The biochemical basis of interactions between Glucocerebrosidase and alpha-synuclein in *GBA*1 mutation carriers

M. Toffoli[1], L. Smith[1], A.H.V. Schapira[1]

[1] Department of Clinical and Movement Neurosciences, University College London Queen Square Institute of Neurology, London, United Kingdom.

Corresponding author:

Professor Anthony H V Schapira, MD, DSc, FRCP, FMedSci

Department of Clinical and Movement Neurosciences, University College London Institute of Neurology, London, UK

Rowland Hill St., London NW3 2PF, UK

Email: a.schapira@ucl.ac.uk

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Abbreviations: A-SYN, alpha-synuclein; BBB, blood brain barrier; BIP, binding immunoglobulin protein; CTSD, Cathepsin D; ER, endoplasmic reticulum; ERAD, endoplasmic-reticulum-associated protein degradation; ERT, enzyme replacement therapy; GC, glucosylceramide; GCase, glucocerebrosidase; GD, Gaucher disease; GS, glucosylsphingosine; LSD, lysosomal storage disorder; PD, NaTC, sodium taurocholate; NN-DNJ, N-(n-nonyl)deoxynojirimycin; Parkinson disease; sapC, saposin C; SMCS Small Molecule Chaperones; SRT, substrate reduction therapy.

Abstract

The discovery of genes involved in familial as well as sporadic forms of Parkinson disease (PD) constitutes an important milestone in understanding this disorder's pathophysiology and potential treatment. Among these genes, *GBA1* is one of the most common and well-studied, but it is still unclear how mutations in *GBA1* translate into an increased risk for developing PD. In this review, we provide an overview of the biochemical and structural relationship between *GBA1* and PD to help understand the recent advances in the development of PD therapies intended to target this pathway.

Introduction

Parkinson disease (PD) is a common neurodegenerative disorder, currently estimated to have a lifetime risk of 3-4%. Therapy for PD is symptomatic and does not influence the progression of the disease. One of the main limitations to the development of disease modifying therapies for PD is the likely etiological and pathogenetic heterogeneity yet with similar clinical and pathological presentations(Balestrino and Schapira 2019). The clinical trials currently investigating potential treatments for PD have focused on those subjects with specific gene mutations(Sardi *et al.* 2018). *GBA1,* the gene causing autosomal recessive Gaucher disease (GD), is an interesting candidate for this role, given the relatively high prevalence of its mutations in PD patients and the existence of drugs for GD that might be effective in treating PD. Although there are some excellent reviews on the topic(Gegg and Schapira 2018; Blandini *et al.* 2019; Stojkovska *et al.* 2018), we thought it opportune to approach this topic from a different angle and so we have focused on both biochemical and molecular structure studies to give perspective and context to the recent and ongoing trials on disease modifying therapies for PD.

Gaucher disease

GD is an autosomal recessive lysosomal storage disorder (LSD) caused by mutations in the gene glucosylceramidase beta (*GBA*1)(Balicki and Beutler 1995; Beutler and Grabowski 2001). Its prevalence has been calculated at 1.75:100,000 in the Australian population (Meikle *et al.* 2016), 1.16:100,000 in the Netherlands(Poorthuis *et al.*) and 2.49:100,000 in the Italian population(Dionisi-Vici *et al.* 2002) and it is commonly regarded as the most common LSD. In the Jewish population the incidence of GD is higher (about 1 in every 800 live births for type 1 GD)(Beutler and Grabowski 2001). *GBA*1, localised to chromosome 1q21, has 11 exons and encodes for the lysosomal enzyme glucocerebrosidase (GCase), that is assembled in the endoplasmic reticulum and transported to the lysosome upon binding to the receptor LIMP-2(Reczek *et al.* 2007).

*GBA*1 mutations can lead to a reduced level of GCase activity with the consequent accumulation of its main substrates, glucosylceramide (GC) and glucosylsphingosine (GS). More than 300 mutations are known to cause GD, encompassing point mutations, splice-site alterations, insertions, deletions, frame shifts and recombinant alleles, with the most common being the point mutations N370S and

L444P(Sidransky *et al.* 2009; Hruska *et al.* 2008). GC accumulates mainly in macrophages and leads to hepatosplenomegaly, bone marrow involvement with consequent anaemia and thrombocytopenia, bone abnormalities, neurological symptoms and other less common manifestations. According to the presence or absence of nervous system involvement, GD is categorized into type 1 (non-neuronopathic), type 2 (acute neuronopathic) and type 3 (chronic neuronopathic)(Beutler and Grabowski 2001). Symptom onset in type 2, the most severe form of GD, is before 6 months of age and death occurs within the first 2 years of life(Mistry *et al.* 2015). Type 3 GD has a later onset and patients usually reach adulthood(Mistry *et al.* 2015). Neurological manifestations in GD include oculomotor apraxia, supranuclear ophthalmoplegia, extrapyramidal features, myoclonic or generalized seizures, cerebellar ataxia, and developmental delay(Tylki-Szymańska *et al.* 2010; Mistry *et al.* 2015). While visceral manifestations are a consequence of GC and GS accumulation in macrophages, neuronal dysfunction and death are considered the main mechanisms for neurological expression, but the exact pathogenesis is not clear. Since substrate accumulation is not observed in GD neurons, it is hypothesized that even low concentrations of GC, GS or both could prove toxic for neurons(Grabowski 2008).

Epidemiological evidence for Parkinson disease related to GBA1

Over the last two decades, growing evidence has pointed toward a relationship between GD and Parkinson disease (PD). The first reports of patients with type 1 GD developing PD were published more than 20 years ago(Neudorfer *et al.* 1996; Tayebi *et al.* 2001; Tayebi *et al.* 2003; Bembi *et al.* 2003), and shifted the classical paradigm that viewed type 1 GD as non-neuronopathic. Subsequent cohort studies highlighted an increased incidence of *GBA*1 mutation carriers (heterozygotes) in PD patients, suggesting that the carrier status is a risk factor for PD(Lwin *et al.* 2004; Ziegler *et al.* 2007; Moraitou *et al.*; Mao *et al.* 2010; Kumar *et al.* 2013; Choi *et al.* 2012). One study compared 5691 PD patients with 4898 controls and estimated an odds ratio for *GBA*1 of 5.43(Sidransky *et al.* 2009). Other genome wide association studies followed, confirming the strong association of *GBA*1 with PD(Do *et al.* 2011; Nalls *et al.* 2014; Simon-Sanchez *et al.* 2009; Satake *et al.* 2009; Chen *et al.* 2014) and making it quantitatively the most common gene associated with PD. The estimated risk of developing PD among GBA1 mutation carriers is between 10 and 30% by the age of 80(O'Regan *et al.* 2017).

GBA1 mutations and PD phenotype

*GBA*1 mutations seem to determine a slight modification of phenotype in PD. Patients with PD+*GBA*1 mutations have been reported to show an earlier age at onset by 4-5 years on average, a higher

prevalence of a more severe akinetic-rigid form of the disease and a worse cognitive profile compared to PD patients without *GBA*1 mutations(Zhang *et al.* 2015; Zhao *et al.* 2016; Goker-Alpan *et al.* 2008). Moreover, PD risk can be stratified among carriers according to their genotype: a recent meta-analysis aggregating 31 studies (11.453 cases and 14.565 controls) showed that odds ratios for developing PD are 2.84-3.07 for mild mutations and 10.28-15.49 for severe mutations(Gan-Or *et al.* 2015); severe mutation carriers also had a 5 years younger age at onset of PD than mild mutations carriers(Gan-Or *et al.* 2015).

GCase structure

A comprehensive structural review of GCase is beyond the scope of this article and can be found in the literature(Lieberman 2011; Kacher *et al.* 2008; Smith *et al.* 2017). The X-ray structure of GCase was first described in 2003(Dvir *et al.* 2003). Since then the enzyme has been studied under different conditions, to eliminate concerns about possible structural diversity using different protein sources and media(Kacher *et al.* 2008). GCase is a 497 amino acid protein(Horowitz *et al.* 1989) with 3 discontinuous domains(Lieberman 2011). Domain 1 (residues 1–27 and 383–414) comprises an antiparallel beta-sheet (2 N terminal beta-strands and 2 antiparallel beta-strands from the insertion between alpha-helix 8 and beta-strand 8 of domain 3) and a structural loop. Domain 1 is connected to domain 2 by a long loop, and has a tight interaction with domain 3, whose active site is capped by the loop in domain 1(Kacher *et al.* 2008). Domain 2 (residues 30–75 and 431–497) is an 8-stranded beta-barrel with a structure that resembles an immunoglobulin (Ig) fold and is connected to domain 3 by a hinge; structures similar to domain 2 can be observed in other glycosidases, but their function is unknown(Kacher *et al.* 2008). Domain 3 (residues 76–381 and 416–430) is a $(\beta/\alpha)_8$ triosephosphate isomerase (TIM) barrel containing the active site(Kacher *et al.* 2008). The active site is a catalytic dyad consisting of the nucleophile residue Glu 340 and the general acid-base residue Glu 235. It is capped by 3 loops, named Loop 1, Loop 2 and Loop 3. These loops are capable of some flexibility(Kacher *et al.* 2008) and this is in part responsible for GCase's pH dependency. In particular, loop 3 can rearrange from an extended to a helical conformation, which is stabilized in an acidic pH, conferring GCase a higher affinity for substrate(Lieberman 2011). Domain 3 and domain 1 contain cysteine residues, which are thought to be important for enzyme activity; in particular, Cys 342, is in close vicinity to the active site so may play a role in stabilization(Liou *et al.* 2006). It has to be noted that some papers refer to domain 2 as domain 3 and vice versa and to loop 1 as loop 3 and vice versa; in our review, we use the nomenclature which is most consistent with previous publications. Another key structural aspect of GCase is its N-linked glycosylation. GCase consists of five potential Asn residues, the first four of which are almost always

occupied (Asn 19, Asn 59, Asn 146, Asn 270 and Asn 462) and glycosylation is vital for the formation of an enzymatically active enzyme(Berg-Fussman *et al.* 1993; Pol-Fachin *et al.* 2016).

Figure 1: Crystal structure of glucocerebrosidase at pH 5.5 (PDB code 3GXI) created using PYMOL (http://www.pymol.org) and corresponding sequence. *Domain I is shown in green. Domain II is shown in blue. Domain III is shown in pink. The active site catalytic residues Glu 235 and Glu 340 are shown as ball-and-stick models, or underlined in the sequence. The five N-glycosylation sites (Asn 19, Asn 59, Asn 146, Asn 270 and Asn 462) are shown as purple spheres. The free cysteine residues are shown as yellow spheres. Four common mutations, L444P, N370S, E326K and 84GG are labelled as red spheres.*

Pathophysiology of GCase-A-syn interaction

Numerous hypotheses have been advanced on the possible ways by which GCase mutations induce α-synuclein (A-SYN) accumulation, but none fully explain experimental data. It is likely that multiple mechanisms have a role and that different *GBA*1 mutations may preferentially lead to different pathogenic pathways. It is also possible that these mutations act differently according to whether they are monoallelic or biallelic.

Substrate accumulation

GCase activity is reduced in the brain and in the blood of PD+*GBA*1 and sporadic PD compared to controls (Gegg *et al.* 2012; Alcalay *et al.* 2015). It is postulated that the accumulation of GC and GS, derived from reduced GCase activity, may prompt A-SYN accumulation by interfering with its homeostasis in the lysosomes. In support of this hypothesis, experimental data show increased A-SYN levels in both cells(Manning-Boğ *et al.* 2009; Cleeter *et al.* 2013) and rats(Xu *et al.* 2011; Manning-Boğ *et al.* 2009) treated with the GCase inhibitor conduritol B epoxide (CBE), which is capable of reducing GCase activity to less than 5% of untreated controls(Cleeter *et al.* 2013), although this result was not replicated in other studies(Dermentzaki *et al.* 2013; Cullen *et al.* 2011). Moreover, *in vitro* studies reported that high concentrations of GC result in enhanced formation of A-SYN amyloid fibrils(Mazzulli *et al.* 2011) and proteinase K resistant A-SYN(Suzuki *et al.* 2015). Interestingly, a recent *in vitro* study found that while GC promotes the formation of long filaments of A-SYN, GS does not(Taguchi *et al.* 2017). A direct pathogenic effect has also been proposed after observing that human-derived

dopaminergic pluripotent stem cells with *GBA1* mutations display accumulation of A-SYN, that can be reversed by treatment with an inhibitor of GC-synthase and consequent reduction of the levels of GC and GS(Kim *et al.* 2018). This might be explained by the observation in iPSCs that soluble high molecular weight polymers of A-SYN, when in a GC and GS rich environment, show unique structural properties, that make them more prone to recruit A-SYN monomers into insoluble toxic, and that reduction of GC and GS levels can reverse this process(Zunke *et al.* 2018). Another possible explanation derives from the knowledge that A-SYN synaptic localization is mediated by lipid rafts(Fortin *et al.* 2004) and, although the physiological effect of this interaction is not clear, a change in lipid levels could alter A-SYN trafficking. That said, although in animal models there is evidence of accumulation of GCase substrate(Suzuki *et al.* 2015), there is some controversy regarding lipid accumulation in the brain of human PD cases. While no evidence of GC and GS accumulation was observed in the putamen, cerebellum and primary motor cortex of PD patients with *GBA*1 mutations in two studies(Gegg *et al.* 2015; Clark *et al.* 2015), another study showed evidence of GS accumulation in the substantia nigra and hippocampus of PD patients without *GBA*1 mutations(Rocha *et al.* 2015). Importantly, no GC accumulation was observed in the brain of PD cases without *GBA*1 mutations(Boutin *et al.* 2016).

Inhibition of autophagy

Autophagy is one of the mechanisms the cell has to disassemble unwanted components. Three types of autophagy are recognised: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). It has been shown that reducing CMA by decreasing LAMP2A levels in dopaminergic cells leads to an increase in A-SYN levels(Alvarez-Erviti *et al.* 2010) and that activation of autophagy by beclin 1 reduce deposits of A-SYN in a cellular model(Spencer *et al.* 2009), suggesting autophagy is a relevant factor in A-SYN clearance and PD pathology. Supporting this theory, impairment of the autophagic pathway has been observed in the brains of idiopathic PD patients(Alvarez-Erviti *et al.* 2010) and the accumulation of autophagosomes linked to lysosomal depletion and dysfunction(Dehay *et al.* 2010). Two studies demonstrated that *GBA*1 knockdown causes downregulation of autophagy via inactivation of protein phosphatase 2A (PP2A) both *in vitro* and *in vivo*(Du *et al.* 2015) and that ceramide can stimulate autophagy(Scarlatti *et al.* 2004). Another hypothesis suggests that GC can stabilize lipid enriched liquid domains, which are essential for autophagy. To support this, decreased membrane fluidity was observed in GCase deficient fibroblasts(Varela *et al.* 2016), although it is not clear whether this process takes place in other cell types, like neurons.

Depletion of functional lysosomes

In GD cellular and *drosophila* models mTOR activity is reduced(Kinghorn *et al.* 2016; Magalhaes *et al.* 2015). Since mTOR is key to the reformation of lysosomes after macroautophagy (a process called autophagic lysosome reformation), it is postulated that *GBA*1 mutations could cause a depletion of functional lysosomes in cells. In neurons and SH-SY5Y cells with decreased GCase activity, the impairment of the autophagic lysosome reformation process was associated with increased levels of A-SYN(Magalhaes *et al.* 2015).

Saturation of the ubiquitin-proteasome pathway

GCase is normally folded in the endoplasmic reticulum (ER), glycosylated in four of its five potential N-glycosylation sites in the Golgi and then transported to the lysosomes(Glickman and Kornfeld 1993; Ericksonss *et al.* 1985; Leonova and Grabowski 2000). Some *GBA*1 mutations result in a misfolded GCase that fails to reach the lysosomes, triggers the endoplasmic-reticulum-associated protein degradation (ERAD) and is targeted by the ubiquitin-proteasome pathway(Ron and Horowitz 2005; Bendikov-Bar and Horowitz 2012; Maor *et al.* 2013), which could become saturated and unable to respond to accumulation of other proteins, like A-SYN. There is now a growing body of evidence in support of activation of the ubiquitin proteasome pathway by mutant GCase(Ron and Horowitz 2005; Bendikov-Bar and Horowitz 2012; Maor *et al.* 2013; Lu *et al.* 2010). In particular two recent studies found increased levels of binding immunoglobulin protein (BiP) and Calnexin associated with GBA mutations in human midbrain neurons derived from PD patients as well as in *Drosophila* models(Fernandes *et al.* 2016; Sanchez-Martinez *et al.* 2016) and another one observed higher levels of intracellular calcium from the ER in neurons derived from GBA-PD patients(Schöndorf *et al.* 2014). However, a minority of studies failed to show a relationship between GBA mutations and ERAD/ubiquitin-proteasome pathway activation(Cullen *et al.* 2011; Farfel-Becker *et al.* 2009; Enquist *et al.* 2007).

The negative feedback

Both *in vitro* and *in vivo* studies have shown that increased levels of A-SYN result in a decrease of GCase activity(Alcalay *et al.* 2015; Mazzulli *et al.* 2011; Gegg *et al.* 2012). One possible explanation of this effect is that A-SYN might impair GCase trafficking, as suggested by the observation that the fraction of GCase that is retained in the ER can be reduced by lowering A-SYN levels and, conversely, overexpression of wild-type or mutant A-SYN can cause accumulation of GCase in the ER in H4 cells, primary cortical neurons and even in brain tissue(Mazzulli *et al.* 2011). An alternative explanation could come from the observation that when interacting with A-SYN, GCase is lifted from the membrane, distancing its active site from the lipid bilayer(Yap *et al.* 2015). GCase activity relies on its interaction with membrane phosphatidylserine lipids(Yap *et al.* 2015) and hence this process may limit the catalytic function of the GCase enzyme. Given that reduced GCase activity can increase A-SYN levels, it is possible that these mechanisms are part of a self-amplifying circle where a rise in A-SYN levels reduces GCase activity, and therefore results in a continued increase in A-SYN levels.

Inhibition of alpha-synucleases

Cathepsin D (CTSD) has a role in A-SYN degradation, as is shown by the augmented proteolysis of A-SYN in a cell culture with overexpression of CTSD and increased insoluble A-SYN in extracts from brains of CTSD knockdown mice(Cullen *et al.* 2009; Sevlever *et al.* 2008). Ceramide acts as a second messenger for CTSD, binding to it and inducing its proteolytic activity(Heinrich *et al.* 2000). A reduction in GCase activity and in ceramide production could then prevent cathepsin D activation and consequently increase insoluble A-SYN levels.

Physical linkage between GCase and A-SYN

Experimental data show that GCase and A-SYN directly interact at lysosomal pH(Yap *et al.* 2011). A-SYN is degraded in lysosomes via CMA(Cuervo *et al.* 2004) and mutations in A-SYN can alter this process(Xilouri *et al.* 2009). A recent study has tried to clarify the structural relationship between the GCase and A-SYN on a lipid bilayer by the mean of neutron reflectometry and, among other results, has shown that approximately half of A-SYN alpha helix (completely embedded in the lipid bilayer of lysosomes) is lifted after the interaction with GCase. The authors have postulated that this movement of A-SYN, operated by GCase, could expose a portion of A-SYN to lysosomal proteases, enhancing its

degradation(Yap *et al.* 2015). Some mutations, like N370S (*see below*), could interfere with this process, increasing A-SYN accumulation.

Mitochondrial impairment

The role of mitochondria in the development of PD is well documented in the literature(Schapira *et al.* 2011; Zaltieri *et al.* 2015). The explanation of this link likely lies in the complex interplay between oxidative stress and A-SYN aggregation. Several studies have shown that reactive oxygen species (ROS) can lead, both directly and indirectly, to A-SYN accumulation. Incubation of A-SYN with H_2O_2 and cytochrome C, or treatment of cultured cells with ROS can lead to A-SYN aggregation(Hashimoto *et al.* 1999; Ostrerova-Golts *et al.* 2000). Moreover, ROS can lead to A-SYN translocation into the nucleus(Xu *et al.* 2006), where the interaction with histones can enhance the formation of insoluble fibrils(Goers *et al.* 2003). On the other hand, high concentration or misfolding of A-SYN can lead to ROS formation in cultured cells(Junn and Mouradian 2002) and increase susceptibility to ROS toxicity in mice(Nieto *et al.* 2006). All this evidence suggests that A-SYN pathology and oxidative stress are linked by a self-perpetuating cycle, where one can promote the other and *vice versa*(Dias *et al.* 2013). Since mitochondria are both a major source and target of ROS, it is likely that they also play a role in PD pathogenesis. The first observations that the mitochondrial complex 1 inhibitor MPTP can cause a parkinsonian syndrome in man(Langston and Ballard 1983) were followed by evidence of reduced complex 1 activity in *post mortem* brain samples of PD patients compared to controls(Schapira *et al.* 1990). Moreover, A-SYN can localise to the mitochondrial membrane and interact directly with mitochondrial complex 1, decreasing its activity(Chinta *et al.* 2010; Li *et al.* 2007). The association between mitochondrial malfunction and alpha-synucleinopathies is also supported by the involvement of PINK1, PARK2 and PARK7, all causative loci for PD, in mitochondrial turnover(Corti *et al.* 2011). In a recent experiment, GCase suppression via CBE and shRNA in SH-SY5Y cells caused a decline in mitochondrial membrane potential, fragmentation of mitochondria, a reduction of ATP synthesis and an increase in free radical generation. In the same experiment, A-SYN levels were significantly higher in *GBA*1 knockdown cells than in controls(Cleeter *et al.* 2013). Similar results have been obtained in fibroblasts from GD patients (L444P/L444P)(de la Mata *et al.* 2015) and mouse models with *GBA*1 knockout(Osellame *et al.* 2013). These data point toward an association between GCase deficiency, mitochondrial dysfunction and alpha-synucleinopathies, but a causative link is yet to be demonstrated and the majority of the *in vivo* evidence of mitochondrial dysfunction derives from *GBA*1 knock-out

models instead of *GBA*1 mutations. The leading pathological hypotheses encompass a combination of 1- reduced mitophagy with a mechanism similar to that described for reduced autophagy, 2- reduced biosynthesis of CoQ_{10} (by altering the lipid/sterol metabolism or by lysosomal impairment), 3- direct damage on mitochondria by A-SYN accumulation, 4- alteration of the control of ER on calcium homeostasis, 5- neuroinflammation damage via activation of the glia by GC accumulation(Gegg and Schapira 2016).

The role of the glia and A-SYN secretion

A-SYN pathology outside of neurons has been investigated in recent years. A study on rat neuronal and astrocyte primary cultures showed that both cell types can take up A-SYN aggregates, although astrocytes far more efficiently , and that A-SYN can travel from astrocytes to neurons, suggesting that spread of A-SYN can occur from cell to cell in a prion-like fashion(Cavaliere *et al.* 2017). In contrast, in the same year another study showed that in mice cortical cultures αSyn is transferred from neurons to astrocytes and also astrocytes to astrocytes, but not from astrocytes to neurons. The authors deducted that astrocytes must play an active role in clearing A-SYN deposits rather than in spreading A-SYN pathology(Loria *et al.* 2017). In any case, the extracellular compartment and the glia are believed to have a central role in A-SYN pathology. GCase deficient neurons show increased levels of A-SYN in the extracellular compartment(Fernandes *et al.* 2016) and mutant GCase and reduced GCase activity can alter secretion and exosome release of A-SYN in mice(Papadopoulos *et al.* 2018). This may directly link GCase alterations and the spreading of A-SYN pathology. In this regard, it is interesting to note that *GBA*1 deficient animal models show increased microglial activation (Keatinge *et al.* 2015; Soria *et al.* 2017) and higher levels of Cathepsin proteases (Vitner *et al.* 2010) and that *GBA*1-KO astrocytes showed decreased autophagy and mitochondrial dysfunction(Osellame and Duchen 2013).

Figure 2*: Possible mechanisms that link GCase alterations and alpha-synuclein accumulation.*

a. *Deficit of GCase activity leads to accumulation of its substrates GC and GS. This might alter the homeostasis of A-syn in the lysosomes.*
b. *GBA1 deficiency causes downregulation of autophagy, one of the key processes to remove A-syn from cells.*
c. *After macroautophagy, lysosomes are reformed. GBA1 mutations interfere with this process, reducing lysosomal reformation and thus the number of functional lysososmes.*
d. *Mutant (misfolded) GCase can saturate the ubiquitin-proteasome pathway, reducing its activity of degradation of A-syn*
e. *Complex structural interactions between A-syn and GCase can lead to a negative feedback where increased A-syn levels impair the activity of GCase and vice versa.*

f. *Mutant GCase has been shown to damage mitochondrial activity and structure, a process that can lead to/aggravate synucleinopathies*
g. *Mutant GCase is believed to interfere with the excretion of A-syn, increasing the spread of A-syn pathology.*

Abbreviations: GCase Glucocerebrosidase; GC glucosylceramide; GS glucosylsphingosine; A-syn alpha-synuclein.

How the most common GCase mutations may be linked to PD

N370S

N370S is one of the most common *GBA*1 mutations causing type 1 GD, the most prevalent among Ashkenazi Jews(Beutler *et al.* 2005) and is the one in which structural features are better understood. The structure of N370S mutant GCase was resolved in 2010; this clarified how the substitution of the Asn residue, that is 13 Å from the catalytic Glu 340 could cause the observed reduction in GCase activity(Wei *et al.* 2011). The main difference between WT GCase and the N370S mutant is the behavior of loop 3: normally loop 3 can exist in both extended and alpha-helical conformation; when it assumes the alpha-helical conformation Trp 312 and Trp 378 are in contact with Asn 370 while Tyr 313 binds to Glu 340; in the extended conformation Tyr 313 binds instead to Glu 235. Loop 3 of the N370S mutant is observed only in the extended conformation, with Tyr 313 bound to Glu325 and Trp 312 and Trp 378 forming a hydrogen bond with Ser 370. Overall, the N370S mutant has a more rigid structure that does not show the small changes at different pH observed with the WT GCase(Wei *et al.* 2011). Additionally, *in vitro* and *in silico* data suggest that N370S alters the interaction between GCase and its cofactor saposin C (SapC) (Romero *et al.* 2019; Salvioli *et al.* 2005). SapC is an 84- residue protein that is vital for the enzymatic activity of GCase, as demonstrated by the rare GD like syndrome caused by sapC deficiency(Tamargo *et al.* 2012). The evidence of a protective role of sapC against GCase inhibition by A-SYN is provided by a recent study, where GCase activity was inhibited by A-SYN *in vitro* and then rescued by the addition of sapC(Yap *et al.* 2013). SapC enhances the bond of WT GCase to the lipidic membrane and its activity, but is unable to do so in the presence of the N370S mutation(Salvioli *et al.* 2005). The *in silico* analysis supported this finding showing that loop 3 of WT GCase assumes an extended conformation (more unstable) in a simulation without SapC, while is in the helical conformation (more stable) when SapC is added to the model. N370S GCase keeps the extended conformation of loop 3 even in the presence of SapC thus preventing SapC from stabilising GCase(Romero *et al.* 2019). Moreover, *in vitro* data indicate that N370S mutant GCase has a lower affinity for A-SYN compared to WT GCase(Yap *et al.* 2011) and upon binding, GCase lifts A-SYN from the membrane exposing an important region of A-

SYN to proteases(Yap *et al.* 2015). By reducing GCase affinity for A-SYN, the N370S mutation could then lower A-SYN degradation and thereby increase A-SYN accumulation.

L444P

L444P is another frequent mutation causing neuronopathic GD (types 2 and 3)(Beutler *et al.* 2005), but it is still unclear how it leads to GCase deficiency. An interesting hypothesis derives from the analysis of the interaction between GCase and sapC and postulates that L444P could amplify A-SYN inhibition of GCase by preventing the bond between GCase and sapC. L444P can be found in the Ig-like domain 2 of GCase and is located between two Asp residues (443 and 445) that should interact with Lys 26 of sapC according to an *in silico* analysis(Atrian *et al.* 2007). Substitutions of residue 444 of GCase could then disrupt the interaction of Asp443 and Asp 445 with Lys 26, finally hindering the bond between GCase and sapC(Atrian *et al.* 2007). The same *in silico* study cited in the previous paragraph showed that, like N370S GCase, L444P GCase also loses the ability to be stabilised and retains an extended loop 3 conformation even in the presence of SapC(Romero *et al.* 2019).

Therapeutic strategies

Enzyme replacement

Currently, GD treatment is based mainly on enzyme replacement therapy (ERT) in the form of periodic infusions of GCase. Given the hypothesized importance of reduced GCase activity to the etiology of *GBA*1 PD, it presents an inviting means of disease intervention. Three commercially available forms of the enzyme exist: imiglucerase (Cerezyme), Taliglucerase alfa (Elelyso) and Velaglucerase alfa (VPRIV). The three enzymes have similar overall activity(Brumshtein *et al.* 2010). Unfortunately, these drugs are not able to cross the blood brain barrier (BBB) due to their size and the absence of mannose receptors on the brain endothelium(Schueler *et al.* 2002), so they are effective only in the treatment of the non-neuropathic type 1 GD and are not suitable for halting the progression of parkinsonism and neurodegeneration(Horowitz *et al.* 2016). New techniques to overcome these limits are under development, e.g. the use of HIV or tetanus virus proteins to facilitate GCase uptake in neurons(Gramlich *et al.* 2016).

Substrate reduction

An alternative strategy for the therapy of GD is substrate reduction therapy (SRT), that act by inhibiting the enzyme GC synthase, thus reducing the levels of GC, the main substrate of GCase. Two SRT drugs have been developed and commercialised: miglustat and eliglustat. The main advantages of SRT over ERT is the oral availability and the potential to cross the BBB(Platt *et al.* 1997). This second feature makes SRT a possible candidate to treat GBA-related PD. Studies of a substrate inhibitor compound (GZ667161) on a murine model of neuronopathic GD displayed reduction of substrate accumulation, extended life span, improved cognitive outcomes and reduction of A-SYN aggregates(Sardi *et al.* 2017; Cabrera-Salazar *et al.* 2012). These findings led to the testing of venglustat, an analog of GZ667161, on PD patients with GBA mutations in a phase 2 trial (ongoing - ClinicalTrials.gov identifier: NCT02906020). Important disadvantages of SRT are the limited experience with their use and the high cost(Horowitz *et al.* 2016). SRT is also linked to significant adverse effects, the most prominent being gastrointestinal problems and neurological symptoms like tremor and peripheral neuropathy(Hollak *et al.* 2009). Moreover, since SRT acts by reducing substrate accumulation and does not directly interact with GCase, it does not prevent the other potential mechanisms proposed to have a role in the development of PD in GD and whether substrate accumulation plays a role in the development of PD in GBA carriers is still a subject of controversy (*see section "Substrate Accumulation"*).

Small Molecule Chaperones (SMCs)

Small molecule chaperones (SMCs) are small proteins that bind to other macromolecular structures and influence their covalent folding or unfolding. Pharmacological SMCs can be specifically designed to bind to a given enzyme and improve its stability, activity and translocation to the target organelle. As noted above, GCase is folded in the ER and then translocated to the lysosomes(Ericksonss *et al.* 1985; Leonova and Grabowski 2000). Some mutant GCase proteins may maintain intrinsic activity, but they are unable to reach the lysosomes to exert their function; SMCs could then facilitate mutant GCase translocation to the lysosomes, increasing overall enzyme activity and reducing the load upon the ubiquitin-proteasome and autophagy pathways. One of the most interesting observations of SMCs is their potential ability to cross the BBB(Luan *et al.* 2013). SMCs can be divided into inhibitory and non-inhibitory.

Inhibitory chaperones

Inhibitory SMCs are competitive inhibitors of GCase. They bind to the active site of the enzyme in the ER and aid translocation to the lysosomes, where GCase and the SMC dissociate in the low pH, supported by the higher substrate concentration. The first molecule that has been employed for this purpose is the iminosugar N-(n-nonyl)deoxynojirimycin (NN-DNJ). Iminosugars are sugar analogs where one oxygen atom in the ring of the structure is replaced by a nitrogen atom; they have a similar structure to GC and therefore bind to the active site of GCase. In 2002 it was demonstrated that NN-DNJ is able to increase GCase activity in fibroblasts of GD patients with the N370S mutation(Sawkar *et al.* 2002). N370S is likely to change the behaviour of loop 3 of GCase, preventing it from assuming a helical conformation at lysosomal pH and modifying its interactions with loop 1 and the active site(Offman *et al.* 2010). The main problem with iminosugars such as GCase SMCs is that they have a high selectivity for GCase and so they may maintain some inhibitory activity inside the lysosome. Isofagomine, another iminosugar, showed promising results in *in vitro* studies(Steet *et al.* 2006), but failed to show improvement of GD symptoms in a subsequent trial (even though it showed increased GCase activity in white blood cells)(Steet *et al.* 2006). Other iminosugars have been tested but none have shown a favorable chaperone/inhibitory ratio(Mena-Barragán *et al.* 2016). As a class of SMCs, iminosugars have two main limitations that make them difficult to titrate: their (off target) activity on enzymes other than GCase (like alpha-glucosidase, alpha-galactosidase, and beta-hexosaminidase) and their high GCase inhibitory activity.

In 2007, three non iminosugar structural classes with potential SMCs activity were identified by the use of quantitative high-throughput screening: aminoquinolines, sulfonamides, and triazines. Of these, the aminoquinoline structural class showed a 40-90% increase in GCase activity, higher GCase lysosomal localization in N370S mutant human fibroblasts and a slight increase in GCase activity compared to WT controls(Zheng *et al.* 2007). One of the most interesting recent development in the field of inhibitory SMCs is the discovery of the chaperoning activity of ambroxol (a drug used to treat airway mucus hypersecretion) by the use of a thermal denaturation assay with wild type GCase(Maegawa *et al.* 2009). Its advantage over other putative agents is that it has an excellent and well established safety profile, which allows a much more rapid progression through regulatory frameworks to clinical trials. Ambroxol is able to reduce total and phosphorylated A-SYN levels in transgenic mice overexpressing A-SYN(Migdalska-Richards *et al.* 2016). A recent pilot study showed that high dose oral ambroxol permeated the blood-brain barrier, decreased glucosylsphingosine levels in the cerebrospinal fluid and improved neurological symptoms in 5 neuronopathic GD patients(Narita *et al.* 2016). These findings

have prompted the initiation of clinical trials with ambroxol to assess brain penetration, efficacy and safety (ClinicalTrials.gov identifiers: NCT02941822, NCT02914366).

Overall, inhibitory SMCs constitute a promising class of drugs for the treatment of GD and its neuropathic complications; it is important to remember that their efficacy is likely to be less in *GBA*1 mutations that alter the active site of the enzyme, that they are unlikely to be active against null mutations and that it is not clear what are the implications of their intrinsic inhibitory activity. Indeed, the ideal inhibitory SMCs should have sufficient inhibitory power to bind to GCase (and traffic it to the lysosome), but not high enough to significantly reduce the activity of the enzyme, which make its development challenging.

Non-inhibitory Chaperones

The abovementioned high throughput screenings all used the currently available commercial recombinant GCase. This has some limitations: the use of sodium taurocholate (NaTC) to activate GCase shifts the optimal pH of GCase to 5.9 and SapC and phosphatidyl-serine, natural cofactors of GCase, are not present in the reaction; these conditions may lead to a conformation of GCase that is not that of the lysosomal enzyme. Moreover, the use of WT GCase with high concentrations of NaTC creates the conditions to maximize the activity of the enzyme and makes it difficult to identify molecules that act as activators of GCase. Recently, a new high throughput screening using spleen homogenates of patients with the N370S/N370S mutation in place of the recombinant WT GCase led to the identification of activators of the enzyme with chaperone activity(Goldin *et al.* 2012). One class of compounds, the pyrazolopyrimides, showed chaperoning activity comparable to that of isofagomine, a potent inhibitory SMCs, in patient fibroblasts(Patnaik *et al.* 2012). Other compounds were tested on a novel macrophage model of GD and dopaminergic neurons, and showed increased GCase activity and lower glycolipid storage(Aflaki *et al.* 2014; Aflaki *et al.* 2016). Interestingly, two of these compounds were also able to lower A-SYN levels and toxicity in dopaminergic neurons from PD patients(Aflaki *et al.* 2016; Mazzulli *et al.* 2016). A clinical trial in the Netherlands is testing a non-inhibitory SMC on PD-GBA patients (ongoing - Nederlands Trial Register: NTR6960). Non-inhibitory SMCs have some potential advantages over inhibitory SMCs: they are easier to dose since there is no need to balance the chaperone and inhibitory activities and their enhancing effect on GCase can synergize with their chaperoning properties to increase overall enzyme activity. On the other hand, since these compounds are most likely allosteric binders, their affinity may vary with the changing conformation of mutant GCase and, as inhibitory SMCs, they are not active against null mutations.

Gene therapy

All the aforementioned treatment strategies require the administration of drugs for the entire life of patients. Gene therapy is an attractive therapeutic option and its recent developments look promising. X-linked severe combined immunodeficiency, Wiskott-Aldrich syndrome and adenosine deaminase deficiency have already been treated by infusion of autologous hematopoietic stem cells genetically treated *ex vivo* by the use of viral vectors(Aiuti *et al.* 2013; Cavazzana-Calvo *et al.* 2010; Cartier *et al.* 2009). The same approach has been tried in a mouse model of GD using a gammaretroviral vector with the viral promoter spleen focus–forming virus (SFFV) to insert a functional *GBA*1 gene in lineage-marker depleted bone marrow cells; upon reinfusion of the treated cells, normalisation of GCase activity and complete disappearance of Gaucher cells infiltration in the bone marrow were observed(Enquist *et al.* 2007). SFFV carries a high risk for insertional mutations and shows supraphysiological expression levels(Montini *et al.* 2009; Zychlinski *et al.* 2008), so recently the same approach has been tried with a self-inactivating lentiviral vector and a different cellular promoter; results on mice with GD showed that the infusion of a low percentage of genetically treated macrophages was able to rescue the GD phenotype(Dahl *et al.* 2015). If successfully transposed to humans, this therapy could cure type 1 GD, whereas it is unclear how it would affect type 2 and 3 GD and the risk for PD.

Conclusions

PD is a severe and common neurodegenerative disorder, with a prevalence that, with an ageing of the population, is likely to rise in the near future. The growing evidence for a link between PD and *GBA*1 (now considered quantitatively the most frequently involved gene in PD) provides novel perspectives for PD treatment. However, much is yet to be understood regarding the molecular basis that underlies the increased risk for PD in GBA1 mutation carriers, and also why some and not others with the mutation develop PD. Improved insight into the structural features of GCase and of its interaction with A-SYN is needed to improve our understanding of both Parkinson and of Gaucher diseases and to facilitate the development of therapies for both.

Conflict of interests

MT is an investigator in the MOVES-PD trial of Venglustat, AHVS has consulted with Sanofi, Kyowa, Prevail and Inflazome. He is a PI on the MOVES-PD study. MT, LS and AHVS have been involved in the aim-PD study as investigators and PI.

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