

## PROJECT SUMMARY

The pathological accumulation of alpha-synuclein ( $\alpha$ -syn) is believed to play a major role in Parkinson's disease (PD) pathogenesis. The autophagy-lysosome pathway (ALP) is responsible for the lysosomal degradation of  $\alpha$ -syn. ALP dysfunction is documented in PD and its inhibition induces accumulation of  $\alpha$ -syn aggregates, implicating its role in PD pathogenesis. Thus it is our goal to identify novel lysosome targets that enhance the autophagic clearance of  $\alpha$ -syn. The glycosphingolipid metabolism pathway is coordinately regulated by several lysosomal enzymes and has received recent focus as a therapeutic target for PD. Alpha-Galactosidase A ( $\alpha$ -Gal A) is a soluble lysosomal enzyme in this pathway and its mutations cause the lysosomal disorder Fabry disease. Evidence from our lab indicates for the first time a novel association between  $\alpha$ -Gal A activity and  $\alpha$ -syn. Alpha-Gal A deficiency causes the aberrant accumulation of  $\alpha$ -syn aggregates and ALP markers in mouse brain, indicating that  $\alpha$ -Gal A regulates  $\alpha$ -syn metabolism. Our preliminary data also indicates a direct relationship between  $\alpha$ -Gal A and PD pathology as demonstrated by significantly reduced  $\alpha$ -Gal A enzymatic activity in PD brain specimens that harbor pathologic accumulation of  $\alpha$ -syn. While other glycosphingolipid metabolizing enzymes have been targeted for PD, there remain several critical gaps in our knowledge of how this pathway regulates  $\alpha$ -syn clearance and  $\alpha$ -syn-associated neurotoxicity. In addition, therapeutics that are clinically approved to treat Fabry disease or show promise in clinical trials, increase  $\alpha$ -Gal A levels and activity, but their efficacy for PD has not yet been explored. Thus to help bridge these critical gaps, our proposal has two Specific Aims. In Aim 1 we will use *in vitro* model systems to test the hypothesis that increasing  $\alpha$ -Gal A enhances the autophagic clearance of  $\alpha$ -syn. Effects of  $\alpha$ -Gal A over-expression will be assessed on endogenous  $\alpha$ -syn in SH-SY5Y human neuroblastoma cells or in cultured neurons from  $\alpha$ -Gal A transgenic mice, or conditionally over-expressed  $\alpha$ -syn in M17 human neuroblastoma cells. We will selectively inhibit or induce the ALP to confirm its selectivity for mediating  $\alpha$ -Gal A function. We will also determine if increasing  $\alpha$ -Gal A enhances function/levels of lysosome-associated proteins important for  $\alpha$ -syn clearance. In Aim 2 we will use  $\alpha$ -Gal A transgenic and wild-type mice, and primary neuron cultures from these mice to test the hypothesis that increasing  $\alpha$ -Gal A attenuates  $\alpha$ -syn-induced neurotoxicity. We will determine if increasing  $\alpha$ -Gal A activity attenuates neuropathology resulting from the exogenous addition of  $\alpha$ -syn pre-formed fibrils, including formation of endogenous  $\alpha$ -syn aggregates and neuron loss. We will also pharmacologically inhibit and stimulate the ALP to confirm its selectivity for mediating  $\alpha$ -Gal A function. Successful completion of this study will provide important insights on  $\alpha$ -Gal A regulation of  $\alpha$ -syn clearance, demonstrate for the first time that  $\alpha$ -Gal A can be targeted to reduce  $\alpha$ -syn-associated neurotoxicity, and justify future studies of clinically approved and newly developed  $\alpha$ -Gal A therapeutics for treating PD.

Parkinson's disease (PD) is a major health issue affecting upwards of one million Americans and because current therapies only provide symptomatic relief there is a critical need for novel therapeutics that delay or attenuate disease pathogenesis. The goal of this application is to confirm that increasing activity of the lysosomal enzyme alpha-Galactosidase A provides therapeutic benefit in pre-clinical models of PD. Successful completion of this study will justify the future study of alpha-Galactosidase A-associated therapeutics already used in humans, for their novel role in attenuating PD pathogenesis.

## SPECIFIC AIMS

Parkinson's disease (PD) represents a class of diseases called synucleinopathies that are defined by the pathological accumulation of alpha-synuclein ( $\alpha$ -syn) aggregate species. It is widely believed that  $\alpha$ -syn regulates PD pathogenesis and that its aberrant accumulation may be due in part to age and disease-related compromises in its degradation. The autophagy-lysosome pathway (ALP) is responsible for the lysosomal degradation of  $\alpha$ -syn. ALP dysfunction itself has been documented in PD and may contribute to  $\alpha$ -syn pathogenesis, as demonstrated by the pathologic accumulation of  $\alpha$ -syn aggregates upon experimental inhibition of the ALP. Autophagy-inducing therapies have been tested clinically for PD but these studies have met with limited success. However, it is logical to predict that promoting lysosome function as a primary therapeutic approach would be more beneficial for PD, as inducing autophagy in the face of lysosome dysfunction could exacerbate cell death rather than prevent it. ***As the lysosome is ultimately responsible for the autophagic degradation of  $\alpha$ -syn, our goal is to identify novel therapeutic targets for PD that attenuate  $\alpha$ -syn-associated toxicity through enhancement of lysosome function.***

The glycosphingolipid metabolism pathway has recently come into focus as a therapeutic target for PD and involves the coordinate regulation of several lysosomal enzymes. Alpha-Galactosidase A ( $\alpha$ -Gal A) is a soluble lysosomal enzyme that hydrolyzes glycosphingolipids in this pathway and is best known for its mutations that cause the lysosomal storage disorder Fabry disease. ***Emerging evidence from our laboratory indicates for the first time a novel association between  $\alpha$ -Gal A activity and  $\alpha$ -syn accumulation.*** We have recently published that  $\alpha$ -Gal A deficiency induces formation of  $\alpha$ -syn aggregate species in mouse brain concomitant with the aberrant accumulation of ALP markers, indicating that  $\alpha$ -Gal A directly or indirectly regulates  $\alpha$ -syn metabolism. Importantly, we have also observed significantly reduced  $\alpha$ -Gal A enzymatic activity in brain specimens from PD patients that also harbor the pathologic accumulation of  $\alpha$ -syn, indicating direct relevance of  $\alpha$ -Gal A activity to PD brain pathology. Other glycosphingolipid metabolizing enzymes, such as glucocerebrosidase (GCase) have been targeted for their potential as PD therapeutics and we also see reduced GCase activity in our PD brain specimens. However, there remain several critical gaps in our understanding of how the glycosphingolipid metabolism pathway regulates PD pathogenesis.

Another important feature of this proposal is that there already exist therapeutics for treating  $\alpha$ -Gal A deficiency in Fabry disease, including enzyme replacement or molecular chaperone therapy that are either clinically approved or which demonstrate promise in clinical trials that act by increasing  $\alpha$ -Gal A activity. Our findings, therefore suggest a potentially important and novel use for such therapeutics that enhance "normal" endogenous  $\alpha$ -Gal A activity in PD patients; however, to date there have been **no** studies to determine whether such therapies are efficacious in treating PD. To help bridge this critical translational gap we have established models of wild-type  $\alpha$ -Gal A over-expression *in vitro* and *in vivo*, including a novel transgenic mouse that constitutively over-expresses wild-type  $\alpha$ -Gal A to determine the impact of experimentally increasing  $\alpha$ -Gal A on  $\alpha$ -syn clearance and neurotoxicity. These findings indicate that a focused investigation of  $\alpha$ -Gal A will provide novel and significant advances in our knowledge of the glycosphingolipid metabolism pathway as a therapeutic target for PD. We thus propose the following two Specific Aims:

**Aim 1) Test the hypothesis that increasing  $\alpha$ -Gal A enhances the autophagic clearance of  $\alpha$ -syn** Using *in vitro* model systems we will determine if  $\alpha$ -Gal A over-expression enhances the clearance of endogenous  $\alpha$ -syn in SH-SY5Y human neuroblastoma cells or in primary neuron cultures, or of conditionally over-expressed  $\alpha$ -syn in M17 human neuroblastoma cells. The ALP will be inhibited to determine its selectivity in mediating  $\alpha$ -Gal A function, and induced to determine if increasing  $\alpha$ -Gal A activity further enhances  $\alpha$ -syn clearance. We will also investigate whether increasing  $\alpha$ -Gal A enhances function/levels of lysosome-associated proteins important for the autophagic clearance of  $\alpha$ -syn. Finally we will over-express inactive  $\alpha$ -Gal A mutants to confirm that promoting  $\alpha$ -syn clearance via increasing  $\alpha$ -Gal A is activity-dependent.

**Aim 2) Test the hypothesis that increasing  $\alpha$ -Gal A attenuates  $\alpha$ -syn-induced neurotoxicity** We will use  $\alpha$ -Gal A transgenic and wild-type mice to determine if increasing  $\alpha$ -Gal A activity *in vivo* attenuates the formation of endogenous  $\alpha$ -syn aggregates and neuron loss that results from the exogenous addition of  $\alpha$ -syn pre-formed fibrils (PFFs). We will also treat cultured neurons from  $\alpha$ -Gal A transgenic and wild-type mice with  $\alpha$ -syn PFFs to substantiate our *in vivo* findings and to determine if  $\alpha$ -Gal A regulation of  $\alpha$ -syn fibril-mediated toxicity is ALP-dependent.

***The successful completion of this study will provide important insights on how  $\alpha$ -Gal A regulates  $\alpha$ -syn clearance and will demonstrate for the first time that  $\alpha$ -Gal A can be targeted to reduce  $\alpha$ -syn-associated neurotoxicity. Promising results would also justify the investigation of clinically approved and novel  $\alpha$ -Gal A-associated therapeutics for treating PD.***

## RESEARCH STRATEGY

### A. Significance

PD is the second most common neurodegenerative disease and the most common movement disorder, with symptoms of tremor, rigidity and bradykinesia resulting from the progressive loss of substantia nigra dopaminergic neurons (1). It is estimated that as many as one million Americans suffer from PD, representing 1-2% of the U.S. population over the age of 65 and 4-5% over the age of 85 (2). In addition to the profound impact of PD on patients and their families there is also a significant economic impact. Annual estimated medical costs for PD exceed 8 billion dollars in the U.S. alone, and the non-medical burden (e.g. lost employment) is estimated to exceed 6 billion dollars annually (2). These costs are predicted to further rise over the next 25 years with increases in numbers of diagnosed patients and cost of their treatment. Another major factor contributing to the debilitating nature of PD is that currently approved therapeutics only treat symptoms rather than delay or prevent disease progression (2). **Thus the prevalence and cost of PD, combined with lack of effective treatments, highlight the critical need to identify new therapeutic targets.**

PD represents a class of diseases called synucleinopathies, defined by the accumulation of inclusions called Lewy bodies or Lewy neurites made of insoluble  $\alpha$ -syn aggregates (3). Alpha-syn aggregate pathology correlates temporally and spatially with PD progression as it is typically limited to lower brain regions early in disease prior to its spread in later disease to motor and cognitive centers (4, 5). Alpha-syn is ordinarily a soluble, natively unfolded monomer but a multi-step process of its folding and oligomerization causes formation of insoluble fibrils *in vitro* similar to those present in PD brain (6). Moreover, mutations and replications in  $\alpha$ -syn in familial PD support a causal role for  $\alpha$ -syn for PD pathogenesis (7, 8). Although we still have a limited understanding of how  $\alpha$ -syn regulates PD pathogenesis, ample pre-clinical *in vitro* and *in vivo* evidence indicates the neurotoxic potential of  $\alpha$ -syn aggregate species (9-15). Importantly, therapeutics aimed at promoting  $\alpha$ -syn clearance present an attractive target in particular those that improve ALP function, as this pathway is responsible for the high capacity clearance of  $\alpha$ -syn (16). Significant to this application, the ALP is disrupted not only with normal brain aging but also in PD brain (17-22). Experimental inhibition of the ALP exacerbates and induction of the ALP attenuates respectively  $\alpha$ -syn aggregate formation and associated toxicity (13, 15, 19, 20, 23-29). **Deficits in the ALP thus offer a logical explanation for the accumulation of pathologic  $\alpha$ -syn species in PD and justify its continued study for therapeutics development.**

PD therapeutics that induce autophagy have had little success in clinical trials (30, 31) and have known side effects arguing against their chronic use (32). The combined use of autophagy inducers and lysosome inhibitors to kill cancer cells (33) suggests that targeting autophagy induction as a monotherapy in PD may not only be ineffective under conditions of lysosome dysfunction in PD but may exacerbate neuron loss. This rationale has led us to investigate the lysosome as a primary target for promoting  $\alpha$ -syn clearance in PD. Preclinical studies suggest that the glycosphingolipid metabolism pathway, which involves coordinated regulation of several lysosomal enzymes, may be a useful therapeutic target for PD (34). Alpha-Gal A is a soluble lysosomal enzyme that hydrolyzes glycosphingolipids in this pathway and its mutations cause the lysosomal storage disorder Fabry disease (35). We now have novel data indicating the potential for  $\alpha$ -Gal A as a target to enhance  $\alpha$ -syn clearance. We recently identified ALP dysfunction in  $\alpha$ -Gal A-deficient mouse brain that corresponds with  $\alpha$ -syn pathology (36). Our preliminary data (Fig. 1) indicate significant  $\alpha$ -Gal A enzymatic deficiency in PD brain specimens that harbor pathologic accumulation of  $\alpha$ -syn (5, 37), corroborating previous reports of  $\alpha$ -Gal A deficiency in PD patient leukocytes (38, 39), and the demonstration of Parkinsonism in a patient with Fabry disease (40). Our data also indicate significant GCCase deficiency in PD brain specimens (Fig. 1) similar to previous reports (20, 41-42). GCCase hydrolyzes glycosphingolipids that are metabolized in the same pathway as  $\alpha$ -Gal A and its mutations are a strong risk factor for PD (43, 44). However, the mechanisms by which GCCase and other glycosphingolipid metabolism enzymes regulate  $\alpha$ -syn clearance are still very unclear and command further investigation (45). Therapeutics for  $\alpha$ -Gal A are already used clinically for Fabry disease and would provide a ready source of agents to test for PD upon validation of  $\alpha$ -Gal A as a therapeutic target. Enzyme replacement therapy (ERT) has been used for over a decade and is generally well tolerated by Fabry patients, though its use for PD would require further development to promote CNS penetration (46). Molecular chaperone therapy (migalstat/AT-1001) is currently in clinical trials for Fabry disease and initial reports suggest both its safety and efficacy (47, 48). Migalstat binds to  $\alpha$ -Gal A and promotes its trafficking to the lysosome, in turn increasing its activity (49). Migalstat also crosses the blood brain barrier in mice following oral administration suggesting its feasibility for PD (50). However, as migalstat is a competitive inhibitor of  $\alpha$ -Gal A (49), rigorous pharmacokinetics and pharmacodynamics studies would be required to validate its safety and efficacy that are beyond the scope of this proposal. Thus we have established  $\alpha$ -Gal A over-expression *in vitro* (Fig. 2) and will use a novel transgenic mouse that over-expresses

human  $\alpha$ -Gal A (51) to test our hypotheses that increasing  $\alpha$ -Gal A promotes the autophagic clearance of  $\alpha$ -syn and attenuates its associated toxicity. **We propose that increasing  $\alpha$ -Gal A activity will enhance lysosome function and provide significant knowledge in how the glycosphingolipid metabolism pathway regulates  $\alpha$ -syn clearance and associated toxicity. These studies could potentially identify a novel therapeutic target within the ALP for treating PD.**

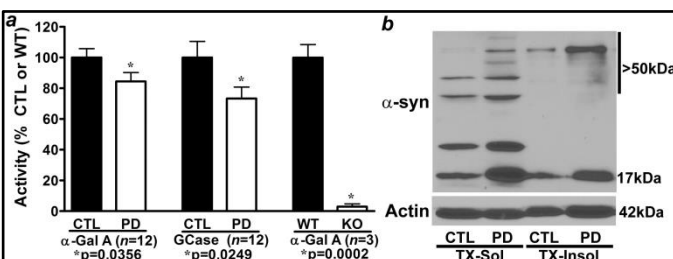
## B. Innovation

The glycosphingolipid metabolism pathway has been studied previously for its therapeutic potential in treating PD (20; 34; 45; 52; 53). Our proposal is innovative because we have identified a novel relationship with a previously unappreciated member of this pathway, the soluble lysosomal enzyme  $\alpha$ -Gal A, for its potential as a therapeutic target for attenuating  $\alpha$ -syn pathogenesis in PD. Our recently published findings of  $\alpha$ -Gal A-deficient mouse brain indicate  $\alpha$ -syn pathology concomitant with ALP dysfunction (36), suggesting that  $\alpha$ -Gal A regulates  $\alpha$ -syn metabolism. In addition, our preliminary data indicate for the first time a significant reduction of  $\alpha$ -Gal A enzymatic activity in PD brain in association with  $\alpha$ -syn accumulation (Fig. 1). However, whether  $\alpha$ -Gal A itself can be targeted to attenuate accumulation of pathologic  $\alpha$ -syn species and their associated neurotoxicity has not been investigated. Thus our hypotheses that increasing  $\alpha$ -Gal A promotes the autophagic clearance of  $\alpha$ -syn and attenuates  $\alpha$ -syn-associated neurotoxicity are highly innovative. In addition, our proposal will identify ALP-associated molecules whose function/levels is increased as a result of increasing  $\alpha$ -Gal A activity, thus providing downstream targets to validate enhancement of  $\alpha$ -Gal A function and which will be explored further in future applications. Adding to this innovation is the potential to validate  $\alpha$ -Gal A for its ability to attenuate  $\alpha$ -syn-associated pathogenesis and disease spread in PD, as existing therapies for PD are only efficacious in their ability to treat symptoms and not attenuate neurodegeneration (1). Adding technical innovation to our project are the proposed use of wild-type (WT; Fig. 2) and mutant  $\alpha$ -Gal A over-expression vectors (54) and a novel  $\alpha$ -Gal A transgenic mouse (51) to determine the importance of increasing  $\alpha$ -Gal A activity in promoting  $\alpha$ -syn clearance and reducing  $\alpha$ -syn-associated toxicity. **Thus the successful completion of this project will justify the future development and testing of innovative therapies that effectively attenuate the pathogenic potential of  $\alpha$ -syn in PD through enhancement of  $\alpha$ -Gal A function.**

## C. Approach

### Preliminary Results

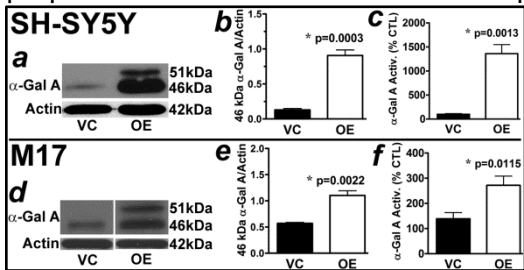
**Alpha-Gal A deficiency in mouse brain and human PD brain** We recently published that the ALP is disrupted in the brains of  $\alpha$ -Gal A-deficient mice as demonstrated by increased immunoreactivity for LC3 and LAMP-1, markers for autophagosomes and lysosomes, respectively as well as the accumulation of lipid inclusions observed by electron microscopy (36). In this study we also observed the accumulation of aggregates positive for  $\alpha$ -syn phosphorylated at serine 129 (p129S- $\alpha$ -syn), a pathological species of  $\alpha$ -syn that accumulates in PD brain (37). These p129S- $\alpha$ -syn aggregates co-localized with ubiquitin and LC3, suggesting that disruption of the ALP correlates with alterations in  $\alpha$ -syn metabolism. As a logical extension to these studies we were recently funded to investigate if  $\alpha$ -Gal A deficiency also occurred in PD brain in a partnership with the Arizona Parkinson's disease Consortium and the Michael J Fox Foundation. We compared frozen specimens classified by a unified staging system, from PD patients exhibiting robust cortical synucleinopathy (stage IV) to those of control patients not diagnosed with PD and lacking any synucleinopathy (stage 0). Our preliminary data indicate a significant reduction of  $\alpha$ -Gal A enzymatic activity in sucrose buffer homogenates prepared from late-stage PD temporal cortex (Fig. 1). Representative western blot analysis indicates increased accumulation of total  $\alpha$ -syn in Triton X-soluble and -insoluble (further solubilized with SDS and urea) PD brain homogenates (Fig. 1), corroborating previous reports of increased  $\alpha$ -syn in these specimens (5, 37). We also indicate significantly reduced GCCase activity in these same PD brain homogenates, reductions similar



**Fig. 1 a)** Alpha-Gal A and GCCase enzymatic activity (nmol/mg/hr, expressed as % CTL) in sucrose buffer homogenates from late-stage PD temporal cortex with known  $\alpha$ -syn pathology (5, 37) are significantly reduced to 84% and 73% respectively of CTL patient levels ( $n=12$  patients/group). Alpha-Gal A activity in  $\alpha$ -Gal A knock-out (KO) mouse brain homogenates is 3% of WT control levels ( $n=3$  mice/group), demonstrating assay specificity. N-acetyl-D-galactosamine and conduritol B-epoxide (Sigma) were added to inhibit non-specific enzymatic activity for  $\alpha$ -Gal A and GCCase, respectively as reported (20, 49, 50). \* $p<0.05$ , t test. **b)** Representative western blot analysis indicates that compared to control (CTL) brain, PD brain exhibited increased  $\alpha$ -syn monomer (17 kDa) and high molecular weight  $\alpha$ -syn species (>50 kDa) that were soluble in Triton X (TX-Sol) or SDS and urea (TX-Insol). Actin (42 kDa) served as loading control.

to those previously reported in PD brain (20, 41, 42). Our demonstration that  $\alpha$ -Gal A deficiency in PD brain is similar in magnitude to GCase deficiency bolsters our rationale for targeting  $\alpha$ -Gal A, as GCase is gaining acceptance as a therapeutic target for PD (20; 34, 45; 52; 53). **These findings support a promising link between  $\alpha$ -Gal A deficiency,  $\alpha$ -syn and PD and are the basis for our hypothesis that increasing  $\alpha$ -Gal A will promote the autophagic clearance of  $\alpha$ -syn and attenuate its associated neurotoxicity.**

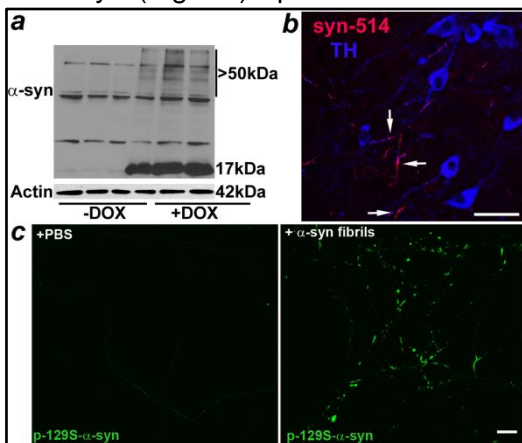
**Over-expression of  $\alpha$ -Gal A in human neuroblastoma cells** To demonstrate feasibility for studies proposed in Aim 1 we induced over-expression of human  $\alpha$ -Gal A (in pCMV6 plasmid vector) in differentiated



**Fig. 2** Western blot analysis of SH-SY5Y (**a-b**) and M17 (**d-e**) cell lysates indicates increase in 51 kDa precursor and 46 kDa active  $\alpha$ -Gal A species (**a, d**) following transient transfection for 24h with human  $\alpha$ -Gal A over-expression vector (OE) compared to vector control (VC). The 46 kDa  $\alpha$ -Gal A active species was quantified in SH-SY5Y (**b**) and M17 (**e**) lysates. Actin (42 kDa) served as the loading control. **c, f** Assay for  $\alpha$ -Gal A enzymatic activity (nmol/mg/hr, expressed as % CTL) indicates robust increase following OE compared to VC. \* $p < 0.05$ , t test.

SH-SY5Y and SK-N-BE (2)-M17 (M17) human neuroblastoma cells (Fig.2). Preliminary results indicate robust increases in the 46 kDa active species of  $\alpha$ -Gal A (55) and a concomitant increase in  $\alpha$ -Gal A enzymatic activity (Fig. 2). **Together these data provide proof of principle support for our hypothesis that increasing  $\alpha$ -Gal A attenuates the autophagic clearance of  $\alpha$ -syn.**

**Proof-of-principle  $\alpha$ -syn studies** We have generated preliminary data to assess  $\alpha$ -syn clearance in Aim 1 and  $\alpha$ -syn aggregate formation in Aim 2. Our lab uses M17 cells that conditionally over-express human WT  $\alpha$ -syn (Fig. 3a) upon treatment with doxycycline (DOX) from our collaborator Dr. Talene Yacoubian (see



**Fig. 3** (**a**) Western blot indicates conditional over-expression of human WT  $\alpha$ -syn, both 17 kDa monomer and >50 kDa high molecular weight species in M17 cell lysates following addition with 10  $\mu$ g/ml doxycycline (+DOX) for 7 days; (**b**) pilot IHC study reveals nitrated  $\alpha$ -syn (syn-514) (arrows) in the substantia nigra measured 6 months following intrastriatal injection of  $\alpha$ -syn PFFs (TH=tyrosine hydroxylase used to stain dopaminergic neurons); (**c**) pilot ICC study reveals increased p129S- $\alpha$ -syn in cultured hippocampal neurons treated for 14 days with  $\alpha$ -syn PFFs (right) compared to PBS vehicle control (left). Scale bars=20  $\mu$ m.

letter of support). Over-expression of  $\alpha$ -syn in M17 cells is indicated by western blot detection of increased 17 kDa monomer, and high molecular weight  $\alpha$ -syn (>50 kDa) that are believed to represent pathological  $\alpha$ -syn species (29). With our co-investigator Dr. Laura Volpicelli-Daley we indicate by immunohistochemistry (IHC) of mouse substantia nigra following intrastriatal injection of  $\alpha$ -syn pre-formed fibrils (PFFs) (Fig. 3b) or by immunocytochemistry (ICC) of cultured hippocampal neurons following exogenous addition of  $\alpha$ -syn PFFs (Fig. 3c) the accumulation of “pathological”  $\alpha$ -syn species, including nitrated  $\alpha$ -syn (syn-514, Fig. 3b) and p129S- $\alpha$ -syn (Fig. 3c). Such  $\alpha$ -syn aggregate species are observed in both of these models concomitant with neuron loss (12; 14) and have been shown to be resistant to autophagic clearance (56). **Together these preliminary results provide the foundation for our proposed use of these  $\alpha$ -syn models to test the potential therapeutic benefit of  $\alpha$ -Gal A enhancement.**

### **Aim 1 Test the hypothesis that increasing $\alpha$ -Gal A enhances the autophagic clearance of $\alpha$ -syn**

**Rationale** The ALP is responsible for the high-capacity degradation of  $\alpha$ -syn and is disrupted in PD brain (16-22). Thus identifying novel targets that promote the autophagic clearance of  $\alpha$ -syn in PD would be a significant advance in the development of PD therapeutics. Our preliminary data indicate a novel relationship between the pathological accumulation of  $\alpha$ -syn and  $\alpha$ -Gal A deficiency both in mouse brain (36) and in PD brain (Fig. 1). However, whether increasing  $\alpha$ -Gal A activity reduces  $\alpha$ -syn accumulation and clearance, and in a manner that requires the ALP have not been investigated. Experiments outlined in this Aim will use *in vitro* over-expression model systems to test our hypothesis that increasing  $\alpha$ -Gal A activity promotes the autophagic clearance of  $\alpha$ -syn, thus confirming its value as a therapeutic target for PD.

### **Aim 1 Experimental Design**

**Neuroblastoma cells** Differentiated SH-SY5Y human neuroblastoma cells will be used to monitor endogenous  $\alpha$ -syn, which we have shown to accumulate following ALP inhibition (26; 28; 29). We will also use an M17 human neuroblastoma cell line that conditionally over-expresses  $\alpha$ -syn (Fig. 3a), a gift of Dr. Talene Yacoubian

(see letter of support). M17 cells will be treated for 7 days with 10 µg/ml DOX to induce α-syn over-expression, followed by α-Gal A over-expression and/or experimental manipulation of the ALP to monitor the clearance of over-expressed α-syn upon DOX removal. Both cell lines exhibit transient over-expression of WT α-Gal A levels at 48h by western blot (Sigma HPA000237) and activity assay (Fig. 2) following nucleofection of cDNA in pCMV6 vector using methods we have shown previously (29). The lysosomal co-localization of over-expressed α-Gal A will be confirmed by its immunocytochemical (ICC) co-localization with lysosomal membrane protein LAMP-1 (U. Iowa Hybridoma bank) using methods as previously described (28).

**Primary neuron cultures from α-Gal A transgenic mice** To confirm that the effects of α-Gal A over-expression on endogenous α-syn in SH-SY5Y also occur in neurons (23) we will assess primary cultures of cortical neurons generated from embryonic day 16 WT or α-Gal A transgenic mice. We will soon receive breeding pairs of transgenic mice that over-express human WT α-Gal A (51) to our lab from Dr. Robert J. Desnick via an approved MTA between UAB and the Icahn School of Medicine at Mount Sinai (see letter of support). Brains from six-month-old α-Gal A transgenic mice were shown previously to exhibit an 11-fold increase in α-Gal A enzymatic activity, with mice lacking any noticeable phenotype (51). Genotyping will be performed as previously described (51). Prior to assessment of α-syn neurotoxicity we will confirm that cultured neurons from α-Gal A transgenic mice exhibit a significant increase in α-Gal A activity (as in Fig. 2).

**Detection of α-syn** Cells will be fixed with 4% paraformaldehyde (PF) plus 1% Triton X-100 for ICC detection of α-syn. Triton X removes soluble proteins during fixation, thus purifying for detection of insoluble α-syn species (12). Fixed cells will be probed for total α-syn (BD Biosciences syn-1) or α-syn species known to localize to insoluble α-syn inclusions including anti-p129S-α-syn (MJFF-Abcam ab168381) or nitrated α-syn (clone 514, Biologend MMS-556R), alone or co-labeled with anti-ubiquitin (Sigma U0508) (12; 14; 36; 57). Western blot will be performed to detect detergent-soluble vs. -insoluble fractions of α-syn monomer (17 kDa) and high molecular weight “pathological” species (>50 kDa) as we have shown previously (26; 58). Total α-syn (Santa Cruz sc-7011, C-20 clone), p129S-α-syn and nitrated α-syn will be assessed by western blot analysis.

**Aim 1-Experiment 1. Confirm that increasing α-Gal A activity reduces α-syn accumulation** We will assess effects of α-Gal A over-expression on endogenous α-syn in SH-SY5Y and cortical neurons, and the rate of clearance of over-expressed α-syn following its conditional over-expression in M17 cells. The ALP will be inhibited to determine its selectivity for regulating α-Gal A-dependent metabolism of α-syn. We will inhibit macroautophagy induction by siRNA knockdown of the autophagy-inducing gene Atg7 (by siRNA nucleofection simultaneous with α-Gal A over-expression) in neuroblastoma cells, lentiviral shRNA-mediated knockdown of Atg7 in cultured cortical neurons (see letter of support from Dr. Jeonga Kim for Atg7 lentiviral constructs). Effects of inhibiting macroautophagy induction will be substantiated by treatment with 3-methyl adenine, which inhibits class III PI3-kinase (26). We will inhibit ALP completion by treatment with chloroquine (26) and bafilomycin A1 (15, 23, 26, 29), agents that increase α-syn through disruption of lysosomal pH, or by treatment with pepstatin A, which selectively inhibits cathepsin D-mediated degradation of α-syn (25; 59). We will also determine if inducing macroautophagy induction via treatment with rapamycin or trehalose (24) promotes additive or synergistic reductions in α-syn levels when combined with increasing α-Gal A activity.

**Aim 1-Experiment 2. Determine if increasing α-Gal A activity promotes ALP function** We will also investigate ALP-dependent mechanism(s) by which increasing α-Gal A activity regulates the autophagic clearance of α-syn. Autophagic flux will be measured by western blot for autophagy markers LC3-II (Sigma L7543) and p62 (Sigma P0067) in the presence or absence of lysosome inhibitors (29; 60). We will assess lysosome function by measuring activities of lysosomal enzymes including cathepsin D, which selectively hydrolyzes α-syn (25; 59) and GCase, which we (Fig. 1) and others demonstrate to be deficient in PD brain in association with pathological α-syn accumulation (20, 41, 42), and by measurement of acidic vesicle pH using LysoTracker Red as we have shown previously (28; 29; 61). Finally, number/size of lysosomes will be assessed by measuring LAMP-1 by ICC and western blot analysis (28; 29; 36).

**Aim 1-Experiment 3. Confirm that protective effects of α-Gal A are activity-dependent** To address the putative requirement for α-Gal A activity we will detect α-syn following nucleofection with vectors that over-express mutant forms of α-Gal A found in Fabry disease, including D93G and C142Y, which both inhibit active site function and reduce α-Gal A activity below that of non-transfected levels in HEK cells (54). Dr. Derralynn Hughes (University College, London, see letter of support) will graciously provide our lab with these vectors.

**Expected Outcomes & Alternative Approaches** We predict that increasing α-Gal A activity *in vitro* will attenuate the accumulation of endogenous and over-expressed α-syn. We also predict that α-Gal A regulates the autophagic clearance of α-syn, as would be demonstrated by loss of function following ALP inhibition, and potentially further gain of function following pharmacological induction of macroautophagy. Such results would

be corroborated by showing that increasing  $\alpha$ -Gal A enhances autophagic flux. If we demonstrate in particular that increasing  $\alpha$ -Gal A shifts the dose responsiveness for inhibition of  $\alpha$ -syn degradation by pepstatin A this would suggest that  $\alpha$ -Gal A may regulate the function of cathepsin D, an interpretation that would be corroborated upon demonstration of its increased activity. In addition, if we demonstrate that  $\alpha$ -Gal A over-expression increases GCase activity and LAMP-1 levels concomitant with promoting autophagic clearance of  $\alpha$ -syn, we would investigate in future studies the potential for  $\alpha$ -Gal A to specifically regulate transcription factor EB (TFEB)-induced lysosome biogenesis (62) as a mechanism for promoting autophagic clearance of  $\alpha$ -syn. Finally, we predict that the effects of  $\alpha$ -Gal A over-expression are activity-dependent, as would be confirmed by over-expression of mutant forms of  $\alpha$ -Gal A that lack enzymatic activity (54). Over-expression of  $\alpha$ -Gal A mutants may actually promote the deleterious accumulation of  $\alpha$ -syn as we observed in  $\alpha$ -Gal A-deficient mouse brain (36). In contrast it is possible that a protective effect of  $\alpha$ -Gal A protein may be revealed that is independent of its activity, the mechanism(s) by which this occurs would be explored in future funding applications. It is possible that there are inherent differences in the ability of transient vs. constitutive over-expression of  $\alpha$ -Gal A to regulate the autophagic clearance of  $\alpha$ -syn. Such differences could be explained by relative differences in activity induced by transient vs. constitutive  $\alpha$ -Gal A over-expression that could be tested by performing a DNA dose-response study for transient over-expression. Alternatively there may be differences in compensatory regulation of lysosome function induced by transient vs. constitutive over-expression of  $\alpha$ -Gal A that may also explain discrepancies in regulating the autophagic clearance of  $\alpha$ -syn. Finally, if  $\alpha$ -Gal A over-expression reduces the accumulation of endogenous or over-expressed  $\alpha$ -syn but in a manner not regulated by macroautophagy we would consider exploring alternate modes of  $\alpha$ -syn degradation including chaperone-mediated autophagy (CMA) and the ubiquitin-proteasome system (UPS) (16; 63).

**Timetable** We predict Experiment 1 will be completed during Year 1 of funding, and Experiments 2-3 will be completed during Year 2 of funding.

### **Aim 2 Test the hypothesis that increasing $\alpha$ -Gal A attenuates $\alpha$ -syn-induced neurotoxicity**

**Rationale** Alpha-syn is widely believed to contribute to PD pathogenesis, and identifying novel targets that attenuate its associated neurotoxicity would significantly advance PD therapeutics development. To complement our *in vitro* assessment for the autophagic clearance of  $\alpha$ -syn proposed in Aim 1, we will treat  $\alpha$ -Gal A WT and transgenic mice with  $\alpha$ -syn PFFs, which induce the robust formation of endogenous  $\alpha$ -syn aggregates and neuron loss (11, 14). To corroborate our *in vivo* findings we will treat neurons cultured from these mice with  $\alpha$ -syn PFFs to confirm *in vitro* that  $\alpha$ -Gal A regulates formation of endogenous  $\alpha$ -syn aggregates and neuron loss (12, 64), and to confirm that the effects of  $\alpha$ -Gal A are ALP-dependent.

### **Aim 2 Experimental Design**

**Aim 2-Experiment 1. Determine if increasing  $\alpha$ -Gal A in vivo attenuates  $\alpha$ -syn PFF-mediated neuropathology** To confirm that  $\alpha$ -Gal A transgenic mice exhibit increased  $\alpha$ -Gal A in the midbrain similar to that observed in whole brain (51) we will demonstrate in a pilot activity assay study (Fig. 1) a significant increase in  $\alpha$ -Gal A activity in midbrain homogenates, and by robust increases in  $\alpha$ -Gal A as observed by IHC specific to tyrosine-hydroxylase-positive nigrostriatal dopaminergic neurons. Alpha-syn PFFs will be prepared from recombinant mouse  $\alpha$ -syn as previously described by our research team (64). Deeply anesthetized 8 week-old male  $\alpha$ -Gal A transgenic or WT mice will receive a stereotaxic 2.5  $\mu$ L micro-injection of either PBS vehicle or 5  $\mu$ g of  $\alpha$ -syn PFFs in PBS into the right dorsal striatum as previously described (14), using equipment from the laboratory of Dr. David G. Standaert (see letter of support). Mice will be euthanized 6 months later, a time that coincides with robust  $\alpha$ -syn aggregate formation and neuron loss (14). Mice within each group will be randomly assigned for biochemistry or IHC. For biochemical analysis the right (injected) and left (non-injected) ventral midbrains are rapidly frozen for preparation of detergent-soluble and -insoluble fractions (26; 58) to detect  $\alpha$ -syn species as described in Aim 1. For IHC detection mice will be perfused with 4% PF, with frozen, free-floating sections prepared spanning the substantia nigra. Sections will be probed for chromogenic detection of tyrosine hydroxylase (Pel-Freez) to assess neuron loss. Pathological  $\alpha$ -syn species including p129S- $\alpha$ -syn (MJFF-Abcam ab168381) or nitrated  $\alpha$ -syn (clone 514, Biogen MMS-556R) will be assessed separately or co-localized with ubiquitin as described in Aim 1, as well as total  $\alpha$ -syn (BD Biosciences syn-1) that is resistant to degradation by proteinase K (DAKO) as previously described (51; 65).

**Aim 2-Experiment 2. Confirm that  $\alpha$ -Gal A attenuates  $\alpha$ -syn PFF-mediated neuropathology in vitro** We will use primary hippocampal neuron cultures from embryonic day 16  $\alpha$ -Gal A-transgenic or WT mice as they exhibit robust formation of endogenous  $\alpha$ -syn pathological species (Fig. 3) and neuron loss (12) following treatment with  $\alpha$ -syn PFFs. Neuron cultures will be treated with  $\alpha$ -syn mouse PFFs at 1-5  $\mu$ g/ml PFFs starting at day in vitro (DIV) 5 as previously described (12; 64). Neurons will be harvested at incremental time points



from DIV5-19 to track levels and progression of  $\alpha$ -syn pathology (12), including ICC and western blot analysis of detergent-soluble and -insoluble total  $\alpha$ -syn, p129S- $\alpha$ -syn or nitrated  $\alpha$ -syn, and ICC analysis of  $\alpha$ -syn species co-localized to ubiquitin, as described in Aim 1. Neuron loss will be quantified by ICC detection of NeuN-positive neurons as previously reported (12; 36) beginning at DIV15, a time shown to coincide with significant  $\alpha$ -syn PFFs-induced neuron loss (12). Increased  $\alpha$ -Gal A activity will be confirmed as in Aim 1.

**Aim 2-Experiment 3. Determine if the ALP regulates effects of  $\alpha$ -Gal A on  $\alpha$ -syn PFF-mediated neuropathology *in vitro*** As we predict in Aim 1 that  $\alpha$ -Gal A over-expression promotes ALP function, we will determine if pharmacologically inhibiting macroautophagy induction or lysosomal completion (as described in Aim 1) in primary neuron cultures attenuates, or if inducing macroautophagy by treatment with rapamycin and trehalose (as described in Aim 1) further enhances, protection afforded by increasing  $\alpha$ -Gal A activity on reducing  $\alpha$ -syn aggregate formation and neuron loss.

**Expected Outcomes & Alternative Approaches** We predict that increasing  $\alpha$ -Gal A activity *in vivo* will attenuate TH-positive neuron loss and numbers/size of pathological  $\alpha$ -syn species (and their co-localization with ubiquitin), and levels of high-MW  $\alpha$ -syn species by western blot in mouse substantia nigra that result from the intrastriatal injection of  $\alpha$ -syn PFFs (12, 14). These results are predicted to be corroborated by *in vitro* results in primary neuron cultures from  $\alpha$ -Gal A transgenic mice indicating a delay and/or attenuation of neuron loss and numbers, size or co-localization of  $\alpha$ -syn aggregate species induced by the exogenous addition of  $\alpha$ -syn PFFs. Finally we predict that these effects of  $\alpha$ -Gal A are ALP-dependent, as would be demonstrated by loss of function following inhibition of macroautophagy and potential gain of function following treatment with inducers of macroautophagy. Together these results would complement findings observed in Aim 1 demonstrating that  $\alpha$ -Gal A promotes the autophagic clearance of  $\alpha$ -syn, and would confirm the therapeutic potential of increasing  $\alpha$ -Gal A in accepted  $\alpha$ -syn models of PD. It is also possible that increasing  $\alpha$ -Gal A may not protect against  $\alpha$ -syn PFF-induced aggregate formation or neuron loss, or may even exacerbate neuropathology suggesting its neurotoxic potential, despite the available literature reporting a lack of a deleterious CNS phenotype following  $\alpha$ -Gal A over-expression (51; 66). Such findings would be carefully compared to those obtained in Aim 1, as differences in the outcome of these studies could be explained by the different methods proposed to increase endogenous  $\alpha$ -syn. Finally, if as in Aim 1 the protective effects of  $\alpha$ -Gal A appear to be regulated independent of macroautophagy we would consider exploring alternative methods of  $\alpha$ -syn degradation including CMA and the UPS (16; 63).

**Timetable** We predict Experiment 1 biochemistry studies and Experiment 2 will be completed during Year 1 of funding. Experiment 1 IHC studies and Experiment 3 are predicted to be completed during Year 2 of funding.

**Statistics for Aims 1-2** For *in vitro* studies, values within a single experiment will be generated by averaging replicates from at least three different wells/dishes and will be expressed as percent vehicle or vector control (for  $\alpha$ -Gal A enhancement) for any given time point/treatment. For *in vivo* studies, each mouse is considered an “*n*” of 1. Quantitative analysis of ICC mean fluorescence intensity will be performed using ImageJ (NIH). Alpha-syn aggregates  $\geq 1 \mu\text{m}$  in size will be quantified as well as their distribution by size using the “cell counter” plugin, and co-localization analysis for  $\alpha$ -syn species and ubiquitin will be performed using the “Coloc 2” plugin, as described previously by our lab and others (36; 67). For western blot analysis, protein band intensities will be expressed relative to actin loading control. We will use unbiased stereology according to the optical fractionator principle (68) to quantify by IHC numbers of tyrosine hydroxylase-positive neurons as previously described (56; 69). Effects of treatment and time will be measured by Student’s paired t-test (one comparison between two groups), one-factor ANOVA (one comparison between multiple groups) or two-factor ANOVA (e.g. time vs. treatment interactions between groups) as appropriate, with post hoc analysis performed using Bonferroni’s test (significance set *a priori* at  $p < 0.05$ ). ICC Co-localization analysis will be performed using the Manders-Costes threshold analysis as described previously by our lab (36). For all experiments, descriptive statistics will be reported as mean  $\pm$  SEM. Based on our published reports (12; 14; 26; 28; 29; 36) our power analysis predicts a need for at least six independent experiments for *in vitro* assay endpoints, and an *n* of six mice per treatment group for each endpoint (biochemical vs. IHC, thus each mouse treatment group will be comprised of 12 experimental mice) to achieve the statistical power necessary to test for significance.

**Summary** This application utilizes a highly integrated *in vitro* and *in vivo* approach of  $\alpha$ -Gal A over-expression to test our innovative hypothesis that  $\alpha$ -Gal A regulates the autophagic clearance of  $\alpha$ -syn and its associated neurotoxicity. Successful completion of this study will confirm that  $\alpha$ -Gal A regulates  $\alpha$ -syn-associated toxicity and will provide a firm foundation for future testing of  $\alpha$ -Gal A-associated therapeutics already used in humans for their novel treatment of PD, as well development of new therapeutics that attenuate  $\alpha$ -syn-associated pathogenesis in PD through their promotion of  $\alpha$ -Gal A function.

**SUMMARY STATEMENT**  
( Privileged Communication )

*Release Date:* 03/16/2015

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*Application Number:* 1 R21 NS093435-01

**Principal Investigator**

**SHACKA, JOHN J PHD**

**Applicant Organization: UNIVERSITY OF ALABAMA AT BIRMINGHAM**

*Review Group:* **CMND**  
**Cellular and Molecular Biology of Neurodegeneration Study Section**

*Meeting Date:* **02/23/2015**  
*Council:* **MAY 2015**  
*Requested Start:* **07/01/2015**

*RFA/PA:* **PA13-303**  
*PCC:* **SUTHEMND**

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*Project Title:* **Alpha-Galactosidase A: a novel target for reducing alpha-synuclein toxicity**

*SRG Action:* **Impact Score: 39 Percentile: 22 +**

*Next Steps:* **Visit [http://grants.nih.gov/grants/next\\_steps.htm](http://grants.nih.gov/grants/next_steps.htm)**

**Human Subjects: 10-No human subjects involved**

**Animal Subjects: 30-Vertebrate animals involved - no SRG concerns noted**

<b>Project Year</b>	<b>Direct Costs Requested</b>	<b>Estimated Total Cost</b>
<b>1</b>		
<b>2</b>		
<hr/>		
<b>TOTAL</b>		

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**ADMINISTRATIVE BUDGET NOTE:** The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the **COMMITTEE BUDGET RECOMMENDATIONS** section.

## 1R21NS093435-01 SHACKA, JOHN

### VERTEBRATE ANIMAL UNACCEPTABLE

**RESUME AND SUMMARY OF DISCUSSION:** This R21 application proposes to investigate the mechanism by which Alpha-Galactosidase A ( $\alpha$ -Gal A), a lysosomal enzyme of the glycosphingolipid metabolism pathway, may enhance autophagic clearance of alpha-synuclein ( $\alpha$ -syn), which accumulation is considered a hallmark of Parkinson's disease (PD) pathogenesis. The experimental design relies on approaches in cella and in vivo to investigate autophagic clearance of  $\alpha$ -syn, and to determine whether  $\alpha$ -Gal A overexpression attenuate neuropathology following stereotaxic cerebral injection of exogenous alpha-synuclein pre-formed fibrils. Although assigned reviewers generally recognized the appeal of a connection between lysosome dysfunction and synucleinopathy as well as the importance of better understanding ways to manipulate autophagy-lysosome pathways to impact alpha-synuclein expression, strong difference of opinions were expressed with respect to the significance and potential impact of the proposed studies. On the one hand, some reviewers considered the application as innovative, supported by compelling preliminary data, particularly in vivo, and valued its proof-of-concept nature to assess whether existing  $\alpha$ -Gal modifying therapies may be leveraged for PD. On the other hand, a significant concern was expressed about the lack of compelling data associating  $\alpha$ -Gal A with PD, including the lack of CNS neurodegenerative pathology in juvenile onset of Fabry's. Shortcomings in the experimental design were also noted, including a single time point in the animal studies, and the reliance on very high  $\alpha$ -Gal A overexpression. Notwithstanding the strong expertise of the investigative team in lysosome and synuclein biology, no consensus could be reached at the end of the discussion. The final scoring range was broad and the overall impact score reflected individual weighting of the strengths and weaknesses of an otherwise very good application.

**DESCRIPTION (provided by applicant):** The pathological accumulation of alpha-synuclein ( $\alpha$ -syn) is believed to play a major role in Parkinson's disease (PD) pathogenesis. The autophagy-lysosome pathway (ALP) is responsible for the lysosomal degradation of  $\alpha$ -syn. ALP dysfunction is documented in PD and its inhibition induces accumulation of  $\alpha$ -syn aggregates, implicating its role in PD pathogenesis. Thus it is our goal to identify novel lysosome targets that enhance the autophagic clearance of  $\alpha$ -syn. The glycosphingolipid metabolism pathway is coordinately regulated by several lysosomal enzymes and has received recent focus as a therapeutic target for PD. Alpha-Galactosidase A ( $\alpha$ -Gal A) is a soluble lysosomal enzyme in this pathway and its mutations cause the lysosomal disorder Fabry disease. Evidence from our lab indicates for the first time a novel association between  $\alpha$ -Gal A activity and  $\alpha$ -syn. Alpha-Gal A deficiency causes the aberrant accumulation of  $\alpha$ -syn aggregates and ALP markers in mouse brain, indicating that  $\alpha$ -Gal A regulates  $\alpha$ -syn metabolism. Our preliminary data also indicates a direct relationship between  $\alpha$ -Gal A and PD pathology as demonstrated by significantly reduced  $\alpha$ -Gal A enzymatic activity in PD brain specimens that harbor pathologic accumulation of  $\alpha$ -syn. While other glycosphingolipid metabolizing enzymes have been targeted for PD, there remain several critical gaps in our knowledge of how this pathway regulates  $\alpha$ -syn clearance and  $\alpha$ -syn-associated neurotoxicity. In addition, therapeutics that are clinically approved to treat Fabry disease or show promise in clinical trials, increase  $\alpha$ -Gal A levels and activity, but their efficacy for PD has not yet been explored. Thus to help bridge these critical gaps, our proposal has two Specific Aims. In Aim 1 we will use in vitro model systems to test the hypothesis that increasing  $\alpha$ -Gal A enhances the autophagic clearance of  $\alpha$ -syn. Effects of  $\alpha$ -Gal A over-expression will be assessed on endogenous  $\alpha$ -syn in SH-SY5Y human neuroblastoma cells or in cultured neurons from  $\alpha$ -Gal A transgenic mice, or conditionally over-expressed  $\alpha$ -syn in M17 human neuroblastoma cells. We will selectively inhibit or induce the ALP to confirm its selectivity for mediating  $\alpha$ -Gal A function. We will also determine if increasing  $\alpha$ -Gal A enhances function/levels of lysosome-associated proteins important for  $\alpha$ -syn clearance. In Aim 2 we will use  $\alpha$ -Gal A transgenic and wild-type mice, and primary neuron cultures from these mice to test the hypothesis that increasing  $\alpha$ -Gal A attenuates  $\alpha$ -syn-induced

neurotoxicity. We will determine if increasing  $\alpha$ -Gal A activity attenuates neuropathology resulting from the exogenous addition of  $\alpha$ -syn pre-formed fibrils, including formation of endogenous  $\alpha$ -syn aggregates and neuron loss. We will also pharmacologically inhibit and stimulate the ALP to confirm its selectivity for mediating  $\alpha$ -Gal A function. Successful completion of this study will provide important insights on  $\alpha$ -Gal A regulation of  $\alpha$ -syn clearance, demonstrate for the first time that  $\alpha$ -Gal A can be targeted to reduce  $\alpha$ -syn-associated neurotoxicity, and justify future studies of clinically approved and newly developed  $\alpha$ -Gal A therapeutics for treating PD.

**PUBLIC HEALTH RELEVANCE:** Parkinson's disease (PD) is a major health issue affecting upwards of one million Americans and because current therapies only provide symptomatic relief there is a critical need for novel therapeutics that delay or attenuate disease pathogenesis. The goal of this application is to confirm that increasing activity of the lysosomal enzyme alpha-Galactosidase A provides therapeutic benefit in pre-clinical models of PD. Successful completion of this study will justify the future study of alpha-Galactosidase A-associated therapeutics already used in humans, for their novel role in attenuating PD pathogenesis.

#### **CRITIQUE 1:**

Significance: 6

Investigator(s): 3

Innovation: 4

Approach: 6

Environment: 2

**Overall Impact:** The overall goals of this application are to investigate if increasing alpha-galactosidase A levels, a soluble lysosomal enzyme, can alter alpha-synuclein expression and aggregate formation by modulating autophagy-lysosome pathway. First, it is proposed to use in vitro cells systems to test the effects of increasing alpha-galactosidase A expression on enhancing the autophagic clearance of  $\alpha$ -synuclein. Secondly, alpha-galactosidase A transgenic compared to wild-type mice will be used to determine if alpha-galactosidase A over expression can attenuate neuropathology resulting from stereotaxic cerebral injection of exogenous alpha-synuclein pre-formed fibrils. Similarly, primary neuron cultures from these mice will be used to test that increasing alpha-galactosidase A expression can attenuate alpha-synuclein aggregation and induced neurotoxicity by the addition of exogenous alpha-synuclein fibrils.

However, there are a number of issues that raise important concerns about the impact of the proposed studies and the ability to interpret the data generated. For example, the clinical relevance of the high levels of alpha-galactosidase A expression that will be used for both cell culture and mouse studies (>11 fold). In addition, the investigators do not seem to be entirely familiar with the tools that they are using and are proposing to use. For example, throughout the application syn-514 is described as a nitration specific antibody alpha-synuclein when in fact it is an antibody to alpha-synuclein antibody that can react with all synuclein proteins.

#### **Significance:**

##### **Strengths**

- To try to find new ways to manipulate autophagy-lysosome pathways to impact alpha-synuclein expression could be a promising therapeutic approach

##### **Weaknesses**

- The data associating alpha-galactosidase A with Parkinson's disease (PD) are not compelling. In fact, patients with Fabry disease caused by mutations in the alpha-galactosidase A gene present with brain vascular diseases and peripheral complications but no CNS neurodegenerative diseases. Also there is no clear link between carriers of alpha-galactosidase

A genetic mutations and risk to develop Parkinson's disease. In addition, mice null for alpha-galactosidase A do not seem to develop bona fide alpha-synuclein pathology.

**Investigator(s):**

**Strengths**

- The principal investigator and co-investigator have the skills to conduct most of the proposed research.

**Weaknesses**

- Recent productivity of the principal investigator has been modest.

**Innovation:**

**Strengths**

- The proposed idea of a link between alpha-galactosidase A and PD is novel.

**Weaknesses**

- Regulation of autophagy to try to regular alpha-synuclein expression is not a new concept.
- No new systems or approaches are used or developed.

**Approach:**

**Strengths**

- Complementary in vitro and in vivo sets of studies.

**Weaknesses**

- A single time point (6 months) is proposed for the in vivo seeding studies which is insufficient to investigate change in time-dependent processes. Thus, important alterations in progression of alpha-synuclein pathology could be readily missed. Therefore, it would be important to include dose and time-dependent response analyses.
- All the models proposed (either in vitro or in vivo) use very high levels of alpha-galactosidase A overexpression that are not likely to be clinically relevant.

**Environment:**

**Strengths**

- The institution and the research environment are excellent.

**Protections for Human Subjects:**

Not applicable (No Human Subject)

**Vertebrate Animals:**

Unacceptable

- Animal numbers are justified. Surgical procedures including post-operation pain management and animal wellbeing monitoring are not adequately described.

**Budget and Period of Support:**

Recommend as requested

**CRITIQUE 2:**

Significance: 2

Investigator(s): 2

Innovation: 2

Approach: 3

Environment: 1

**Overall Impact:** The potential interplay between  $\alpha$ -syn and lysosomal function is highly significant and the proposed work and model innovative. The preliminary data are strong and supportive of the hypothesis. Understanding how lysosome biology may be modulated to promote degradation of  $\alpha$ -syn is important. This may implicate the integrity of another lysosomal protein as critical to maintenance of proper lysosomal proteolysis, in a specific disease-relevant context.

**Significance:**

**Strengths**

- Addressing major emerging interest in lysosome dysfunction and synucleinopathy in novel way
- Solid preliminary data support disease-relevance

**Investigator(s):**

**Strengths**

- Well-suited to perform the proposed studies
- Has assembled excellent team for support

**Innovation:**

**Strengths**

- Novel target and preliminary data
- Testing the effects of improving lysosomal function (outside of broad induction of macroautophagy) on  $\alpha$ -syn clearance is innovative

**Approach:**

**Strengths**

- Multiple cell models
- $\alpha$ GalA inactive control
- thoughtful consideration of secondary effects on lysosomal proteases
- test a genetic modifier of PFF transmission model

**Weaknesses**

- Endogenous  $\alpha$ -syn “aggregates” are not observed in mice thus one cannot study their clearance (Aim 2), perhaps insoluble  $\alpha$ -syn in aged mice would be more practical.
- Greater attention to characterizing changes in  $\alpha$ -syn is advised (multiple antibodies to control for non-specific HMW bands, sequential fractionation, etc).
- In vivo and primary neuron focus is perhaps too heavily emphasized in “transmission” models and not intrinsic turnover of  $\alpha$ -syn. A stronger focus on primary neurons in Aim 1 would be advised

**Environment:**

**Strengths**

- Excellent with complementary expertise available

**Protections for Human Subjects:**

Not applicable (No Human Subject)

**Vertebrate Animals:**

Acceptable

**Budget and Period of Support:**

Recommend as requested

### CRITIQUE 3:

Significance: 1  
Investigator(s): 2  
Innovation: 2  
Approach: 3  
Environment: 2

**Overall Impact:** This application aims to test the novel hypothesis that alpha-galactosidase, a lysosomal enzyme that degrades glycosphingolipids, can enhance autophagic clearance of alpha-synuclein and attenuate the toxicity of alpha-synuclein fibrils in cell lines, primary neurons and  $\alpha$ -Gal transgenic mice. The potential significance is high given that genetic accumulations of wild type  $\alpha$ -synuclein cause Parkinson's disease and linkages between Gaucher's carriers and PD. The principal investigator and co-investigator are experts in lysosomal biology and neuronal transmission of alpha-synuclein fibrils, respectively. The plan includes important studies of alpha-synuclein clearance in primary neuron cultures and  $\alpha$ -Gal transgenic mice. If the hypothesis is correct, this could lead to new neuroprotective directions for sporadic PD. Each Aim includes important steps to address causality. There are some concerns with defining pathological species to be cleared in Aim 1, but these are minor issues as the heart of the application is the in vivo and ex vivo studies of Aim 2. The Significance and Investigators criteria outweigh minor concerns in Approaches.

#### Significance: Strengths

- Because accumulation of wild type  $\alpha$ -synuclein contributes to dominant familial PD and  $\alpha$ -synuclein polymorphisms increase risk of sporadic PD, addressing cellular mechanisms that can reduce its levels within neurons would be highly significant
- That glycosphingolipid metabolism is implicated by GBA associations further strengthens the rationale to study enzymes in their metabolic pathway.
- Given that degradation systems in neurons may differ from tumor cell lines, the inclusion of primary neurons in both Aims is an important component.
- There are case reports implicating Parkinsonism in Fabry's patients and existing enzyme replacement or molecular chaperone therapies may speed up clinical translation.

#### Investigator(s): Strengths

- Strong team of investigators with expertise in lysosomal neuropathology and with transcellular spread of alpha-synuclein fibrils.

#### Innovation: Strengths

- Proof of concept for whether  $\alpha$ -Gal modifying therapies that exist may be leveraged for PD
- Use of  $\alpha$ -Gal mutant to demonstrate if its activity or other potential interactions are causative
- This line of work may also help provide additional support for a currently controversial mechanism for the spread of PD pathology.

#### Approach: Strengths

- Preliminary data show decreases in both  $\alpha$ -Gal and GCase activities in human PD brains, correlating with increased HMW species of  $\alpha$ -syn.

- The  $\alpha$ -Gal A overexpression systems are already in place in vitro and in vivo. Techniques to assess syn aggregation, clearance and spread of pathological species are in place.
- Aim 1 will establish role of ALP using specific molecular and pharmacological manipulations as well as pinpoint whether or not the  $\alpha$ -Gal enzymatic activity is essential, to support use of existing therapeutics to enhance  $\alpha$ -Gal activity.
- Aim 2 addresses whether  $\alpha$ -Gal is effective on pre-formed alpha-synuclein fibrils, an important consideration many autophagy-based studies have not established that effects of rapamycin, for instance, are directly due to fibril clearance or occur through translational mechanisms.

#### **Weaknesses**

- In Aim 1. It is unclear how the studies with differentiated SH-SY5Y cells or wild type versus  $\alpha$ -Gal OE mice will help with the hypothesis. Whether it is a goal to reduce soluble monomers or oligomers of  $\alpha$ Syn that is in its normal synaptic location is unclear. It seems that the value of this assay would be improved by demonstration of pathological oligomers, aggregates or modified species.
- Although increased  $\alpha$ Syn with M17 induction can allow for more easy assessment of  $\alpha$ -Syn clearance, how will rate of clearance be distinguished from kinetics of  $\alpha$ -syn induction is unclear. Some more details would be helpful.

#### **Environment:**

##### **Strengths**

- The environment at UAb is highly supportive with strong inter-departmental neuroscience interactions.

#### **Protections for Human Subjects:**

Not applicable (No Human Subject)

#### **Vertebrate Animals:**

Acceptable

#### **Budget and Period of Support:**

Recommend as requested

**THE FOLLOWING SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE, OR REVIEWER'S WRITTEN CRITIQUES, ON THE FOLLOWING ISSUES:**

**VERTEBRATE ANIMAL (Resume):** Unacceptable. Surgical procedures including post-operation pain management and animal wellbeing monitoring are not adequately described.

**COMMITTEE BUDGET RECOMMENDATIONS:** The budget was recommended as requested.

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**+ Derived from the range of percentile values calculated for the study section that reviewed this application.**

**NIH has modified its policy regarding the receipt of resubmissions (amended applications). See Guide Notice NOT-OD-14-074 at <http://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-074.html>. The impact/priority**



**score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see [http://grants.nih.gov/grants/peer\\_review\\_process.htm#scoring](http://grants.nih.gov/grants/peer_review_process.htm#scoring).**

## MEETING ROSTER

### Cellular and Molecular Biology of Neurodegeneration Study Section Molecular, Cellular and Developmental Neuroscience Integrated Review Group CENTER FOR SCIENTIFIC REVIEW CMND

February 23, 2015 - February 24, 2015

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