Published in final edited form as:

Cell Tissue Res. 2018 July ; 373(1): 51–60. doi:10.1007/s00441-017-2704-y.

Molecular Mechanisms of α -Synuclein and *GBA1* in Parkinson's Disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized pathologically by the presence of Lewy bodies comprised of insoluble alpha-synuclein. Pathological, clinical and genetic studies demonstrate that mutations in the *GBA1* gene, which encodes the lysosomal enzyme glucocerebrosidase (GCase) that is deficient in Gaucher's disease, are important risk factors for the development of PD. The molecular mechanism for the association between these two diseases is not completely understood. We discuss several possible mechanisms that may lead to *GBA1*-related neuronal death and alpha-synuclein accumulation including disruptions in lipid metabolism, protein trafficking, and impaired protein quality control mechanisms. Elucidating the mechanism between GCase and alpha-synuclein may provide insight into potential therapeutic pathways for PD and related synucleinopathies.

Keywords

neurodegeneration; alpha-synuclein; lysosomal dysfunction; glucosylceramide; protein aggregation

Parkinson's Disease

Parkinson's disease (PD) is the most prevalent motor disease and second most common neurodegenerative disorder, affecting about 1% of people over 60 years of age (Nussbaum and Ellis, 2003). The incidence of PD increases with age, and men are more likely to develop PD compared to women. Although the average age of onset is 60, rare early-onset cases can occur with diagnosis at age 40 or younger that are often a result of genetic mutations (Quinn, et al., 1987). The clinical features of PD include motor deficits such as a distinctive resting tremor, slowness of movement (bradykinesia), stiffness in the limbs or trunk, and impaired balance and coordination. The motor symptoms of PD are likely the result of decreased dopamine neurotransmitter levels due to a progressive loss of

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Conflict of Interest

JRM and DK are co-founders of Lysosomal Therapeutics, INC, a company that uses lysosomal biology to develop treatments for neurodegenerative disease.

dopaminergic neurons within the substantia nigra pars compacta (SNpc) region of the midbrain. In addition to the SNpc, neurodegeneration has been documented in many other circumscribed regions of the nervous system (Alexander, 2004).

The recognition of the role of dopamine in PD led to the development of the dopamine precursor levodopa (L-DOPA) in 1961 (Birkmayer and Hornykiewicz, 1961) as dopamine replacement therapy for patients. While this treatment can ameliorate the symptoms of PD, it does not stop disease progression. As the disease advances, the increase in dosage and prolonged use of L-DOPA causes patients to experience side effects of the drug, including dyskinesias (spontaneous, involuntary movements) and hallucinations. Currently, no therapy exists for treating the underlying neurodegeneration in PD.

α -Synuclein as a key player in PD pathogenesis

The first clues into the molecular etiology of PD occurred as a result of genetic analysis of a family with autosomal dominant inheritance of PD, called the Contursi kindred. This led to the identification of a mutation at residue 53 from alanine to threonine (A53T) in the *SNCA* gene that encodes alpha-synuclein (α -syn) (Polymeropoulos, et al., 1997; Spillantini, et al., 1995), a small 140 amino acid (AA) pre-synaptic protein. Critical to the pathogenic mechanism of α -syn, A53T and other familial-linked point mutations result in accelerated oligomerization or fibrillization of the protein (Conway, et al., 1998), indicating a probable gain-in-toxic function mediated by protein aggregation. Further support of this hypothesis comes from the study of experimental animal models where overexpression of α -syn in drosophila or mice results in α -syn pathology and neurotoxicity that is similar to that observed in PD (Maries, et al., 2003). Perhaps the most critical evidence for a role of α -syn accumulation in disease comes from PD families that harbor a duplication or triplication in the *SNCA* genomic region (Chartier-Harlin, et al., 2004; Singleton, et al., 2003). These patients demonstrate PD histopathology and a dose-dependent clinical phenotype that presents much earlier in triplication carriers than those with a duplication (Singleton and Gwinn-Hardy, 2004). Together, genetic analysis of rare familial forms of PD has unequivocally implicated α -syn accumulation to disease pathogenesis.

There is significant evidence that α -syn plays a role not only in rare familial forms of PD, but also in sporadic PD. After the discovery of the A53T mutation, pathological studies showed that α -syn is a major component of Lewy body inclusions that histopathologically define sporadic and most genetic forms of the disease (Spillantini, et al., 1997). Classic Lewy bodies are spherical cytoplasmic inclusions 8–30 μ m in diameter with a dense eosinophilic core surrounded by a pale-staining halo of radiating fibrils (Baba, et al., 1998).

Ultrastructurally, these inclusions show a central electron-dense filamentous core with a loose fibrillary rim (Forno, 1996). The filamentous α -syn within Lewy inclusions exhibits properties similar to other disease-linked amyloid proteins including amyloid- β and tau, which contain a cross- β structure and are insoluble in mild non-ionic detergents (Duda, et al., 2002; Serpell, et al., 2000). Prior to the discovery of α -syn, pathologists mainly used traditional histological stains such as eosin or anti-ubiquitin antibodies to identify inclusions (Kuzuhara, et al., 1988; Pollanen, et al., 1993). Multiple anti- α -syn antibodies have since been generated against aggregated or oxidized forms of the protein (Baba, et al., 1998;

Duda, et al., 2000). Collectively, a-syn antibodies remain as the best tools to identify Lewy inclusions in the nervous system, and have allowed for the discovery of a family of neurodegenerative diseases defined by synuclein inclusions, termed synucleinopathies. The soundly documented feature of Lewy inclusions comprised of aggregated a-syn is direct evidence that protein homeostasis (proteostasis) is disrupted in the PD brain.

Genetics implicates common pathways in the etiology of PD that converge on protein clearance pathways

Since the initial discovery of *SNCA* mutations as a cause for familial PD (Polymeropoulos, et al., 1997), other studies over the past 20 years have indicated a strong role for genetic factors in the etiology of PD. Additional rare, monogenic forms have been identified with autosomal recessive modes of inheritance including Parkin, PINK1, ATP13A2, and DJ-1 (Scott, et al., 2017). These mutations often lead to early-onset PD and the recessive mode of inheritance indicates that neurodegeneration likely occurs through a loss-of-function mechanism. Interestingly, the putative normal function of these genes all converge on the mitochondrial / lysosomal system. For example, both Parkin and PINK1 play important roles in initiating autophagic clearance of damaged mitochondria via lysosomes (i.e. mitophagy) (Youle and Narendra, 2011). ATP13A2 is thought to be essential for proper functioning of the lysosome since its depletion leads to lysosomal dysfunction and a-syn accumulation (Schultheis, et al., 2013; Tsunemi and Krainc, 2014), and DJ-1 is thought to protect mitochondria from oxidative damage (Guzman, et al., 2010).

Highly penetrant mutations resulting in other rare forms of familial PD occur through autosomal dominant modes of inheritance, likely with gain-in-toxic-function mechanisms. Additional point mutations in *SNCA* have been discovered including A30P, E46K, H50Q, G51D, most of which accelerate assembly kinetics of the protein (Conway, et al., 1998; Cullen, et al., 2011; Ghosh, et al., 2013; Greenbaum, et al., 2005; Rutherford, et al., 2014). Mutations in leucine-rich repeat kinase 2 (LRRK2) represent the most common form of familial PD, including the G2019S mutation that promotes kinase activity. Previous studies have identified interactions of LRRK2 with multiple Rab proteins, that result in Rab over-phosphorylation and functional deregulation (Cookson, 2016; Steger, et al., 2016). Since Rab proteins are key mediators of protein trafficking (Fig. 1), this may explain how LRRK2 mutations negatively influence vesicular transport and the autophagic-lysosomal pathway. Consistent with the importance of disrupted trafficking in PD pathogenesis, mutations in a distinct trafficking component called VPS35 that mediates endosome-to-Golgi trafficking also causes autosomal dominant PD (Vilarino-Guell, et al., 2011; Zimprich, et al., 2011). A-syn has emerged as a key mechanistic link between multiple genetic factors that function in vesicular trafficking, since its overexpression can disrupt endoplasmic reticulum (ER)-to-Golgi trafficking and the maturation of proteins through the early secretory pathway (Cooper, et al., 2006; Gitler, et al., 2008). An important down-stream consequence of a-syn-induced trafficking disruption is lysosomal dysfunction, which occurs through impeding the maturation of several hydrolases resulting in depleted lysosomes (Mazzulli, et al., 2011; Mazzulli, et al., 2016a). Together, genetic analysis of familial PD has implicated a role for

mitochondria, protein trafficking, and lysosomes as key pathways involved in disease progression.

Lysosomal dysfunction is linked to PD pathogenesis through mutations in lysosomal *GBA1*

In addition to monogenic forms that directly or indirectly implicate protein clearance pathways in PD pathogenesis, mutations in lysosomal hydrolases are also emerging as critical risk factors for the disease. This is best described through the link of *GBA1* mutations in PD, which was discovered through clinical observations of Parkinsonian symptoms in patients with the lysosomal storage disorder, type I adult-onset Gaucher disease (GD) (Neudorfer, et al., 1996). Most patients with type I GD present mainly with visceral symptoms, including enlargement of the spleen and liver, bone abnormalities, and macrophages containing accumulated lysosomal substrates (Grabowski, 2008). At the time, the finding of neurological involvement in these GD patients was unusual since the majority of type I GD patients were not widely known to present with neurological symptoms. However, more severe forms of GD have been classified as types 2 and 3 with infantile or adolescent onset and often present with prominent neurodegeneration (Grabowski, 2008). Patients with GD harbor loss-of-function mutations in the *GBA1* gene that encodes lysosomal glucocerebrosidase (GCase), a hydrolase enzyme that catalyzes the breakdown of glucosylceramide (GluCer) into glucose and ceramide (Brady, et al., 1965). Since many of the known *GBA1* mutations, including N370S, L444P, and 84GG, reduce the stability and / or enzymatic function of the enzyme (Grace, et al., 1994), lysosomes accumulate the undigested substrate GluCer and other lipids which leads to compromised lysosomal function (Fig. 1).

After the discovery of PD symptoms present in type I GD patients, clinicians also noted that first-degree relatives of GD patients with heterozygote mutations in *GBA1* presented with Parkinsonism at a higher than expected frequency, suggesting that heterozygote carriers were also at risk (Goker-Alpan, et al., 2004). Validation studies were subsequently performed on a large population of idiopathic PD patients, demonstrating that 5–10% of patients harbored at least one *GBA1* mutation (Sidransky, et al., 2009). The clinical progression of PD patients with *GBA1* mutations is often more rapid compared to patients without mutations, and there is a greater risk for cognitive impairment (Brockmann, et al., 2011; McNeill, et al., 2012; Neumann, et al., 2009; Sidransky, et al., 2009; Tan, et al., 2007). Brains of *GBA1*-PD patients show a reduction in GCase in the substantia nigra, putamen, amygdala, and cerebellum (Gegg, et al., 2012). Like idiopathic PD, the pathology of *GBA1*-PD patient brains also involves the loss of nigral dopamine neurons and the presence of Lewy bodies and neurites (Westbroek, et al., 2011). It is now widely accepted that *GBA1* mutations represent one of the strongest genetic risk factors for PD. Interestingly, *GBA1* mutations have also been linked to another synucleinopathy, dementia with Lewy bodies (Nalls, et al., 2013). Further establishing the relationship of *GBA1* to synucleinopathies, Lewy body pathology has been documented in GD patients with neurological symptoms (Wong, et al., 2004) strongly suggesting that α -syn plays a key role in the neurodegenerative process of these patients.

Mechanistic links between GCase and a-syn

Since the discovery of clinical, genetic, and pathological links between GD and PD, recent work has focused on understanding the mechanistic role of GCase in the development of synucleinopathies. Because all of the known *GBA1* mutations result in loss-of-enzymatic function, initial studies were focused on determining if reduction in GCase activity could alter a-syn levels. Studies on the cellular clearance of physiological and pathological forms of a-syn indicated an important role for the lysosomal system, suggesting that lysosomal dysfunction could lead to a-syn aggregation (Cuervo, et al., 2004; Lee, et al., 2004; Vogiatzi, et al., 2008). Initial pharmacological studies using conduritol-b-epoxide (CBE) to inhibit lysosomal GCase demonstrated that reducing enzymatic activity alone was sufficient to induce a-syn accumulation in neuronal cell lines and mice (Manning-Bog, et al., 2009). Later, work from our group and others had shown that reduction of GCase activity, either through shRNA knock-down, CBE treatment, or endogenously expressed GD-causing mutations in iPS neurons, resulted in the formation of pathological a-syn similar to that observed in GD and PD brain (Cleeter, et al., 2013; Mazzulli, et al., 2011; Rocha, et al., 2015a; Rockenstein, et al., 2016; Schondorf, et al., 2014; Xu, et al., 2011). Reducing GCase activity caused a general decline in the rate of lysosomal proteolysis, however a-syn was found to selectively accumulate indicating an intimate relationship between a-syn and GCase (Mazzulli, et al., 2011). This specificity is thought to occur through a direct interaction between the accumulated GluCer substrate and a-syn, since GluCer was found to directly influence the aggregation rate of oligomeric intermediates and fibril formation *in vitro* (Fig. 1; (Mazzulli, et al., 2011). Similar to the effect of dopamine on a-syn aggregation (Conway, et al., 2001; Mazzulli, et al., 2006), GluCer was found to kinetically stabilize oligomeric intermediate a-syn species (Fig. 1; Mazzulli, et al., 2011). Interestingly, this effect was only observed under acidic conditions similar to that expected in lysosomal compartments (pH 5.0), indicating that the a-syn accumulation observed in patient neurons was likely initiated in lysosomes that accumulate GluCer (Mazzulli, et al., 2011). In addition to defects in the lysosomal system, cells that express *GBA1* mutations also show disruptions in the mitochondrial pathway and altered calcium homeostasis (Osellame, et al., 2013; Schondorf, et al., 2014), indicating that multiple cellular pathways may converge to culminate in a-syn aggregation (Fig. 1).

While lysosomal dysfunction and lipid accumulation may play a role in a-syn accumulation in patients with homozygous mutations in *GBA1*, there is considerable debate regarding whether this same mechanism of action may occur in heterozygote carriers. This is partly due to the fact that some studies have shown that lipid substrate accumulation does not occur in the brains of patients with *GBA1*-PD (Gegg, et al., 2015). It is also assumed, mainly from cell culture and animal model studies, that 50% of the remaining wild-type (wt) GCase is sufficient to maintain normal GluCer levels. However, it is possible that other pathogenic factors, such as aging and exposure to environmental toxins, contribute to a decline in activity of the remaining wt GCase in heterozygote *GBA1* carriers. Consistent with this, studies have shown that GCase activity in sporadic PD brain declines with aging and is accompanied by substrate accumulation when measured from circumscribed brain regions (Rocha, et al., 2015a). In long-lived PD patient-derived iPS neuronal models that were aged

for >400 days, we found an age-dependent decline in lysosomal and GCase activity within neurons that harbor two copies of wt *GBA1* (Mazzulli, et al., 2016a). Detection of this phenotype in iPS cultures may have been facilitated by the fact that measurements were made from near-homogenous populations of midbrain neurons, whereas activity / substrate measurements from patient brains or *GBA1* heterozygote mouse models were done on mixed populations of cells (Gegg, et al., 2015).

Another factor that may contribute to a decline in wt GCase activity is aggregated α -syn. During studies on the clearance rate of α -syn, it was serendipitously discovered that the glycosylation patterns of GCase were altered in response to changes in α -syn expression levels (Mazzulli, et al., 2011). As α -syn levels were reduced, a reduction in the low molecular weight ER-form of GCase occurred concomitantly with an increase in glycosylated high molecular weight forms. Consistent with previous studies showing that α -syn disrupted the early secretory pathway and could block ER-Golgi trafficking (Cooper, et al., 2006; Gosavi, et al., 2002), these data suggested that α -syn could also disrupt the trafficking of GCase (Fig. 1). Subsequent studies in primary neuronal cultures and patient-derived iPS neurons validated that overexpression of α -syn caused the accumulation of an immature GCase form that was sensitive to endoglycosidase H and reduced the amount of enzyme that reached the lysosomal compartment (Chung, et al., 2013; Mazzulli, et al., 2011; Mazzulli, et al., 2016a). Additionally, analysis of pathologically-confirmed idiopathic PD brain harboring wt GCase showed a reduction of GCase activity in lysosome-enriched fractions, while the activity within ER-derived microsomes was slightly elevated (Mazzulli, et al., 2011). Reduced GCase activity in sporadic or *GBA1*-PD brain was subsequently confirmed by other groups (Gegg, et al., 2012; Murphy, et al., 2014; Rocha, et al., 2015a), and alterations in GCase maturity were observed in the brains of patients expressing A53T α -syn (Chung, et al., 2013). Collectively, these data suggested that α -syn did not initially cause an overall reduction in GCase protein in the cell, but instead altered its organelle distribution with specific reduction in lysosomal compartments (Mazzulli, et al., 2011; Mazzulli, et al., 2016a). Compartment-specific activity assays done in *SNCA* triplication PD iPS neurons harboring wt GCase demonstrated an age-dependent decline in activity that was sufficient to cause GluCer and glucosylsphingosine (GluSph) accumulation, although to a lesser degree compared to GD patient midbrain neurons (Mazzulli, et al., 2016a). In addition to disruptions in GCase trafficking, previous studies have shown that α -syn can directly interact with GCase and reduce its activity (Yap, et al., 2013). Together, this indicates that GCase activity can be reduced in the absence of *GBA1* mutations through other factors such as aging and α -syn accumulation, implying that reduced GCase activity may play an important role in both *GBA1*-PD and sporadic PD.

In addition to mechanisms that involve loss-of-GCase function, toxicity may also occur through a gain-in-toxic function mechanism. Some studies have shown that overexpression of *GBA1* mutants in cell lines, without a reduction in activity, could lead to α -syn accumulation (Cullen, et al., 2011). Since mutant GCase is improperly folded in the ER, it is possible that chronic mutant protein expression overwhelms the folding machinery and results in ER stress followed by cell death (Fig. 1). Indeed, ER stress is suspected to play a prominent role in PD, possibly as a result of α -syn accumulation, in the presence or absence of *GBA1* mutations (Chung, et al., 2013; Colla, et al., 2012; Hoozemans, et al., 2007). Other

studies indicated that ER stress occurs in novel iPS midbrain models of *GBA1*-PD expressing N370S/wt GCse, along with lysosomal dysfunction and a-syn accumulation (Fernandes, et al., 2016). Interestingly, the same report documented elevations in certain GluCer species with C16 and C24 aliphatic chain lengths, while other species were not changed or reduced. Although the contribution of distinct GluCer species to a-syn pathology is not known, it is possible that certain forms more potently disrupt lysosomal function or rapidly convert a-syn into amyloid fibrils. In other studies, *GBA1*-PD iPS cultures that were purified to homogeneity also showed alterations in GluCer profiles compared to isogenic corrected controls, indicating that heterozygote mutations are sufficient to cause lipid accumulation (Schondorf, et al., 2014). Since there is evidence for both gain- and loss-of-function mechanisms in *GBA1*-linked PD, it is possible that a combination of factors, including toxicity induced by the expression the mutant protein alone and reduced GCse function in the lysosome, lead to cell death in PD.

It is important to note that *GBA1* mutant-induced cell death can also occur independently of a-syn, since pathology can occur in non-neuronal cells that do not express a-syn within the viscera of GD patients. Previous studies have shown that GluCer accumulation causes alterations in ER morphology and enhanced cytosolic calcium release from ER stores, an effect that can be restored by inhibiting the GluCer synthase enzyme ((Korkotian, et al., 1999; Liou, et al., 2016). Disruptions in GluCer metabolism are expected to result in altered lipid composition in multiple sub-cellular organelles and the plasma membrane, with putative deleterious down-stream effects on multiple cellular pathways. Because GluCer is a precursor for multiple sphingolipids and gangliosides that play an important role in lipid raft composition, GluCer accumulation may affect essential processes such as synaptic transmission or other signaling pathways that rely on lipid rafts. The consequence of these changes and how they may lead to cell death are unknown and should be the focus of future investigations.

A critical factor in regulating GCse activity is lysosomal integral membrane protein-2 (LIMP-2), a ubiquitously expressed transmembrane glycoprotein that is responsible for trafficking GCse to the lysosome (Reczek, et al., 2007). The interaction of GCse and LIMP-2 begins in the ER, followed by trafficking of the GCse-LIMP-2 complex to the lysosomes via the Golgi apparatus (Blanz, et al., 2010; Reczek, et al., 2007). Genome wide association studies have shown that single-nucleotide polymorphisms (SNPs) within the SCARB2 gene that encodes LIMP-2 are associated with PD (Do, et al., 2011; Hopfner, et al., 2013; Michelakakis, et al., 2012). Additionally, genetic variations in the LIMP-2 locus have been associated with dementia with Lewy bodies (Bras, et al., 2014). Further evidence for an involvement in LIMP-2 in the development of synucleinopathies comes from mouse models of LIMP-2 deficiency. Initial LIMP-2 knock-out lines were developed in a background that is naturally depleted of a-syn (Harlan B16-J strain) and exhibited a normal lifespan with mild neurological changes (Berkovic, et al., 2008). Interestingly, when LIMP-2 knock-out mice were backcrossed into a line that had normal levels of a-syn, the animals demonstrated a prominent neurodegenerative phenotype and did not survive longer than 10 months (Rothaug, et al., 2014), indicating a role for a-syn in LIMP-2 $-/-$ mediated toxicity. Pathological analysis showed that LIMP-2 $-/-$ resulted in lysosomal dysfunction, lipid storage, and accumulation of insoluble a-syn inclusions in the brain (Rothaug, et al., 2014).

Similar to the effect of *GBA1* knock-out, LIMP-2 deficient mice also accumulated soluble oligomeric forms of a-syn (Rothaug, et al., 2014). Importantly, overexpression of LIMP-2 reduced a-syn levels, indicating a potential therapeutic pathway that is independent of direct lysosomal GCase activation (Fig. 1; (Rothaug, et al., 2014). These data further solidify the connection between reduced GCase activity and a-syn accumulation, since disruption of activity occurred through an independent pathway that did not require direct *GBA1* mutations.

Therapeutic approaches for *GBA1*-related PD

Since all known *GBA1* mutations effect the stability and / or activity of GCase, most therapeutic efforts have focused on replacing the wt form through either overexpression or by stabilizing the mutant forms through small-molecule chaperones. While enzyme replacement therapy has remained the standard treatment for visceral symptoms of GD, the enzyme does not pass the blood-brain barrier and therefore has no effect on the CNS. In experimental models, the first demonstration that *GBA1* mutant-induced synucleinopathy could be rescued by overexpression of wt *GBA1* occurred through AAV overexpression in a GD mouse model (Sardi, et al., 2011). Subsequently, similar effects were observed after wt *GBA1* was overexpressed in A53T a-syn mice that do not have *GBA1* mutations, indicating that increasing GCase activity could rescue both mutant and non-mutant *GBA1* synucleinopathy mice (Fig. 1; Rocha, et al., 2015b; Rockenstein, et al., 2016; Sardi, et al., 2013). In addition to strategies focused on replacement of the mutant protein, small molecule GCase activators and chaperones have potential as therapies for GD and PD. Iminosugars such as isofagomine bind to the enzyme's active site within the ER at pH 7.4 and stabilize the mutant protein by acting as a chaperone-like molecule (Fig. 1). This enhanced stability promotes ER-exit and trafficking of GCase to lysosomes where the iminosugar is more likely to dissociate in the acidic environment (Steet, et al., 2006; Valenzano, et al., 2011). Because isofagomine is an active site inhibitor and relies on an efficient wash-out in the lysosome in order to become active, therapeutic development for this compound and other iminosugars has remained complicated.

An alternative to enzyme replacement therapy for GD is substrate reduction therapy through inhibition of glycosphingolipid synthesis. As of now, compounds aimed at reducing the accumulation of lipids such as eliglustat and miglustat have had limited utility and effectiveness in neuronopathic GD patients due to their inability to pass the blood-brain barrier (Sybertz and Krainc, 2014). Given the overlap between PD and GD, some of the therapeutic strategies might be applicable to both diseases. A global Phase 2 study currently underway is testing GZ/SAR402671, also known as ibiglustat, as a potential therapy for PD patients harboring *GBA1* mutations. Ibiglustat is a small-molecule inhibitor of GluCer synthase that is able to cross the blood-brain barrier, and is currently in a Phase 2 trial for neuronopathic GD type 3 patients. Preclinical trials with a closely related GluCer synthase inhibitor GZ667161 showed that both A53T a-syn overexpressing mice and mice carrying homozygous *GBA1* D409V mutations had reduced pathological aggregate accumulation (e.g. a-syn, ubiquitin, and tau) compared to age-matched control mice (Sardi, et al., 2017). Additionally, treatment with GZ667161 ameliorated cognitive impairment in both of these

mouse models (Sardi, et al., 2017), indicating a potential for clinical success of GluCer synthase inhibitors in PD patients (Fig. 1).

Direct targeting of GCase through allosteric activators

Recently, compounds that directly activate the GCase enzyme have been identified through high-throughput screening (HTS) methods. Putative allosteric binders were identified through screening compound libraries using spleen lysates of GD patients harboring N370S / N370S mutations (Patnaik, et al., 2012). An important distinction of this screening method compared to those used previously was the use of tissue homogenate that contained the enzyme's natural activating factors such as saposin C. Previous screening methods utilized the recombinant purified enzyme, which requires the addition of artificial activators such as sodium taurocholate or other bile salts in order to achieve detectable enzymatic turnover. Since the assay conditions are designed to achieve optimal activity, identifying small-molecule allosteric compounds that can further activate the protein is difficult. However, using the spleen-lysate HTS method, the addition of these artificial activators is not required, and consequently a series of small molecule activators were discovered that potently activate and promote the translocation of mutant GCase into lysosomes (Fig. 1; (Aflaki, et al., 2014; Patnaik, et al., 2012). Application of some of these pyrazolopyrimidine analog molecules to patient-derived midbrain neurons harboring *GBA1* mutations resulted in enhancement of lysosomal targeting and activity, at levels that were sufficient to reduce lipid substrates, reduce amyloid pathology, and reverse the neurodegenerative phenotypes (Aflaki, et al., 2016; Mazzulli, et al., 2016b). Furthermore, these compounds were found to activate wt GCase in midbrain neurons that accumulate a-syn through either *SNCA* triplication, A53T mutation, ATP13A2 mutation, as well as in idiopathic PD lines (Mazzulli, et al., 2016b). This indicates that GCase activation and lipid substrate reduction may be a useful therapeutic option for multiple synucleinopathies.

Indirect targeting of GCase through proteostasis enhancement

Other pathways that can rescue GCase activity levels in GD patient cells have broadly focused on enhancing proteostasis, mainly through improving function at the ER. For example, proteasome inhibitors can function as proteostasis regulators through enhancing expression of the cell's endogenous chaperones, as well as inhibiting ER associated degradation (ERAD) (Mu, et al., 2008). Proteostasis regulators can work in conjunction with small-molecule chaperones to provide enhanced enzymatic rescue, even in cells expressing severely unstable GCase mutants such as L444P (Mu, et al., 2008). Other agents that function as proteostasis regulators do so by increasing calcium levels in the ER. Compounds such as dantrolene or diltiazem block ryanodine receptors at the ER, thereby enhancing the activity of calcium-dependent chaperones such as calnexin and grp78 (Fig. 1). Interestingly, diltiazem is an FDA approved drug used to treat hypertension, and is correlated with reduced risk for developing PD (Ritz, et al., 2010). Therefore, it is possible that further development of proteostasis regulators that exploit and enhance the endogenous quality control machinery of the cell may prove beneficial for *GBA1*-linked synucleinopathies as well as other lysosomal storage diseases characterized by destabilizing loss-of-function mutations.

Therapeutic targets that enhance protein homeostasis may also provide benefit in PD, since ER stress has been shown in a variety of models of PD that accumulate a-syn. The subsequent activation of the unfolded protein response (UPR), as detected by immunoreactivity of UPR markers, has been shown in the substantia nigra of post-mortem PD brains (Hoozemans, et al., 2007). Neurotoxin (6-OHDA and MPP+) cell models of PD also show an induction of genes involved in ER stress and UPR such as ER chaperones and elements of the ubiquitin-proteasome system (UPS) (Holtz and O'Malley, 2003). Furthermore, both *SNCA* mutation and triplication models show ER stress (Chung, et al., 2013; Colla, et al., 2012). Collectively, these data demonstrate that a-syn induces ER stress, and indicate a potential for therapeutic intervention.

Increased ER stress has also been found in iPS-derived neuronal models from PD patients carrying *GBA1* mutations (Fernandes, et al., 2016). Expression of human mutant GCases in drosophila models has been shown to lead to a loss of dopaminergic neurons, a progressive PD-like locomotor defect, abnormal GCase aggregates in the ER and increased levels of ER stress reporters (Sanchez-Martinez, et al., 2016). Importantly, the ER stress and motor deficits could be rescued with isofagomine or ambroxol, another pharmacological GCase chaperone (Sanchez-Martinez, et al., 2016). Ambroxol is already FDA-approved for the treatment of respiratory diseases, and is currently in Phase 2 clinical trials as a potential treatment for the cognitive and motor symptoms in PD dementia patients. Additionally, ambroxol will be tested in an upcoming Phase 2 study for treatment of PD patients with and without *GBA1* mutations.

A direct link between *GBA1*-PD and dysfunctional ERAD has been provided by the observation that misfolded GCase can directly interact with Parkin (Ron, et al., 2010). Parkin is an E3 ubiquitin ligase that is up-regulated in response to ER stress (Imai, et al., 2000), suggesting that it is involved in the ERAD of misfolded ER proteins. Evidence shows that misfolded / mutant GCases are retained in the ER and are therefore able to trigger UPS and ERAD (Bendikov-Bar, et al., 2011; Mu, et al., 2008; Ron and Horowitz, 2005). Furthermore, the degree of ERAD of mutant GCase variants correlates with and is one of the factors that has been suggested to determine GD severity (Ron, et al., 2010). Taken together, these studies demonstrate the potential for enhancement of proteostasis as a therapeutic approach to *GBA1*-PD and other synucleinopathies.

Acknowledgments

This work was supported by the National Institute of Neurological Disorders and Stroke grant R01NS092823 (JRM) and R01NS076054 (DK).

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Figure 1.

Potential gain- and loss-of-function mechanisms for the role of *GBA1* pathogenesis in PD. (a) Misfolded mutant GCase (e.g. N370S, L444P) in the ER causes ER stress and may lead to α-syn aggregation and inhibition of ER-to-Golgi protein trafficking. (b) Loss of GCase function leads to accumulation of lipids such as GluCer, which directly interacts with and stabilizes α-syn oligomers in the lysosomal compartment. The consequent α-syn accumulation inhibits ER-to-Golgi GCase trafficking, resulting in a positive feedforward loop of self-propagating disease. Loss of GCase function may also cause general lysosomal dysfunction, defective mitophagic and autophagic clearance pathways, and Ca²⁺ homeostasis dysfunction. Potential for therapeutic interventions involving multiple pathways are being explored such as ER, trafficking machinery, reduced GluCer synthesis, and direct activation of GCase within lysosomes.