Prosaposin Reduces α-Synuclein in Cells and Saposin C Dislodges it from Glucosylceramide-enriched Lipid Membranes

Rika Kojima1 · Mark Zurbruegg1 · Tianyi Li1 · Wojciech Paslawski1 · Xiaoqun Zhang1 · Per Svenningsson1,2

Received: 13 July 2022 / Accepted: 10 September 2022 / Published online: 24 September 2022
© The Author(s) 2022

Abstract Parkinson’s disease (PD) is the second most common progressive neurodegenerative disorder affecting over 1% of the 65+ age population. Saposin C, a lysosomal protein required for the normal activity of glucocerebrosidase (GCase), may serve as a disease modifier in PD. Saposin C is cleaved from its precursor, Prosaposin (PSAP), which is secreted as an uncleaved protein and exerts neuroprotective effects. In this study, we aim to elucidate the neuroprotective roles of PSAP and saposin C in PD by evaluating their effects on α-synuclein accumulation in human neuroblastoma cells. Stable overexpression of PSAP reduced monomeric α-synuclein levels in SH-SY5Y cells, while PSAP knockdown by small interfering RNA led to the opposite effect, and those effects were independent of GCase activity. Autophagy flux was decreased by stable PSAP overexpression. Furthermore, a flow-through assay revealed that recombinant saposin C was able to detach α-synuclein from artificial glucosylceramide-enriched lipid membranes at the lysosomal pH. Taken together, our findings provide further evidence that PSAP and saposin C as key proteins involved in α-synuclein clearance by dislodging it from lipid membranes.

Keywords α-Synuclein · Autophagy · Glucocerebrosidase · Prosaposin · Saposin C

Introduction Parkinson’s disease (PD) is the second most common progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra (Poewe et al. 2017). The dopamine deficiency caused by the dopaminergic cell death results in motor symptoms particularly rest tremors, rigidity, and bradykinesia. Those symptoms can be counteracted by dopamine replacement therapies, especially in the early stages of the disease (Lees et al. 2009). However, the effect of the medication wears off as the disease progresses (Armstrong and Okun 2020). Although many patients are coping with the disease by controlling symptoms with the help of medications or deep brain stimulation, there are still no treatments that can slow down the progression of PD.

The causes of the specific neuronal cell death remain unknown, but misfolding and aggregation of α-synuclein are involved in PD pathology (Braak et al. 2003; Meade and Fairlie 2019). α-Synuclein is a major component of Lewy bodies, which are inclusion bodies found in affected neurons of PD patients (Lashuel et al. 2012). α-Synuclein is part of the SNARE complex and its normal function relates to neurotransmitter release (Burré et al. 2010) and some mutations or multiplications in the SNCA gene, which encodes α-synuclein, are known to cause PD (Polymeropoulos et al. 1997).

Mutations in the GBA1 gene are the most common risk factor for developing PD (Do et al. 2019). The GBA1 gene encodes glucocerebrosidase (GCase), a lysosomal protein which breaks down glucocerebroside into glucose and ceramide. More than 495 mutations in the GBA1 gene are known to cause impairment in glycosphingolipid metabolism, and GBA1 mutation homozygosity leads to Gaucher’s disease (GD) (Behl et al. 2021). Even asymptomatic heterozygote GBA1 mutation carriers have a higher risk of developing PD, which is comparable to GD patients (Avenali et al. 2020). Despite the fact that their GCase activity is strongly reduced, only a smaller set of GD patients develop PD symptoms (Alcalay et al. 2014). Although reduced GCase activity is
associated with both idiopathic and familial PD (Alcalay et al. 2015), it is still largely unknown how GCase contributes to the development of PD.

Prosaposin (PSAP) is a neurotrophic and neuroprotective factor that can be found in various body fluids such as milk and cerebrospinal fluid (Hineno et al. 1991; O’Brien et al. 1994; Sikora et al. 2007). In addition to its neuroprotective roles, PSAP is cleaved in the late endosomes/lysosomes and serves as a precursor of saposins A–D, which are essential activators for several lysosomal hydrolases (Kishimoto et al. 1992; Leonova et al. 1996). Complete loss of PSAP leads to a neonatally lethal phenotype in both mice and humans (Harzer et al. 1989; Fujita et al. 1996), indicating that PSAP plays an important role in normal cellular functions.

Among four saposins, saposin C, which is required for a normal function of GCase, has been suggested as a disease modifier of GD (Tamargo et al. 2012). Deficiency in saposin C is known to cause neuropathological types of GD (Vaccaro et al. 2010; Tamargo et al. 2012). Yap et al. have shown that saposin C protects GCase against α-synuclein inhibition by displacing α-synuclein from lipid membranes (Yap et al. 2013). Also, previous studies have reported that the neuroprotective effect of PSAP is attributed to a short amino acid sequence in the N-terminal part of the saposin C region (O’Brien et al. 1995; Gao et al. 2013). TX14(a), a 14-mer prosaptide derived from the neurotrophic region in the saposin C domain of PSAP has neuroprotective effects equivalent to full-length PSAP (Hiraiwa et al. 1997b; Tsuboi et al. 1998). Some studies argue that the neuroprotective effects of prosaptides are mediated by the orphan G-protein coupled receptors, GPR37 and GPR37L1, but no consensus has been reached on this argument (Meyer et al. 2013; Leinartaité and Svenningsson 2017; Giddens et al. 2017; Liu et al. 2018).

In this study, we showed that overexpression of PSAP reduced monomeric α-synuclein levels in SH-SY5Y cells while PSAP knockdown by siRNA lead to the opposite effect, and those effects were independent of GCase activity. Moreover, we found that recombinant saposin C was able to dislodge α-synuclein from the artificial glucosylceramide-enriched vesicles at the lysosomal pH 5.4. Our findings suggest that PSAP and saposin C work as neuroprotective factors at least partly by inhibiting α-synuclein aggregation via replacing it from the lysosomal membrane.

**Materials and Methods**

**Chemicals**

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Merck KGaA) and were of analytical grade. All solutions were made using Milli-Q deionized water (Millipore).

**Cell Culture**

SH-SY5Y cells were purchased from American Type Culture Collection (CRL-2266™, 70019544) and grown in a 1:1 mixing of MEM (11095–080, Gibco) and F-12 K (21127–022, Gibco) containing 10% FBS (10500–064, Gibco), NEAA (11140–035, Gibco), Sodium Pyruvate (11360–039, Gibco), and Penicillin–Streptomycin (100U/mL, 15140–122, Gibco). Cells were split 1:5 every 3–5 days.

**Generation of Transgenic Cell Lines**

PSAP-GFP (HG16224-ACG, Sino Biological) and control EGFP (13031, Addgene) plasmids underwent amplification in E. coli and were purified using plasmid extraction kits (21413, Qiagen). Transfection into SH-SY5Y cells was done using Lipofectamine 2000 (11668019, Invitrogen) as per manufacturer instructions. PSAP-OE cells were selected by 200 μg/mL of Hygromycin B (10687010, Gibco) for 30 days. EGFP-OE cells were selected by 300 μg/mL of Geneticin (10131027, Gibco) for 30 days. Stable overexpression of EGFP or PSAP-GFP has been confirmed by Western blot and observation under a fluorescent microscope. Cells were subsequently kept in low concentration antibiotics (100 μg/mL) during experiments. GFP fluorescence was examined routinely during experiments to ensure consistent protein expression.

**GCase Activity Assay**

Cells were washed with PBS and lysed in TNT buffer (100 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.2% Triton X-100) containing protease inhibitor (cOmplete™ Mini, 04693159001, Roche) for 30 min on ice. Cells were spun down at 16,000×g for 10 min at 4 °C and supernatant was collected. Total protein content in lysates was determined by bichronic acid (BCA) assay (23225, Thermo Scientific). For non-specific GBA activity, 30 μg of the total protein was transferred into an Eppendorf tube and mixed with the activity assay buffer (Citrate–Phosphate buffer pH 5.4, 1% bovine serum albumin, 0.25% Triton X-100, 0.25% mM Sodium taurocholate, 0.1% EDTA) and 1 mM 4-methylumbelliferyl β-d-glucopyranoside (44059, Glycosynth). The Eppendorf tubes were inverted gently multiple times to ensure equal distribution of reagents and then incubated for 2 h at 37 °C. The reaction was terminated by the addition of 25% 1 M Glycine–NaOH buffer (pH 10.5). The reaction product was loaded into a black 96 well plate in duplicate. Fluorescence was measured on a Tecan Spark 10 M (Tecan, Switzerland).
(Ex: 360 / Em: 449). For GCcase activity, 30 μg of the total protein was mixed with the activity assay buffer and incubated with 5 nM AMP-dNM (10010332, Cayman Chemical) for 30 min at room temperature. Then, 1 mM 4-methylumbelliferyl β-d-glucopyranoside (M3633, Sigma-Aldrich) was added to the lysates. The reaction mixture was loaded into a black 96 well plate in duplicate and the fluorescence was measured on a Tecan Spark 10 M every 15 min for 9 h at 37 °C. GCcase activity was determined by analyzing the relative slopes calculated from 0 to 6 h measurements.

### Live-cell Imaging with Confocal Microscopy

Cells were seeded on an 8 well-chambered coverglass (155411, Thermo Fisher Scientific) and imaged on a Carl Zeiss LSM 880 confocal microscope using a 40×1.4 NA oil immersion objective. Before the imaging was performed, cell culture media was replaced with phenol red-free media. 50 nM LysoTracker™ Deep Red (L12492, Invitrogen) was directly added to the cell culture media and incubated for 15–60 min before the image acquisition. For RAB5/RAB7 co-localization experiment, RAB5-RFP or RAB7-RFP plasmids (14437 and 14436, Addgene) were transfected to PSAP-OE cells 24 h before the experiment using Lipofectamine 2000 (11668019, Invitrogen) as per manufacturer instructions.

### Pharmacological Treatments

Pharmacological treatments were done by replacing media containing each drug. Condruritol B epoxide (CBE) (C5424, Sigma-Aldrich), Bafilomycin A1 (B1793, Sigma-Aldrich), or MG132 (1748, Tocris) was added to the cell culture media in the cell culture media containing CBE, Bafilomycin A1, or MG132 for 5 days, 2 h, or 4 h, respectively. For CBE treatment, old cell culture media was replaced with fresh media containing CBE after 72 h. After the treatment, cells were collected and subjected to Western blot and/or GCcase activity assay.

### siRNA Transfection

**PSAP** siRNA (s529240, Thermo Fisher Scientific), and Negative control siRNA, (AM4611, Thermo Fisher Scientific) were diluted in Opti-MEM (11058021, Thermo Fisher Scientific) and mixed with Lipofectamine RNAiMax (13778030, Thermo Fisher Scientific). The mixture was mixed by tapping and incubated for 5 min at room temperature. Old media was removed and fresh media containing 10 nM siRNA was added. Cells were cultured for 10 days. Cells were split upon confluence. New siRNA/Lipofectamine was added every 72 or 96 h. After 10 days, cells were collected and subjected to Western blot and real-time PCR analyses.

### Western Blot

Cell lysates were extracted in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitor (cOmplete™ Mini, 04693159001, Roche). The protein content of samples was determined by BCA assay (23225, Thermo Fisher Scientific). Samples were diluted to equal protein concentrations in lysis buffer and mixed with 4×SDS-loading buffer (1610747, Bio-rad). 10 or 20 μg of the sample was loaded into each well. The semi-dry transfer was done at 2.5 A for 11 min onto the PVDF membrane (1704275, Bio-Rad). Membranes containing α-synuclein were placed in 4% PFA, 0.02% glutaraldehyde for 30 min and subsequently washed 3 times with TBS-T. Membranes were then blocked in 5% bovine serum albumin (A9647, Sigma-Aldrich) or Intercept™ (TBS) Blocking buffer (927–60001, LI-COR). Primary antibodies were applied overnight at 4 °C, except for β-actin which was applied for 2 h at room temperature. Antibodies used in the study are indicated in Table 1.

The signals from target proteins were read on LI-COR Odyssey CLx (LI-COR Biosciences) or ChemiDoc™ MP Imaging System (Bio-rad). Western blot quantification was done by Image Studio ver 5.2. Obtained signals from each target protein were normalized to the signals from the housekeeping proteins (β-actin or GAPDH) in the same lane. Relative signal intensities represented in the percentage of the control group were calculated by each membrane. Then, the obtained relative signal intensities from each replicated experiment were integrated and statistically analyzed using GraphPad Prism v9.00, 7.03 or 5.04. The original whole blots are in Supplementary Fig. 1.

### Real-time PCR

RNA was extracted with RNeasy Plus Mini Kit (74134, Qiagen). RNA concentration and quality were determined using Spark 10 M with a NanoQuant inset. QuantiTect Reverse Transcription Kit (205311, Qiagen) was used for cDNA synthesis. Real-time PCR was performed using Sso Advanced Universal SYBR green supermix (1725272, Bio-rad) with 2.5–5 ng of cDNA per reaction. hRPL19 was used as an internal control to which the relative expression of SNCA and PSAP mRNA was normalized. The following primers were used: hRPL19 5′-ATGTATACAGCCTG TACCTG-3′- and 5′-TTCTTTGCTCTTCTCCTTG-3′-, hSNCA 5′-ACCAACAGGGTGTGGCAGAAG-3′- and
5′-CTTGCTCTTTGGTCTTCTCAGCC-3′, hPSAP 5′-CCCCGTCCTGGACTGAAAG-3′ and 5′-TATGTC GCAGGGAAGGATT-3′. The reaction was initiated at 95 °C for 30 s and then 39 cycles of 95 °C for 15 s followed by 60 °C for 30 s. Melt curve analysis was performed to ensure the specificity of amplicons. Ct-values were converted to RNA expression levels using the 2^{-ΔΔCt} method.

α-Synuclein ELISA
Extracellular α-synuclein concentration was measured using the Human α-Synuclein ELISA Kit (#844101, BioLegend) by following the manufacturer’s instructions. Obtained α-synuclein concentrations from 200 µL of undiluted cell culture media were normalized to total protein concentration from cells. For sample collection, cells were seeded on a 24 well plate and grown at 80–90% confluence. After the medium collection, cells were lysed with RIPA buffer and total protein was determined by BCA assay for normalization.

Generation of Glucosylceramide Lipid Vesicles
Vesicles were generated as previously described (Taguchi et al. 2017). Shortly, glucosylceramide and phosphocholine lipids were obtained from Avanti polar lipids (131304P, 840051P, Avanti Polar Lipids). Lipids were dissolved in a 2:1 chloroform/methanol mixture and dried in a glass vial under a nitrogen stream. Dried lipids were rehydrated in either 0.1 M phosphate buffer (pH 7.4) or citrate phosphate buffer (pH 5.4) for 1 h at room temperature to an end concentration of 138 µM with 25:75% experimental glucosylceramide: phosphocholine ratio. Vials were briefly vortexed and placed in an ultrasonic cleaning bath for 30 min. The lipids underwent two freeze/thaw cycles and were placed back in the bath for another 60 min. Lipids were extruded immediately to generate 100 nm diameter vesicles using the Avanti lipid extruder (610023, Avanti Polar Lipids).

Generation of Recombinant A-synuclein and Saposin C
α-Synuclein was prepared as previously described (Paslawski et al. 2016). Saposin C vector pET16b (Ahn et al. 2006), containing the insert coding for human saposin C, was expressed in E. coli Rosetta™ 2 (DE3) competent cells using an autoinduction method. The cells’ pellet was collected by centrifugation, suspended in the osmotic shock buffer (20 mM Tris–HCl, pH 7.2, 40% sucrose), incubated for 10 min and centrifuged again. Next, cells were treated with ice-cold deionized water, supplemented with saturated MgCl₂ solution and briefly incubated on ice. The soluble fraction was collected, and the majority of proteins were depleted by adjusting pH to 3.5 with 1 M HCl. The remaining supernatant was collected by centrifugation and pH was adjusted to 7.5 with 1 M NaOH. The solution was filtered and fractionated on a Q-Sepharose column. Fractions containing saposin C were identified by SDS-PAGE and/or Western Blot, pooled together, dialyzed against deionized water and lyophilized. Obtained protein powder was suspended in 1× PBS and fractionated using Superose 6 size exclusion column connected to the ÄKTA Explorer system (GE Healthcare). Afterwards, fractions containing saposin C were identified by SDS-PAGE and/or Western Blot, pooled together, dialyzed against deionized water and concentration was determined using Nanodrop ND-1000 (Thermo Fisher Scientific). Finally, the protein was aliquoted, lyophilized and stored at −20 °C.

Table 1 Antibodies used for Western blot

| Antibody          | Host     | Company        | Cat. #       | Dilution factor |
|-------------------|----------|----------------|--------------|-----------------|
| Primary antibodies|          |                |              |                 |
| Anti-α-synuclein  | Rabbit   | SantaCruz      | sc-7011-R    | 1:2000          |
| Anti-PSAP         | Rabbit   | Atlas antibodies| HPA004426 | 1:1000          |
| Anti-GCase        | Rabbit   | Abcam          | Ab128879     | 1:1000          |
| Anti-LC3b         | Rabbit   | Cell Signaling | 2775S        | 1:1000          |
| Anti-SQSTM1/p62   | Rabbit   | Cell Signaling | 5114S        | 1:1000          |
| Anti-β-actin      | Mouse    | Sigma-Aldrich  | A2228        | 1:10,000        |
| Anti-GAPDH (6C5)  | Mouse    | SantaCruz      | sc-32233     | 1:1000          |
| Anti-GFP          | Rabbit   | Abcam          | AB6556       | 1:3000          |
| Secondary antibodies|       |                |              |                 |
| IRDye 800CW Goat Anti-Rabbit IgG | Goat | Licor | P/N 926–32211 | 1:20,000 |
| IRDye 680RD Goat Anti-Mouse IgG | Goat | Licor | P/N 926–68070 | 1:20,000 |

2316 Journal of Molecular Neuroscience (2022) 72:2313–2325
Flow-through Assay

Fresh lipid vesicles were transferred to a clear 96 well plate and incubated in the presence of 5 μM recombinant α-synuclein and 5 μM experimental protein. Short synthetic saposin C peptides (TX14(a)) were obtained from Synpeptide and their purity and identity were verified by mass-spectrometry (Shanghai, China). Plates were incubated at 37 °C, with 95% humidity, 5% CO₂ for 2 h. Incubated vesicles were loaded onto Ultraceł 100 kDa spin filters (UFC510096, Merck-Millipore). Samples were spun at 14,000 × g for 30 min. Spin filters were washed by the addition of extra buffer followed by a 10-min spin at 14,000 × g. Spin filters were inverted in a new collection tube and the retained fraction was collected by spinning at 1000 × g for 2 min. Retained and flow-through fractions are mixed with 5× loading buffer, heated to 95 °C immediately after spinning and subjected to Western blot analysis.

Statistics

GraphPad Prism v9.00, 7.03 or 5.04 was used for the generation of all graphs and statistical analysis. Error bars represent the SEM. Student’s t-test or Mann–Whitney U test was used for pairwise comparisons depending on the normality of distribution. One-way ANOVA with Dunnett’s multiple comparison test or two-way ANOVA with Tukey’s multiple comparison test was used to compare multiple variables. Significance was set as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

Results

Overexpression of PSAP Lowers Levels of A-synuclein

To investigate the effect of PSAP expression on α-synuclein accumulation, we generated SH-SY5Y cells stably overexpressing PSAP (PSAP-OE cells). We used the plasmid containing green fluorescent protein (GFP)–linked PSAP, which allows us to monitor intercellular trafficking of exogenous PSAP. As a control, SH-SY5Y cells stably overexpressing enhanced-GFP (EGFP-OE cells) were also created. Overexpression of PSAP-GFP or EGFP from the plasmids was confirmed by Western blot (Fig. 1a). To check whether the exogenous PSAP-GFP is properly trafficked in the cells, live-cell imaging was performed. The lysosome was stained with LysoTracker, a fluorescent dye for labeling and trafficking the lysosome in live cells (Fig. 1b). Co-localization of PSAP-GFP and LysoTracker was observed in most of the cells, which indicates proper targeting of the lysosome. In addition, we also detected that a part of PSAP-GFP was co-localized with RAB5 and RAB7, which are early and late endosome marker proteins, respectively (Chavrier et al. 1990), suggesting that the exogenous PSAP was secreted into the cell culture medium and taken up by the neighboring cells (Supplementary Fig. 2).

Next, α-synuclein levels in PSAP-OE and EGFP-OE cells were examined by Western blot. We showed that monomeric α-synuclein levels were significantly decreased in PSAP-OE cells compared with control EGFP-OE cells (Fig. 1c, d; Mann–Whitney U test: p = 0.0175). On the other hand, mRNA levels of SNCA were not affected by PSAP overexpression, suggesting that PSAP decrease the amount of α-synuclein at protein levels independent of gene transcription (Fig. 1e; Mann–Whitney U test: p = 0.6991). To examine α-synuclein secretion in EGFP-OE and PSAP-OE cells, α-synuclein levels in the cell culture medium were analyzed by enzyme-linked immunosorbent assay (ELISA). Extracellular α-synuclein was significantly decreased in PSAP-OE cells (Fig. 1f, Mann–Whitney U test: p = 0.0011). Thus, we showed that overexpression of PSAP lowers both intra- and extracellular α-synuclein levels without changing mRNA levels.

As Saposin C is known to enhance GCase activity (Weiler et al. 1993), we demonstrated the effects of PSAP overexpression on GCase expression and activity. The expression levels of GCase were not changed by PSAP overexpression (Fig. 1g, h; Mann–Whitney U test: p = 0.2345). Then, we examined non-specific GBA activity and specific GCase activity by treating the cells with 5 nM AMP-deoxynojirimycin (AMP-dNM), a specific GBA2 inhibitor (Overkleeft et al. 1998; Schöndorf et al. 2014) (Fig. 1i; two-way ANOVA with Tukey’s multiple comparison test: EGFP Control vs. PSAP Control: p = 0.7938; EGFP Control vs. EGFP AMP-dNM: p < 0.0001; PSAP Control vs. PSAP AMP-dNM: p = 0.8055; EGFP AMP-dNM vs. PSAP AMP-dNM: p = 0.0005).

GCase activity, represented as AMP-dNM unaffected fraction of the non-specific GBA activity, was 34% in EGFP-OE cells and 90% in PSAP-OE cells. Considering that PSAP overexpression does not alter GCase levels and non-specific GBA activity, this result suggests that PSAP may be able to compensate for disrupted GBA2 enzyme activity by promoting GCase activity.

The Decrease in Levels of A-synuclein is GCase Activity Independent

To investigate whether GCase activity is accounting for the reduction of α-synuclein levels in PSAP-OE cells, we used Conduritol B epoxide (CBE), an irreversible inhibitor of GCase, to suppress GCase activity in the cells. PSAP-OE cells were treated with CBE for 5 days. We confirmed that 100 µM CBE treatment potently decreased
Overexpression of Prosaposin (PSAP) lowers levels of α-synuclein. (a) Representative Western Blot for anti-GFP (Left) and anti-PSAP (Right) in EGFP-Overexpressing (EGFP-OE) cells and PSAP-Overexpressing (PSAP-OE) cells showing PSAP-GFP at 105 kDa, endogenous PSAP at 70 kDa, and control EGFP at 32 kDa. (b) Representative live-cell images of PSAP-OE cells for PSAP-GFP co-localized with the lysosome (Green: PSAP-GFP, Magenta: LysoTracker, scale bar 5 µm). (c) Representative Western Blot for anti-α-synuclein and anti-β-actin in EGFP-OE cells and PSAP-OE cells. (d) Quantification of Western Blot analysis showing levels of α-synuclein in EGFP-OE cells (n=10) and PSAP-OE cells (n=8). (e) Real-time PCR analysis showing mRNA levels of SNCA in EGFP-OE cells (n=6) and PSAP-OE cells (n=6). (f) Quantification of α-synuclein levels in cell culture media of EGFP-OE cells (n=8) and PSAP-OE cells (n=8) measured by enzyme-linked immunosorbent assay. (g) Representative Western blot for anti-Glucocerebrosidase (GCase) and anti-GAPDH in EGFP-OE cells and PSAP-OE cells. (h) Quantification of Western blot analysis showing levels of GCase in EGFP-OE cells (n=8) and PSAP-OE cells (n=8). (i) Non-specific GBA activity and specific GCase activity analysis in EGFP-OE cells (n=8) and PSAP-OE cells (n=8) treated with or without AMP-deoxynojirimycin. Data are expressed as mean ± SEM. n.s: non-significant, p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001, Mann–Whitney U test (d, e, f, h) and Two-way ANOVA with Tukey’s multiple comparison test (i)

Overexpression of PSAP Affects Autophagy

Previous reports claimed that PSAP and saposin C deficiency increases autophagy (Vaccaro et al. 2010; Motta et al. 2016). To investigate whether PSAP overexpression has effects on autophagy, we checked the levels of LC3-I and LC3-II, common autophagosome markers to monitor autophagy flux, under basal and autophagy inhibition conditions (Tanida et al. 2008). EGFP-OE and PSAP-OE cells were incubated for 2 h with dimethyl sulfoxide (DMSO) or 100 nM Bafilomycin A1, a V-ATPase inhibitor blocking autophagosome-lysosome fusion and lysosomal acidification (Mauvezin and Neufeld 2015). Bafilomycin A1 treatment increased LC3-II levels in both cell lines, but the upregulation of autophagy was significantly weaker in PSAP-OE cells (Fig. 3a, b; two-way ANOVA with Tukey’s
multiple comparison test: EGFP DMSO vs. PSAP DMSO: \( p = 0.2896 \); EGFP Bafilomycin A1 vs. PSAP Bafilomycin A1: \( p = 0.0036 \) compared with EGFP-OE cells. The ratio of LC3-II/LC3-I, which is also commonly used as an indicator of autophagy flux, showed the same tendency (Fig. 3a, c; two-way ANOVA with Tukey’s multiple comparison test: EGFP DMSO vs. PSAP DMSO: \( p = 0.2621 \); EGFP Bafilomycin A1 vs. PSAP Bafilomycin A1: \( p = 0.0130 \)). We examined the levels of p62, a ubiquitin-binding protein which serves as another autophagy marker (Supplementary Fig. 3). There were no significant changes in p62 levels between EGFP- and PSAP-OE cells under basal or autophagy inhibition conditions. Also, p62 levels were not affected by Bafilomycin A1 treatment in either cell line. While LC3-II levels were substantially affected in PSAP-OE cells, the effect of PSAP overexpression on p62 levels was barely detected. This could be due to the nature of the complex regulations of p62 levels compared with LC3, which was argued in previous publications (Liu et al. 2016; Klionsky et al. 2021).

To sum, overexpression of PSAP alters autophagy in the opposite direction of what has been reported in PSAP deficient cells.

Based on our observation that PSAP overexpression decreased both intracellular and extracellular \( \alpha \)-synuclein without affecting mRNA levels, we considered that degradation of \( \alpha \)-synuclein is upregulated in PSAP-OE cells. Since \( \alpha \)-synuclein is known to be degraded by both autophagy and the ubiquitin–proteasome system, we investigated the degradation of \( \alpha \)-synuclein in both pathways. First, to examine autophagy–lysosome-dependent degradation of \( \alpha \)-synuclein, cells were treated with Bafilomycin A1 and \( \alpha \)-synuclein levels were quantified by Western blot (Fig. 3d–f; Unpaired Student’s \( t \)-test: (Fig. 3e); \( F(6) = 1.590, p = 0.1630 \)). While LC3 accumulation was observed in Fig. 3a, the levels of \( \alpha \)-synuclein were not changed in EGFP-OE and PSAP-OE cells. Next, to investigate the ubiquitin–proteasome–dependent degradation of \( \alpha \)-synuclein, the cells were treated with MG132, a reversible proteasomal inhibitor, and \( \alpha \)-synuclein levels were quantified by Western blot (Fig. 3g–i; Unpaired Student’s \( t \)-test: (Fig. 3h); \( F(6) = 0.4943, p = 0.6387 \) and (Fig. 3i); \( F(6) = 1.880, p = 0.1092 \)). Similar to Bafilomycin A1 treatment, MG132 treatment did not affect \( \alpha \)-synuclein degradation. These results indicate that the reduced levels of \( \alpha \)-synuclein in PSAP-OE cells were not caused by direct upregulation of autophagy or ubiquitin–proteasome system.

**Saposin C Can Detach \( \alpha \)-synuclein From Glucosylceramide–enriched Lipid Vesicles**

**Knock-down of PSAP Increases Levels of \( \alpha \)-synuclein**

Given the result that the overexpression of PSAP reduced \( \alpha \)-synuclein levels, we hypothesized that a decrease in PSAP levels would result in increased levels of \( \alpha \)-synuclein. To test this hypothesis, we used siRNA targeting PSAP mRNA. First, we optimized siRNA concentration by using the non-specific GBA activity assay as a readout (Supplementary Fig. 4). PSAP knockdown caused a dose-dependent decrease in non-specific GBA activity. We decided to take 10 nM as the highest concentration of siRNA that did not show visible negative effects on cell growth while still decreasing GBA activity. WT SH-SY5Y cells were treated over 10 days with three subsequent 10 nM control siRNA or siPSAP transfections. Western blot confirmed reduced expression of PSAP in siPSAP treated cells (Fig. 4a, b; Mann–Whitney \( U \) test: \( p = 0.0006 \)). Knockdown of PSAP...
was also validated at mRNA levels (Fig. 4c; Mann–Whitney U test: \( p = 0.0207 \)). As we hypothesized, PSAP knockdown significantly increased \( \alpha \)-synuclein levels (Fig. 4d, e; Mann–Whitney U test: \( p = 0.0047 \)). However, mRNA levels of \( \text{SNCA} \) were decreased in siPSAP-treated cells, indicating the existence of feedback inhibition of \( \text{SNCA} \) transcription (Fig. 4f; Mann–Whitney U test: \( p = 0.0499 \)).

Saposin C Can Detach \( \alpha \)-synuclein From Glucosylceramide-enriched Lipid Vesicles

Based on previous reports showing that saposin C can interrupt GCase-\( \alpha \)-synuclein interactions (Yap et al. 2013; Gruschus et al. 2015), we hypothesized that there is a direct competition between saposin C and \( \alpha \)-synuclein. To test
the hypothesis, we created artificial lipid vesicles and incubated them with recombinant α-synuclein and saposin C to examine how they influence each other’s levels in vitro. The vesicles consisted of glucosylceramide and phosphocholine, and either in a pH 7.4 phosphate buffer or a pH 5.4 citrate–phosphate buffer, which are cytoplasmic pH or lysosomal pH, respectively. Addition of recombinant saposin C, but not a control peptide or the neuroprotective prosaptide (TX14(a)), caused α-synuclein to be dislodged from the artificial vesicles at lysosomal pH 5.4 (Fig. 5a, c–e; One-way ANOVA with Dunnett’s multiple comparison test: (Fig. 5c); F (2, 6) = 5.076, p = 0.0513; Control vs. TX14(a): p = 0.9372; Control vs. Saposin C: p = 0.0405. (Fig. 5d); F (2, 6) = 5.076, p = 0.0513; Control vs. TX14(a): p = 0.9372; Control vs. Saposin C: p = 0.0405. (Fig. 5e); F (2, 6) = 6.203, p = 0.0346; Control vs. TX14(a): p = 0.9550; Control vs. Saposin C: p = 0.0340), but not at cytoplasmic pH 7.4 (Fig. 5b, f–h; One-way ANOVA with Dunnett’s multiple comparison test: (Fig. 5f); F (2, 6) = 0.5646, p = 0.5961; Control vs. TX14(a): p = 0.9716; Control vs. Saposin C: p = 0.4755). This result indicates that saposin C prevents α-synuclein from binding to glucosylceramide-enriched lipid membranes.

**Discussion**

Our study shows that PSAP regulates the levels of α-synuclein, which is attributed to saposin C’s function to promote α-synuclein clearance by dissociating α-synuclein from the lipid membranes. We demonstrate that α-synuclein levels are decreased/increased by PSAP overexpression/knockdown, which strongly suggests that PSAP is playing an important role in α-synuclein regulation. We also show that mRNA transcription of the SNCA gene is not affected by PSAP overexpression, which indicates that the regulation of α-synuclein by PSAP occurs at the translational
or post-translational level, rather than the transcriptional level. Furthermore, we revealed that PSAP-OE cells showed altered autophagy, recapitulating the involvement of PSAP in autophagy-lysosomal function (Motta et al. 2016). The association between lysosomal storage disease and autophagy deficits has been shown in many studies (Ward et al. 2016; Seranova et al. 2017). It was revealed that lack of saposin C triggers enhanced autophagy, which results from reduced levels and enzymatic activities of cathepsin B and cathepsin D (CTSD) (Tatti et al. 2012). CTSD is an endopeptidase that cleaves PSAP into four saposins (Hiraiwa et al. 1997a). Aside from its property to cleave PSAP, CTSD is also responsible for α-synuclein degradation (Sevlever et al. 2008). Recently, it was proposed that the network of three lysosomal proteins, CTSD, PSAP, and progranulin, is associated with α-synuclein clearance (Tayebi et al. 2020). Based on our findings and literature, it is possible that PSAP may enhance α-synuclein degradation via interaction with CTSD. Further investigation of the connection between the network proteins and α-synuclein degradation may provide us with a key to understanding α-synuclein pathology in PD patients.

According to the fact that mutations in the GBA1 gene are the common risk factors for PD, and saposin C is an activator of GCase, the link between GCase with saposin C in PD has been explored (Yap et al. 2013; Ouled Amar Bencheikh et al. 2018). Some studies reported that glucosylceramide, a substrate of GCase, is important for lipid raft formation and serves as a site for α-synuclein aggregation (Mazzulli et al. 2016; Taguchi et al. 2017; Zunke et al. 2018). In addition, it was demonstrated that saposin C binds to GCase competitively with α-synuclein both in solution and on the lipid vesicle, which protects GCase from α-synuclein inhibition (Yap et al. 2013). Based on these observations, we investigated the interaction between α-synuclein, saposin C, and glucosylceramide by generating an intra-lysosomal environment in vitro. Our results show that saposin C promotes the dissociation of α-synuclein from glucosylceramide-rich lipid vesicles in acidic conditions. This indicates that saposin C may prevent α-synuclein aggregation or accumulation by detaching it from the lipid membranes in the lysosome, which leads to efficient α-synuclein clearance. Thus, we suggest that the observed decrease in α-synuclein levels in PSAP-OE
cells is attributed to the ability of saposin C to counteract α-synuclein forming aggregations on the lysosomal membrane and promotes efficient removal of α-synuclein.

However, since we overexpressed or knocked down the whole PSAP in the cells, there is a possibility that other saposins may also contribute to decreasing α-synuclein levels. For example, a recent study revealed the association between saposin D mutation and PD (Oji et al. 2020). Aggregation of α-synuclein was observed in skin fibroblast and induced-pluripotent stem cell–derived dopaminergic neurons from saposin D mutant PD patients (Oji et al. 2020). Therefore, the specific roles of saposin C in PD pathology require further investigation. One opportunity to explore saposin C roles is to generate saposin C–specific knockout cells by introducing mutations in the saposin C domain of PSAP (Vaccaro et al. 2010).

A limitation of this study may be that we decided to use undifferentiated SH-SY5Y cells to study the neuroprotective roles of PSAP and saposin C. Although differentiated cells are considered a better model for PD, it has been reported that most of the genes belonging to the major PD pathways and modules are intact in an undifferentiated SH-SY5Y cell line (Krishna et al. 2014). We, therefore, believe that our results provide further evidence that increased levels of PSAP, and particularly saposin C, may counteract α-synuclein accumulation and that strategies to elevate PSAP and saposin C may be beneficial against PD.

Conclusion

Our results conclude that altered levels of PSAP, particularly saposin C, could underlie pathophysiological events of PD. Our findings propose that increasing PSAP levels may counteract α-synuclein pathology by enhancing α-synuclein clearance, which contributes to preventing the development of PD.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12031-022-02066-y.

Acknowledgements The saposin C vector was a generous gift from Gil Privé.

Author Contribution Rika Kojima: Validation, Formal analysis, Investigation, Writing—Reviews & Editing. Mark Zurbruegg: Conceptualization, Investigation, Writing—Original Draft. Tianyi Li: Investigation, Supervision. Wojciech Paslawski: Resources, Supervision. Xiaoyun Zhang: Supervision, Writing—Reviews & Editing. Per Svenningsson: Conceptualization, Supervision, Project administration, Writing—Reviews & Editing, Funding acquisition.

Funding Open access funding provided by Karolinska Institute. This work was supported by a consolidator grant from ERC (PROGSY 649116), van Geest Foundation and Lexa International/Nordstjernan to T.L., W.P., X.Z., and P.S.. R.K. and M.Z. were supported by Karolinska Institutet KID program. R.K. was supported by a CZND grant. P.S. is a Wallenberg Clinical Scholar.

Data Availability The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests R.K., M.Z., T.L., W.P., and X.Z. have no competing interests. P.S. has received honoraria from Shire and Sanofi Genzyme.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Ahn VE, Leyko P, Alattia J-R et al (2006) Crystal structures of saposins A and C. Protein Sci 15:1849–1857. https://doi.org/10.1110/ps.06255606

Alcalay RN, Dinur T, Quinn T et al (2014) Comparison of Parkinson risk in Ashkenazi Jewish patients with Gaucher disease and GBA heterozygotes. JAMA Neurol 71:752–757. https://doi.org/10.1001/jamaneurol.2014.313

Alcalay RN, Levy OA, Waters CC et al (2015) Glucocerebrosidase activity in Parkinson’s disease with and without GBA mutations. Brain 138:2648–2658. https://doi.org/10.1093/brain/awv179

Armstrong MJ, Okun MS (2020) Diagnosis and treatment of Parkinson disease: a review. JAMA 323:548–560. https://doi.org/10.1001/jama.2019.22360

Avenali M, Blandini F, Cerri S (2020) Glucocerebrosidase defects as a major risk factor for Parkinson’s disease. Front Aging Neurosci 12:97. https://doi.org/10.3389/FNAGL.2020.00097/BIBTEX

Behl T, Kaur G, Fratila O et al (2021) Cross-talks among GBA mutations, glucocerebrosidase, and α-synuclein in GBA-associated Parkinson’s disease and their targeted therapeutic approaches: a comprehensive review. Transl Neurodegener 10(10):1–13. https://doi.org/10.1186/S40035-020-00226-X

Braak H, Del Tredici K, Rüb U et al (2003) Staging of brain pathology related to sporadic Parkinson’s disease. Neurobiol Aging 24:197–211. https://doi.org/10.1016/S0197-4580(02)00065-9

Burré J, Sharma M, Tsutsenis T et al (2010) α-Synuclein promotes SNARe-complex assembly in vivo and in vitro. Science 329:1663. https://doi.org/10.1126/SCIENCE.1195227

Chavrier P, Parton RG, Hauri HP et al (1990) Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell 62:317–329. https://doi.org/10.1016/0092-8674(90)90369-P
Dermentzaki G, Dimitriou E, Xilouri M et al (2013) Loss of β-glucocerebrosidase activity does not affect alpha-synuclein levels or lysosomal function in neuronal cells. PLoS ONE. https://doi.org/10.1371/journal.pone.0060674

Do J, McKinney C, Sharma P (2019) Sidransky E (2019) Glucocerebrosidase and its relevance to Parkinson disease. Mol Neurodegener 14(14):1–16. https://doi.org/10.1186/S13024-019-0336-2

Fujita N, Suzuki K, Vanier MT et al (1996) Targeted disruption of the mouse sphingolipid activator protein gene: a complex phenotype, including severe leukodystrophy and wide-spread storage of multiple sphingolipids. Hum Mol Genet 5:711–725. https://doi.org/10.1093/hmg/5.7.711

Gao HL, Li C, Nunnari J, McKinney C, Sharma P (2019) Sidransky E (2019) Glucocerebrosidase and its relevance to Parkinson disease. Mol Neurodegener 14(14):1–16. https://doi.org/10.1186/S13024-019-0336-2

Lashuel HA

Klionsky DJ, Abdel-Aziz AK, Abdelfatah S et al (2021) Guidelines for Parkinson’s disease − lessons and emerging principles. Mol Neurodegener 14(14):1–14. https://doi.org/10.1186/S13024-019-0329-1

Meyer RC, Giddens MM, Schaeffer SA, Hall RA (2013) GPR37 and GPR37L1 are receptors for the neuroprotective and glioprotective factors prosapptide and prosaposin. Proc Natl Acad Sci U S A 110:9529–9534. https://doi.org/10.1073/pnas.1219004110

Mota M, Tatti M, Furlan F et al (2016) Clinical, biochemical and molecular characterization of prosaposin deficiency. Clin Genet 90:220–229. https://doi.org/10.1111/CGE.12755

O’Brien JS, Carson GS, Seo H et al (1995) Identification of the neurotrophic factor sequence of prosaposin. FASEB J 9:681–685. https://doi.org/10.1096/fasebj.9.8.7768361

O’Brien JS, Carson GS, Seo HC et al (1994) Identification of prosaposin as a neurotrophic factor. Proc Natl Acad Sci U S A 91:9593–9596. https://doi.org/10.1073/pnas.91.20.9593

Ouled Amar Bencheikh B, Leveille E, Ruskey JA et al (2018) Sequencing of the GBA coactivator, Saposin C, in Parkinson disease. Neurobiol Aging 72:187.e1-187.e3. https://doi.org/10.1016/j.neurobiaging.2018.06.034

Ovekleef HS, Renkema GH, Neele J et al (1998) Generation of specific deoxynojirimycin-type inhibitors of the non-lysosomal glucosylceramidase. J Biol Chem 273:26522–26527. https://doi.org/10.1074/jbc.273.41.26522

Pawlski W, Lorenzen N, Otzen DE (2016) Formation and characterization of α-synuclein oligomers. In: Methods in Molecular Biology. Humana Press Inc., pp 133–150

Poewe W, Seppi K, Tanner CM et al (2017) Parkinson disease. Nat Rev Dis Prim 3(1):1–21. https://doi.org/10.1038/nrdp.2017.13

Polymeropoulos MH, Lavedan C, Leroy E et al (1997) Mutation in the α-synuclein gene identified in families with Parkinson disease. Science 276(5306):2045–2047. https://doi.org/10.1126/science.276.5321.2045

Schönwetter DC, Aureli M, McAllister FE et al (2014) IPSC-derived neurons from GBA1-associated Parkinson’s disease patients show autophagic defects and impaired calcium homeostasis. Nat Commun 5:1–17. https://doi.org/10.1038/ncomms5028

Seronova E, Connolly KJ, Zatyka M et al (2017) Dysregulation of autophagy as a common mechanism in lysosomal storage diseases. Essays Biochem 61:733–749. https://doi.org/10.1042/EB20170055

Selever D, Jiang P, Yen SHC (2008) Cathepsin D is the main lysosomal enzyme involved in the degradation of alpha-synuclein and...
generation of its carboxy-terminally truncated species. Biochemistry 47:9678–9687. https://doi.org/10.1021/BI800699V
Sikora J, Harzer K, Elleder M (2007) Neurolysosomal pathology in human prosaposin deficiency suggests essential trophic function of prosaposin. Acta Neuropathol 113:163–175. https://doi.org/10.1007/s00401-006-0148-7
Taguchi YV, Liu J, Ruan J et al (2017) Glucosylsphingosine promotes α-synuclein pathology in mutant GBA-associated Parkinson’s disease. J Neurosci 37:9617–9631. https://doi.org/10.1523/JNEUROSCI.1525-17.2017
Tamargo RJ, Velayati A, Goldin E, Sidransky E (2012) The role of saposin C in Gaucher disease. Mol Genet Metab 106:257–263. https://doi.org/10.1016/j.ymgme.2012.04.024
Tanida I, Ueno T, Kominami E (2008) LC3 and autophagy. Methods Mol Biol 445:77–88. https://doi.org/10.1007/978-1-59745-157-4_4
Tatti M, Motta M, Di Bartolomeo S et al (2012) Cathepsin-mediated regulation of autophagy in saposin C deficiency. 9:241–243. https://doi.org/10.4161/AUTO.22557
Tayebi N, Lopez G, Do J, Sidransky E (2020) Pro-cathepsin D, Prosaposin, and Progranulin: lysosomal networks in Parkinsonism. Trends Mol Med 26:913–923
Tsui K, Hiraiwa M, O’Brien JS (1998) Prosaposin prevents programmed cell death of rat cerebellar granule neurons in culture. Dev Brain Res 110:249–255. https://doi.org/10.1016/S0165-3806(98)00109-6
Vaccaro AM, Motta M, Tatti M et al (2010) Saposin C mutations in Gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting. Hum Mol Genet 19:2987–2997. https://doi.org/10.1093/hmg/ddq204
Ward C, Martinez-Lopez N, Otten EG et al (2016) Autophagy, lipophagy and lysosomal lipid storage disorders. Biochim Biophys Acta - Mol Cell Biol Lipids 1861:269–284. https://doi.org/10.1016/J.BBALIP.2016.01.006
Weiler S, Carson W, Lee Y et al (1993) Synthesis and characterization of a bioactive 82-residue sphingolipid activator protein, saposin C. J Mol Neurosci 4:161–172. https://doi.org/10.1007/BF02782499
Yap TL, Grasuuch JM, Velayati A et al (2013) Saposin C protects glucocerebrosidase against α-synuclein inhibition. Biochemistry 52:7161–7163. https://doi.org/10.1021/bi401191v
Zunke F, Moise AC, Belur NR et al (2018) Reversible conformational conversion of α-synuclein into toxic assemblies by glucosylceramide. Neuron 97:92-107.e10. https://doi.org/10.1016/j.neuron.2017.12.012

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.