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# Investigating the Regulation of Alpha-Synuclein Clearance by Alpha-Galactosidase-A in Parkinson Disease

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## ABSTRACT

Parkinson Disease (PD), the second most common neurodegenerative disease, affects 1% of Americans over the age of 65. PD pathology is characterized by dopaminergic neuron loss and accumulation of insoluble alpha-synuclein ( $\alpha$ syn) aggregates. Pre-clinical studies suggest that the aberrant accumulation  $\alpha$ syn contributes to PD pathogenesis. The autophagy lysosomal pathway (ALP) is a metabolism pathway capable of high capacity clearance of  $\alpha$ syn. Thus targeting the clearance of  $\alpha$ syn, in particular by enhancing ALP function, could be a valuable treatment option for PD. We have shown previously that brains of mice deficient in the lysosomal enzyme alpha-Galactosidase A ( $\alpha$ GalA) exhibit the pathological accumulation of  $\alpha$ syn concomitant with ALP dysfunction. **These findings led us to hypothesize that increasing  $\alpha$ GalA activity would enhance  $\alpha$ syn clearance.** To test this hypothesis, we examined if increasing  $\alpha$ GalA activity using recombinant  $\alpha$ GalA (Fabrazyme, Genzyme Corp.) accelerates the clearance of conditionally over-expressed  $\alpha$ syn in M17 human neuroblastoma cells. Fabrazyme treatment increased the level and activity of  $\alpha$ GalA in M17 cells and promoted the clearance of over-expressed  $\alpha$ syn. These data suggest the utility of  $\alpha$ GalA activity as a therapeutic target for promoting the clearance of  $\alpha$ syn in a preclinical model of PD.

Keywords: Alpha-Galactosidase A, Alpha-Synuclein, Parkinson Disease, Autophagy Lysosomal Pathway

## INTRODUCTION

Parkinson Disease (PD), the second most common neurodegenerative disease, affects 1% of Americans over 65 and this number is expected to rise<sup>1</sup>. This disease not only affects patients and their families, but also has a profound economic impact. Expenditures on PD medical care in the U.S. exceed 8 billion dollars annually; additional economic impact comes from lost employment and other non-medical factors associated with the disease<sup>1</sup>. PD is characterized by classic motor symptoms such as resting tremor, rigidity, and bradykinesia that result from the progressive degeneration of dopaminergic neurons in the substantia nigra. PD is also characterized by non-motor symptoms such as depression, personality changes, and cognitive impairment<sup>2</sup>. Approved therapies for PD treat symptoms but do not prevent or delay disease progression. Faced with these challenges, the

discovery of novel therapeutic targets is critical for the future treatment of PD.

PD is a member of the class of disorders called synucleinopathies, characterized by the inclusion of accumulated insoluble  $\alpha$ syn aggregates known as Lewy bodies<sup>3</sup>. The function of  $\alpha$ syn is not well known, but it is believed to play a role in regulating vesicular neurotransmitter release<sup>4</sup>. Normally,  $\alpha$ syn is a soluble monomer, but *in vitro* studies show it is able to undergo a process of folding and oligomerization, yielding insoluble fibrils similar to what are seen in PD brain<sup>5</sup>. The aberrant accumulation of monomeric  $\alpha$ syn is believed to promote its misfolding into insoluble species, the accumulation of which is evident in synucleinopathies like PD. Studies of mutations in  $\alpha$ syn genes, such as SNCA triplication, in familial PD provide support for the pathogenic role of  $\alpha$ syn in PD<sup>6</sup>. Further evidence supporting a role for  $\alpha$ syn in PD pathogenesis is supported by the Unified Lewy Body Staging system, in which the incidence of  $\alpha$ syn-containing Lewy bodies progressing from lower to higher brain regions correlates with the progression of PD symptoms<sup>2</sup>. While the mechanisms by which  $\alpha$ syn regulates PD pathogenesis are poorly understood, its neurotoxic potential has been shown in numerous *in vitro* and *in vivo* studies<sup>7-11</sup>. Therefore, therapeutics aimed at promoting the high-capacity clearance of  $\alpha$ syn, could be valuable as a method to reduce  $\alpha$ syn pathology in PD<sup>12</sup>.

The ALP is an intracellular degradation pathway that maintains energy balance and organellar quality control and is responsible for the high capacity clearance of  $\alpha$ syn<sup>13</sup>. Notably, function of the ALP has been shown to decline with normal brain aging and is disrupted in PD brain<sup>14-19</sup>. Experimental inhibition of the ALP has been shown to increase  $\alpha$ syn accumulation while its experimental induction has been shown to attenuate  $\alpha$ syn accumulation<sup>11,19-26</sup>. Together these studies suggest that ALP dysfunction may contribute to  $\alpha$ syn-associated pathogenesis in PD and that the ALP could be targeted in the development of PD therapeutics.

The glycosphingolipid metabolism pathway involves the action of several lysosomal enzymes and has been shown previously to be disrupted in PD<sup>17,27</sup>. Alpha Galactosidase A ( $\alpha$ GalA) is a soluble lysosomal enzyme in this pathway. Its deficiency, caused by many different mutations in the human GLA gene that encode  $\alpha$ GalA, results in Fabry disease, a

lipidosis type of lysosomal storage disorder<sup>28</sup>. Our lab has shown that brains of mice deficient in  $\alpha$ GalA exhibit the pathologic accumulation of  $\alpha$ syn concomitant with alterations to ALP markers suggesting that  $\alpha$ GalA regulates  $\alpha$ syn metabolism, potentially through disruption of ALP function<sup>20</sup>. These findings led us to propose  $\alpha$ GalA as a candidate therapeutic target for promoting the effective clearance of  $\alpha$ syn.

In the present study, we tested whether increasing  $\alpha$ GalA activity in a neuronal cell line increases the clearance of over-expressed  $\alpha$ syn, to determine if  $\alpha$ GalA is a useful therapeutic target for PD. Using this model system, we evaluated an *in vitro* cell model of the PD synuclein pathology disease state. We also investigated whether increasing  $\alpha$ GalA activity promotes the clearance of over-expressed  $\alpha$ syn.

## MATERIALS AND METHODS

### Cell culture maintenance and lysate collection

We obtained an M17 cell line with conditional tetracycline inducible (tet-on) expression of the gene encoding human wild type  $\alpha$ syn as a generous gift from Dr. Talene Yacoubian (UAB Dept. of Neurology) and as previously reported<sup>29</sup>. M17 human neuroblastoma cells were maintained in MEM media with 10% fetal bovine serum and 500  $\mu$ g ml<sup>-1</sup> G418 (for selection pressure) and allowed to grow between passages to 70–80% confluency. Cells were treated with the tetracycline derivative, Doxycycline (Dox; 10  $\mu$ g ml<sup>-1</sup>) for up to 7 days to drive the conditional over-expression of  $\alpha$ syn. To assess clearance of over-expressed  $\alpha$ syn, Dox was removed from the media and the cells were allowed to continue to grow for up to 7 days in its absence.

### $\alpha$ GalA increase by Fabrazyme

Our lab has recently obtained the pharmaceutical Fabrazyme (Fz; Genzyme Corp.), a type of recombinant enzyme replacement therapy (ERT) that is approved for human use in the treatment of Fabry disease<sup>30</sup>. Fz was administered in culture media at concentrations ranging from 1–100  $\mu$ g ml<sup>-1</sup> for 24–72 hours. Following Fz treatment, culture media was removed and cells were washed with 1X PBS to remove extracellular Fz. Whole cell lysates were then prepared in a 25 mM HEPES lysis buffer containing 1% SDS, 1% Triton X-100, 1% protease inhibitor cocktail (Sigma P8340), and 1% each of two different phosphatase inhibitors (Sigma P5726 and P0044).

### Detection of $\alpha$ GalA activity

Whole cell lysates were assessed for  $\alpha$ GalA activity using a fluorometric activity assay as previously described<sup>31</sup>. Active  $\alpha$ GalA enzyme cleaves the assay substrate, 4-Methylumbelliferone  $\alpha$ -D-galactopyranoside (Sigma M7633) to 4-Methylumbelliferone (Sigma M1381). N-acetyl-D-galactosamine (Sigma A2795) was used to inhibit the non-specific activity of  $\alpha$ Galactosidase B

(N-acetyl- $\alpha$ -D-Galactosaminidase), which also cleaves the 4-Methylumbelliferone  $\alpha$ -D-galactopyranoside substrate<sup>32</sup>. Fluorescence of the cleavage product 4-Methylumbelliferone was measured at 460 nm. Activity is reported as units of nmol converted substrate/mg protein in sample/hour. Proteins were quantified using the bicinchoninic acid (BCA) protein assay as previously reported<sup>25-26</sup>.

### Detection of $\alpha$ GalA and $\alpha$ syn protein levels

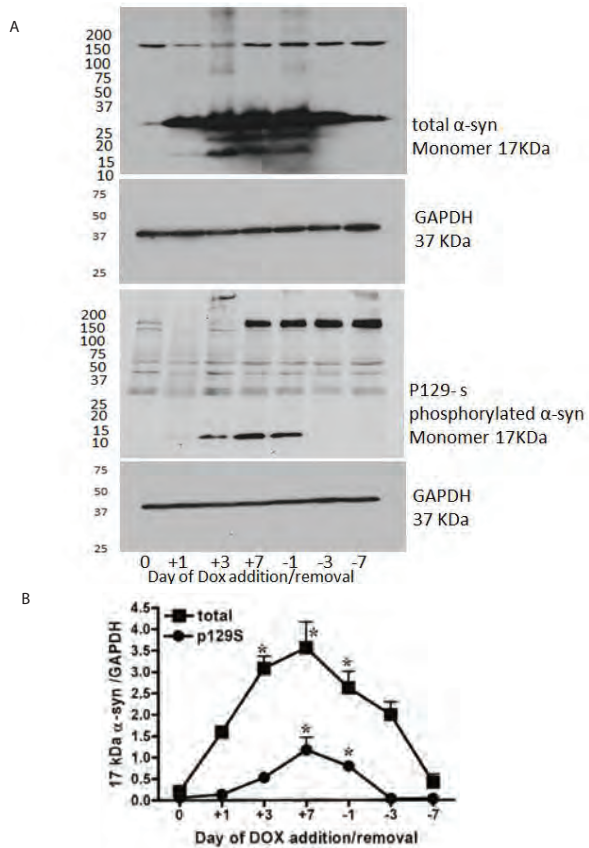
Western blot of whole cell lysates was used to detect the levels of proteins of interest using methods as previously described<sup>23,25-26</sup>. Antibodies used include Santa Cruz sc-7011C-20 clone and BD Biosciences 610787 to detect total  $\alpha$ syn monomer (17 kDa), Abcam ab168381 to detect  $\alpha$ syn phosphorylated at serine 129 (P129S- $\alpha$ syn) (17 kDa)<sup>33</sup>, and Genetex GTX101178 N1C2 to detect  $\alpha$ GalA active species (46 kDa)<sup>34</sup>. Side by side comparisons of Santa Cruz and BD Biosciences antibodies for total  $\alpha$ syn indicated no difference in detection of 17 kDa monomer (data not shown). Gels were run with 20–25  $\mu$ g protein per lane with equal loading across each gel using an N of 3–4 per data point. Blots were probed for antibodies of interest then stripped using Restore™ Western Blot Stripping Buffer (Thermo Scientific 21059) and re-probed for either actin (Sigma 1978; 42 kDa) or GAPDH (Cell Signaling 2118; 37 kDa) loading controls. Films were scanned and quantified using UN-SCAN-IT™ software (Orem, UT).

## STATISTICS

Statistical analyses were completed using either Student's t-test for comparisons between two treatment groups, or one-way analysis of variance (ANOVA) for comparisons between multiple treatment groups. Post hoc analysis was performed using Bonferroni's test with significance set *a priori* at  $p < 0.05$ .

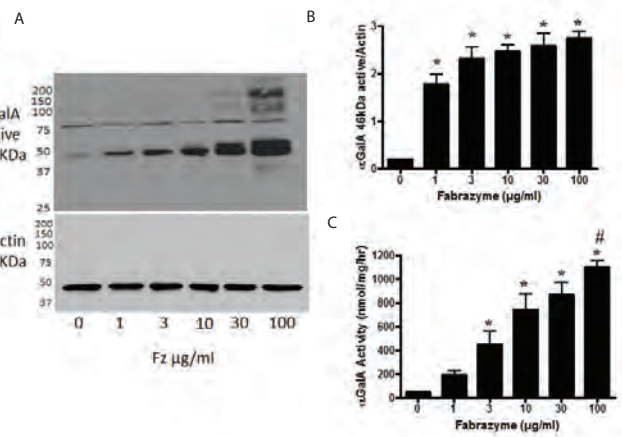
## RESULTS

First, we established a working *in vitro* model of regulated, conditional over-expression of  $\alpha$ syn in which we could monitor its clearance over time. We chose the M17 human neuroblastoma cell line, an immortalized cell line with neuronal processes, commonly accepted as an *in vitro* model for neurons<sup>29</sup>. Treatment with Dox significantly increased levels of total  $\alpha$ syn 17 kDa monomer after 3–7 days (Day 3; Day 7) (Fig. 1). We also probed for P129S- $\alpha$ syn (17 kDa), a pathologic species of  $\alpha$ syn known to accumulate in PD brain and localize to Lewy bodies, and found that it was significantly increased after 7 days of Dox treatment (Fig. 1). The increases in both species were still present at 1 day (Day -1) after Dox removal (Fig. 1). Between 1 and 3 days after Dox removal (Day -1; Day -3) the levels of both total and phosphorylated  $\alpha$ syn began to decline, and by 7 days after Dox removal (Day -7) they returned to approximately basal levels (Fig. 1).

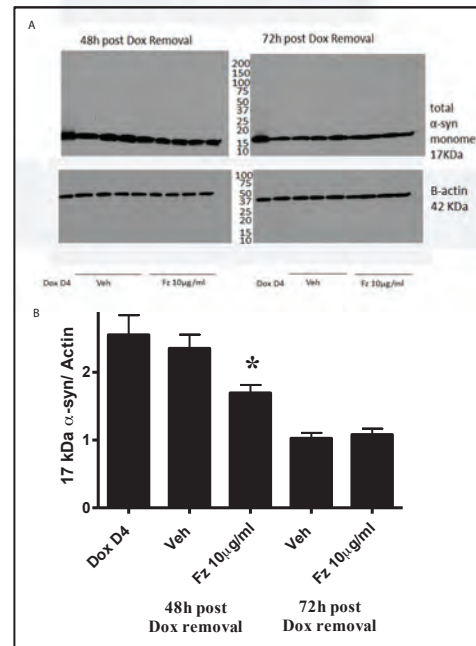


**Fig 1 | Relationship of  $\alpha$ GalA activity and  $\alpha$ syn protein quantity.** (a, b) Western blot indicates conditional over-expression and clearance of human WT  $\alpha$ syn in M17 cell lysates following treatment with  $10 \mu\text{g ml}^{-1}$  doxycycline (DOX) for 7 days, followed by DOX removal for 7 days, measured by 17 kDa species for total  $\alpha$ syn (BD "syn-1") and p129S- $\alpha$ syn. Asterisks indicate days of peak levels for each  $\alpha$ syn species. GAPDH (37 kDa) served as loading control. All data are expressed as mean  $\pm$  SD,  $n = 3$  replicates for each data point. \* $p < 0.05$ , 1-way ANOVA and Bonferroni's post hoc test.

After establishing the M17 cell model of transient, over-expression of  $\alpha$ syn, we treated cells with Fz to confirm the pharmacological increase of  $\alpha$ GalA *in vitro*. We first performed a Fz dose-response for  $\alpha$ GalA levels and activity following its addition to M17 cell culture media for 24 hr. Treatment of M17 cells with Fz significantly increased  $\alpha$ GalA levels and activity (Fig. 2). Concentrations of Fz ranging from 1–100  $\mu\text{g ml}^{-1}$  significantly increased levels of protein, as measured by western blot analysis (Fig. 2B) and activity as measured by fluorimetric activity assay (Fig. 2C) compared to cells treated with vehicle control. The increase in activity was concentration dependent with significant increases observed with concentrations ranging from 3–100  $\mu\text{g ml}^{-1}$ , and the greatest increase in activity observed at 100  $\mu\text{g ml}^{-1}$  (Fig. 2C).



**Fig 2 | Fabrazyme increases  $\alpha$ GalA.** (a) Western blot analysis of whole cell lysates shows an increase of  $\alpha$ GalA 46 kDa active species in M17 cells following administration of Fabrazyme (Fz) at 1, 3, 10, 30, or 100  $\mu\text{g ml}^{-1}$  compared to vehicle control. (b) The 46 kDa active species was quantified relative to actin loading control. The assay for  $\alpha$ GalA enzymatic activity (nm/mg/hr, expressed as % CTL was increased in  $\alpha$ GalA (c) following Fz administration. All data are expressed as mean  $\pm$  SD,  $n = 4$  replicates for each data point. \* $p < 0.05$ , 1-way ANOVA and Bonferroni's post hoc test (difference from control, 0). # maximal increase in activity.



**Fig 3: Increasing  $\alpha$ GalA activity increases clearance of over-expressed  $\alpha$ syn.** (a) Western blot analysis of  $\alpha$ syn in M17 whole cell lysates shows increased clearance of  $\alpha$ syn 17 kDa monomer after 48 and 72 hours of Dox induced over-expressed with administration of Fz ( $10 \mu\text{g ml}^{-1}$ ) for 48 and 72 hours at the removal of Dox compared to vehicle control. (b) Monomer was quantified relative to actin loading control. All data are expressed as mean  $\pm$  SD,  $n = 4$  replicates for each data point. \* $p < 0.05$ , t-test and Bonferroni's post hoc test (difference from control, VEH).

Our next goal was to determine if increasing  $\alpha$ GalA activity regulated the clearance of over-expressed  $\alpha$ syn. Over-expression of  $\alpha$ syn was induced by four days of Dox treatment and  $\alpha$ syn levels were evaluated for 72 hours after removal of Dox in the presence or absence of Fz. Treatment with Fz in culture media significantly decreased levels of total  $\alpha$ syn monomer in M17 cells compared to vehicle controls at 48, but not 72 hours after Dox removal (Fig. 3).

## DISCUSSION

PD has a drastic impact on the health of millions of Americans and creates a major financial burden on the healthcare system. Reports of ALP dysfunction in PD along with the discovery of the pathogenic accumulation of  $\alpha$ syn in  $\alpha$ GalA deficient mouse brains led us to investigate the therapeutic potential of increasing  $\alpha$ GalA in a preclinical model of  $\alpha$ syn accumulation in PD. We established an *in vitro* model of transient  $\alpha$ syn over-expression and pharmacological enhancement of  $\alpha$ GalA to provide proof-of-principle validation of  $\alpha$ GalA as a therapeutic target for  $\alpha$ syn pathology in PD. We found that increasing  $\alpha$ GalA activity by Fz accelerates the clearance of over-expressed  $\alpha$ syn in our M17 model of transient  $\alpha$ syn over-expression.

We confirmed that the addition of Dox to culture media for 7 days induced a significant, but transient increase in  $\alpha$ syn, making it an effective model for studying the relationship between increasing  $\alpha$ GalA activity and the clearance of  $\alpha$ syn. Using an *in vitro* system with an immortalized cell line limits the extent to which these experimental results can be generalized to the brain, but future studies in our laboratory will investigate the same “clearance” effect of  $\alpha$ GalA on  $\alpha$ syn in our *in vitro* primary neuron culture model and an *in vivo* mouse model of  $\alpha$ syn over-expression.

The concentration-dependent increase in  $\alpha$ GalA activity resulting from Fz treatment are believed to be internalized, not just found in the culture media, as cells were washed with PBS prior to lysis. Preliminary immunocytochemistry data from our lab indicates the localization of increased  $\alpha$ GalA by Fz to lysosomes (data not shown), suggesting that Fz is indeed internalized and reaching the lysosome. Although we found significant effects of Fz treatment at concentrations  $\geq 1 \mu\text{g ml}^{-1}$ , Fz is known to be internalized in cells via the mannose 6-phosphate receptor pathway at concentrations up to  $1 \mu\text{g ml}^{-1}$  (35). This suggests that higher concentrations may be internalized via different mechanism(s). As such, ongoing studies in our laboratory are focused on the therapeutic potential of Fz concentrations  $\leq 1 \mu\text{g ml}^{-1}$ , in addition to confirming the mechanisms by which low and high concentrations of Fz are internalized in M17 cells and its lysosomal localization subsequent to internalization.

Our *in vitro* model system was used to evaluate the effect of increasing  $\alpha$ GalA on the clearance of over-expressed  $\alpha$ syn. Fz significantly decreased total levels of over-expressed

total  $\alpha$ syn when it was added for 48 hr after Dox removal, supporting our hypothesis that increasing  $\alpha$ GalA activity accelerates the clearance of over-expressed  $\alpha$ syn. Further studies are necessary to elucidate the mechanism(s) by which  $\alpha$ GalA regulates  $\alpha$ syn, including the functional examination of ALP-specific markers. One possible mechanism by which  $\alpha$ GalA may promote the clearance of  $\alpha$ syn is by regulating levels/activity of cathepsin D, a lysosomal aspartic acid protease that has been shown to proteolytically degrade  $\alpha$ syn<sup>36</sup>. This and other mechanisms of ALP function will be explored in future studies. The current study focused on clearance of soluble  $\alpha$ syn monomers. Future studies will also determine if increasing  $\alpha$ GalA activity promotes the clearance of existing insoluble  $\alpha$ syn aggregates, and/or prevents them from accumulating in the first place.

Induction of autophagy as a therapy for PD has had few successes in clinical trials and has known side effects<sup>37-39</sup>. In addition, the induction of autophagy concurrent with the inhibition of lysosomal function has been used as a therapy for killing cancer cells, suggesting it may further increase the stress to neurons in PD if autophagy is induced without proper lysosome function<sup>40</sup>. This suggests that inducing autophagy in PD under conditions of lysosomal dysfunction would not only be ineffective, but could in fact exacerbate neurodegeneration. Evidence for the involvement of the ALP in PD pathology is ample. Cathepsin D (CD) deficiency decreases both lysosome and proteasome function and leads to  $\alpha$ syn accumulation<sup>22</sup>. *ATP13A2* gene mutations in autosomal dominant PD cause multiple impairments of lysosome function including substrate degradation and autophagosome processing<sup>19</sup>. Inhibition of lysosomal acidification by bafilomycin A1 increases toxicity of  $\alpha$ syn aggregates<sup>24</sup>, knock-down of *ATP6V0C* H+ pump increases accumulation of  $\alpha$ syn<sup>26</sup>, and pathogenic lysosome depletion has been shown in PD<sup>16</sup>. Lastly, glucocerebrosidase (GBA) activity and  $\alpha$ syn accumulation interact bidirectionally in synucleinopathies including Gaucher Disease and PD<sup>17</sup>. Our data suggest that  $\alpha$ GalA, another lysosomal enzyme in the glycosphingolipid metabolism pathway with GBA, is also capable of affecting  $\alpha$ syn levels through a yet unknown mechanism. Our lab is focused on the continued investigation of  $\alpha$ GalA and lysosome function as therapeutic targets for attenuating  $\alpha$ syn-associated pathogenesis in PD.

## CONCLUSION

We established an *in vitro* model of inducible  $\alpha$ syn over-expression to determine if pharmacologically increasing the activity of  $\alpha$ GalA affects its clearance. Fabrazyme increases levels and activity of  $\alpha$ GalA in M17 human neuroblastoma cells and promotes the clearance of over-expressed  $\alpha$ syn in these cells. ***These data provide support for the continued investigation of  $\alpha$ GalA as a therapeutic target for PD.***

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