nine weeks after unilateral AAV-A53T-αSyn injections. Based on our earlier study by Svarcbahs et al. (2016), KYP-2047 treatment was initiated at a time of significant symptoms (4-weeks post-injection), and at time of mild symptoms (2-weeks post-injection) to reflect the human PD where the diagnosis is typically based on motor symptoms. However, unlike in the model based on wild type AAV αSyn injection (Svarcbahs et al., 2016), the motor deficit could not be improved with chronic PREP inhibition in either of the treatment setups. Although KYP-2047 did not have effects on mouse behavior in the cylinder test or in the 21-h locomotor activity test, it had significant impact on total oligomeric αSyn (detected by αSynO5 antibody) and particularly on proteinase K resistant αSyn species after 4-week treatment. AAV-A53T-αSyn caused an increase in the αSynO5 staining in the SN and STR at the 2-week treatment setup after the AAV-A53T-αSyn injections, but PREP inhibitor treatment did not result in changes in the αSynO5 levels at this time point when detected by IHC. However, our WB results from the 2-week treatment setup showed that PREP inhibitor treatment reduced certain αSynO5 levels in the SN. The effect by KYP-2047 was seen in RIPA soluble monomeric αSyn in WB, and as IHC reveals various forms of αSyn aggregates (Brannstrom et al., 2014), it
appears that PREP inhibition was able to induce clearance of more soluble and possibly monomeric forms of αSyn. Furthermore, PREP inhibition did not reduce the PK-resistant αSyn aggregates after 2-week treatment, but only after the longer-lasting 4-week treatment. Therefore, the effect of KYP-2047 on PK-resistant αSyn forms after the 4-week treatment may arise from the ability to reduce smaller αSyn forms that can act as seeds for more aggregated, insoluble aggregates. This is supported by an earlier study using less aggregation-prone AAV-Wt-αSyn, where only a small amount of PK-resistant αSyn species were seen, and where the 4-week treatment by KYP-2047 had significantly smaller effect on the PK-resistant αSyn oligomers compared to the total αSyn oligomers (Svarcbahs et al., 2016). KYP-2047 can also induce autophagy that further boosts the degradation of αSyn oligomers and even preformed fibrils (Rostami et al., 2020; Savolainen et al., 2014; Svarcbahs et al., 2016). This was also seen in the current study since the other protein accumulation marker, the autophagy receptor p62 (Lamark et al., 2017), was also significantly decreased after PREP inhibition in the nigral tissue soluble fraction. Earlier data about the effect of KYP-2047 on performed αSyn fibrils (Rostami et al., 2020) and our current results on PK-resistant αSyn species suggests that KYP-2047-induced autophagy is particularly important in the degradation of larger oligomeric species. Another interesting finding was the reduced levels of the marker for oxidative stress, 4-HNE modified proteins, after PREP inhibitor treatment. This in line with previous reports, as it has already been shown that PREP inhibition is able to reduce oxidative stress in cellular models (Eteläinen et al., 2021; Puttonen et al., 2006). Notably, even though we managed to decrease the αSyn oligomers and proteinase K resistant oligomers with the longer, 4-week KYP-2047 treatment, we failed to protect the nigrostriatal TH− neurons that were significantly decreased in SN. A significant decrease was not seen in the 2-week setup, which suggests a progressive nature for the model and neuronal damage. In the SN, TH− OD showed a more pronounced decrease than the TH+ neuron count, which may arise from the down-regulating effect of αSyn on TH (Alerte et al., 2008; Lou et al., 2010). However, the TH− decrease was not as evident in striatum, and to study the functionality of the striatal dopaminergic system, and to verify the dopamine depletion, we performed a microdialysis study, which to our knowledge, was implemented for the first time in an AAV-A53T-αSyn mouse model. A significant difference was seen in the n-amphetamine-sulphate-induced release of extracellular dopamine levels, indicating disturbances in dopaminergic neurotransmission and/or dopaminergic cell death after the AAV-A53T-αSyn injections. This suggests to abnormalities in the dopamine transporter (DAT) functions that are implicated in the pathogenesis of PD (Kisos et al., 2014). Direct protein-protein interaction between αSyn and DAT has been reported to downregulate DAT activity via clathrin-mediated endocytosis (Bellucci et al., 2011; Sorkina et al., 2005, 2013; Wersinger and Sidhu, 2005), and Kisos et al. (2014) demonstrated that the DAT-αSyn interaction resulted in lower DAT levels on the cell membranes in the brain of transgenic A53T-αSyn mice, that is in line with our microdialysis results. Since TH− immunoreactivity was not significantly reduced in the striatum, it is likely that behavioral deficits are partially due to disturbances in DAT function, and not solely due to the degeneration of the nigrostriatal dopaminergic neurons. Tissue HPLC data from the 4-week treatment setup confirmed TH− immunostaining and microdialysis results, as we could observe reduced tissue levels of striatal dopamine and its metabolites (DOPAC and HVA) after the A53T-αSyn overexpression. Then again, PREP inhibitor did not have effects on dopamine release or dopaminergic markers that is in line with TH− and behavioral results.

5. Conclusions

Our results showed that although KYP-2047 did not reduce soluble αSyn oligomers it was protective in a cell culture model of αSyn aggregation, whereas in vivo, more than 50% reduction in proteinase K resistant αSyn oligomers did not provide protection for the nigrostriatal dopaminergic system. Therefore, it appears that KYP-2047 can reduce toxic forms of αSyn in cells that were not detected by soluble αSyn ELISA assay. Anle138b that blocks the oligomerization process at early phase of αSyn aggregation was effective and protective in the cell culture assay. Therefore, it could have been possible that earlier initiation of the KYP-2047 treatment in vivo would also have blocked the rapidly forming A53T-αSyn oligomers at early stage (Conway et al., 2000), and resulted in a different outcome. However, this would not have been a disease-like situation, as PD is generally diagnosed based on already existing movement impairments with the ongoing αSyn aggregation process. When considering a potential drug treatment for PD and other synucleinopathies, our results in cellular and in vivo models strongly suggest that it is crucial to further characterize αSyn aggregation process, and identify the toxic forms of αSyn as the removal of random αSyn forms does not provide neuroprotection. This should be taken into consideration particularly with antibody therapies that are often epitope or structure specific. Our results also indicate that best effects could be achieved when the treatment is initiated at the time of toxic insult or early in the pathophysiological process. However, this would require earlier biomarkers for PD.

Ethics approval

Animal experiments were conducted according to the 3R principles of the EU directive 2010/63/EU regarding the care and use of experimental animals and following the local laws and regulations (Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes (497/2013), Government Degree on the Protection of Animals Used for Scientific or Educational Purposes (564/2013)). The experiment protocols were authorized by the national Animal Experiment Board of Finland (ESAVI/42235/2019).

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Tony S. Eteläinen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. Tommi P. Kilpeläinen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. Samuli Auno: Data curation, Investigation, Validation, Writing – review & editing. Francesca De Lorenzo: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. Johanna K. Uhari-Vaananen: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Validation, Writing – review & editing. Ulrika H. Julku: Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing. Timo T. Myöhänen: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.
Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability

Data will be made available on request.

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List of abbreviations

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References

4-HNE 4-hydroxynonenal
5-HT 5-hydroxytryptamine;
5-HIAA 5-hydroxyindoleacetic acid
AAV adeno-associated virus
α-syn alpha-synuclein
αSynOS oligomer-specific αsyn
CBA β-actin promoter
CMV cytomegalovirus
DAB di-aminobenzidine;
DOPAC 3,4-Dihydroxyphenylacetic acid
ELISA enzyme-linked immunosorbent assay
HVA homovanillic acid
IHC immunohistochemistry
i.p. intra peritoneal
KYP-2047 4-phenylbutanoyl-1-prolyl-2(S)-cyanopyrrolidine;
LDH lactate hydrogenase
MTT 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide;
N2A Neuro2A murine neuroblastoma cell culture
OD optical density
PD Parkinson’s disease
PREP prolyl oligopeptidase
PK proteinase K
ROS reactive oxygen species
SN substantia nigra pars compacta
TH tyrosine hydroxylase-positive
WB Western blot

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2022.109213.

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Data availability

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List of abbreviations

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