PROJECT SUMMARY

The pathological accumulation of alpha-synuclein (α-syn) is believed to play a major role in Parkinson's disease (PD) pathogenesis. The autophagy-lysosome pathway (ALP) provides for the high-capacity clearance of α-syn and its dysfunction is well-documented in PD. Inhibiting the ALP has been shown to induce α-syn accumulation. Conversely, excess α-syn has been shown to inhibit the ALP. Because the lysosome is critical for α-syn clearance we believe its continued investigation will further delineate mechanisms of PD pathogenesis and foster development of PD therapeutics. Alpha-Galactosidase A (α-Gal A) is a soluble lysosomal enzyme, with mutations causing the rare lysosomal disorder Fabry disease. While it is unknown if α-syn accumulates in Fabry patients, our analysis of postmortem PD brains indicates a decrease in α-Gal A activity specific to specimens with increased α-syn pathology. Our preliminary data also indicate reduced α-Gal A activity in neuroblastoma cells following the conditional over-expression of α-syn. Together with our report of α-syn pathology and altered ALP markers in α-Gal A-deficient mouse brain, these findings suggest a strong link between α-Gal A deficiency and α-syn accumulation. However, whether α-Gal A deficiency exacerbates the neurotoxic potential of α-syn is unknown. Increasing α-Gal A activity via enzyme replacement therapy (ERT) is clinically approved therapy for Fabry disease. Because ERT has limited CNS bioavailability, there is a critical gap in understanding its potential for treating PD. To help bridge this gap we developed novel research tools to increase α-Gal A activity in neuronal systems, including its dose-responsive increase in neuronal cells via ERT, and transgenic mice that exhibit two-fold increases in α-Gal A brain activity. Our preliminary data in neuroblastoma cells shows that α-Gal A ERT enhances the clearance of over-expressed α-syn. However, whether increasing α-Gal A activity attenuates α-syn-associated neurotoxicity has not been tested. Taken together, we hypothesize that α-syn-associated neurotoxicity is exacerbated by α-Gal A deficiency and is attenuated by increasing α-Gal A activity. In Aim 1 we will determine if α-Gal A-deficiency in primary neuron cultures exacerbates neurotoxicity resulting from the exogenous addition of α-syn pre-formed fibrils (PFFs) in a manner concomitant with ALP disruption. We will also determine if α-Gal A–deficient mice exhibit exacerbated loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra following AAV2-mediated over-expression of human wild-type α-syn. In Aim 2 we will determine if α-syn PFF-mediated neurotoxicity in primary neuron cultures is attenuated by α-Gal A ERT or the transgenic over-expression of α-Gal A and if this protection is regulated by the ALP. We will also determine if α-Gal A over-expressing mice exhibit a reduction in TH-positive neuron loss resulting from AAV2-α-syn. If our hypothesis is correct, it would suggest that α-Gal A deficiency regulates α-syn pathogenesis, a mechanism worthy of future investigation, and would accelerate the development of therapeutics for PD that act by increasing CNS α-Gal A activity.

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 the lysosomal enzyme alpha-Galactosidase A regulates the neurotoxic potential of alpha-synuclein, a protein that accumulates in PD brain and may contribute to disease spread. Successful completion of this study will justify a heightened focus on alpha-Galactosidase A for its contribution to PD pathogenesis and for targeting this enzyme as a therapeutic.

INTRODUCTION

This is the A1 resubmission of application 1 R21 NS093435-01, "Alpha-Galactosidase A: a Novel Target for Reducing alpha-synuclein Toxicity", reviewed in March, 2015 by the CMND study section. The first submission received a 22 percentile (impact score 39). I am gratified by the many positive comments indicated for our work. However, there were several important issues raised by the Reviewers that collectively diminished enthusiasm for our proposal. **After careful consideration I have thoroughly addressed the concerns raised by the three Reviewers. I believe addressing these concerns has significantly improved our A1 application.** As our resubmission has undergone substantial revisions, changes are not marked in the text. Below I have itemized specific critiques raised by the Reviewers and our response. References numbered below can be found in our attached Reference List.

1. Critique: Reviewer 1 cites a "…lack of compelling data associating α-Gal A with PD" and "…no clear link between carriers of alpha-galactosidase A genetic mutations and risk to develop Parkinson's disease."

Response: Preliminary results, described in detail in the revised proposal suggest there is in fact a strong link between α-Gal A, α-syn and PD. First, analysis of postmortem temporal cortex specimens from human PD reveals there is significant α-Gal A deficiency, but only in specimens from late-stage PD that also harbor significant, pathologic accumulation of α-syn *(Fig. 1)*. Corroborating our findings in postmortem brain, we now include a letter of support from our colleague, Dr. Roy Alcalay (Columbia), whose unpublished data indicate significantly decreased α-Gal A activity in dried blood spots of patients with sporadic PD, evidence supporting the putative role of α-Gal A as a premortem PD biomarker. In addition, our preliminary results in an α-syn over-expressing M17 cell line *(Fig. 2)* demonstrate a bidirectional, reciprocal modulation of α-Gal A activity and α-syn: over-expression of α-syn leads to a significant reduction in α-Gal A activity, while pharmacologically increasing α-Gal A activity enhances clearance of α-syn. We concur that at present there is no established genetic association between α-Gal A mutations and the risk for PD, but there is little data on this: a search of the database PDGENE.org reports "There are no eligible published association studies for this gene." The link to glucocerebrosidase (GCase), now well recognized, emerged out of chance observation of Parkinsonism in GBA mutation carriers. To determine if there is in fact an association with α-Gal A would require a large-scale epidemiologic study in PD patients and of Parkinsonism in Fabry disease patients and their relatives. If our hypothesis that α-Gal A regulates α-syn neurotoxicity is correct, it would justify an effort of this kind.

2. Critique: Reviewer 1 suggests "…mice null for α-Gal A do not seem to develop bona fide α-syn pathology."

Response: While these mice do not develop classic Lewy bodies, they do demonstrate clear cut p129S α-syn-positive axonal spheroids in the hindbrain (45). Synucleinopathy without definitive Lewy pathology is found in many mouse models deficient in lysosomal enzymes including cathepsin D (25) and glucocerebrosidase (58; 59), though the relevance of these enzymes to the pathological accumulation of α-syn in PD is widely accepted. Similarly, Lewy pathology is not observed in α-syn transgenic over-expression models. Importantly, our study to date of α-Gal A-deficient mice (45) has not considered the effect of concurrent α-syn over-expression. Thus in Aim 1 of our resubmission we propose to use α-Gal A-deficient mice and primary neuron cultures from these mice to critically test the hypothesis that α-Gal A deficiency exacerbates α-syn-associated neurotoxicity, an important set of experiments that complement our existing hypothesis that increasing α-Gal A activity attenuates α-syn-associated neurotoxicity.

3. Critique: Reviewer 1 indicated that **"**All the models proposed…use very high levels of α-Gal A that are not likely to be clinically relevant."

Response: To address this concern, we will use pharmacological modulation of α-Gal A activity through use of Fabrazyme® (Genzyme Corp.; see attached letter). This will allow us to explore the effect of increasing α-Gal A activity *in vitro* over a range of different doses *(Figs. 2-3)*. We will also use a new line of transgenic mice that over-express human α-Gal A for *in vitro* and *in vivo* studies. These mice exhibit only a two-fold increase in brain α-Gal A enzymatic activity *(Fig. 3)*, thus more clinically relevant for analysis as a putative therapeutic.

4. Critique: Reviewer 2 indicated that our approach was "…too heavily emphasized in transmission models."

Response: In place of the *in vivo* mouse fibril model proposed in the first submission, we are now proposing to assess the effects of α-Gal A deficiency and human GLA over-expression in mice in which α-syn is expressed using an AAV vector, a robust *in vivo* model of α-syn neurotoxicity well characterized in our lab and in that of our Collaborator, Dr. David G. Standaert (66-68).

5. Response to miscellaneous critiques: Our proposed *in vitro* studies are now focused solely on primary neuron cultures and not neuroblastoma cell lines. We are now using multiple antibodies for detection of both endogenous and p129S-α-syn. Finally, in response to deficiencies in our Vertebrate Animals section we have more thoroughly described surgical procedures, post-operation and post-pain management.

SPECIFIC AIMS

Parkinson's disease (PD) represents a class of diseases called synucleinopathies that are defined by the pathological accumulation of alpha-synuclein (α-syn) species. It is widely believed that α-syn regulates PD pathogenesis and that its aberrant accumulation is due in part to age and disease-related compromises in its degradation. The autophagy-lysosome pathway (ALP) is responsible for the high-capacity clearance of α-syn and its dysfunction is well documented in PD. Not only has inhibiting the ALP been shown to increase α-syn accumulation and toxicity, but excess α-syn has been shown to inhibit the ALP, thus reinforcing the concept of improving ALP function as a therapeutic strategy for PD. Autophagy-inducing therapies have been tested clinically for PD but to date they have met with limited success. However, it is logical to predict that targeting the lysosome could be more beneficial for PD, as inducing autophagy without also improving lysosome function may not necessarily enhance α-syn clearance. **As the lysosome is crucial for α-syn clearance, we believe its continued investigation will further delineate mechanisms of PD pathogenesis and accelerate the development of effective PD therapeutics.**

Lysosomal enzymes have received recent focus for their role in PD pathogenesis and therapy. Alpha-Galactosidase A (α-Gal A) is a soluble lysosomal enzyme that metabolizes glycosphingolipids and is best known for its mutations that define the rare lysosomal storage disorder Fabry disease. While it is unknown if Fabry patients exhibit α-syn neuropathology, our analysis of postmortem PD brains indicates a significant decrease in α-Gal A activity found only in late-stage specimens with significantly increased α-syn pathology. In addition, our preliminary *in vitro* data indicate significantly decreased α-Gal A activity in M17 human neuroblastoma cells following their conditional over-expression of α-syn. Furthermore, we have previously shown that α-Gal A-deficient mouse brains exhibit at baseline the pathological accumulation of α-syn and ALP markers, suggesting the potential for α-Gal A to regulate the clearance of α-syn. **Together our preliminary results support a strong link between α-syn accumulation and α-Gal A deficiency. However, whether α-syn-associated neurotoxicity is exacerbated by α-Gal A deficiency has not been previously tested.**

Increasing α-Gal A activity via enzyme replacement therapy (ERT) is clinically approved for the safe and effective treatment of Fabry patients. A gene therapy approach to increase α-Gal A activity is also being explored in preclinical studies. Because increasing α-Gal A activity in the human CNS remains an obstacle, there is a critical gap in knowledge for its potential to treat PD. To help bridge this gap we have established novel pre-clinical research tools for increasing α-Gal A activity in neuronal model systems, including dose-responsive increases in α-Gal A activity in primary neuron cultures by α-Gal A ERT and transgenic mice that exhibit two-fold increases in brain α-Gal A activity. Our preliminary data indicate α-Gal A ERT enhances the clearance of conditionally over-expressed α-syn, supporting the concept of increasing α-Gal A activity as a strategy to regulate α-syn metabolism. **However, whether increasing α-Gal A activity attenuates the neurotoxic potential of α-syn is unknown and requires further investigation.**

Taken together, we hypothesize that α-syn-associated neurotoxicity is exacerbated by α-Gal A deficiency and is attenuated by increasing α-Gal A activity. To test this hypothesis we will utilize two established models of α-syn neurotoxicity: 1) exogenous addition of α-syn pre-formed fibrils (PFFs) to primary neuron cultures, which causes accumulation of endogenous α-syn species and neuron loss; and 2) AAV2-mediated over-expression of human wild-type α-syn in mouse substantia nigra, which causes the robust loss of tyrosine hydroxylase (TH)-positive neurons. Using these models we propose the following two Specific Aims:

Aim 1) Determine if α-Gal A deficiency exacerbates α-syn-associated neurotoxicity. We will use α-Gal A-deficient and wild type (WT) primary neuron cultures to quantify toxicity caused by α-syn PFFs. We will also determine if α-Gal A deficiency alters ALP function *in vitro* and if α-syn PFFs exacerbate these alterations. Finally, α-Gal A-deficient and WT mice will be used to quantify TH-positive neuron loss caused by AAV2-α-syn.

Aim 2) Determine if increasing α-Gal A activity attenuates α-syn-associated neurotoxicity. We will increase α-Gal A activity pharmacologically by α-Gal A ERT or genetically in neuron cultures from α-Gal A transgenic mice to assess toxicity caused by α-syn PFFs. We will also selectively induce or inhibit the ALP *in vitro* to determine its role in mediating the response to increased α-Gal A activity. Finally, α-Gal A transgenic over-expressing and WT mice will be used to quantify TH-positive neuron loss caused by AAV2-α-syn.

Completion of this study will delineate the role of α-Gal A in pre-clinical α-syn models of PD. If our hypothesis is correct, it would implicate α-Gal A deficiency as a candidate mechanism of α-syn pathogenesis in PD worthy of further investigation, and would justify the future development of therapeutics for PD that increase CNS α-Gal A activity. A successful outcome would also provide the rationale for future large-scale epidemiologic studies that address the potential association of α-Gal A mutations in PD patients, and of Parkinsonism in Fabry disease patients and their relatives.

RESEARCH STRATEGY

A. Significance

PD is the second most common neurodegenerative disease and the most common movement disorder, with motor symptoms caused by the progressive loss of substantia nigra dopaminergic neurons (1). As many as one million Americans are estimated to have PD, representing 1-2% of the U.S. population over 65 and 4-5% over 85 (2). In addition to the profound impact of PD on patients and their families there is a significant economic impact. Annual estimated U.S. medical costs for PD exceed 8 billion dollars, and annual non-medical costs (e.g. lost employment) are estimated to exceed 6 billion dollars (2). These costs are predicted to further rise in the next 25 years with increasing numbers of diagnosed patients and the 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, as well as the lack of effective treatments, highlight the critical need to identify new therapeutic targets.***

In addition to substantia nigra neuron loss, PD is defined by the pathologic accumulation of α-syn-containing Lewy body inclusions and neurites (3). Alpha-syn pathology correlates temporally and spatially with PD progression as it is typically limited to lower brain regions in early disease prior to its later spread to motor and cognitive centers (4, 5). While α-syn is ordinarily a soluble, natively unfolded monomer, a multi-step *in vitro* process of its folding and oligomerization produces insoluble fibrils similar to those present in PD brain (6). Moreover, α-syn mutations and replications in familial PD support its causal role for PD pathogenesis (7, 8). Although it is still unclear how α-syn regulates PD pathogenesis, ample pre-clinical evidence indicates the neurotoxic potential of α-syn (9-15). The high-capacity clearance of α-syn is regulated by the ALP (16) and its function is compromised in normal aging brain and PD brain (17-22). Alpha-syn accumulation and toxicity are exacerbated by experimental inhibition of the ALP and attenuated by ALP induction (13, 15, 19, 20, 23-29). Conversely, it has also been shown that excess α-syn inhibits the ALP (20, 30-32). Together these findings underscore a continued need to study this pathway for PD. Autophagy-inducing therapeutics have had little success in PD clinical trials (33, 34) and their known side effects argue against their chronic use (35). The combined use of autophagy inducers and lysosome inhibitors to kill cancer cells (36) suggests autophagy induction as a monotherapy for PD may be ineffective or detrimental under known conditions of lysosome dysfunction. This rationale has led us to postulate the lysosome as a primary target for promoting α-syn clearance in PD. ***In summary, a growing connection between ALP deficits and α-syn pathology justify further investigation of the lysosome, to further delineate mechanisms of α-syn pathogenesis and to accelerate the development of effective PD therapeutics.***

Studies suggest the glycosphingolipid metabolism pathway, with coordinate regulation of several lysosomal enzymes, is useful for identifying therapeutic targets for PD (37). Alpha-Gal A is a soluble lysosomal enzyme in this pathway and its mutations cause the rare lysosomal storage disorder Fabry disease (38). Fabry patients have been diagnosed with extrapyramidal symptoms of Parkinsonism (39, 40), demonstrating a putative connection between these two diseases. In addition, a polymorphism for α-Gal A has been reported in a PD patient that may compromise its gene expression (41). While it is unknown if Fabry patient brains harbor α-syn pathology, neuropathology has been documented in motor and non-motor brain regions affected by PD (42-44). Importantly, our preliminary results now establish a novel, strong link between α-Gal A deficiency and α-syn accumulation as demonstrated by the pathologic accumulation of α-syn concomitant with disruption of ALP markers in α-Gal A-deficient mouse brains (45); significantly reduced α-Gal A activity specific to postmortem PD brain specimens that also harbor the pathologic accumulation of α-syn (5, 46) ***(Fig. 1)***; and significantly reduced α-Gal A activity in lysates from M17 human neuroblastoma cells that conditionally over-express α-syn ***(Fig. 2)***. Our findings are further substantiated by the report of α-Gal A deficiency in dried blood spots collected from hundreds of PD patients (unpublished data from Dr. Roy Alcalay, Columbia Univ.; see attached letter). ***However, whether α-Gal A deficiency exacerbates the neurotoxic potential of α-syn has not been investigated, thus providing the rationale to test this hypothesis in Aim 1 of our proposal.***

Therapeutics that replace α-Gal A in Fabry disease would provide ready sources for testing the utility of α-Gal A as a therapeutic target for PD. Enzyme replacement therapy (ERT) is well tolerated by Fabry patients, though its use for PD requires further development to promote its CNS penetration (47). Chaperone therapy (e.g. migalistat) increases α-Gal activity by promoting its trafficking to lysosomes (48) and is in clinical trials for Fabry disease. (49, 50). While a preclinical study indicates its CNS bioavailability (51) migalistat is a known competitive inhibitor of α-Gal A (48), thus requiring extensive pharmacologic analyses that are beyond the scope of this proposal. Virus-mediated gene therapy is also being explored in pre-clinical models as a way to increase α-Gal A in Fabry disease (52, 53). We have now established ERT as a research tool to dose-responsively increase α-Gal A activity *in vitro* ***(Figs. 2-3)***. Importantly, our results indicate ERT accelerates the

clearance of over-expressed α-syn in M17 cells **(Fig. 3)**. We have also established a genetic approach via development of transgenic mice that over-express human *GLA* and exhibit a ~2-fold increase in brain α-Gal A activity **(Fig. 3)**. *These newly developed pre-clinical tools will be used to test our novel hypothesis in Aim 2 of our proposal that increasing α-Gal A activity attenuates α-syn-associated neurotoxicity.*

B. Innovation

Our proposal is innovative for several reasons. Our published data in α-Gal A deficient mice (45) and preliminary data from postmortem PD brain and cells that conditionally over-express α-syn **(Figs. 1-2)** identify a novel, strong link between α-Gal A deficiency and the aberrant accumulation of α-syn. In addition, our preliminary data indicate α-Gal A ERT increases the clearance of over-expressed α-Gal in neuroblastoma cells. However, whether α-Gal A activity regulates α-syn-associated neurotoxicity has not been tested. Thus our global hypothesis that α-syn-associated neurotoxicity is exacerbated by α-Gal A deficiency and is attenuated by increasing α-Gal A activity is highly innovative. In Aim 1 of our proposal we will test for the first time the potential for α-syn-induced neurotoxicity to be exacerbated in α-Gal A-deficient mice and in primary neuron cultures from these mice. In addition, we have developed innovative pre-clinical research tools to be used in Aim 2 that pharmacologically and genetically increase α-Gal A activity in neuronal model systems **(Figs. 2-3)**. Finally, adding to the innovation of our proposal is the potential to validate α-Gal A for its ability to attenuate α-syn-associated pathogenesis and disease spread in PD, as existing therapies for PD are only able to treat symptoms and not attenuate neurodegeneration (1).

C. Approach
Preliminary Results

Relationship between α-Gal A deficiency and α-syn accumulation Through a grant with the Michael J Fox Foundation and the Arizona Parkinson's disease Consortium (Dr. Thomas Beach, Director), we investigated α-Gal A in postmortem PD and its relation to α-syn pathology. Temporal cortex homogenates were prepared from control patients (stage 0), those with Incidental Lewy Body Disease (ILDB) or with different stages of idiopathic PD (IIa-IV) as defined by relative Lewy body pathology (5, 46). We observed a significant

~20% decrease in α-Gal A levels and activity **(Fig. 1c, d)** only in specimens from late stage PD with significantly increased α-syn phosphorylated at serine 129 (p129S-α-syn) **(Fig. 1b)**, a pathological species of α-syn localized to Lewy bodies of PD brain (46). These findings are corroborated by unpublished data shared to us by Dr. Roy Alcalay (Columbia Univ.; see attached letter) whose analysis of dried blood spots obtained premortem from hundreds of PD patients indicates a significant decrease in α-Gal A activity. Our observed reductions in α-Gal A activity are similar in magnitude to those observed for glucocerebrosidase (GCase) in idiopathic PD (20, 54, 55). GCase is in the same lipid metabolism pathway as α-Gal A. Large-scale epidemiologic studies have identified mutations for the human GBA1 gene that encodes GCase as PD risk factors (56, 57), and results of preclinical studies suggest the utility of GCase as a therapeutic target for PD (20, 37, 58-61). Our findings suggest that α-Gal A may be a useful therapeutic target for PD similar to GCase. In addition, we have established in our lab a model of conditional over-expression/clearance of human wild-type (WT) α-syn in M17 human neuroblastoma cells via on/off treatment with doxycycline **(DOX, Fig. 2)** as previously described (62). Significant

Fig. 1 Decrease in α-Gal A levels/activity is specific to late stage PD brain specimens with robust α-syn pathology Sucrose buffer homogenates (+1% triton X-100) were prepared from temporal cortex specimens of neurologically normal control patients (CTL, stage 0), or patients staged relative to Lewy body pathology: stage IIa PD/ILDB (limited to brainstem and olfactory bulb); stage III (sparse in temporal cortex) or stage IV PD "late stage" PD (robust in temporal cortex and throughout the brain) (5). **a)** Representative western blots for 17 kDa p129S-α-syn and α-Gal A 46 kDa "active" species (74). Data were normalized to Actin (42 kDa) and expressed as fold stage 0 CTL **(b, c)**. α-Gal A activity (nmol/mg/hr, expressed as % CTL) is significantly reduced to 81% of control (CTL) patient levels. N-acetyl-D-galactosamine (Sigma) was added to inhibit non-specific enzymatic activity as reported (48, 51). All data are expressed as mean±SD, *n*=10-12 cases/stage. *$p<0.05$, compared to all other stages, 1 way ANOVA and Bonferroni's post-hoc test.

Fig. 2 Over-expression of α-syn correlates with reduced α-Gal A activity and its clearance is enhanced by increasing α-Gal A activity (a, b) Western blot indicates conditional over-expression and clearance of human WT α-syn in M17 cell lysates following treatment with 10 µg/ml doxycycline (DOX) for 7 days, followed by DOX removal for 7 days, measured by 17 kDa species for total α-syn (BD "syn-1") and p129S-α-syn (abcam 168381). Asterisks indicate days of peak levels for each α-syn species. GAPDH (37 kDa) served as loading control. **c)** Significant decreases in α-Gal A activity (asterisks) were observed in M17 lysates on days of peak α-syn over-expression. **d)** Dose-responsive increases in α-Gal A activity (fold control) observed following 24h treatment of M17 cells with 1-10 µg/ml Fabrazyme (Fz). **e)** 48h treatment with Fz (10 µg/ml) after DOX removal significantly enhanced clearance of over-expressed total α-syn (BD syn-1). Actin (42 kDa) served as loading control. All data are expressed as mean±SD, n≥ 3 replicates for each data point. *p<0.05, 1-way ANOVA and Bonferroni's post hoc test **(b-c)** and t-test **(e)**.

decreases in α-Gal A activity were observed **(Fig. 2c)** at time points corresponding with maximal increases in α-syn species **(Fig. 2a-b)**, reinforcing the connection between α-syn accumulation and α-Gal A deficiency. Finally, we recently reported the pathological accumulation of p129S-α-syn in α-Gal A-deficient mouse hindbrain, concomitant with alterations in ALP markers (45). While these findings suggest α-Gal A regulates α-syn metabolism, whether these mice exhibit greater sensitivity to the deleterious effects of α-syn over-expression has not been investigated. **We will use α-Gal A-deficient mice and primary neuron cultures from these mice to test our novel hypothesis in Aim 1 that α-Gal A deficiency exacerbates α-syn-associated neurotoxicity.**
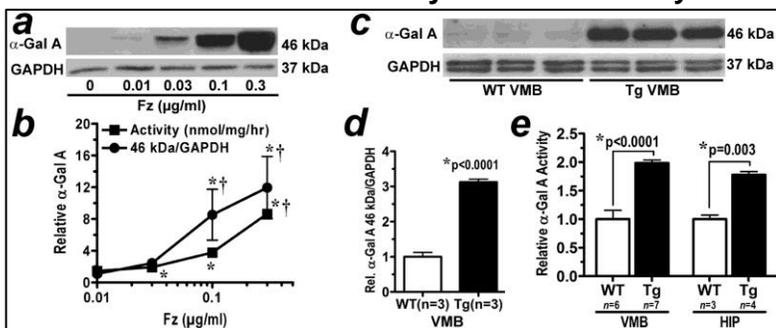
Fig. 3 Increased α-Gal A activity in mouse primary neuron cultures and mouse brain (a, b) Dose-responsive increases in α-Gal A 46 kDa "active" (74) species (western blot, normalized to GAPDH) and activity observed after 24h treatment of day in vitro 5 WT hippocampal neuron cultures with 0.01-0.3 µg/ml Fabrazyme (Fz). *p<0.05 vs. 0 µg/ml, 1-way ANOVA & Bonferroni's post hoc test; "†" signifies dose(s) of maximal increase. **c-d)** Ventral midbrain homogenates from 4-6-week-old transgenic (Tg) mice over-expressing human WT α-Gal A genomic DNA indicate a ~3-fold increase in the 46 kDa α-Gal A "active" species (normalized to GAPDH and expressed relative to WT control). **e)** α-Gal A activity in Tg brain homogenates (relative to WT) is ~2-fold higher in ventral midbrain and ~1.8-fold higher in hippocampus. *p<0.05, t-test. All data expressed as mean±SD and represent mice from two separate litters.

Increasing α-Gal A in neuronal model systems
We have now established pharmacological and genetic methods to increase α-Gal A activity in neuronal cells. We are using Fabrazyme® (Fz) ERT from Genzyme Corp. (see attached letter) as an in vitro tool to dose-responsively increase α-Gal A activity in M17 cells **(Fig 2d)** and primary neuron cultures **(Fig. 3a-b)**. Importantly, preliminary results from M17 cells indicate enhanced clearance of over-expressed α-syn following treatment with Fz **(Fig. 2e)**, suggesting α-Gal A can be targeted to promote α-syn clearance. **As a logical extension of this result we will determine in Aim 2 if increasing α-Gal A activity in primary neuron cultures dose-responsively attenuates α-syn-associated neurotoxicity.** In addition, through collaboration with Dr. Robert J. Desnick at Mt. Sinai (see attached letter) we are now breeding transgenic mice at UAB that over-express genomic DNA for human WT *GLA* and exhibit a ~two-fold increase in activity in α-Gal A homogenates from ventral midbrain and hippocampus **(Fig. 3)**. New founders were produced out of necessity as archived sperm from previous founders (63) was not viable. **These mice and primary neuron cultures from these mice will also be used in Aim 2 to test our novel hypothesis that increasing α-Gal A activity attenuates α-syn-associated neurotoxicity.**

Aim 1 Determine if α-Gal A deficiency exacerbates α-syn-associated neurotoxicity

Rationale Our published (45) and preliminary **(Figs. 1-2)** data indicate a novel relationship between α-syn accumulation and α-Gal A deficiency. However, whether α-Gal A deficiency itself potentiates α-syn neurotoxicity has not been previously tested. Our previous study of α-Gal A-deficient mice (45) did not investigate neurotoxicity resulting from α-syn over-expression, thus providing a novel and readily available
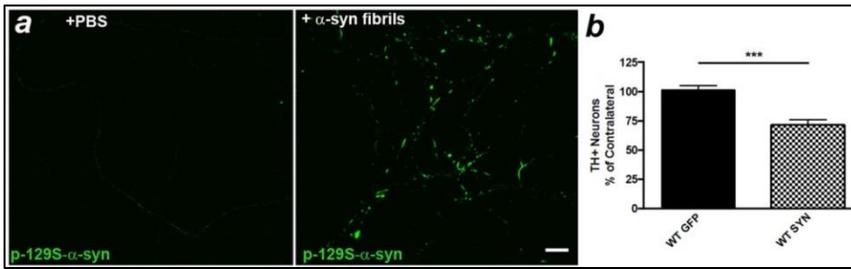
Fig. 4 Proof of principle α-syn toxicity assays a) ICC reveals increased p129S-α-syn (Abcam ab168381) in primary hippocampal cultures treated for 14 days with α-syn PFFs (right) compared to PBS vehicle control (left). Scale bars=20 µm. **b)** ~25% loss of TH-positive neurons in mouse substantia nigra observed 6 mo following infection with AAV2-WT-α-syn compared to AAV2-WT-GFP (expressed as % uninfected contralateral control) ***p<0.05, t test.

model for this Aim. We will use two models of α-syn neurotoxicity **(Fig. 4)** that are well-established by our research team and are amenable to testing in α-Gal A-deficient mice. First we will treat primary mouse neuron cultures with α-syn pre-formed fibrils (PFFs), which induce robust accumulation of endogenous α-syn and neuron loss (12, 64, 65). ALP function will also be assessed in an attempt to delineate a mechanism(s) by which α-Gal A deficiency putatively exacerbates α-syn PFF neurotoxicity. As a complementary *in vivo* approach we will quantify loss of tyrosine hydroxylase (TH)-positive neurons caused by AAV2-mediated over-expression of human wild-type α-syn in mouse substantia nigra. This model is well-established in our lab (66) and that of our UAB Collaborator, Dr. David Standaert (see letter) (66-68), who will assist in our experimental design and data interpretation. **Successful completion of this Aim will determine the potential for α-Gal A deficiency as a mechanism of α-syn pathogenesis in pre-clinical models of PD.**

Alpha-Gal A-deficient mice Alpha-Gal A deficient mice were generated previously by insertion of a *neo* cassette in exon 3 of the mouse *Gla* gene (69). They lack α-Gal A enzymatic activity but have a normal lifespan (45; 69). Mice and embryos from timed-pregnant mice will be genotyped as previously described (45).

Aim 1-Experiment 1 Determine if α-Gal A deficiency exacerbates neurotoxicity induced by α-syn pre-formed fibrils (PFF) We will use the α-syn PFF model of *in vitro* neurotoxicity established by our co-investigator Dr. Laura Volpicelli-Daley, whereby the exogenous addition of α-syn PFFs to primary hippocampal neuron cultures induces the pathological accumulation of endogenous α-syn species **(Fig. 4)** and concomitant neuron loss (12, 64). PFFs will be prepared from recombinant mouse α-syn as we have previously described (64). WT or α-Gal A-deficient primary neuron cultures will be prepared from embryonic day 16 embryos and treated with α-syn mouse PFFs at 1-5 µg/ml 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 α-syn pathology (12), including immunocytochemistry (ICC) and western blot analysis of p129S-α-syn (MJFF-Abcam ab168381; Biolegend 825701/clone m81A) or total α-syn (BD Biosciences syn-1; Santa Cruz sc-7011/clone C-20), and ICC analysis of α-syn species co-localized to ubiquitin (Sigma U0508) (12, 14, 45, 70). For ICC, cells will be fixed with 4% paraformaldehyde (PF) and 1% Triton X-100 to remove soluble proteins during fixation thus purifying for detection of insoluble α-syn species (12). Neuron loss beginning at DIV15 will be quantified by ICC detection of NeuN-positive neurons as previously reported (12, 45).

Aim 1-Experiment 2 Assessment of ALP function To determine if ALP dysfunction is a candidate mechanism by which α-Gal A deficiency putatively exacerbates α-syn PFF toxicity, we will first probe via western blot for the autophagosome marker LC3-II (Sigma L7543) and the autophagy receptor p62 (Sigma P0067). If changes in LC3-II and p62 levels are observed we will next assay for autophagic flux by western blot analysis of LC3-II following maximal inhibition of lysosome function with chloroquine or bafilomycin A1 (15, 23, 26, 29). No further change in LC3-II would suggest inhibition of autophagic flux, whereas its further increase would suggest compensatory autophagy induction (71). Autophagy induction will be assessed by western blot analysis of de-phosphorylation (stimulation) or increased phosphorylation (inhibition) of the mTOR substrate p70S6K (Thr 389; CST 9202/9205) (71).

Aim 1-Experiment 3 Determine if over-expression of α-Gal A in mice attenuates neuron loss caused by the targeted over-expression of α-syn Plasmids encoding GFP and human WT α-syn (66, 68) will be packaged in AAV serotype 2 (AAV2) by the UNC vector core (Chapel Hill, NC) at high titer (≥10^{12} vg/ml). We propose six experimental groups: naïve 1) WT and 2) α-Gal A-deficient mice to control for surgery; AAV2-GFP in 3) WT and 4) α-Gal A-deficient mice to control for AAV infection; AAV2-α-syn in 5) WT and 6) α-Gal A-deficient mice. Craniectomies will be performed on deeply anesthetized eight-week-old male mice, followed by syringe insertion into the substantia nigra and microinjection of AAV as previously described (66-68). Mice will be euthanized via trans-cardial perfusion with 4% PF under deep isoflurane anesthesia at six months following AAV-α-syn infection, a time coinciding with significant loss of TH-positive neurons **(Fig. 4)** (66, 67). Frozen, free-floating sections will be prepared spanning the entire midbrain. Every fifth section spanning the substantia

nigra will be probed for chromogenic detection of rabbit anti-TH (Pel-Freez) to assess neuron loss. Unbiased stereology will be used to quantify TH-positive neurons in the right (infected) and left (non-infected) substantia nigra according to the optical fractionator principle (72) and as previously described (66, 67).

Expected Outcomes, Potential Pitfalls and Alternative Approaches We predict α-Gal A deficiency will exacerbate and/or accelerate neuron loss and the abundance/size of endogenous α-syn species in primary neuron cultures following the exogenous addition of α-syn PFFs. We also predict that α-Gal A deficiency will exacerbate TH-positive neuron loss resulting from AAV2-mediated over-expression of human wild-type α-syn in mouse substantia nigra. These results would support α-Gal A deficiency as a mechanism of α-syn pathogenesis in PD, thus warranting future investigation of the putative reciprocal relationship between α-Gal A deficiency and the pathological accumulation of α-syn. As α-Gal A is not completely deficient in PD, logical follow-up experiments may include partial knockdown α-Gal A gene expression using a lentiviral approach. As α-Gal A deficiency in mice is X-linked (45), female heterozygous and WT mice could be tested in future studies to examine the effect of α-Gal A gene dosage on α-syn toxicity. If our *in vitro* studies indicate α-Gal A deficiency inhibits the ALP, and is further inhibited by α-syn PFFs, the ALP would be more rigorously explored as a mechanism in future studies. If α-Gal A deficiency minimally affects ALP function at baseline, yet still exacerbates α-syn neurotoxicity, we would consider investigating the ubiquitin proteasome system (UPS) as an alternative method of degrading α-syn (73). It is possible that α-Gal A deficiency may not exacerbate α-syn-associated neurotoxicity *in vitro* or *in vivo*, despite our *in vivo* findings of p129S-α-syn pathology in α-Gal A-deficient mice under basal conditions (45). However, such negative results would not preclude the potential for therapeutic benefit by increasing α-Gal A activity in studies proposed in Aim 2, in particular if increasing α-Gal A activity promotes differential neuronal survival responses to α-syn insults.

Aim 2 **Determine if increasing α-Gal A activity attenuates α-syn-associated neurotoxicity**

Rationale Our preliminary data indicate a novel relationship between α-syn accumulation and α-Gal A deficiency ***(Figs. 1-2)*** and suggest that increasing α-Gal A activity enhances α-syn clearance ***(Fig. 2)***. However, whether α-Gal A can be targeted to attenuate the neurotoxic potential of α-syn has not been investigated. We will critically assess the potential for increasing α-Gal A activity in attenuating *in vitro* neurotoxicity mediated by α-syn PFFs and TH-positive neuron loss *in vivo* resulting from AAV2-mediated over-expression of human wild-type α-syn. In this aim we will also selectively stimulate and inhibit the ALP *in vitro* to determine its role in regulating the putative protection afforded by increasing α-Gal A activity. **Successful completion of this Aim will critically evaluate the utility of α-Gal A as a therapeutic target for PD.**

Experimental methods to increase α-Gal A activity We have validated two distinct methods for increasing α-Gal A activity in neuronal model systems. Our "pharmacologic" approach involves treatment of WT primary neuron cultures with Fz ERT, which increases in α-Gal A levels and activity over a range of doses ***(Figs. 2-3)***. Our "genetic" approach involves use of transgenic mice that over-express genomic DNA for human α-Gal A obtained from our Collaborator, Dr. Robert Desnick (see letter). We are actively breeding these mice in our colony, which exhibit a ~2-fold increase in α-Gal A activity in mouse ventral midbrain and hippocampus homogenates ***(Fig. 3)***, levels that could be reasonably achieved by future α-Gal A therapeutics. Prior to assessment of α-syn neurotoxicity we will confirm that α-Gal A transgenic primary neuron cultures exhibit significantly increased α-Gal A activity as observed in brain homogenates. Mice will be genotyped as previously described (63). Increased α-Gal A activity will be confirmed by activity assay ***(Figs. 1-3)*** (48, 51), and corroborated by western blot to detect α-Gal A 46 kDa "active" species (Gene Tex GTX101178) (74). The lysosomal 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) as previously described (28).

Aim 2-Experiment 1 **Determine if increasing α-Gal A attenuates neurotoxicity induced by α-syn pre-formed fibrils (PFF)** We will use the α-syn PFF model of *in vitro* neurotoxicity (see Aim 1-Experiment 1 for details). Increased α-Gal A activity will be achieved either by using α-Gal A transgenic vs. WT primary neuron cultures or WT neuron cultures treated either prior to, simultaneously with or subsequent to the exogenous addition of α-syn PFFs. Neurons will be harvested at incremental time points from DIV5-19 and assessed for α-syn pathology and neuron loss as described in Aim 1-Experiment 1.

Aim 2-Experiment 2 **Determine if the putative protection afforded by increasing α-Gal A activity is ALP-dependent.** Using the above *in vitro* α-syn PFF model, we will assess the effects of increasing α-Gal A activity in response to selective inhibition of macroautophagy induction by lentiviral knockdown of the genes *Atg7* or *Becn1* **(Fig. 5)** or by treatment with the class III PI3-kinase inhibitor 3-methyl adenine (26), in the presence vs. absence of increased α-Gal A activity. ALP completion will be inhibited by treatment with the lysosome inhibitors chloroquine or bafilomycin A1 (15, 23, 26, 29). We will also determine if inducing macroautophagy by
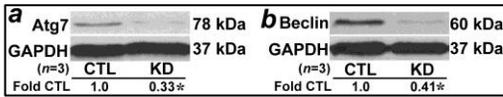
Fig. 5 Western blot of hippocampal neuron lysates treated on DIV5 for 24h with lentivirus (M0I=5, Dharmacon) for empty vector CTL or shRNA (KD) for **a)** Atg7 (CST 8558) or **b)** Beclin-1 (CST 3738). Fold CTL (*n*=3, relative to GAPDH) is quantified for Atg7 and Beclin-1 KD. *p<0.05, t-test.

treatment with rapamycin or trehalose (24) offers additive or synergystic protection in combination with increased α-Gal A activity.

***Aim 2-Experiment 3** Determine if increased α-Gal A activity in mice attenuates neuron loss caused by the targeted over-expression of α-syn* Using methods described in Aim 1-Experiment 3 and as previously described (66-68), we propose six experimental groups of eight-week old mice: naïve 1) WT and 2) α-Gal A transgenic mice to control for surgery; AAV2-GFP in 3) WT and 4) α-Gal A transgenic mice to control for AAV infection; AAV2-α-syn in 5) WT and 6) α-Gal A transgenic mice. Mice will be euthanized six months after AAV infection and processed for quantification of TH-positive neurons in substantia nigra sections.

Expected Outcomes, Potential Pitfalls and Alternative Approaches We predict that increasing α-Gal A activity in primary neuron cultures will delay or attenuate neuron loss and the abundance/size of endogenous α-syn species following the exogenous addition of α-syn PFFs. If neuroprotection is observed when Fz is added after α-syn fibrils, this could suggest the utility of increasing α-Gal A activity for management of PD progression. We also predict that the putative protective effects of increasing α-Gal A are ALP-dependent, as would be demonstrated by loss of function upon inhibition of macroautophagy and gain of function upon induction of macroautophagy. These results would justify the future study of the ALP as a candidate mechanism of α-Gal A neuroprotection, in particular if in Aim 1 ALP dysfunction is observed by α-Gal A deficiency and is further exacerbated by α-syn PFFs. If α-Gal A protection appears to be regulated independent of macroautophagy we would consider studying alternative methods of degrading α-syn including chaperone mediated autophagy and the UPS (16, 73). We also predict that transgenic over-expression of α-Gal A will attenuate TH-positive neuron loss in the mouse substantia nigra caused by AAV2-mediated over-expression of human WT α-syn. Together with promising *in vitro* findings, this would support our hypothesis that increasing α-Gal A attenuates α-syn neurotoxicity in pre-clinical models of PD and would justify the future development of therapeutics for PD that increase CNS α-Gal A activity. It is possible that increasing α-Gal A may not attenuate α-syn-associated neurotoxicity *in vitro* or *in vivo*, or despite a lack of published evidence indicating its deleterious effects on the CNS (53, 63) may even exacerbate neurotoxicity. Such results would dampen enthusiasm for α-Gal A as a therapeutic target for PD. If, however, α-Gal A-mediated protection is limited to ERT, this could be explained by differential responses of neurons to acute (Fz) vs. constitutive (transgenic over-expression) increases in α-Gal A activity, or by protection limited to higher doses, with increases in activity not attainable in these transgenic mice. Such results would argue as an alternative approach the future development of conditional α-Gal A over-expressing mice or transgenic mice that constitutively express higher levels of α-Gal A activity, for their subsequent examination of α-syn neurotoxicity.

Statistics for Aims 1-2 Values from replicates within a single *in vitro* experiment will be averaged and expressed as percent vehicle (Fz) or WT (α-Gal A deficiency or over-expression) for any given time point/treatment. For western blot analysis, protein band intensities will be expressed relative to actin loading control. For all experiments, descriptive statistics will be reported as mean ± SD. Differences will be tested for significance using Student's paired t-test (one comparison between two groups), one-factor ANOVA (one comparison between multiple groups) or two-factor ANOVA (e.g. treatment vs. genotype interactions between groups), with post hoc analysis performed using Bonferroni's test (significance set *a priori* at $p < 0.05$). ICC co-localization analyses will be performed using the Manders-Costes threshold analysis as described previously (45). Based on our published reports, our power analysis predicts a need for at least six independent experiments for *in vitro* α-syn PFF assays (12), and 12 mice/group for *in vivo* AAV2-α-syn experiments to demonstrate significant loss of TH-positive neurons and significant recovery of this loss (66, 67).

Timetable Aim 1: *in vitro* α-syn PFF studies will be completed during Year 1 and *in vivo* AAV studies will be started in Year 1 and finished in Year 2. **Aim 2:** *in vitro* α-syn PFF studies will be completed during Year 2 and *in vivo* AAV studies will be started in Year 1 and finished in Year 2.

Summary This study will critically test our novel hypothesis that α-syn-associated neurotoxicity is exacerbated by α-Gal A deficiency and is attenuated by increasing α-Gal A activity. If our hypothesis is correct, it would warrant further study of α-Gal A deficiency as a candidate mechanism of α-syn pathogenesis and would justify the development of PD therapeutics that increase CNS α-Gal A activity. A successful outcome would also provide a strong rationale for future large-scale epidemiologic studies that address the potential association of α-Gal A mutations in PD patients, and of Parkinsonism in Fabry disease patients and their relatives.

SUMMARY STATEMENT

Application Number: **1 R21 NS093435-01A1**

Principal Investigator

SHACKA, JOHN J

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

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

Meeting Date:	**02/25/2016**	*RFA/PA:*	**PA13-303**
Council:	**MAY 2016**	*PCC:*	**SUTHEMND**
Requested Start:	**07/01/2016**		

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

SRG Action: **Impact Score:16   Percentile:2 +**
Next Steps: **Visit 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**
Clinical Research - not NIH-defined Phase III Trial

Project Year	Direct Costs Requested	Estimated Total Cost
1		
2		
TOTAL		

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.

1R21NS093435-01A1 SHACKA, JOHN

RESUME AND SUMMARY OF DISCUSSION: This is a resubmission of an R21 application proposing to investigate the mechanism by which Alpha-Galactosidase A (α-Gal A), a lysosomal enzyme of the glycosphingolipid metabolism pathway, may enhance autophagic clearance of alpha-synuclein (α-syn), in the context of Parkinson's disease (PD) pathogenesis. Discussion was brief, primarily focusing on the several strengths of the application. These included the high significance of this area of investigation and its high relevance to PD pathology given the potential of α-Gal A emerging as a target for therapeutic intervention, the multiple model system, convincing responses to previous review, and overall the appealing proof-of-concept nature of the application, which was deemed particularly appropriate for the R21 program. Weaknesses were minor if not negligible and included a concern about the timing of toxicity. A very strong support emerged from the discussion for this now outstanding to exceptional application.

DESCRIPTION (provided by applicant): The pathological accumulation of alpha-synuclein (α-syn) is believed to play a major role in Parkinson's disease (PD) pathogenesis. The autophagy-lysosome pathway (ALP) provides for the high-capacity clearance of α-syn and its dysfunction is well-documented in PD. Inhibiting the ALP has been shown to induce α-syn accumulation. Conversely, excess α-syn has been shown to inhibit the ALP. Because the lysosome is critical for α-syn clearance we believe its continued investigation will further delineate mechanisms of PD pathogenesis and foster development of PD therapeutics. Alpha-Galactosidase A (α-Gal A) is a soluble lysosomal enzyme, with mutations causing the rare lysosomal disorder Fabry disease. While it is unknown if α- syn accumulates in Fabry patients, our analysis of postmortem PD brains indicates a decrease in α-Gal A activity specific to specimens with increased α-syn pathology. Our preliminary data also indicate reduced α-Gal A activity in neuroblastoma cells following the conditional over-expression of α-syn. Together with our report of α-syn pathology and altered ALP markers in α-Gal A-deficient mouse brain, these findings suggest a strong link between α-Gal A deficiency and α-syn accumulation. However, whether α-Gal A deficiency exacerbates the neurotoxic potential of α-syn is unknown. Increasing α-Gal A activity via enzyme replacement therapy (ERT) is clinically approved therapy for Fabry disease. Because ERT has limited CNS bioavailability, there is a critical gap in understanding its potential for treating PD. To help bridge this gap we developed novel research tools to increase α-Gal A activity in neuronal systems, including its dose-responsive increase in neuronal cells via ERT, and transgenic mice that exhibit two-fold increases in α-Gal A brain activity. Our preliminary data in neuroblastoma cells shows that α-Gal A ERT enhances the clearance of over-expressed α-syn. However, whether increasing α-Gal A activity attenuates α-syn-associated neurotoxicity has not been tested. Taken together, we hypothesize that α-syn-associated neurotoxicity is exacerbated by α-Gal A deficiency and is attenuated by increasing α-Gal A activity. In Aim 1 we will determine if α-Gal A-deficiency in primary neuron cultures exacerbates neurotoxicity resulting from the exogenous addition of α-syn pre-formed fibrils (PFFs) in a manner concomitant with ALP disruption. We will also determine if α-Gal A–deficient mice exhibit exacerbated loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra following AAV2-mediated over- expression of human wild-type α-syn. In Aim 2 we will determine if α-syn PFF-mediated neurotoxicity in primary neuron cultures is attenuated by α-Gal A ERT or the transgenic over-expression of α-Gal A and if this protection is regulated by the ALP. We will also determine if α-Gal A over-expressing mice exhibit a reduction in TH-positive neuron loss resulting from AAV2-α-syn. If our hypothesis is correct, it would suggest that α-Gal A deficiency regulates α-syn pathogenesis, a mechanism worthy of future investigation, and would accelerate the development of therapeutics for PD that act by increasing CNS α-Gal A activity.

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 the lysosomal enzyme alpha- Galactosidase A regulates the neurotoxic potential of alpha-synuclein, a protein that accumulates in PD brain and may contribute to disease spread. Successful

completion of this study will justify a heightened focus on alpha-Galactosidase A for its contribution to PD pathogenesis and for targeting this enzyme as a therapeutic.

CRITIQUE 1

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

Overall Impact: This is a revised application from an established investigator seeking to examine the relationship between alpha-Gal A activity and the metabolism of alpha-synuclein. This application is based on evidence from this investigator (and a colleague/expert in the field) taken from postmortem brain/tissue suggesting decreased alpha-Gal A activity in PD patients. Here, the investigator will examine the lysosomal consequences of reduced alpha-Gal A activity, ask whether augmented alpha-Gal A activity can reduce the toxicity of synuclein and if so, if this rescue is dependent on autophagy. The hypotheses are novel and innovative, and most of the methods are appropriate. There is an excellent team assembled for this project.

Significance:
Strengths
- Emerging evidence suggests a genetic and pathologic link between lysosomal enzymes, alpha-synuclein metabolism, and PD.
- This application seeks to identify means to improve alpha-synuclein metabolism.

Weaknesses
- The application could be better aimed to understand the actual mechanisms underlying synuclein accumulation, lysosomal deficiency, and alpha-Gal A activity.
- Although the data from patients are supportive, the application and/or preliminary data could have better addressed whether synuclein affects alpha-Gal A or vice versa (and not likely both, although that possibility is entertained).

Investigator(s):
Strengths
- Outstanding team assembled.

Innovation:
Strengths
- The use of genetic and enzyme replacement strategies are innovative.
- Novel target and hypotheses.

Approach:
Strengths
- Solid panel of reagents and appropriate use of models.

Weaknesses
- Most of the application is descriptive in nature as the direct influence of altered alpha-Gal A activity on lysosome function is not interrogated.
- Effort in Aim 2 to silence ATG7 and Beclin1, or treat 3-MA, really only addresses the autophagy related turnover of synuclein, but not adequately the lysosomal degradation as a whole and therefore does not mechanistically address how one lysosomal enzyme a-Gal A, effects protease activity within the lysosome, which persists as a major unaddressed question.

Lysosome morphology, pH, synuclein protease activities and stability are likely more important and not studied.

Environment:
Strengths
- Uniquely supportive of proposed studies.

Protections for Human Subjects:
Not Applicable (No Human Subjects)

Vertebrate Animals:
YES, all five points addressed

Biohazards:
Not Applicable (No Biohazards)

Resubmission:
- Responsive to previous review

Resource Sharing Plans:
Acceptable

Budget and Period of Support:
Recommend as Requested

CRITIQUE 2

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

Overall Impact: This is a resubmission of an R21 to study the potential therapeutic value of manipulating a-Gal in protecting primary neuron and mouse models of a-Syn-mediated neurodegeneration. There was already significant support for the initial submission and the current submission is even stronger with more physiological levels of a-Gal to be tested and elimination of SH-SY5Y studies in favor of primary neurons. If this exploratory work continues to prove positive, this could open the field to new mechanisms and therapies for PD.

Significance:
Strengths
- As lysosomal dysfunction and aSyn accumulation are implicated in familial and sporadic PD, determining whether lysosome based treatments that are already in clinical use can confer protection would be highly significant.
- The investigator provide additional information to further strengthen the link between reduced a-Gal function and Parkinson's disease patients. Even though there is no as yet studied genetic association, preliminary data indicate that a-Gal may interrupt part of the pathogenic pathway and this would represent a potential high yield gain ideal for an exploratory application.

Investigator(s):

Strengths
- Strong team of experts in lysosomal pathology (Shacka), PFF toxicity (Volpicelli-Daley), and AAV model of a-syn toxicity (Standaert).

Innovation:
Strengths
- Leveraging existing a-Gal therapies to correct lysosomal deficiencies in PD.

Approach:
Strengths
- Major improvements in testing more physiologically achievable levels of a-Gal have been developed, to include both Fabrazyme titrations and a new Tg line with 2-fold overexpression.
- The tumor cell work has been dropped and the studies are now focused on coordinated in vivo and primary neuron work.
- The two questions to be addressed: whether a-Gal deficiency as observed in postmortem PD brains and in blood of sporadic PD patients exacerbates aSyn toxicity and whether enhanced a-Gal confers protection in culture and AAV models of synucleinopathy.

Environment:
- Outstanding

Protections for Human Subjects:
Not Applicable (No Human Subjects)

Vertebrate Animals:
YES, all five points addressed

Resource Sharing Plans:
Acceptable

Budget and Period of Support:
Recommend as Requested

CRITIQUE 3

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

Overall Impact: This resubmitted application seeks to elucidate the role of alpha-galactose A (α-Gal A), a lysosomal enzyme, in the autophagic clearance of alpha-synuclein (α-syn). The preliminary data suggest that α-Gal A may contribute to α-syn aggregate clearance. This application employs primary neurons and mouse models in which α-Gal A levels are increased or decreased to address the question of how α-Gal A enzyme activity impacts α-syn clearance. The application has been significantly revised with the addition of data showing decreased activity of α-Gal A in the blood of patients with sporadic Parkinson's disease. A transgenic mouse expressing lower, more physiologically relevant levels of α-Gal A overexpression (2-fold) has also been added. The inclusion of α-Gal A deficient mice and primary neurons is likely to elucidate the role of this enzyme in α-syn clearance. The research team has complementary expertise in α-syn seeding models and autophagic clearance of

proteins and is well-qualified to conduct the proposed research. The team is likely to produce meaningful results on the role of α-Gal A in Parkinson's disease. Findings have the potential to impact treatment strategies for synucleinopathies.

Significance:
Strengths
- Clearance of α-syn aggregates through autophagic pathways is well-established. This research focuses on the level and function of a specific enzyme, α-Gal A, which may be dysregulated in PD patients, compromising the clearance of α-syn.
- If the hypothesis is correct, findings would have implications for PD therapeutic strategies.
- The multitude of experiments planned using physiologically relevant models is likely to answer the question of whether α-Gal A plays a major role in degrading α-syn.
- The role of specific mediators of protein aggregate clearance by autophagy are poorly characterized for most protein-misfolding disorders, and thus findings may be relevant for other diseases in addition to synucleinopathies..

Investigator(s):
Strengths
- The principal investigator is an expert in autophagy and is well-suited to conduct these experiments. All necessary tools are established in his laboratory.
- Co-investigator (Volpicelli-Daley) has developed the model for the induction of α-syn inclusions in cell culture or in the brain of mice using preformed α-syn fibrils and adds expertise in PD.

Innovation:
Strengths
- The hypothesis that α-Gal A has a key role in PD is conceptually innovative.

Approach:
Strengths
- The experiments are thoughtfully considered and the application is well-written.
- The assessment of patient samples from collaborator (Alcalay) provides a necessary comparison to human disease.
- The inclusion of α-Gal A deficient mice and primary neurons is an important addition to the revised application.
- The use of AAV to overexpress α-syn is also likely to help define the role of α-Gal A in the clearance of endogenous α-syn.

Weaknesses
- The mechanism underlying the decrease in α-Gal A in late stage disease is unclear, and it is possible that the decrease of α-Gal A and other lysosomal enzymes occurs in the end stage of many neurologic diseases. Measurements of additional lysosomal enzyme levels and assessing α-Gal A levels in patients with other neurologic diseases would aid in the interpretation of the findings.

Environment:
Strengths
- The research environment, facilities, and collaborators at the University of Alabama at Birmingham are outstanding.

Protections for Human Subjects:
Not Applicable (No Human Subjects)

Vertebrate Animals:

YES, all five points addressed

Biohazards:
Not Applicable (No Biohazards)

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 REVIEWERS' WRITTEN CRITIQUES, ON THE FOLLOWING ISSUES:

VERTEBRATE ANIMAL (Resume): The use of vertebrate animals is appropriately justified and scientifically acceptable.

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

Footnotes for 1 R21 NS093435-01A1; PI Name: SHACKA, JOHN J

+ 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.

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

CMND
02/25/2016 - 02/26/2016

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