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The Cholesterol 24-Hydroxylase Enzyme, CYP46A1, Reduces Overexpressed Alpha-Synuclein Proteins in Human Cellular Models of Parkinson's Disease

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Abstract

Background: A growing body of evidence suggests a correlation between cholesterol metabolism and the pathogenesis of Parkinson's Disease (PD). We and others have demonstrated that the activation of the cholesterol 24-hydroxylase enzyme, CYP46A1, responsible for converting cholesterol to 24S-Hydroxycholesterol (24-OHC) in the brain, is an effective therapeutic strategy for several neurodegenerative diseases as Alzheimer's disease, huntington's disease and spinocerebellar ataxia type 3. Nevertheless, there is still much to be elucidated regarding the role of CYP46A1 in PD. Alpha-Synuclein, the symbol of pathological protein of PD, exhibits a pronounced affinity for binding to lipid membranes, especially in cholesterol-rich regions and contains a high-affinity cholesterol-binding motif in the 67-78 a region. To further our understanding of CYP46A1's actions, we assessed not only the expression of wild-type alpha-syn but also that of the *A53T* alpha-syn mutant associated with the familial form of the disease as well as mutants lacking presumed cholesterol-binding sites in conjunction with CYP46A1.

Methods: We established several models of PD in human neuroblastoma SH-SY5Y cells through transient transfection with either wild-type or mutant alpha-syn or by inhibiting key genes involved in the disease using Small Interfering RNA (siRNA). The effects of CYP46A1 co-transfection on the alpha-syn expression were then analyzed by Western blot.

Results: Overexpression of human CYP46A1 leads to a significant reduction in wild-type alpha-syn protein levels in SH-SY5Y cells. Our investigations suggest that this decrease is mediated through the autophagy-lysosomal pathway. Additionally, our findings indicate that CYP46A1 may also reduce the levels of alpha-syn proteins carrying mutations in the cholesterol-binding domain or at the residue *A53T*, which is associated with familial pathology. Moreover, CYP46A1 retains its functionality in a cellular model of PD associated with *GBA1*. The gene *GBA1* is involved in lipid metabolism and its deficiency represents the most prevalent genetic factor associated with an elevated risk of PD.

Conclusion: These findings offer valuable insights into the pathogenesis of PD and reveal potential therapeutic avenues involving CYP46A1, highlighting its ability to reduce alpha-syn expression, which could benefit patients with PD.

Keywords: Parkinson's disease; CYP46A1; Alpha-synuclein; Autophagy; Human neuroblastoma cells (SH-SY5Y)

Introduction

Alpha-Synuclein (aSyn) plays a central role in the development of Parkinson's Disease (PD) [1]. A key characteristic of PD is the degeneration of dopaminergic neurons in the substantia nigra pars compacta. The remaining neurons display an accumulation of alpha-syn proteins, forming aggregates in Lewy bodies. The *SNCA* gene encodes a 14.5-kDa protein, alpha-syn, which has been implicated as an important factor in synaptic vesicle trafficking and dopamine release.

However, its physiological function remains incompletely understood. Overexpression of the alpha-syn protein, resulting from duplications or triplications of the *SNCA* locus or point mutations in the *SNCA* gene, is associated with familial forms of PD [2-4]. While many of these genetic alterations are either rare or confer a variable risk for developing PD, they highlight that the exclusive overexpression of alpha-syn can contribute to the onset of the disease. The spread of aggregated alpha-syn from cell to cell is currently considered as a mechanism explaining the pathological progression of the disease. Due to the aberrant assembly of alpha-syn being a common feature

in the development of PD, significant efforts have been dedicated to understanding and inhibiting this phenomenon.

Recent findings indicate that Lewy bodies in addition of the high level of alpha-syn proteins are also characterized by a high lipid content and degenerated organelles suggesting that lipids, which are the main constituents of cell membranes, may contribute to the development of the disease [5,6]. Over the last two decades, accumulating evidence suggests a transient binding of alpha-syn to lipid membranes particularly in cholesterol-rich regions [7-10]. Cholesterol may affect the binding

of alpha-syn to the membrane and its abnormal aggregation [10-12]. Studies have also demonstrated that alpha-syn significantly stimulates cholesterol efflux [13].

The brain, being the highest cholesterol-rich organ in mammals, maintains a major balance of cholesterols levels through a precisely regulated process called brain cholesterol homeostasis. Cholesterol, is an integral component of membranes, contributing to their structure and function and acts as a precursor of metabolites involved in various metabolic pathways. The cholesterol is mainly found in the myelin sheaths of the oligodendrocytes, as well as in the membranes of glial cells and neurons. Astrocytes synthesize cholesterol and export it through Adenosine Triphosphate (ATP)-binding cassette transporters, primarily *via* ATP Binding Cassette Subfamily a Member 1 (ABCA1) transporter. The transportation of cholesterol to neurons is facilitated by *APOE*, which is also synthesized by astrocytes [14].

Studies have shown disruptions in this balance during neurodegenerative diseases such as Alzheimer's Disease (AD) [15-17], Huntington's Disease (HD) [18,19] and Amyo-trophic Lateral Sclerosis (ALS) as well as in brain disorders such as epilepsy [20,21]. Previous research has demonstrated that regulating cholesterol homeostasis through the over expression of cytochrome P450 family 46 subfamily A member 1 (CYP46A1), a brain-specific enzyme that converts cholesterol into 24-Hydroxycholesterol (24-OHC), has protective effects against AD, HD, Spino-Cerebellar Ataxia Type 3 (SCA3) [22-26]. The role of CYP46A1, primarily located in neurons, is major because cholesterol itself cannot cross the Blood-Brain Barrier (BBB), whereas 24-OHC can pass through BBB into the systemic circulation. Acting as a ligand for Liver X Receptors (LXR), 24-OHC subsequently regulates the transcription of cholesterol transport proteins such as ABCA1, which are located on the plasma membrane. Consequently, this process effectively regulates cholesterol efflux. However, these studies have revealed that the effects of CYP46A1 activity extend beyond cholesterol maintenance, leading to a reduction in aggregated proteins implicated in these diseases [27]. These findings underscore the pivotal role of cholesterol turnover and 24-OHC in neurological disorders.

Despite this, only a few studies investigated the role of CYP46A1 in PD. Recent research has revealed reduced levels of CYP46A1 proteins in induced Pluripotent Stem Cell (iPSC)-derived dopaminergic neurons from patients carrying the *SNCA* triplication or the *A53T*, *SNCA* mutation, both associated with familial PD onset [28]. Notably, CYP46A1 overexpression has been observed to elevate levels of 24-OHC and 24 (S), 25-epoxycholesterol (24, 25-EC) in the developing midbrain. This process has been proposed as a potential mechanism for preventing dopaminergic loss in PD [29]. These data suggested that CYP46A1 could be an important factor in PD.

The aim of our study was to investigate whether the overexpression of CYP46A1 could also be beneficial for PD and lead to a decrease in overexpressed alpha-syn. In the present study, we aimed to investigate whether the expression of CYP46A1 could reduce the levels of overexpressed alpha-syn proteins in neuroblastoma SH-SY5Y cells. To deepen our comprehension of CYP46A1's actions, we evaluated not only the expression of wild-type alpha-syn but also the expression of *A53T* alpha-syn mutant associated with the familial form of the disease [2], as well as mutants lacking presumed cholesterol-binding sites, all in the presence of CYP46A1. Additionally, we investigated the impact of CYP46A1 on alpha-syn overexpression by inhibiting autophagic-lysosome functions using autophagic inhibitors and by reducing the expression of the lysosomal enzyme encoded by the *GBA1* gene, which are among the most common genetic factors associated with an increased risk of PD [30,31].

Materials and Methods

Generation of untagged alpha-synuclein constructs

Human alpha-synuclein (WT or *A53T*) cDNA was amplified by PCR from previously described vectors kindly provided by Add gene (plasmids #102361 and 105727). The PCR products were cloned in the HindIII/XbaI sites of the pcDNA3.1 vector using primers described.

Cell culture and co-transfections

Human SH-SY5Y neuroblastoma cells (ATCC: CRL-266), were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax (Gibco), supplemented with 4.5 g/L D-glucose and sodium pyruvate, 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco), in a 5% CO_2 atmosphere at 37°C. Cells were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen), following to the manufacturer's instructions. The pcDNA3-EGFP plasmid was obtained from Add gene (plasmid #13031) while pAAV-HA-CYP46A1 or pCMV-CYP46A1-Flag plasmids were provided by Askbio France. For autophagy inhibition, treatments were initiated either 6 hours or 24 hours post-transfection with 3-MA or BafA1, respectively. Cells were then incubated with 3-MA for 42 hours or with BafA1 for 24 hours. 3-MA (189-490, Millipore) was prepared at a concentration of 50 mM in Opti-MEM (Gibco), sterilized by filtration through a syringe filter with a 0.2 μM pore size and added to a final concentration of 5 mM in the cell medium. BafA1 (B1793, Sigma) was dissolved as a 10 uM solution in DMSO and added to the cell medium to achieve a final concentration of 50 nM. For proteasome inhibition, MG132 (474790, Sigma) was prepared as a 20 mM solution in DMSO. Cells were treated with 20 μM MG132 42 hours post-transfection for 6 hours. The concentrations of these inhibitors were selected based on data from other studies and preliminary experiments.

Protein extraction and western-blot analysis

Cell pellets were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors for 30 min at 4°C. Whole lysates were clarified by centrifugation at 10,000 rpm for 15 min. Protein concentrations were quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) with Bovine Serum Albumin (BSA) standards for comparison. Equal volumes of each sample were separated by SDS-PAGE. Protein samples were denatured for 3 min at 95°C and separated into 4%-20% SDS-polyacrylamide gels, then transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% BSA in Tris-Buffered Saline (TBS) pH 8 for 1 h, followed by overnight incubation with primary antibodies (dilution 1:1000) at 4°C. After washing three times for 10 min each in TBS with 0.1% Tween-20, membranes were incubated with Li-COR fluorescent secondary antibodies (dilution 1:6000) for 1 hour at room temperature and washed three more times with TBS with 0.1% Tween-20. Finally, images of the membranes were captured using a Li-COR Odyssey fluorescence scanner. The protein ladder used was precision plus protein standards Kaleidoscope ladder, 10 to 250 kD (Bio-Rad).

Immunofluorescence microscopy

For immunostaining, SH-SY5Y cells were cultured on glass coverslips. Twenty-four hours post-transfection, cells were washed twice with Phosphate-Buffered Saline (PBS), fixed with 4% paraformaldehyde in PBS, quenched for 10 min with 0.1 M glycine in

PBS and permeabilized for 30 min at room temperature with 0.05% saponin, PBS, 0.5% bovine serum albumin. The coverslips were incubated for 2 h at room temperature with the primary antibodies (see below) at dilution 1:250 in PBS containing 0.5% BSA, 0.05% saponin. After washing, cells were subsequently incubated for 2 hours at room temperature with the secondary antibodies (see below) at dilution 1:500, covered from light. Cells were washed three times with PBS and mounted using Vectashield with DAPI or without (Vector Laboratories). Fluorescent images of cells were captured with a 60X objective using a Zeiss Apotome 2 microscope with the structured illumination system equipped of a Zeiss Axiocam camera. Image acquisition was performed with the ZEN 2 (Zeiss) software. The images were merged and analyzed using Image J.

Antibodies

The following primary antibodies were used: Rabbit anti-actin (Sigma, A2066), rabbit anti-GFP (Sigma, SAB301138), rabbit anti-HA (Cell signaling, 3724), mouse anti-alpha-synuclein antibody (Invitrogen, AHB0261), mouse anti-Flag (Sigma, F3165), mouse anti-ubiquitin (Invitrogen, 14-6078-82), mouse anti-Lamp2A (Abcam, Ab25631), rabbit anti-LC3B (Abcam, 51520), rabbit anti-*GBA1* (Sigma, G4171). Secondary antibodies for immunoblotting were appropriate goat anti-mouse or anti-rabbit IRDye 680RD or 800CW IgG (Odyssey LI-COR). For immunofluorescence labelling Alexa-488 or Alexa-555, conjugated donkey, anti-mouse or anti-rabbit, (life technologies) were used.

RNA isolation and quantitative PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), treated with DNase I and reverse-transcribed using the one script qPCR RT Kit (Ozyme). cDNA was amplified using SYBR Green on the Roche Light Cycler 480 for quantification. The relative expression levels of mRNA were normalized to S18 ribosomal RNA levels (RibS18).

The following primers were used: Human CYP46A1 forward (5'CGAGTCCTGAGTCGGTTAAGAAGTT3') and re-verse (5'AGTCTGGAGCGCACGGTACAT3'), human alpha-syn forward (5'ACCAAACAGGGTGTGGCAGAAG3') and reverse (5'CTTGCTCTTTGGTCTTCTCAGCC3') and human Ribs18 forward (5'GAGGATGAGGTGGAACGTGT3') and reverse (5'GGACCTGGCTGTATTTTCCA3'). The program consisted of an initial hot start cycle at 95°C for 2 min, followed by 45 cycles at 95°C for 10 s, 65°C for 20 s and 72°C for 20 s with a final extension at 72°C for 10 min. Each sample was assayed in triplicate.

Site-direct mutagenesis

The human alpha-syn-pcDNA3.1 plasmid was subjected to site-directed mutagenesis to introduce specific amino acid changes within the domain spanning residues 67 to 78, as listed in Supplementary Table 1. Various plasmids were generated including: Plasmid 38 with mutations *A69G*, *V70L, T72S, G73R*; plasmid 39 with mutations *A69T, V70R, G73Y, V74W, A76S*; plasmid 40 with mutations *V71S, T72A, T75A, A76S* and plasmid 42 with mutation A69K. Forward and reverse primers, each of 25-40 nucleotides long, were designed to introduce the desired mutations (see Supplementary Table 2). Additional primers were necessary for cloning into the HindIII/XbaI sites of the pcDNA3.1 vector (see Supplementary Table 2). PCR was performed using the mutation primers (forward or reverse), cloning primers (HindIII or XbaI primers) and the alpha-syn-pcDNA3.1 plasmid as a template,

along with a high-fidelity thermostable Taq DNA polymerase (Thermo Fisher, Master Mix Dream Taq Green PCR). The PCR conditions involved an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s and extension at 72°C for 30 s, with a final extension step at 72°C for 7 min. For each construct, a final PCR was conducted using the two PCR fragments isolated on agarose gels with a gel extraction kit (Qiagen), along with the two HindIII and XbaI primers. The resulting PCR product was isolated using the Qiaquick PCR purification kit (Qiagen), digested with HindIII and XbaI and then ligated into the pcDNA3.1 plasmid, which had been previously digested with HindIII and XbaI. The plasmids were transformed into competent bacteria *via* heat shock. Single colonies were selected, grown overnight and plasmid DNA was isolated using a miniprep kit (Qiagen). Sequencing of the plasmids was conducted to confirm the introduced base changes.

Reduction of Lamp2A or *GBA1* expression by RNA silencing

To downregulate the levels of Glucocerebrosidase (GCase) encoded by the *GBA1* gene or Lamp2A proteins, siRNAs were purchased from thermo fisher scientific (Ambion by life technologies). Three siRNAS targeting human *GBA1* (*GBA1*a (GBA-s501316), *GBA1*b (GBA-s534767) and *GBA1*c (GBA-s534768)) were tested. The siRNA targeting human Lamp2A is Lamp2-s8086. As a negative control, siRNA s18483 obtained from Thermofisher was used. The specific siRNAs, at a concentration of 50 nM, were co-transfected with Lipofectamine 2000 (Invitrogen) along with alpha-syn and GFP or CYP46A1 plasmids for 48 hours into SH-SY5Y cells.

Statistical analysis

Statistical analysis were conducted using GraphPad Prism. The specific statistical tests used are indicated in the figure legends. Values are presented as means ± SEM. A p-value of less than 0.05 was considered statistically significant.

Results

Decrease in alpha-syn expression following co-transfection with CYP46A1 in neuroblastoma SH-SY5Y cells

To explore the impact of CYP46A1 on alpha-syn in PD, we generated a plasmid harboring human Wild-Type (WT) alpha-syn cDNA. This construct was then co-transfected with a plasmid expressing either CYP46A1 or GFP into human neuroblastoma SH-SY5Y cells. Forty-eight hours post-transfection, the presence of CYP46A1 led to a significant threefold reduction in the levels of alpha-syn proteins. Immunofluorescence analysis of CYP46A1 and alpha-syn conducted 48 hours post-transfection revealed cytoplasm localization for alpha-syn, characterized by a vesicular pattern. CYP46A1 predominantly exhibited localization around the nuclear membrane and within the cytoplasm, likely associated with the membrane of the endoplasmic reticulum, as previously demonstrated [32]. Next, we investigated whether the reduction in alpha-syn expression occurred at the transcriptional level by performing real-time RT-QPCR analysis. Analysis of alpha-syn mRNA levels from cells co-transfected with alpha-syn and either CYP46A1 or GFP showed no significant difference. These findings suggest that the difference in alpha-syn levels is not attributable to transcriptional regulation by CYP46A1. In conclusion, we have demonstrated that while CYP46A1 does not reduce the level of alpha-syn transcripts, it does affect the level of alpha-syn proteins (Figures 1A-1D).

Impact of CYP46A1 overexpression on *A53T* alpha-syn mutant and mutants devoid of presumed putative cholesterol-binding sites

Some findings underscore the essential role of cholesterol in mediating interactions between physiologically relevant membranes and alpha-syn in neurological diseases. Disruption of this association redistributes alpha-syn away from the synapse and impairs its cellular function [7]. Furthermore, alpha-syn extracellular has been discovered to significantly enhance cholesterol efflux from neuronal cells [13]. We investigated whether interaction of alpha-syn and cholesterol is a prerequisite for the reduction of alpha-syn expression mediated by CYP46A1. Through the utilization of a panel of recombinant fragments and synthetic peptides, it has been discovered that alpha-syn interacts with cholesterol with high affinity through a binding domain called the titled domain [33,34]. To disrupt the interaction between cholesterol and alpha-syn, we engineered mutants within the titled domain (residues 67-78) and assessed the resulting reduction in alpha-syn expression. The amino acid residues of wild-type human alpha-syn involved in cholesterol binding are indicated in bold in the sequence GGA**V**VTGV**T**AVAQ (Supplementary Table 1) [34,35]. Four mutants (*Syn38, Syn39, Syn40* and *Syn42*) were designed by site-directed mutagenesis. Among these mutants, Syn40, was specifically engineered based on the peptide mutant *SynuM53* of alpha-syn, as devised by Crowet and colleagues using molecular modeling methodologies [33]. This mutant is suggested to adopt a parallel orientation to the lipid layer rather than a tilted orientation. Its sequence is GGAVSAGVASVTQ. The other three mutants were designed to alter the essential amino acids identified in these interactions by Fantini et al., (Supplementary Table 1). We also included the *A53T* mutation linked to familial parkinsonism, which has been shown in *in vitro* studies to have no effect on lipid binding [7,36,37]. SH-SY5Y cells were co-transfected with each alpha-syn mutant along with either GFP or CYP46A1, or with wild-type alpha-syn or the *A53T* alpha-syn mutant along with either GFP or CYP46A1. Forty-eight hours post-transfection, we compared the level of alpha-syn protein levels by western blot analysis between the cells co-transfected GFP or CYP46A1. We observed a significant reduction in alpha-syn levels for wild-type or each mutant alpha-syn in the presence of CYP46A1 compared to the presence of GFP. In our study revealed that the decrease of alpha-syn protein levels in cells overexpressing CYP46A1 is not dependent on the interaction between cholesterol and the region 67-78 of alpha-syn. Indeed, CYP46A1 significantly reduced the expression level of alpha-syn mutants at potential cholesterol-binding sites compared to cells co-transfected with GFP. Furthermore, we observed that CYP46A1 can decrease the expression not only of wild-type alpha-syn but also the *A53T* alpha-syn mutant associated with the familial form of the disease (Figures 2A and 2B).

CYP46A1 facilitates the reduction of Wild-Type alpha-syn (WT) protein levels *via* the macroautophagy pathway

Evidence suggests that dysfunction in the autophagy pathway is prevalent in numerous neurodegenerative diseases [38-41]. In PD, alpha-syn has been demonstrated to undergo degradation through macroautophagy, chaperone-mediated autophagy or the proteasome [42-44]. We investigated whether CYP46A1 could enhance macroautophagy, chaperone-mediated autophagy or the proteasome to reduce the expression of alpha-syn proteins in SH-SY5Y cells.

Macroautophagy is a common form of autophagy characterized by the formation of auto phagosome and is effective in removing abnormal proteins and protein aggregates [45]. To investigate the impact of macroautophagy on alpha-syn clearance mediated by CYP46A1, we treated the co-transfected cells with autophagy inhibitors, 3-Methyladenine (3-MA) and Bafilomycin A1 (BafA1). As expected, when cells are co-transfected with alpha-syn and GFP, the presence of these autophagy inhibitors resulted in increased alpha-syn levels. This finding is consistent with the established knowledge that alpha-syn is regulated by macroautophagy. Furthermore, when alpha-syn and CYP46A1 were co-expressed, an increase of alpha-syn proteins was observed in the presence of 3-MA or BafA1. However, this increase in alpha-syn levels was notably lower compared to when alpha-syn and GFP were co-expressed in the presence of the inhibitors. This suggests that CYP46A1 retains its ability to reduce alpha-syn levels even in the presence of autophagy inhibitors. These results were further supported by the quantitative analysis of western blot data from 11 independent experiments.

By inhibiting the fusion of the auto phagosome and the lysosome, BafA1 affects LC3 formation. Through western blot analysis, we observed a significant increase in the LC3B-II/LC3B-I ratio in cells co-expressing alpha-syn and CYP46A1 compared to those co-expressing alpha-syn and GFP, as illustrated in the representative western blot and the data obtained from 11 western blots. These findings strongly suggest that CYP46A1 enhances the induction of macroautophagy of alpha-syn (Figures 3A-3C).

We also examined whether CYP46A1 could influence alpha-syn expression through the Chaperone Mediated Autophagy (CMA) pathway. CMA is a distinctive pathway by which cytosolic protein aggregates are selectively directed to lysosomes for degradation. Wild-type alpha-syn, containing a CMA-targeting motif within its sequence, can undergo degradation in lysosomes *via* the CMA pathway [43]. The substrate-chaperone complex is then transported to lysosomal membranes, where interactions with Lamp2A receptors facilitate CMA activities [46]. To examine whether CYP46A1 can induce the CMA pathway to degrade alpha-syn, SH-SY5Y cells were co-transfected with Lamp2A siRNA or scrambled control siRNA along with alpha-syn accompanied by either GFP or CYP46A1. The reduction of Lamp2A by siRNA is directly related to CMA activity. Lamp2A protein levels were determined by western blotting (Supplementary Figure 1). The protein levels of alpha-syn detected with GFP or with CYP46A1 did not change in cells where Lamp2A protein levels were reduced by 2.5-fold with siRNA compared to cells co-transfected with siRNA control. These results suggest that CYP46A1 enhances alpha-syn degradation through the macroautophagy pathway independently of CMA autophagy (Supplementary Figure 1).

To explore the impact of CYP46A1 on proteasomal activity in the alpha-syn clearance, SH-SY5Y cells were co-transfected with alpha-syn and either GFP or CYP46A1 and subsequently, treated with the proteasome inhibitor MG132 at 20 μM for 6 hours. Our observations revealed an increase in alpha-syn protein levels following MG132 treatment, regardless of the presence of GFP or CYP46A1. This finding is consistent with established knowledge that alpha-syn is degraded not only by autophagy but also by the proteasome [42]. However, the ratio of alpha-syn protein levels in the presence of CYP46A1 compared to those detected with GFP remains unchanged with or without MG132, indicating that CYP46A1 reduces the levels of alpha-syn protein independently of proteasome inhibition (Supplementary Figure 2).
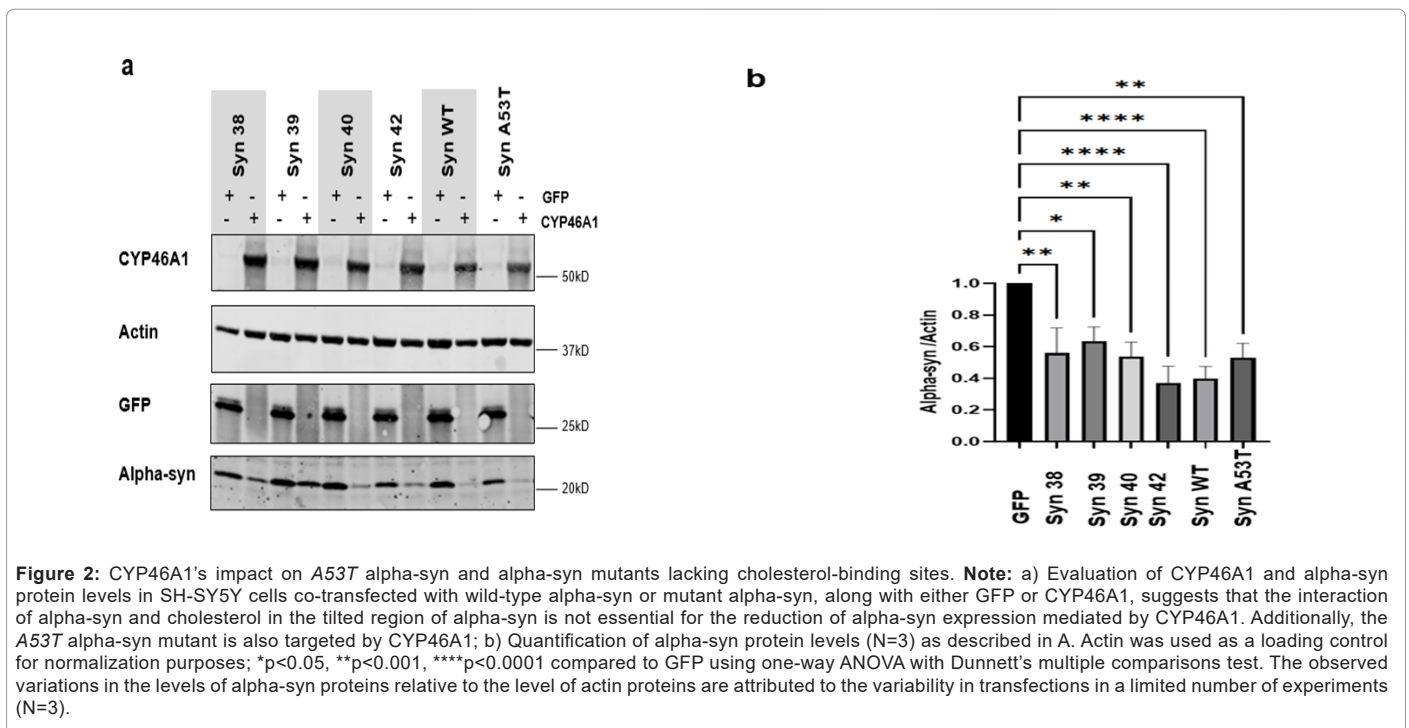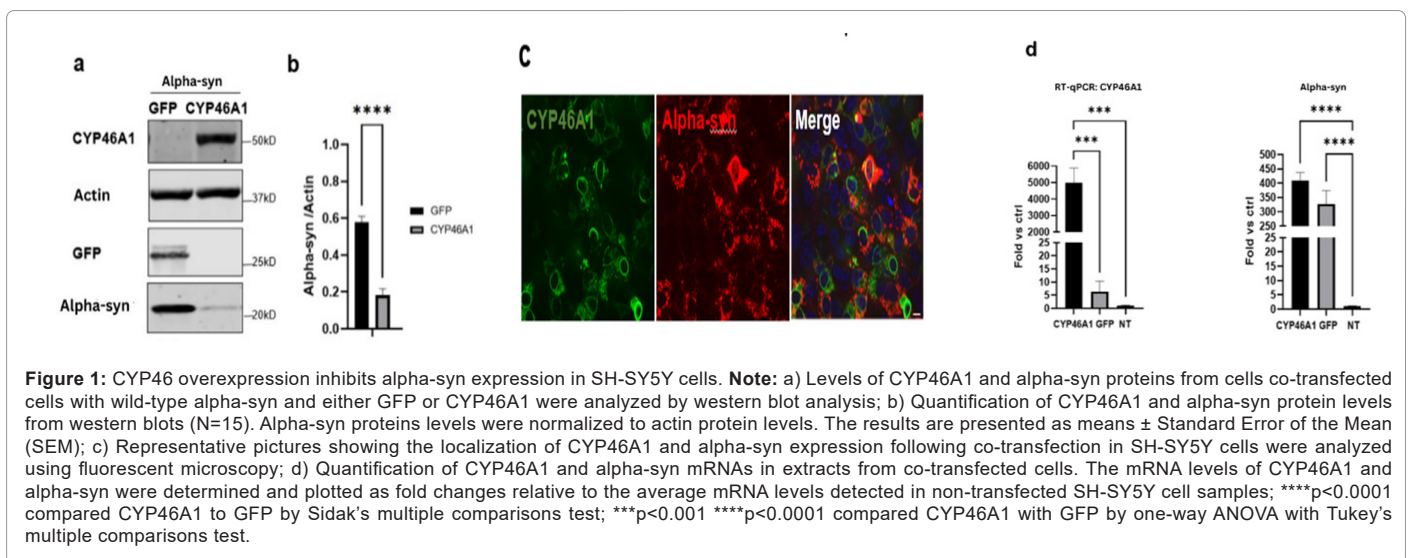
Impact of CYP46A1 on alpha-syn expression in glucocerebrosidase-deficient cells
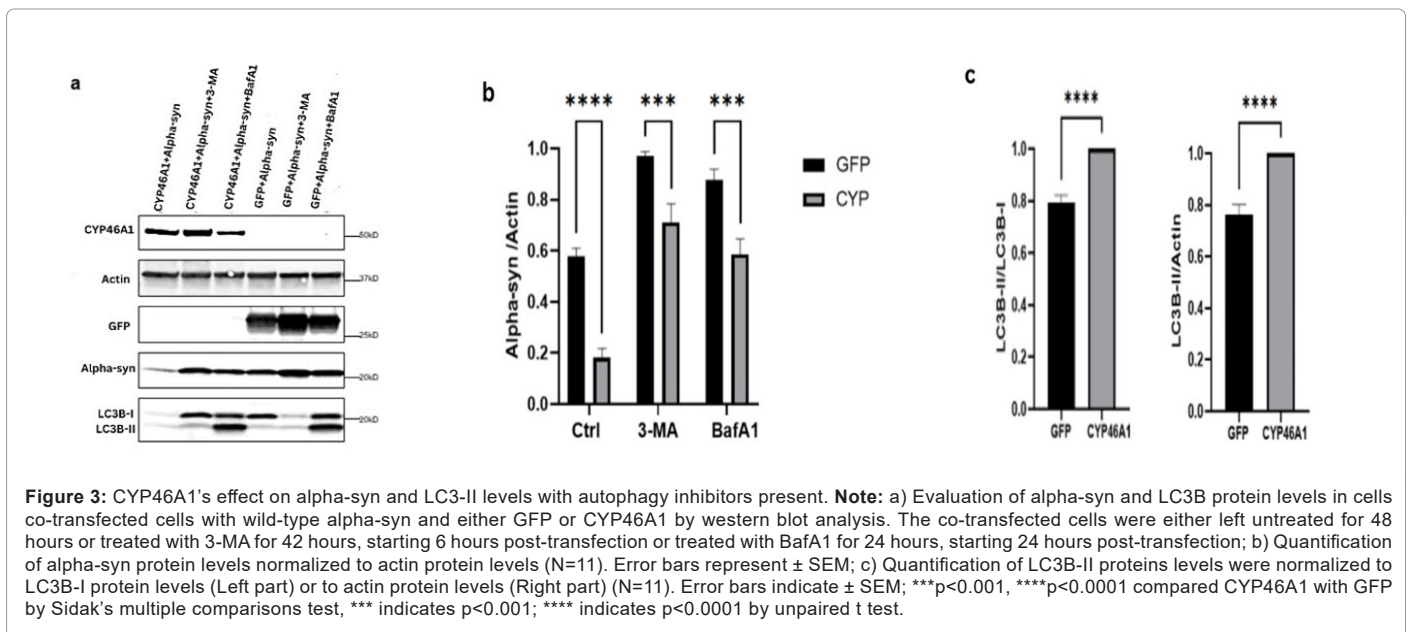
Mutations in the *GBA1* gene constitute the most significant genetic

risk factor numerically for the development of PD, increasing the risk by approximately fourfold [30,47]. *GBA1* gene encodes the lysosomal enzyme Glucocerebrosidase (GCase), which plays an important role in converting glucosylceramide into glucose and ceramide within the lysosome. GCase-deficient cells display disrupted lysosomal recycling and the accumulation of dysfunctional lysosomes. Given that lysosomes serve as the principal degradative compartment within the cell, their biogenesis and recycling are essential for cellular function and the autophagy-lysosomal pathway. The loss of GCase activity exacerbates autophagy impairment and facilitates the accumulation of alpha-syn [48-50].

As our study suggests that the overexpression of CYP46A1 decreases alpha-syn protein levels through the macroautophagy pathway, we aimed to investigate whether CYP46A1 could still reduce alpha-syn expression in PD pathological conditions where the expression of the lysosomal enzyme GCase, which regulates autophagy, is diminished. In this study, SH-SY5Y cells were co-transfected with *GBA1* siRNAs or scrambled control siRNAs along with alpha-syn and either GFP or CYP46A1 for 48 hours. Two out of three *GBA1* siRNAs (si*GBA1*b and 1c) inhibited the *GBA1* gene by factors of 4 and 3, respectively, compared to both scrambled control siRNAs (*siCrtl1* and *siCrtl2*), or *siGBA1a*, as indicated by western blot analysis. The expression levels of alpha-syn detected using CYP46A1 were observed to decrease compared to those detected using GFP. However, no change in alpha-syn expression was noted in cells with *GBA1* deficiency using siRNA *siGBA1b* or *siGBA1c* compared to cells with control siRNA. Our experiments demonstrate that CYP46A1 can still effectively reduce alpha-syn expression, even when the expression of the lysosomal enzyme GCase is reduced (Figure 4).

Figure 1: CYP46 overexpression inhibits alpha-syn expression in SH-SY5Y cells. **Note:** a) Levels of CYP46A1 and alpha-syn proteins from cells co-transfected cells with wild-type alpha-syn and either GFP or CYP46A1 were analyzed by western blot analysis; b) Quantification of CYP46A1 and alpha-syn protein levels from western blots (N=15). Alpha-syn proteins levels were normalized to actin protein levels. The results are presented as means ± Standard Error of the Mean (SEM); c) Representative pictures showing the localization of CYP46A1 and alpha-syn expression following co-transfection in SH-SY5Y cells were analyzed using fluorescent microscopy; d) Quantification of CYP46A1 and alpha-syn mRNAs in extracts from co-transfected cells. The mRNA levels of CYP46A1 and alpha-syn were determined and plotted as fold changes relative to the average mRNA levels detected in non-transfected SH-SY5Y cell samples; ****$p<0.0001$ compared CYP46A1 to GFP by Sidak's multiple comparisons test; ***$p<0.001$ ****$p<0.0001$ compared CYP46A1 with GFP by one-way ANOVA with Tukey's multiple comparisons test.

Figure 2: CYP46A1's impact on *A53T* alpha-syn and alpha-syn mutants lacking cholesterol-binding sites. **Note:** a) Evaluation of CYP46A1 and alpha-syn protein levels in SH-SY5Y cells co-transfected with wild-type alpha-syn or mutant alpha-syn, along with either GFP or CYP46A1, suggests that the interaction of alpha-syn and cholesterol in the tilted region of alpha-syn is not essential for the reduction of alpha-syn expression mediated by CYP46A1. Additionally, the *A53T* alpha-syn mutant is also targeted by CYP46A1; b) Quantification of alpha-syn protein levels (N=3) as described in A. Actin was used as a loading control for normalization purposes; *$p<0.05$, **$p<0.001$, ****$p<0.0001$ compared to GFP using one-way ANOVA with Dunnett's multiple comparisons test. The observed variations in the levels of alpha-syn proteins relative to the level of actin proteins are attributed to the variability in transfections in a limited number of experiments (N=3).

Figure 3: CYP46A1's effect on alpha-syn and LC3-II levels with autophagy inhibitors present. **Note:** a) Evaluation of alpha-syn and LC3B protein levels in cells co-transfected cells with wild-type alpha-syn and either GFP or CYP46A1 by western blot analysis. The co-transfected cells were either left untreated for 48 hours or treated with 3-MA for 42 hours, starting 6 hours post-transfection or treated with BafA1 for 24 hours, starting 24 hours post-transfection; b) Quantification of alpha-syn protein levels normalized to actin protein levels (N=11). Error bars represent ± SEM; c) Quantification of LC3B-II proteins levels were normalized to LC3B-I protein levels (Left part) or to actin protein levels (Right part) (N=11). Error bars indicate ± SEM; ***$p<0.001$, ****$p<0.0001$ compared CYP46A1 with GFP by Sidak's multiple comparisons test, *** indicates $p<0.001$; **** indicates $p<0.0001$ by unpaired t test.

Discussion

Considerable evidence suggests that increased expression of alpha-syn is involved in the pathogenesis of both sporadic and familial Parkinson's Disease (PD), indicating that the observed changes likely represent some of the earliest events in the progression of PD. Defects in cholesterol metabolism have been associated with neurodegenerative diseases [51,52]. Cholesterol's impact on the membrane binding of alpha-syn may facilitate the formation of beta-sheet structures in alpha-syn, thus promoting the generation of abnormal alpha-syn fibrils [10-12]. Several studies have emphasized a close association between alpha-synuclein (alpha-syn) and cholesterol, both intracellularly and extracellularly. Notably, research has identified a high-affinity cholesterol binding domain in the 67-78 amino acid region of alpha-syn [33,34]. While the majority of alpha-syn remains intracellular, extracellular forms of this protein also exist, as it can propagate from cell to cell in a "prion-like" manner [53]. Extracellular alpha-syn has been demonstrated to decrease membrane cholesterol levels and stimulate cholesterol efflux from cells [13,54].

We and others have shown that the activation of CYP46A1 in various brain disease models leads to a decrease in aggregated proteins and improved performance in motor, memory and/or cognitive tests [21-26,55]. However, much less is known about the role of CYP46A1 in PD [27,56]. In our study, we demonstrate that heightened expression of the enzyme CYP46A1, responsible for converting cholesterol into 24S-OHC, results in decreased levels of both wild-type and mutant *A53T* human alpha-syn proteins in neuroblastoma SH-SY5Y cells (Figures 1 and 4). Additionally, we explored various mutations within the amino acids 67-78, which were hypothesized to influence interactions between cholesterol and alpha-syn based on the findings of Crowet et al., and Fantini et al., [33,34]. However, our results indicate that these mutations in the alpha-syn sequence had no discernible impact. CYP46A1 continued to induce a significant reduction in alpha-syn levels, regardless of whether the alpha-syn variant was wild-type or mutant (Figure 2).

These findings suggest that the interplay between alpha-syn and cholesterol, particularly within the 67-78 region, is not essential for CYP46A1's ability to diminish alpha-syn protein levels.

The degradation of alpha-syn is believed to occur through multiple pathways, including macroautophagy, chaperone-mediated autophagy and the proteasome-ubiquitin system [42-44]. Our study suggests that CYP46A1 enhances the macroautophagy pathway, thereby reducing the levels of alpha-syn proteins. Specifically, we observed a significant increase in LC3B-II levels in SH-SY5Y cells overexpressing both CYP46A1 and alpha-syn proteins compared to cells overexpressing GFP and alpha-syn proteins when treated with the autophagy inhibitor BafA1 (Figure 3). These findings are consistent with prior research demonstrating that CYP46A1 promotes autophagy of mutant *ataxin-3* or the huntingtin proteins in SCA3 disease and HD, respectively [25,26,57]. Interestingly, even in the presence of autophagy inhibitors such as 3-MA or BafA1, CYP46A1 retains its ability to decrease the expression of alpha-syn. Hence, overexpression of CYP46A1 appears to be a promising therapeutic strategy for the treating accumulated alpha-syn (Figure 3). Our study did not find any effect of CYP46A1 on CMA pathway or on the proteasome-ubiquitin system for the alpha-syn degradation (Supplementary Figures 1 and 2). Overall, our data suggest that CYP46A1 degrades alpha-syn *via* macroautophagy. However, additional studies focusing on autophagy using molecular or pharmacological approaches in this cellular model could further strengthen the observed results.

Strikingly, one of the most significant genes in PD is *GBA1*, which is involved in lipid metabolism. Variants in this gene are present in 5%-20% of PD patients across different populations. It is well-documented that mutations in the *GBA1* gene can lead to decreased activity of the lysosomal enzyme Glucocerebrosidase (GCase) [30,47,58]. This phenomenon is believed to result from impaired lysosomal function, leading to the accumulation of alpha-syn [48-50]. We conducted co-transfections of SH-SY5Y cells with *GBA1* siRNA, resulting in a fourfold reduction in *GBA1* expression, in combination with human alpha-synuclein and either CYP46A1 or GFP. Our observations revealed that CYP46A1 could reduce the level of alpha-synuclein by the same efficacy, regardless of the expression of *GBA1*. These findings demonstrate that CYP46A1 maintains its ability to suppress alpha-syn expression even in the presence of impaired lysosomal function, as illustrated in Figure 4.
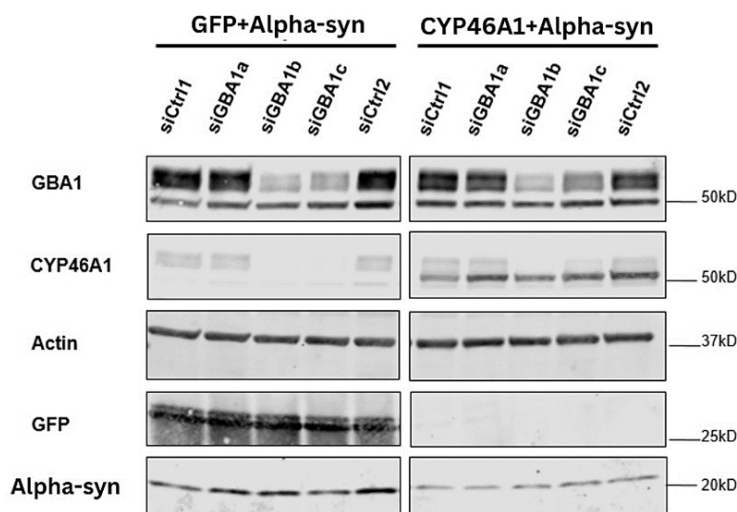
Figure 4: CYP46A1 reduces alpha-syn expression even with *GBA1* inhibited in co-transfected SH-SY5Y cells. SH-SY5Y cells were co-transfected with either siRNAs targeting *GBA1* (si*GBA1*a, si*GBA1*b, or si*GBA1*c) or two different scrambled control siRNAs (siCtrl1, siCtrl2), along with wild-type alpha-syn and either GFP (left panel) or CYP46A1 (right panel) for 48 hours. Cell extracts (20 ug) were subjected to western blot analysis using antibodies specific to human *GBA1*, HA for CYP46A1-HA, actin, GFP and human alpha-syn. The western blot analysis revealed a reduction in *GBA1* expression following transfection with *GBA1* siRNA1b and siRNA1c (lanes 3 and 4) compared to cells transfected with control siRNA (lanes 1 and 5) or *GBA1* siRNA 1 (lane 2). The presence of CYP46A1 leads to decrease in alpha-syn protein levels, regardless of specific siRNA used (right panel), compared to GFP (left panel). These findings suggest that deficiency in GCase does not impede CYP46A1 from inducing degradation of alpha-syn.

Conclusion

This study shows that the role of CYP46A1 extends beyond cholesterol clearance, promoting the reduction of overexpressed alpha-syn. Our *in vitro* results demonstrate its effectiveness across various cellular models of Parkinson's disease, including those with overexpression of alpha-syn with or without *GBA1* deficiency, or overexpression of the *A53T* mutated alpha-syn. This suggests that CYP46A1 holds promise as a therapeutic strategy for PD by mitigating the pathological effects of alpha-syn accumulation. Additional data from the author suggest that overexpression of CYP46A1 may prevent dopaminergic loss in PD. Moving forward, it will be important to ascertain in *in vivo* PD models whether CYP46A1 facilitates neuroprotection and symptom reduction in PD.

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Authors' Contributions

CBG: Conceptualization methodology, validation, formal analysis, investigation, writing-original draft, visualization.

LR and EA: Methodology, validation, formal analysis, investigation, writing, visualization.

FC: Conceptualization, resources, writing, review.

FP: Resources, writing, review and editing, supervision, funding acquisition. All authors have read and approved the final manuscript.

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