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Ambroxol-induced rescue of defective glucocerebrosidase is associated with increased LIMP-2 and saposin C levels in *GBA1* mutant Parkinson's disease cells

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ABSTRACT

Heterozygous mutations in *GBA1* gene, encoding for lysosomal enzyme glucocerebrosidase (GCase), are a major risk factor for sporadic Parkinson's disease (PD). Defective GCase has been reported in fibroblasts of *GBA1*-mutant PD patients and pharmacological chaperone ambroxol has been shown to correct such defect. To further explore this issue, we investigated GCase and elements supporting GCase function and trafficking in fibroblasts from sporadic PD patients — with or without heterozygous *GBA1* mutations — and healthy subjects, in basal conditions and following in vitro exposure to ambroxol. We assessed protein levels of GCase, lysosomal integral membrane protein-2 (LIMP-2), which mediates GCase trafficking to lysosomes, GCase endogenous activator saposin (Sap) C and parkin, which is involved in degradation of defective GCase. We also measured activities of GCase and cathepsin D, which cleaves Sap C from precursor prosaposin. GCase activity was reduced in fibroblasts from *GBA1*-mutant patients and ambroxol corrected this defect. Ambroxol increased cathepsin D activity, GCase and Sap C protein levels in all groups, while LIMP-2 levels were increased only in *GBA1*-mutant PD fibroblasts. Parkin levels were slightly increased only in the PD group without *GBA1* mutations and were not significantly modified by ambroxol. Our study confirms that GCase activity is deficient in fibroblasts of *GBA1*-mutant PD patients and that ambroxol corrects this defect. The drug increased Sap C and LIMP-2 protein levels, without interfering with parkin. These results confirm that chemical chaperone ambroxol modulates lysosomal markers, further highlighting targets that may be exploited for innovative PD therapeutic strategies.

Introduction

Heterozygous mutations in the *GBA1* gene have been recently identified as a major genetic risk factor for the development of sporadic Parkinson's disease (PD) (Sidransky et al., 2009; Sidransky and Lopez, 2012; Asselta et al., 2014) and dementia with Lewy bodies (Nalls et al., 2013; Asselta et al., 2014). Homozygous mutations in the *GBA1* gene cause Gaucher disease (GD); heterozygous mutations occur in 5–10% of sporadic PD cases (Schapira and Gegg, 2013) and impair folding and activity of glucocerebrosidase (GCase), the lysosomal enzyme encoded by the *GBA1* gene. These alterations may impact on lysosomal function and, consequently, on cellular clearance pathways, such as autophagy. Since alpha-synuclein is a substrate of chaperone-mediated autophagy, any alteration in this proteolytic mechanism may favor the pathological intracellular accumulation of this protein and formation of Lewy bodies in PD brains (Alvarez-Erviti et al., 2010; Xilouri and Stefanis, 2015). Indeed, growing evidence is supporting a role for GCase dysfunction in the process of alpha-synuclein accumulation, thereby strengthening the link with PD pathogenesis (Bae et al., 2015; Siebert et al., 2014; Sardi et al., 2015; Schapira, 2015).

Reduced GCase activity, correlated with disease severity, has been detected in fibroblasts derived from patients with GD (Bendikov-Bar et al., 2013). More recently, McNeill et al. (2014) reported reduced activity and protein levels of GCase in fibroblasts from *GBA1* heterozygous mutation carriers, with and without PD. In both GD and *GBA1*-mutant PD patients, these defects were corrected by exposing fibroblasts to ambroxol hydrochloride, a small molecule known for its expectorant and anti-inflammatory activity that may function as a molecular chaperone (Maegawa et al., 2009; McNeill et al., 2014). Ambroxol improved lysosomal biochemistry by modulating the expression of transcription factor EB (TFEB)-associated genes and endoplasmic reticulum (ER) markers (McNeill et al., 2014). Based on these results, ambroxol has been proposed as a potential disease-modifying treatment for PD. In this perspective, the mechanism of action of ambroxol should be further

Abbreviations: PD, Parkinson's disease; GD, Gaucher disease; GCase, glucocerebrosidase; Sap C, saposin C; PSAP, prosaposin; LIMP-2, lysosomal integral membrane 2; ER, endoplasmic reticulum; TFEB, transcription factor EB; GSK3, glycogen synthase kinase 3; UPS, ubiquitin proteasome system.

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characterized. For example, the impact of ambroxol on cellular proteostatic mechanisms and how this may reverberate on GCase efficiency should be clarified.

GCase function and targeting to the lysosomes are supported by endogenous transporters and co-factors. Lysosomal integral membrane protein-2 (LIMP-2), a type III glycoprotein encoded by *SCARB2* gene, is the receptor involved in the lysosomal transport of GCase (Reczek et al., 2007; Rothaug et al., 2014). Saposin C (Sap C) is a substrate presenting co-factor essential in the GCase-dependent hydrolysis of glucosylceramide, which also protects GCase from proteolytic breakdown (Siebert et al., 2014). Sap C — which is defective in a rare variant of GD (Tatti et al., 2011; Motta et al., 2014) — belongs to a family of four small glycoproteins (saposins A–C) originating from the sequential lysosomal proteolysis of precursor prosaposin (PSAP) (Misasi et al., 2009; Hiraiwa et al., 1997; Tamargo et al., 2012). The cleaving enzyme is cathepsin D, a lysosomal aspartyl protease also involved in the processing of alpha-synuclein (McGlinchey and Lee, 2013; Crabtree et al., 2014). Interestingly, mutations in LIMP-2, as well as Sap C deficiency, have been investigated as genetic modifiers in GD and, more recently, in synucleinopathies (Siebert et al., 2014; Sardi et al., 2015).

In fibroblasts from GD patients or PD patients carrying heterozygous *GBA1* mutations, misfolded GCase is retained in the ER, thereby causing ER stress (Westbroek et al., 2011; Tan et al., 2014). ER stress activates the unfolded protein response and modulates proteins that are typically at the cross-road of proteostasis, cell metabolism and viability, such as glycogen synthase kinase 3 (GSK3) and parkin. GSK3 — with the associated signaling pathway involving serine/threonine protein kinase AKT (also known as protein kinase B) and mTOR — is linked to ER stress, which suppresses GSK3-Akt signaling (Chen et al., 2011; Golpich et al., 2015). More importantly, recent reports show that GSK3 inhibition plays a pivotal role in the regulation of lysosomal biogenesis and function in different models of Alzheimer's disease and tauopathies (Nijholt et al., 2013; Parr et al., 2012). Moreover, Ballabio's group has shown that mTOR, one of GSK3 downstream target kinases, interacts with and phosphorylates TFEB on the lysosomal membrane, thereby preventing its translocation to the nucleus, further indicating a connection between these pathways (Settembre et al., 2012). In parallel, parkin, a PD-related E3 ubiquitin ligase whose expression is regulated also by ER stress (Bouman et al., 2011), is involved in mitophagy (Calì et al., 2013) and in the ubiquitination and proteasomal degradation of several substrates, including mutant GCase (Ron et al., 2010; Bendikov-Bar et al., 2014).

We have previously reported proteolytic defects in lymphocytes and fibroblasts from sporadic PD patients, which included reduced proteasome 20S activity and increased levels of parkin and poly-ubiquitinated proteins (Blandini et al., 2006; Ambrosi et al., 2014). The objective of this study was to analyze the impact of *GBA1* mutations in the context of PD by investigating GCase and specific lysosomal factors supporting GCase activity in fibroblasts of PD patients with or without *GBA1* heterozygous mutations or healthy controls. Moreover, we sought to obtain further information on the effects of ambroxol in this context, to confirm its potential as a compound targeting lysosomal dysfunctions that may be central to PD pathogenesis.

Our results confirm that GCase is deficient in *GBA1*-mutant PD cells and that ambroxol enhances GCase activity and protein levels; this effect is associated with increased levels of GCase transporter LIMP-2 and co-factor Sap C.

Subjects and methods

Patients and fibroblast cultures

Fibroblasts were generated from skin biopsies of the upper medial arm of ten sporadic PD patients and seven age- and gender-matched healthy controls (Table 1). Within the PD group, five patients carried L444P (two cases) or N370S (three cases) heterozygous mutations in the *GBA1* gene (PD GBA+); these fibroblasts were obtained from the Telethon Network of Genetic Biobanks (Telethon, Italy). In the other five patients (PD GBA−) and in controls, *GBA1* mutations or polymorphisms were excluded by sequencing all 11 exons of the *GBA1* gene with Genetic Analyzer 3130xl (Applied Biosystems) and comparing data with a reference sequence available at www.ncbi.com (RefSeqGene:NG_009783.1) using Sequencher 4.8 software (GeneCodes, Ann Arbor, MI, USA). The research protocol was approved by the Ethic Committee of the "C. Mondino" National Neurological Institute and informed consent was obtained from all subjects.

Table 1
Clinical and demographic data of subjects enrolled for the study.

	Controls	PD GBA−	PD GBA+
n (gender)	*n* = 7 (3 M, 4 F)	*n* = 5 (3 M, 2 F)	*n* = 5 (3 M, 2 F)
Age (years)	62.8 ± 4.8	65.6 ± 3.1	55.4 ± 11.4
Age at onset (years)	–	51 ± 5.6	42.2 ± 8
Disease duration (years)	–	17.3 ± 3.7	13.2 ± 4.7
UPDRS scale III	–	19.5 ± 3	9.8 ± 7

Two GBA+ PD patients carried the L444P mutation and three the N370S mutation in the *GBA1* gene. All patients were under treatment with levodopa, except one GBA+ patient who was not taking any dopamine-based medication. The five GBA− patients were also taking dopamine agonists as adjunctive therapy. Unified PD rating scale (UPDRS) scores (part III) are reported in the ON phase. Results are expressed as mean ± SD.

All fibroblast strains were cultured in RPMI 1640(Sigma) complemented with 1% streptomycin and penicillin antibiotics and 20% fetal bovine serum (FBS, Sigma). Cells used in the experiments were grown and expanded in flasks up to a maximum of thirteen passages. Cells were treated for 5 days with 60 μM ambroxol (Sigma), according to previous data from McNeill et al. (2014). Ambroxol was dissolved in DMSO and a frozen stock (20 mM) was prepared. For each experimental round, all cell lines were kept in culture, treated and processed together. The medium was changed on the third day during the 5-day session and ambroxol-containing medium was prepared fresh each time.

Western blot analysis

Protein lysates were obtained by resuspending fibroblast pellets in ice-cold lysis buffer (CelLytic, Sigma) containing diluted phosphatase (1:10, Roche) and protease inhibitors (1:25, Roche). After centrifugation, the supernatant was collected and protein concentration was measured using a Bicinchoninic Acid Protein Assay (Sigma). Protein lysates were run on 4–12% gels, transferred onto nitrocellulose membranes (Invitrogen) and western blot was performed. Membranes were blocked (Odyssey blocking buffer, LiCor) and incubated overnight with the following primary antibodies: actin (1:2000), ubiquitin (1:1000), AKT1 (1:500), Sap C (1:1000), GSK3α/β (1:500), GSK3α-Phospho(Ser21) (1:500) (Santa Cruz); GCase (1:1000), Sigma; mTOR (1:1000), phospho-mTOR (1:500), parkin (1:500), phospho-Akt(Ser473) (1:500) and GSK3β-Phospho(Ser9) (1:500) (Cell Signalling); LIMP-2 (1:1000) (Novus Biologicals). As secondary antibodies, IRDye® 700 goat anti-mouse, IRDye® 800 goat anti-rabbit (1:10000) (LiCor, Biosciences) were used. Image analysis of western blots was performed using the fluorescent near-infrared Odyssey® scanner and software (LiCor, Biosciences) and fluorescence was normalized with the corresponding actin signal.

Quantitative real-time PCR

Real-time PCR (rtPCR) was used to quantify mRNA for *GBA1* gene. The expression of *GBA1* transcript was normalized to that of housekeeping gene actin as in McNeill's (2014) work. Total RNA extraction was performed with RNeasy Plus mini kit (Qiagen). The RNA concentration was determined by NanoDrop spectrophotometer ND-1000 (Thermo Scientific) and cDNA was synthesized using the iScript™ cDNA

Synthesis Kit (Bio-Rad). rtPCR was performed in a total volume of 20 μl containing 10 μl of SsoFast™ EvaGreen® Supermix (Bio-Rad); 5 ng of cDNA and 300 nM of forward and reverse primers, using the iCycler5 (Biorad). Amplifications were performed in triplicate. Thermal cycling conditions included 30 s at 98 °C followed by 40 cycles at 98 °C for 5 s, 58 °C for 5 s and 95 °C for 5 s. Results are expressed as relative levels of mRNA, normalized to actin expression.

Fluorescent immunocytochemistry

Cells were plated on coverslips in 12-well plates (10,000 cells/well). Ambroxol treatment was performed for 5 days in the plates. Lysosomes were stained using the Lysotracker (LifeSciences) staining. Cells were incubated with 50 nM of the dye in serum-free medium for 1 h, then fixed with 4% paraformaldehyde, washed, permeabilized and blocked for another hour. Antibodies were diluted in phosphate-buffered saline containing 0.1% Triton X-100 and 1% normal goat serum. Incubation with primary antibody (Sap C, 1:200) was performed overnight at 4 °C and with secondary antibody (anti-rabbit Alexa Fluor596, 1:500) 1 h at room temperature. Coverslips were mounted on slides with DAPI Prolong antifade reagent (Life Sciences) and visualized using a fluorescent microscope (Axioscope).

Enzyme activity assays

GCase activity was assessed in 20 μg of protein lysates for each cell line, using a modification of the protocol described by Vaccaro et al. (2010). A reaction mix was prepared (0.1 M sodium citrate phosphate, pH 5.6; 0.1% Triton X-100; 0.25% sodium taurocholate and 2.5 mM 4-methylumbelliferyl-b-D-glucuronide or 4-MUG) and 200 μl were added to each sample. A standard curve was also prepared using increasing doses of 4-MUG (GCase substrate). Samples and standards were incubated for 1 h at 37 °C on a shaker. The reaction was stopped with 500 μl of 0.1 M glycine, pH 10 solution and fluorescence (excitation: 355 nm; emission: 460 nm) was measured using a microplate reader (Molecular Devices).

Cathepsin D activity was assessed on fibroblast lysates with enzyme-linked immunosorbent commercial kit (BioVision). Briefly, fibroblast pellets were resuspended in cathepsin D lysis buffer, lysed for 10 min shaking on ice and centrifuged at maximum speed. Protein-containing supernatants (20 μl) were loaded together with standards, positive and negative controls in 96-well plates and incubated with a reaction mix (reaction buffer and substrate) following the manufacturer's instructions. Results were expressed as a measure of fluorescence produced after enzymatic cleavage and were detected with a microplate reader (Molecular Devices). Values of fluorescence were normalized to the protein content in each sample.

Activity of 20S proteasome, the catalytic subunit of the 26S proteasome, was assessed on fibroblast lysates with ELISA commercial kit (Enzo Life Science), as previously described (Ambrosi et al., 2014).

Statistics

Statistical evaluation of data was performed using PRISM software. We applied the Kolmogorov–Smirnov test to assess normal distribution of our data. Accordingly, non-parametric (Kruskal–Wallis) or parametric (ANOVA followed by Bonferroni post-hoc) tests were applied to analyze the data. Statistical significance was set at $p < 0.05$.

Results

GCase activity and protein levels

Basal GCase activity was reduced in fibroblasts from PD GBA + patients, with respect to controls, while no significant reduction was observed in fibroblasts from PD GBA − patients. As for GCase protein levels, a slight, non-significant, reduction was observed in PD GBA + fibroblasts with respect to PD GBA − patients and controls (Fig. 1A–C). Compared to baseline, ambroxol treatment (60 μM, for 5 days) increased activity and protein levels in all groups, without significant intergroup differences. Across groups, ambroxol increased GCase activity by 93%, and GCase protein levels by 48%. As a result, post-treatment GCase activity in PD GBA + fibroblasts reached the same levels detected in control fibroblasts at baseline. To investigate whether these changes were caused by an increase in gene expression, we quantified *GBA1* mRNA (Fig. 1D). The 5-day exposure of fibroblasts to 60 μM ambroxol induced a moderate increase in *GBA1* mRNA, which tended to be more pronounced — although non-significantly — in both PD groups (+50%), compared to controls (+20%). Brighter and more diffuse Lysotracker staining, representative of increased functional lysosomal mass, was observed in fibroblasts exposed to ambroxol (Fig. 1E).

PSAP, Sap C and cathepsin D

Basal levels of uncleaved precursor PSAP (Fig. 2A) and cleaved Sap C (Fig. 2B), as well as basal activity of cleaving enzyme cathepsin D (Fig. 2E), were unchanged in patient fibroblasts, regardless of the presence of *GBA1* mutations. Ambroxol significantly increased Sap C protein levels in all groups (Fig. 2B and D), while moderate, non-significant increases were detected in PSAP levels (Fig. 2A). Also, ambroxol induced a significant increase in the enzymatic activity of cathepsin D, in all groups (Fig. 2E).

LIMP-2

Basal protein levels of GCase trafficking mediator LIMP-2 were similar in fibroblasts of both PD groups and controls. Exposure to ambroxol induced moderate increases of LIMP-2 levels in all groups, but only in PD GBA + fibroblasts was the increase (+37%) statistically significant compared to baseline values (Fig. 3A–B).

Parkin, ubiquitinated protein levels and proteasome 20S activity

Parkin protein levels were significantly higher, while proteasome 20S activity was lower, in fibroblasts from PD GBA − patients, with respect to both controls and PD GBA + patients. Ambroxol induced moderate, non-significant increases in parkin levels and proteasome 20S activity, in all groups (Fig. 4A–B, D). Fibroblasts from PD GBA − patients showed a slight, non-significant increase in the levels of ubiquitinated proteins (Fig. 4C), which were not modified by ambroxol treatment.

AKT/GSK3/mTOR signaling pathway

The AKT/GSK3/mTOR signaling pathway is involved in the control of cell proliferation, glucose metabolism, inflammation, autophagy and, according to recent literature, lysosomal biogenesis and function (Nijholt et al., 2013; Parr et al., 2012). Basal expression of GSK3α phosphorylated at Ser21 was significantly increased in fibroblasts from PD GBA − patients, compared to controls, and was normalized by ambroxol treatment (Fig. 5A–B). No differences between the groups, or ambroxol-induced changes, were detected for AKT phosphorylated at Ser473, GSK3β phosphorylated at Ser9 or phosphorylated mTOR, the downstream target of the AKT/GSK3 pathway (Fig. 5C–E).

Discussion

The purpose of this study was to confirm and extend previous findings on GCase defects in fibroblasts of PD patients carrying heterozygous mutations of the *GBA1* gene; a secondary objective was to obtain further information on the effects of chemical chaperone ambroxol in this context. Our results confirm the data recently reported by McNeill et al. (2014), who showed reduced GCase activity in fibroblasts of PD

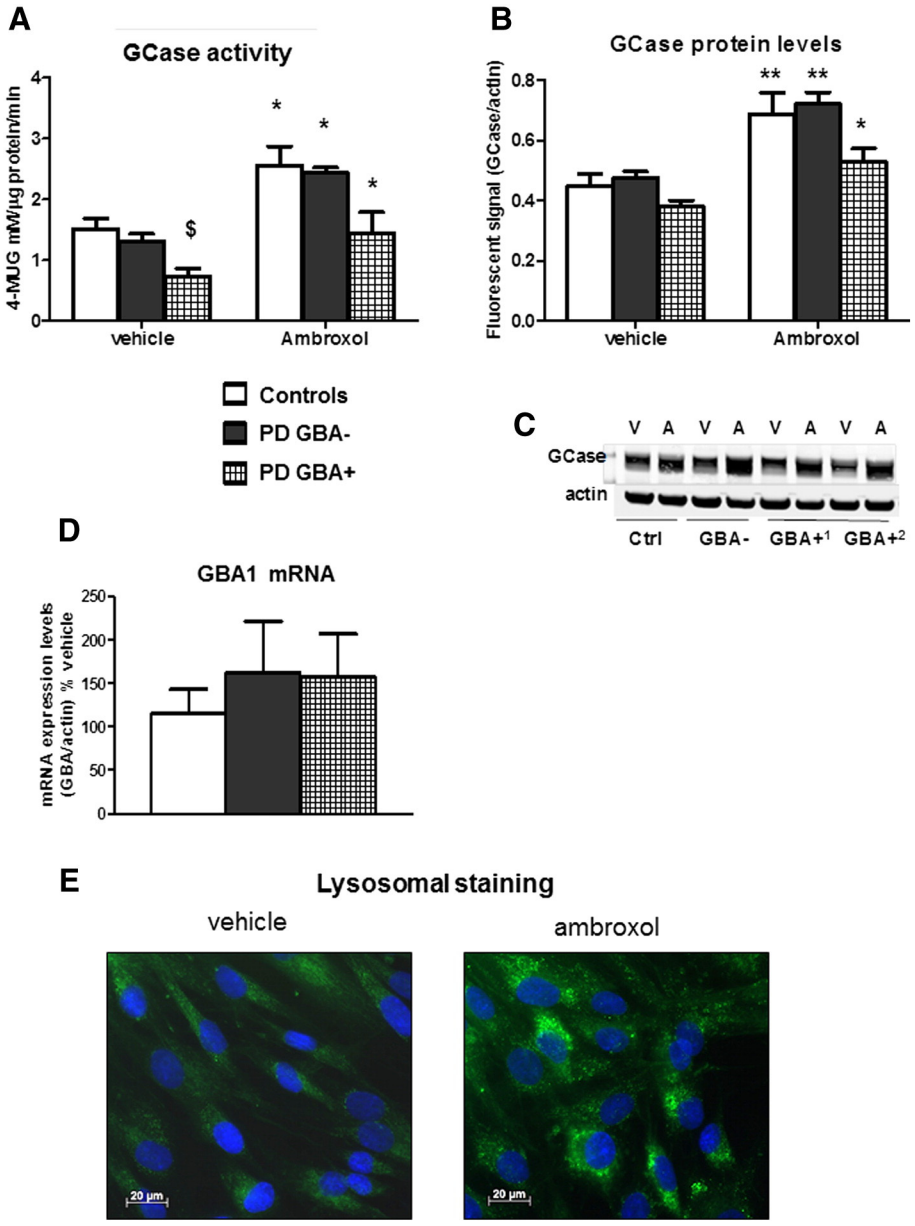

Fig. 1. GCase activity and protein levels at baseline and after ambroxol. GCase activity (A) was significantly reduced in fibroblasts of PD patients with *GBA1* mutations (PD GBA+), compared to both PD patients without *GBA1* mutations (PD GBA−) and controls; protein levels of GCase (B) did not differ significantly between groups. Ambroxol increased GCase protein levels and activity in all groups. C) Representative image of a cropped membrane after western blot for GCase antibody (GCase) in a control subject (Ctrl), one PD patient without *GBA1* mutation (GBA−) and two with *GBA1* mutation (GBA+[1–2]). V: vehicle; A: 60 µM ambroxol (5 days). D) *GBA1* mRNA changes, compared to baseline, following exposure to ambroxol. E) Brighter and more diffuse Lysotracker N26 green staining — suggestive of increased functional lysosomal mass — in control fibroblasts exposed to ambroxol (magnification: 40×; scale bar: 20 µm). Ctrl: $n = 7$; PD GBA−: $n = 5$; PD GBA−: $n = 5$; \$ $p < 0.05$ vs. controls; *$p < 0.05$, **$p < 0.01$ vs. vehicle (Bonferroni post-hoc test).

patients carrying N370S or L444P mutations in *GBA1* gene. Fibroblasts of our *GBA1*-mutant PD patients showed 50% reduction in GCase activity, with respect to both controls and patients without mutations. This defect was rescued following exposure to ambroxol, further confirming Mc Neill's data; the increases in lysosomal mass and LIMP-2 protein levels detected in our cells corroborated the ability of ambroxol to enhance lysosomal function.

One critical point of our study was to investigate elements known to support GCase activity and trafficking to the lysosomes and assess whether ambroxol treatment could modulate their levels. In this frame, the most important role is played by Sap C and LIMP-2. Sap C originates from the cleavage of precursor PSAP operated by cathepsin D (Misasi et al., 2009; Hiraiwa et al., 1997; Tamargo et al., 2012) and its expression is linked to TFEB-associated gene network (Palmieri et al., 2011). In our study, fibroblasts from PD patients did not show altered basal levels of PSAP, Sap C or cathepsin D activity, with respect to controls, regardless of the presence of *GBA1* mutations. This was not surprising, as Sap C deficiency was only reported in an extremely rare variant of GD caused by biallelic mutations in the *PSAP* gene (Grabowski and Horowitz, 1997; Motta et al., 2014). Moreover, Tatti et al. (2012) demonstrated that cathepsin D activity is preserved in fibroblasts from patients with GD, who show GCase defects far more severe than those reported in heterozygous mutant *GBA1* carriers. On the other hand, the fact that ambroxol significantly enhanced cathepsin D activity and Sap C levels provide further interesting insights into the mechanisms of action of the drug. Indeed, ambroxol may enhance the efficiency of the cathepsin D-mediated cleavage of PSAP to saposins, therefore increasing the availability of Sap C, which would further support GCase function. Furthermore, Yap et al. (2013) have demonstrated that Sap C physically prevents the interaction between GCase and

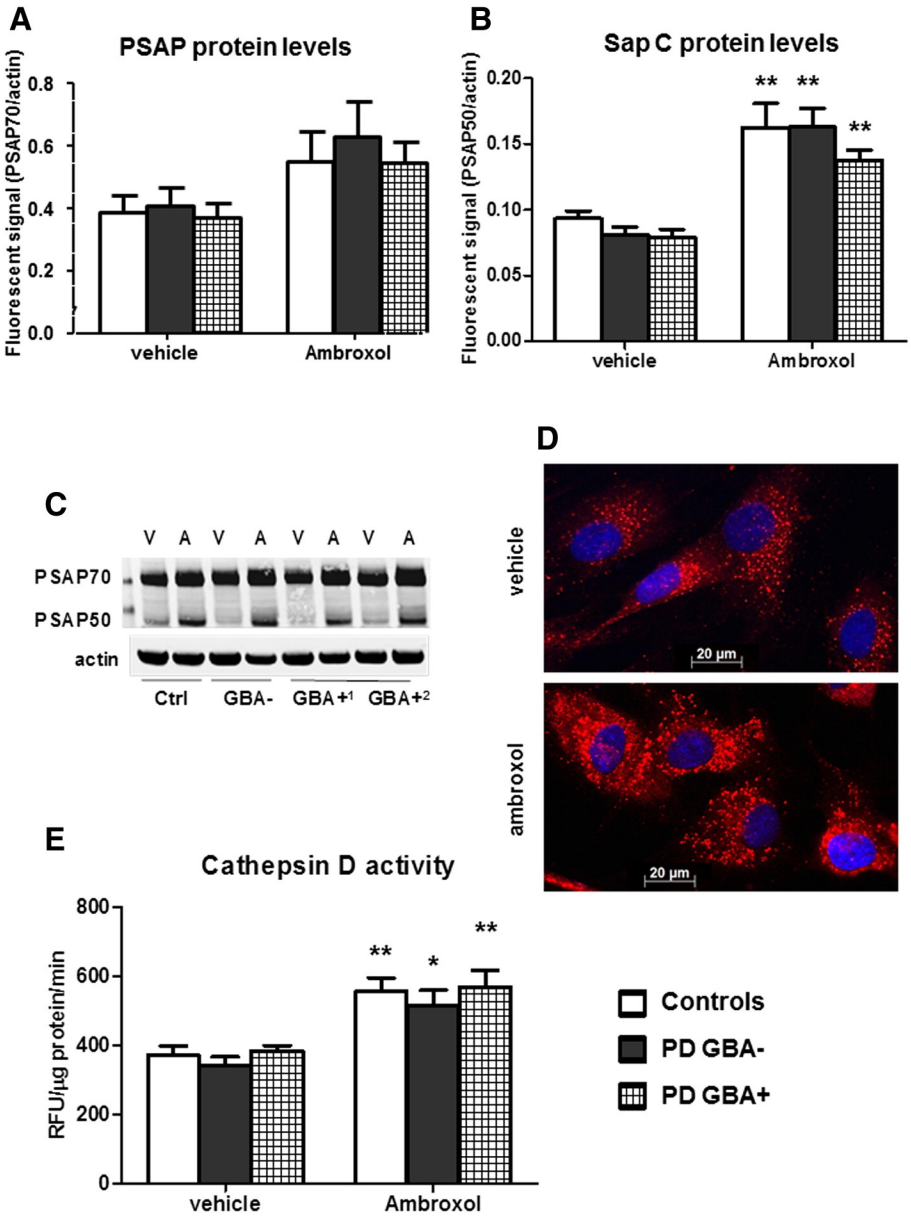

Fig. 2. PSAP, Sap C and cathepsin D activity at baseline and after ambroxol. A) Uncleaved prosaposin (PSAP70) and B) cleaved Sap C (PSAP50) at baseline and after ambroxol. C) Representative image of a membrane after western blot for Sap C in a control subject (Ctrl), one PD patient without *GBA1* mutation (GBA−) and two with *GBA1* mutation (GBA+[1–2]). V: vehicle; A: 60 μM ambroxol (5 days). D) High vacuolization and brighter Sap C staining (red) is shown in the perinuclear area of fibroblasts treated with ambroxol (magnification: 40×; scale bar: 20 μm). E) Cathepsin D activity at baseline and after ambroxol treatment. Ctrl: $n = 7$; PD GBA−: $n = 5$; PD GBA−: $n = 5$; $^{*}p < 0.05$ vs. vehicle; $^{**}p < 0.01$ vs. vehicle (Bonferroni post-hoc test).

alpha-synuclein and avoids alpha-synuclein-mediated inhibition of GCase in a dose-dependent manner (Yap et al., 2013). Reduced GCase activity favors the accumulation of alpha-synuclein in the brain of sporadic PD patients (Murphy et al., 2014). Therefore, small chaperone molecules — such as ambroxol — that are able to boost Sap C expression may also block the vicious cycle that links GCase dysfunction to alpha-synuclein accumulation.

As observed with Sap C, we did not find significant differences in basal levels of GCase transporter LIMP-2 between PD patients (with or without *GBA1* mutation) and healthy subjects; ambroxol moderately increased LIMP-2 levels in all groups, but only in PD GBA+ fibroblasts was the increase statistically significant, with respect to baseline values. Reczek and collaborators have shown that LIMP-2/GCase interaction is preserved in the presence of N370S or L444P mutations in *GBA1* gene; they have also shown that LIMP-2 over-expression can reverse ER retention of mutant GCase (Reczek et al., 2007). This evidence is in line with our results at baseline and with the fact that ambroxol, which plays a role also in supporting translocation of mutant GCase to the lysosomes (Siebert et al., 2014), enhanced protein levels of LIMP-2 selectively in GBA+ fibroblasts.

At basal level, together with the defect of GCase activity in PD GBA+ fibroblasts, we found alterations in other proteostasis markers. The alterations were restricted to PD GBA− patients and were variably influenced by exposure to ambroxol. In line with previous observations of our group, we detected increased parkin levels and reduced activity of catalytic proteasomal subunit 20S in PD GBA− fibroblasts, with respect to controls (Ambrosi et al., 2014), while we did not find modifications of these parameters in PD GBA+ fibroblasts. No information about impaired activity of 20S proteasome in *GBA1* mutant cells is available in the literature. However, mutant GCase causes ER stress and activation of the unfolded protein response, which is associated with activation of the proteasome (Shenkman et al., 2007) and subsequent degradation

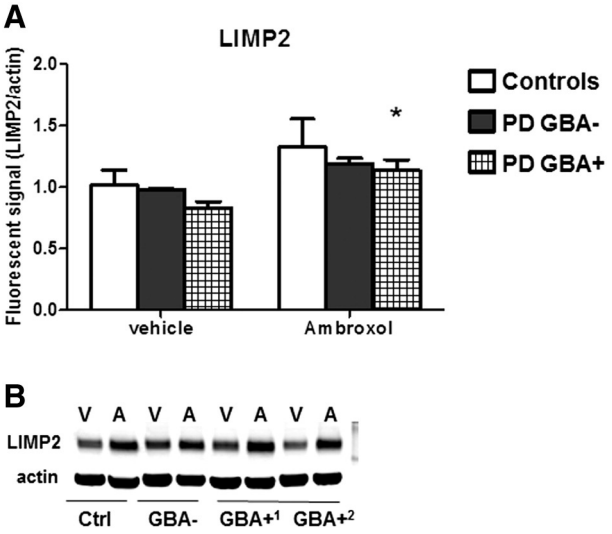

Fig. 3. LIMP-2 protein levels at baseline and after ambroxol. A) LIMP-2 protein levels at baseline and after ambroxol. B) Representative images of a membrane after western blot for LIMP-2 in a control subject (Ctrl), one PD patient without *GBA1* mutation (GBA−) and two with *GBA1* mutation (GBA+[1–2]). V: vehicle; A: 60 μM ambroxol (5 days). Ctrl: $n = 7$; PD GBA−: $n = 5$; PD GBA−: $n = 5$; $^{*}p < 0.05$ vs. vehicle (Bonferroni post-hoc test).

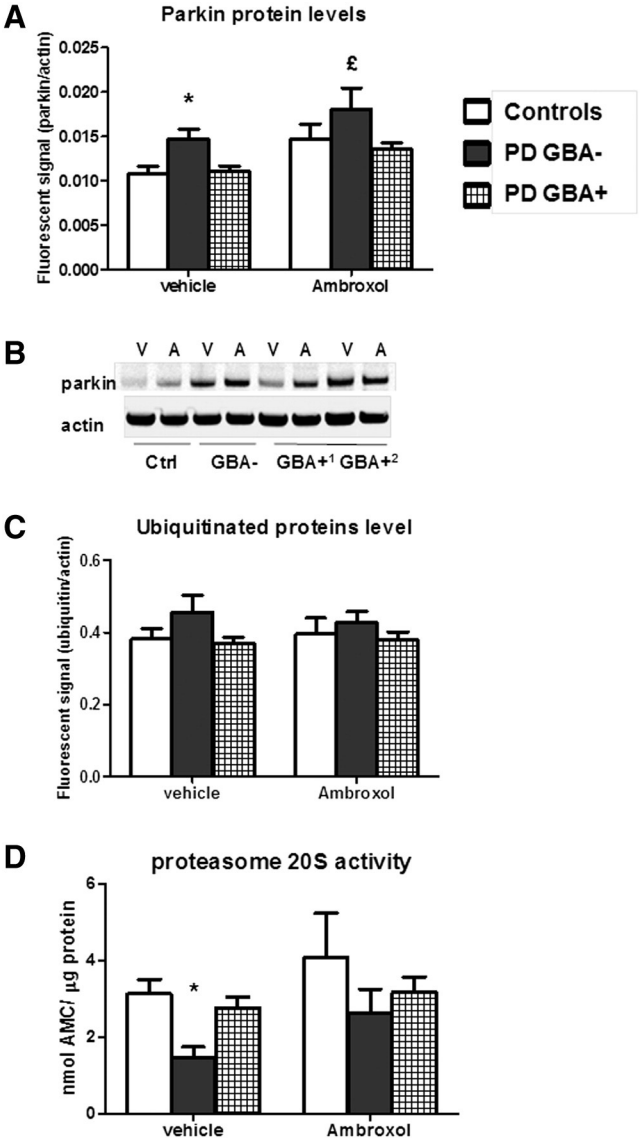

Fig. 4. Parkin, ubiquitinated protein and 20S proteasome activity at baseline and after ambroxol. Parkin (A), ubiquitinated protein levels (C) and proteasome 20S activity (D), at baseline and following exposure to ambroxol. Baseline parkin levels are higher and proteasome 20S activity is lower in PD GBA− patients as compared to the other groups. Ambroxol induces non-significant increases in parkin, poly-ubiquitinated protein levels and proteasome 20S activity in all groups. B) Representative image of cropped membranes after western blot for parkin in a control subject (Ctrl), one PD patient without *GBA1* mutation (GBA−) and two with *GBA1* mutation (GBA+[1-2]). V: vehicle; A: 60 μM ambroxol (5 days). Ctrl: $n = 7$; PD GBA−: $n = 5$; PD GBA−: $n = 5$; $^{*}p < 0.05$ vs. controls and PD GBA+; £$p < 0.05$ vs. PD GBA+ (Bonferroni post-hoc test).

of misfolded GCase (Bendikov-Bar and Horowitz, 2012). Therefore, assuming that a reduction in proteasomal activity — associated with increased parkin levels — might be present in all PD patient cells, the absence of such changes in PD GBA+ fibroblasts may be due to a compensatory increase in proteasomal activity aimed at promoting defective GCase degradation and, more in general, counteracting lysosomal dysfunction in these cells.

At variance with the effects shown on GCase, Sap C, cathepsin D and LIMP-2, ambroxol induced only moderate, non-significant modifications of parkin levels and proteasome 20S activity. These findings indicate a specificity of ambroxol in sustaining endogenous factors that support GCase function and trafficking to the lysosomes, rather than increasing the efficiency of the proteasomal degradative pathway.

Another biochemical alteration restricted to PD GBA− patients regarded GSK3. GSK3 contributes to protein quality control and mediates inflammatory processes that are usually paralleled by activation of ER stress-induced responses (Feng et al., 2013; Zhang and Kaufman, 2008). GSK3 is constitutively activated in resting cells (Golpich et al., 2015; Sutherland et al., 1993; Woodgett, 1994); upon extracellular stimulation, GSK3 can be inactivated by phosphorylation at its N-terminal serine residues Ser21 (GSK3α) and Ser9 (GSK-3β), which triggers downstream signal transduction (Avrahami and Eldar-Finkelman, 2013; Sutherland et al., 1993). We found increased GSK3 phosphorylation at Ser21 in fibroblasts of PD GBA− patients. We had previously observed similar increases in GSK3 phosphorylation in PD lymphocytes, which had been partly explained with the effect of L-Dopa treatment (Armentero et al., 2011). However, fibroblasts are far less susceptible to the effects of circulating drugs, with respect to peripheral blood cells. Furthermore, all patients (with the exception of one PD GBA+ patient) were taking L-Dopa, while GSK3α phosphorylation increased only in PD GBA− patients. Therefore, it is unlikely that L-Dopa induced hyper-phosphorylation of GSK3α. Previous data from Cartelli et al. (2012) showed that GSK3β phosphorylation at Ser9 is reduced in fibroblasts of sporadic PD patients, but no further information on GSK3α (Ser21) phosphorylation in PD fibroblasts is available in the literature. Therefore, on the basis of our experimental data the exact nature of this finding remains difficult to explain. Interestingly, ambroxol reduced phosphorylation of GSK3α (Ser21) only in fibroblasts of PD GBA− patients. This indicates that ambroxol-induced effects on GCase activity and GSK3 phosphorylation are likely unrelated, pointing out an issue that will require further investigation.

Conclusions

In conclusion, our study confirmed that GCase is defective in fibroblasts of *GBA1*-mutant PD patients and that ambroxol is able to correct this defect by increasing both activity and protein levels of GCase. Ambroxol enhanced Sap C protein levels, the essential co-factor promoting GCase activity in the lysosomes, by supporting activity of cathepsin D, which cleaves Sap C from precursor PSAP. Moreover, ambroxol increased LIMP-2 protein, the receptor responsible for

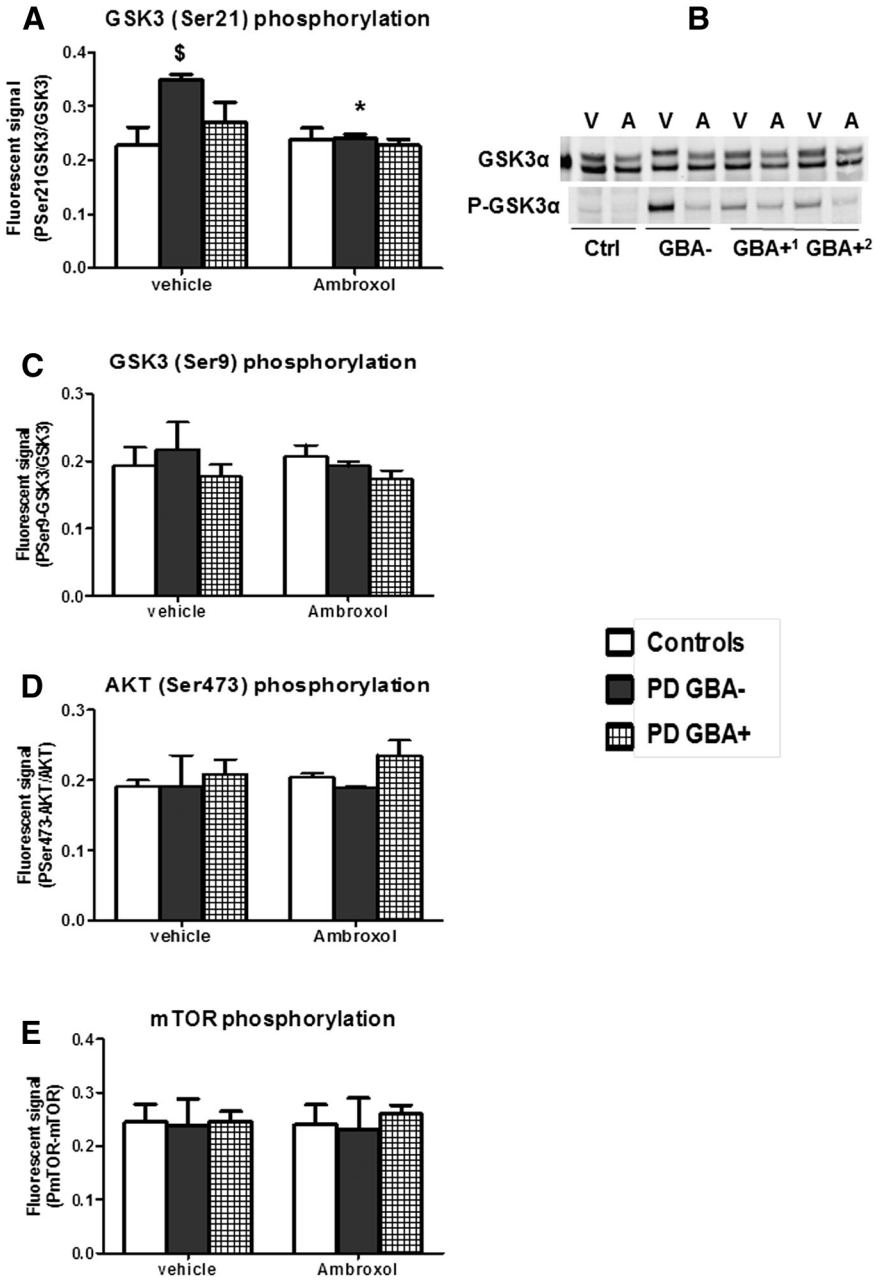

Fig. 5. GSK3/Akt/mTOR pathway activation at baseline and after ambroxol. A) Phosphorylation pattern of GSK3α at Ser21 at baseline and after ambroxol treatment. Fibroblasts of PD patients without *GBA1* mutation (PD GBA−) show higher phosphorylation levels at baseline. This pattern normalizes after ambroxol treatment. B) Representative image of cropped membranes after western blot for GSK3α/P-GSK3α in one control subject (Ctrl), one PD patient without *GBA1* mutation (GBA−) and two PD patients with *GBA1* mutation (GBA+[1] and GBA+[2]). V: vehicle; A: 60 μM ambroxol (5 days). No differences between groups were observed for phosphorylation of GSK3β at Ser9 (C), AKT at Ser473 (D) and mTOR (E), either at baseline or after ambroxol treatment. Ctrl: $n = 7$; PD GBA−: $n = 5$; PD GBA−: $n = 5$; $\$p < 0.05$ vs. controls, $^*p < 0.05$ vs. vehicle (Bonferroni post-hoc test).

targeting GCase to the lysosomes, in *GBA1*-mutant fibroblasts. On the other hand, ambroxol did not interfere with parkin, thereby showing a preferential effect on lysosomal dynamics. The use of molecular chaperones or chaperone modulators, especially in the context of drug repositioning, has been recently proposed as a potential disease-modifying treatment for neurodegenerative diseases associated with proteinopathy, such as PD (Mortiboys et al., 2013; Yang et al., 2014; Ono et al., 2009; Richter et al., 2014). Full understanding of the changes triggered by ambroxol, and possibly other chaperones, in cellular proteostasis will be crucial for better addressing the real potential of these compounds and for identifying new pharmacological strategies and targets for PD.

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References

Alvarez-Erviti, L., Rodriguez-Oroz, M.C., Cooper, J.M., Caballero, C., Ferrer, I., Obeso, J.A., Schapira, A.H.V., 2010. Chaperone-mediated autophagy markers in Parkinson disease brains. Arch. Neurol. 67, 1464–1472.

Ambrosi, G., Ghezzi, C., Sepe, S., Milanese, C., Payan-Gomez, C., Bombardieri, C.R., Armentero, M.-T., Zangaglia, R., Pacchetti, C., Mastroberardino, P.G., Blandini, F., 2014. Bioenergetic and proteolytic defects in fibroblasts from patients with sporadic Parkinson's disease. Biochim. Biophys. Acta 1842, 1385–1394.

Armentero, M.T., Sinforiani, E., Ghezzi, C., Bazzini, E., Levandis, G., Ambrosi, G., Zangaglia, R., Pacchetti, C., Cereda, C., Cova, E., Basso, E., Celi, D., Martignoni, E., Nappi, G., Blandini, F., 2011. Peripheral expression of key regulatory kinases in Alzheimer's disease and Parkinson's disease. Neurobiol. Aging 32, 2142–2151.

Asselta, R., Rimoldi, V., Siri, C., Cilia, R., Guella, I., Tesei, S., Soldà, G., Pezzoli, G., Duga, S., Goldwurm, S., 2014. Glucocerebrosidase mutations in primary parkinsonism. Parkinsonism Relat Disord. 20 (11), 1215–1220.

Avrahami, L., Eldar-Finkelman, H., 2013. GSK-3 and lysosomes meet in Alzheimer's disease. Commun. Integr. Biol. 6, e25179.

Bae, E.J., Yang, N.Y., Lee, C., Lee, H.J., Kim, S., Sardi, S.P., Lee, S.J., 2015. Loss of glucocerebrosidase 1 activity causes lysosomal dysfunction and α-synuclein aggregation. Exp. Mol. Med. 27 (47), e153.

Bendikov-Bar, I., Horowitz, M., 2012. Gaucher disease paradigm: from ERAD to comorbidity. Hum. Mutat. 33 (10), 1398–1407.

Bendikov-Bar, I., Maor, G., Filocamo, M., Horowitz, M., 2013. Ambroxol as a pharmacological chaperone for mutant glucocerebrosidase. Blood Cells Mol. Dis. 50, 141–145.

Bendikov-Bar, I., Rapaport, D., Larisch, S., Horowitz, M., 2014. Parkin-mediated ubiquitination of mutant glucocerebrosidase leads to competition with its substrates PARIS and ARTS. Orphanet J. Rare Dis. 9, 86.

Blandini, F., Sinforiani, E., Pacchetti, C., Samuele, A., Bazzini, E., Zangaglia, R., Nappi, G., Martignoni, E., 2006. Peripheral proteasome and caspase activity in Parkinson disease and Alzheimer disease. Neurology 66, 529–534.

Bouman, L., Schlierf, A., Lutz, A.K., Shan, J., Deinlein, A., Kast, J., Galehdar, Z., Palmisano, V., Patenge, N., Berg, D., Gasser, T., Augustin, R., Trümbach, D., Irrcher, I., Park, D.S., Wurst, W., Kilberg, M.S., Tatzelt, J., Winklhofer, K.F., 2011. Parkin is transcriptionally regulated by ATF4: evidence for an interconnection between mitochondrial stress and ER stress. Cell Death Differ. 18, 769–782.

Calì, T., Ottolini, D., Negro, A., Brini, M., 2013. Enhanced parkin levels favor ER-mitochondria crosstalk and guarantee Ca(2+) transfer to sustain cell bioenergetics. Biochim. Biophys. Acta 1832, 495–508.

Cartelli, D., Goldwurm, S., Casagrande, F., Pezzoli, G., Cappelletti, G., 2012. Microtubule destabilization is shared by genetic and idiopathic Parkinson's disease patient fibroblasts. PLoS One 7 (5), e37467.

Chen, C.-H., Shaikenov, T., Peterson, T.R., Aimbetov, R., Bissenbaev, A.K., Lee, S.-W., Wu, J., Lin, H.-K., Sarbassov, D.D., 2011. ER stress inhibits mTORC2 and Akt signaling through GSK-3β-mediated phosphorylation of rictor. Sci. Signal. 4, ra10.

Crabtree, D., Dodson, M., Ouyang, X., Boyer-Guittaut, M., Liang, Q., Ballestas, M.E., Fineberg, N., Zhang, J., 2014. Over-expression of an inactive mutant cathepsin D increases endogenous alpha-synuclein and cathepsin B activity in SH-SY5Y cells. J. Neurochem. 128, 950–961.

Feng, L., Zhang, D., Fan, C., Ma, C., Yang, W., Meng, Y., Wu, W., Guan, S., Jiang, B., Yang, M., Liu, X., Guo, D., 2013. ER stress-mediated apoptosis induced by celastrol in cancer cells and important role of glycogen synthase kinase-3β in the signal network. Cell Death Dis. 4, e715.

Golpich, M., Amini, E., Hemmati, F., Ibrahim, N.M., Rahmani, B., Mohamed, Z., Raymond, A.A., Dargahi, L., Ghasemi, R., Ahmadiani, A., 2015. Glycogen synthase kinase-3 beta (GSK-3β) signaling: implications for Parkinson's disease. Pharmacol. Res. 97, 16–26.

Grabowski, G.A., Horowitz, M., 1997. Gaucher's disease: molecular, genetic and enzymological aspects. Baillieres Clin. Haematol. 10, 635–656.

Hiraiwa, M., Martin, B.M., Kishimoto, Y., Conner, G.E., Tsuji, S., O'Brien, J.S., 1997. Lysosomal proteolysis of prosaposin, the precursor of saposins (sphingolipid activator proteins): its mechanism and inhibition by ganglioside. Arch. Biochem. Biophys. 341, 17–24.

Maegawa, G.H.B., Tropak, M.B., Buttner, J.D., Rigat, B.A., Fuller, M., Pandit, D., Tang, L., Kornhaber, G.J., Hamuro, Y., Clarke, J.T.R., Mahuran, D.J., 2009. Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. J. Biol. Chem. 284, 23502–23516.

McGlinchey, R.P., Lee, J.C., 2013. Emerging insights into the mechanistic link between α-synuclein and glucocerebrosidase in Parkinson's disease. Biochem. Soc. Trans. 41, 1509–1512.

McNeill, A., Magalhaes, J., Shen, C., Chau, K.-Y., Hughes, D., Mehta, A., Foltynie, T., Cooper, J.M., Abramov, A.Y., Gegg, M., Schapira, A.H.V., 2014. Ambroxol improves lysosomal biochemistry in glucocerebrosidase mutation-linked Parkinson disease cells. Brain 137, 1481–1495.

Misasi, R., Hozumi, I., Inuzuka, T., Capozzi, A., Mattei, V., Kuramoto, Y., Shimeno, H., Soeda, S., Azuma, N., Yamauchi, T., Hiraiwa, M., 2009. Biochemistry and neurobiology of prosaposin: a potential therapeutic neuro-effector. Cent. Nerv. Syst. Agents Med. Chem. 9, 119–131.

Mortiboys, H., Aasly, J., Bandmann, O., 2013. Ursocholanic acid rescues mitochondrial function in common forms of familial Parkinson's disease. Brain 136, 3038–3050.

Motta, M., Camerini, S., Tatti, M., Casella, M., Torreri, P., Crescenzi, M., Tartaglia, M., Salvioli, R., 2014. Gaucher disease due to saposin C deficiency is an inherited lysosomal disease caused by rapidly degraded mutant proteins. Hum. Mol. Genet. 23, 5814–5826.

Murphy, K.E., Gysbers, A.M., Abbott, S.K., Tayebi, N., Kim, W.S., Sidransky, E., Cooper, A., Garner, B., Halliday, G.M., 2014. Reduced glucocerebrosidase is associated with increased α-synuclein in sporadic Parkinson's disease. Brain 137, 834–848.

Nalls, M.A., Duran, R., Lopez, G., Kurzawa-Akanbi, M., McKeith, I.G., et al., 2013. A multicenter study of glucocerebrosidase mutations in dementia with Lewy bodies. JAMA Neurol. 70, 727–735.

Nijholt, D.A.T., Nölle, A., van Haastert, E.S., Edelijn, H., Toonen, R.F., Hoozemans, J.J.M., Scheper, W., 2013. Unfolded protein response activates glycogen synthase kinase-3 via selective lysosomal degradation. Neurobiol. Aging 34, 1759–1771.

Ono, K., Ikemoto, M., Kawarabayashi, T., Ikeda, M., Nishinakagawa, T., Hosokawa, M., Shoji, M., Takahashi, M., Nakashima, M., 2009. A chemical chaperone, sodium 4-phenylbutyric acid, attenuates the pathogenic potency in human alpha-synuclein A30P + A53T transgenic mice. Parkinsonism Relat. Disord. 15, 649–654.

Palmieri, M., Impey, S., Kang, H., di Ronza, A., Pelz, C., Sardiello, M., Ballabio, A., 2011. Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. Hum. Mol. Genet. 20, 3852–3866.

Parr, C., Carzaniga, R., Gentleman, S.M., Van Leuven, F., Walter, J., Sastre, M., 2012. Glycogen synthase kinase 3 inhibition promotes lysosomal biogenesis and autophagic degradation of the amyloid-β precursor protein. Mol. Cell. Biol. 32, 4410–4418.

Reczek, D., Schwake, M., Schröder, J., Hughes, H., Blanz, J., Jin, X., Brondyk, W., Van Patten, S., Edmunds, T., Saftig, P., 2007. LIMP-2 is a receptor for lysosomal mannose-6-phosphate-independent targeting of beta-glucocerebrosidase. Cell 131, 770–783.

Richter, F., Fleming, S.M., Watson, M., Lemesre, V., Pellegrino, L., Ranes, B., Zhu, C., Mortazavi, F., Mulligan, C.K., Sioshansi, P.C., Hean, S., De La Rosa, K., Khanna, R., Flanagan, J., Lockhart, D.J., Wustman, B.A., Clark, S.W., Chesselet, M.-F., 2014. A GCase chaperone improves motor function in a mouse model of synucleinopathy. Neurotherapeutics 11, 840–856.

Ron, I., Rapaport, D., Horowitz, M., 2010. Interaction between parkin and mutant glucocerebrosidase variants: a possible link between Parkinson disease and Gaucher disease. Hum. Mol. Genet. 19, 3771–3781.

Rothaug, M., Zunke, F., Mazzulli, J.R., Schweizer, M., Altmeppen, H., Lüllmann-Rauch, R., Kallemeijn, W.W., Gaspar, P., Aerts, J.M., Glatzel, M., Saftig, P., Krainc, D., Schwake, M., Blanz, J., 2014. LIMP-2 expression is critical for β-glucocerebrosidase activity and α-synuclein clearance. Proc. Natl. Acad. Sci. U. S. A. 111 (43), 15573–15578.

Sardi, S.P., Cheng, S.H., Shihabuddin, L.S., 2015. Gaucher-related synucleinopathies: the examination of sporadic neurodegeneration from a rare (disease) angle. Prog. Neurobiol. 125C, 47–62.

Schapira, A.H., 2015. Glucocerebrosidase and Parkinson disease: recent advances. Mol Cell Neurosci. 66(Pt A), 37–42.

Schapira, A.H.V., Gegg, M.E., 2013. Glucocerebrosidase in the pathogenesis and treatment of Parkinson disease. Proc. Natl. Acad. Sci. U. S. A. 110, 3214–3215.

Settembre, C., Zoncu, R., Medina, D.L., Vetrini, F., Erdin, S., Huynh, T., Ferron, M., Karsenty, G., Vellard, M.C., Facchinetti, V., Sabatini, D.M., Ballabio, A., 2012. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J. 31, 1095–1108.

Shenkman, M., Tolchinsky, S., Lederkremer, G.Z., 2007. ER stress induces alternative nonproteasomal degradation of ER proteins but not of cytosolic ones. Cell Stress Chaperones 12 (4), 373–383.

Sidransky, E., Lopez, G., 2012. The link between the GBA gene and parkinsonism. Lancet Neurol. 11, 986–998.

Sidransky, E., Nalls, M.A., Aasly, J.O., Aharon-Peretz, J., Annesi, G., et al., 2009. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N. Engl. J. Med. 361, 1651–1661.

Siebert, M., Sidransky, E., Westbroek, W., 2014. Glucocerebrosidase is shaking up the synucleinopathies. Brain 137, 1304–1322.

Sutherland, C., Leighton, I.A., Cohen, P., 1993. Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. Biochem. J. 296 (Pt 1), 15–19.

Tamargo, R.J., Velayati, A., Goldin, E., Sidransky, E., 2012. The role of saposin C in Gaucher disease. Mol. Genet. Metab. 106, 257–263.

Tan, Y.L., Genereux, J.C., Pankow, S., Aerts, J.M.F.G., Yates, J.R., Kelly, J.W., 2014. ERdj3 is an endoplasmic reticulum degradation factor for mutant glucocerebrosidase variants linked to Gaucher's disease. Chem. Biol. 21, 967–976.

Tatti, M., Motta, M., Salvioli, R., 2011. Autophagy in Gaucher disease due to saposin C deficiency. Autophagy 7, 94–95.

Tatti, M., Motta, M., Di Bartolomeo, S., Scarpa, S., Cianfanelli, V., Cecconi, F., Salvioli, R., 2012. Reduced cathepsins B and D cause impaired autophagic degradation that can be almost completely restored by overexpression of these two proteases in Sap C-deficient fibroblasts. Hum. Mol. Genet. 21, 5159–5173.

Vaccaro, A.M., Motta, M., Tatti, M., Scarpa, S., Masuelli, L., Bhat, M., Vanier, M.T., Tylki-Szymanska, A., Salvioli, R., 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.

Westbroek, W., Gustafson, A.M., Sidransky, E., 2011. Exploring the link between glucocerebrosidase mutations and parkinsonism. Trends Mol. Med. 17, 485–493.

Woodgett, J.R., 1994. Regulation and functions of the glycogen synthase kinase-3 subfamily. Semin. Cancer Biol. 5, 269–275.

Xilouri, M., Stefanis, L., 2015. Chaperone mediated autophagy to the rescue: a new-fangled target for the treatment of neurodegenerative diseases. Mol Cell Neurosci. 66(Pt A), 29–36.

Yang, C., Swallows, C.L., Zhang, C., Lu, J., Xiao, H., Brady, R.O., Zhuang, Z., 2014. Celastrol increases glucocerebrosidase activity in Gaucher disease by modulating molecular chaperones. Proc. Natl. Acad. Sci. U. S. A. 111, 249–254.

Yap, T.L., Gruschus, J.M., Velayati, A., Sidransky, E., Lee, J.C., 2013. Saposin C protects glucocerebrosidase against α-synuclein inhibition. Biochemistry 52, 7161–7163.

Zhang, K., Kaufman, R.J., 2008. From endoplasmic-reticulum stress to the inflammatory response. Nature 454, 455–462.