Targeting *miR-22-3p* to enhance *GBA1* expression and GCase activity in iPSC-derived models of Parkinson's disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons (DNs) in the substantia nigra and the aggregation of alpha-synuclein (α-syn) containing inclusions. Mutations in the *GBA1* gene, encoding the enzyme glucocerebrosidase (GCase), have been identified in a subset of PD patients, leading to a reduction in its overall enzymatic activity, with a reduction often observed in idiopathic patients. To date, classical therapies for GCase deficiency have been hindered by poor blood-brain barrier (BBB) permeability, limiting their efficacy. Thus, other approaches toward raising GCase levels are needed, leading us to the microRNA-22-3p (*miR-22-3p*) as a potential target to upregulate *GBA1* expression. This study aims to investigate the impact of targeting *miR-22-3p* with an antisense oligonucleotide (ASO) approach on enhancing *GBA1* expression and GCase activity in iPSC-derived DNs. Both overexpression and knockdown of *miR-22-3p* significantly modulated *GBA1* expression in control DNs. Additionally, targeting *miR-22-3p* helped to restore GCase activity in DNs expressing *GBA1* with the PD-associated L444P mutation. Taken together, targeting *miR-22-3p* holds promise for enhancing *GBA1* expression and GCase activity, offering a novel therapeutic approach for PD, particularly in patients with *GBA1* mutations.

Résumé

La maladie de Parkinson est une maladie neurodégénérative caractérisée par la perte des neurones dopaminergiques de la substance noire ainsi que par la présence, au sein de ces cellules, d'inclusions contenant des agrégats d'alpha synucléine. Pour un sous-groupe de patients parkinsoniens, la maladie est causée par des mutations dans le gène *GBA1* ; qui code pour l'enzyme glucocerebrosidase (GCase). Ces mutations conduisent à une diminution de l'activité enzymatique; une baisse par ailleurs observée dans les cas idiopathiques de parkinson. Jusqu'à présent, les stratégies thérapeutiques classiques visant à rétablir l'activité enzymatique de la GCase demeure d'efficacité très restreinte, en raison de la faible perméabilité de la barrière hématoencéphalique aux médicaments. Des stratégies alternatives sont requises et nous avons considéré le *miR-22-3p* comme cible thérapeutique potentielle en vue d'augmenter l'expression du gène *GBA1*. Ce projet vise à déterminer dans quelle mesure le ciblage du *miR-22-3p* par des oligonucléotides antisens permet d'augmenter les niveaux d'expression du gène *GBA1* et de l'activité enzymatique GCase dans des neurones dopaminergiques dérivées de cellules IPS. La sur expression aussi bien que l'atténuation du *miR-22-3p* ont permis de moduler l'expression du gène du gène *GBA1* dans des neurones dopaminergiques contrôles. Par ailleurs, le ciblage du mir22-3P a permis de restaurer l'activité GCase dans des neurones dopaminergiques de patients parkinsoniens portant la mutation L444P dans le gène *GBA1*. Nos résultats suggèrent que les stratégies de modulation de *miR-22-3p* constituent une avenue prometteuse pour la restauration de l'expression du gène *GBA1* et de l'activité GCase dans le contexte de la maladie de parkinson et plus particulièrement pour les patients portant des mutations dans le gène *GBA1*.

Table of Contents

List of Figures

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Experimental design, iPSC-derived cell culture, cell collection, processing, data analysis, text, and figures were performed by the author.

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List of Abbreviations

- 4-MUG, 4-methylumbelliferyl-beta-D-glucuronide
- α-syn, alpha-synuclein
- ASO, antisense oligonucleotide
- BBB, blood-brain barrier
- BSA, bovine serum albumin
- CNS, central nervous system
- CRISPR/Cas9, clustered regulatory interspaced palindromic repeats
- DMEM, dulbecco's modified eagle's medium
- DNA, deoxyribonucleic acid
- EB, embryoid body
- EDDU, Early Drug Discovery Unit
- ER, endoplasmic reticulum
- GBA, glucocerebrosidase gene
- GCase, glucocerebrosidase
- GD, Gaucher disease
- IF, immunofluorescence
- iPSC, induced pluripotent stem cells
- LB, Lewy bodies
- LIMP-2, lysosomal integral membrane protein 2
- LNA, locked nucleic acid
- miR, microRNA
- mRNA, messenger RNA
- PD, Parkinson's disease
- PBS, phosphate-buffered saline
- PI, Power inhibitor
- RT-qPCR, reverse transcription quantitative polymerase chain reaction
- SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
- UTR, untranslated region
- WB, western blot

1. Introduction and Rationale

Parkinson's disease (PD) is a fast-progressing neurodegenerative disease affecting the older population and characterized by a loss of dopaminergic neurons (DNs) and the formation of Lewy bodies (LBs) enriched with pathological α-synuclein (α-syn) [1]. The standard therapies manage symptoms but do not intervene with the propagation of toxic α-syn, With the disease projected to double in prevalence by 2040 due to factors such as the growing aging population, environment, lifestyle, genetics, and healthcare, there is an urgent need for novel therapies that can effectively halt the spread of α-syn and prevent further neurodegeneration [2].

Mutations in the *GBA1* gene, encoding lysosomal enzyme glucocerebrosidase (GCase), result in decreased enzyme activity and represent a common genetic risk factor for PD [3]. In addition to a genetic predisposition, reduced GCase activity is also observed in patients without the *GBA1* mutations and has even been associated with normal aging [4]. Mechanistically, the deficiency in GCase activity leads to lysosomal dysfunction and α-syn accumulation, which further interferes with GCase trafficking and promotes α-syn aggregation [5–7]. Elevating levels of GCase is shown to restore lysosomal health and reduce α-syn levels [8–10]. Given the relationship between GCase and α-syn, enhancing GCase activity offers a promising approach to restoring lysosomal function and alleviating α-syn burden, potentially slowing the PD progression.

Current therapies for GCase deficiency in Gaucher's disease (GD), the most common lysosomal storage disorder, are ineffective for CNS applications due to their inability to penetrate the blood-brain barrier (BBB). Hence, we are addressing the need for targets and molecular approaches to enhance neuronal GCase activity [11]. Several microRNAs (miRs), including *miR-22-3p*, have been identified as negative regulators of *GBA1* expression [12,13]. It is yet to be determined if the knockdown of *miR-22-3p* would have the counteractive effect of enhancing *GBA1* expression, GCase activity, and consequently, α-syn levels. Antisense oligonucleotides (ASOs), a powerful RNA editing tool, offer a novel strategy to manipulate *miR-22-3p* levels, potentially rescuing GCase activity and mitigating α-syn pathology [14]. Thus, this thesis aims to examine the *miR-22-3p*/*GBA1* regulatory network and investigate the impact of targeting *miR-22-3p* with ASOs.

2. Background

2.1 Overview of Parkinson's disease

PD is the second most common neurodegenerative disease after Alzheimer's disease affecting over 6 million people worldwide [2]. Clinically, the disease is defined as a combination of motor and non-motor symptoms [15]. At the early stage of the disease, non-motor symptoms commonly precede motor manifestations and include neurobehavioral changes, cognitive defects, disturbed sleep, sensory impairments, as well as difficulty performing autonomic functions. As the disease progresses motor symptoms appear and include bradykinesia, postural instability, shuffling gait, tremors, muscle stiffness, difficulty walking, and facial paucity [15].

The main pathological hallmark of PD is the development of LBs, intraneuronal inclusions predominately composed of misfolded α-syn that leads to neurodegeneration of DNs in the substantia nigra (SN), which projects to the basal ganglia responsible for motor control [1]. Dopamine depletion due to neuronal loss in basal ganglia leads to dysregulation of motor circuits, resulting in characteristic motor symptoms in PD [15]. Post-mortem examination and neuroimaging techniques suggest that by the time of diagnosis, patients are already likely to suffer from more than 50% loss of DNs which occurs early in the disease course, preceding the onset of overt motor symptoms [11]. During early clinical intervention, patients experience temporary alleviation of motor and neuropsychiatric symptoms. However, α-syn pathology progresses rapidly to advanced stages, where patients experience more severe symptoms and lose responsiveness to treatment, leading to significant immobility and reduced quality of life [11].

There is currently no cure for PD, and existing therapies primarily function by elevating dopamine levels in the brain. Levodopa is a naturally occurring dopamine precursor and its chemically synthesized counterpart is currently the most effective therapy for the management of motor symptoms [16]. Typically given in combination with carbidopa or benserazide, which are inhibitors of the DOPA decarboxylase enzyme responsible for the conversion of levodopa to dopamine, this combination therapy mitigates premature conversion of levodopa outside the brain and alleviates side effects such as nausea and vomiting [17]. Since the initial approval of levodopa over 50 years ago, multiple formulations of immediate-, controlled-, or time-release carbidopa/levodopa have been developed to maximize targeted delivery and minimize the

adverse side effects. In addition to oral capsules, commercial formulations of carbidopa/levodopa, such as DUOPA and Inbrija, can be administered directly into the intestine via pump or inhalation, respectively [18,19]. ND0612 is a new liquid formulation of carbidopa/levodopa designed to be delivered subcutaneously [20]. Other carbidopa/levodopa formulations in the trial include IPX203, a new extended-released oral formulation of a 1:4 carbidopa/levodopa as well as extended-release beads of levodopa alone which was submitted for FDA approval in November 2022 [21]. Levodopa is often combined with other PD medications. These include dopamine agonists, which mimic the effect of dopamine by stimulating D2-like dopamine receptors. Catechol-O-Methyltransferase (COMT) inhibitors block the COMT enzyme, preventing methylation of dopamine, while monoamine oxidase type B (MAO-B) inhibitors hinder oxidative deamination of dopamine by MAO-B enzyme [16]. Such combination therapy prevents the breakdown of dopamine in the brain, leading to higher dopamine availability, and improving PD symptoms. However, despite the efficacy of current therapies in the management of motor symptoms, their long-term use often results in higher doses of the drugs, which result in motor complications such as dyskinesia, nausea/vomiting, sleep disturbances, sedation, and hallucinations [22]. Furthermore, there are no therapies that target α-syn pathology to halt progressive neurodegeneration in PD. As such, there is an urgent need for disease-modifying treatments that can treat PD at the early stages of the disease.

The etiology of PD involves a complex interplay among aging, environmental factors, and genetics. Natural decline of cellular processes important for the maintenance of metabolically demanding neurons and clearance of misfolded proteins is associated with aging which is the greatest risk factor for PD [23]. Environmental exposure to contaminants such as pesticides, metals, and industrial by-products has been associated with PD [24]. Moreover, genetics plays a significant role in PD, contributing to both familial and sporadic cases. Variants associated with PD are grouped based on allele frequency and degree of penetrance [25]. Monogenic or familial PD (5-10% of cases) is associated with rare but high penetrance pathogenic variants that generally cause an early-onset type of disease. On the other hand, common genetic variants with low penetrance individually contribute to a small risk of sporadic PD (95% of cases). With the advent of genetic analysis techniques, genome-wide association studies have identified nearly a hundred genetic loci and variants that are linked to an increased risk of PD [26,27]. These genes include *SNCA*, *PRKN*, *PINK1*, *DJ-1*, *LRRK2* and *GBA1*.

Interestingly, most PD-related genes are involved directly or indirectly in autophagy and lysosomal pathways important for clearing intracellular protein aggregates. Dysregulation of these quality control systems is hypothesized to contribute to the accumulation of protein aggregates observed in PD [25]. Understanding how different genetic variants influence disease pathology can improve the selection of potential therapeutic targets for specific biological pathways impaired in PD.

2.2 Alpha-synuclein in Parkinson's disease

α-syn encoded by the *SNCA* gene is a presynaptic protein of 140 residues that is highly expressed in neuronal tissues, comprising 1% of total cytosolic protein [28]. Although primarily found in neural tissues, it is also expressed in red blood cells, suggesting a role in hematopoiesis [29]. During the late stage of synapse development, α-syn localizes to presynaptic terminals where it associates with synaptic vesicles. Although its function is not well understood, it has been shown that α- plays a role in vesicle formation and trafficking as well as SNARE-mediated exocytosis of neurotransmitters [30].

Mutations in *SNCA* gene were identified as the first genetic cause of familial PD which was confirmed later histologically after the discovery that α-syn is the predominant protein component of LBs [1,31]. Point mutations such as A53T induce accelerated oligomerization of the protein, whereas duplication and triplication of *SNCA* result in a dosage-dependent effect α-syn accumulation which is associated with early-onset PD with a more severe and faster progression [25]. The abnormal α-syn accumulation promotes the formation of misfolded species that subsequently aggregate into neurotoxic species in the cytoplasm [28]. Numerous studies show that α-syn overexpression can disrupt early secretory pathways, resulting in impairment of autophagy and lysosome-mediated degradation pathways important for protein turnover. Overall, α-syn aggregates are found to overwhelm cellular quality-control systems and disrupt biological processes leading to lysosomal dysfunction, ER stress, and mitochondrial impairment [33]. To alleviate cellular stress, affected neurons release α-syn species into brain parenchyma which can further promote the spreading of α-syn pathology to neighboring neurons and glia [34]. Moreover, aggregated α-syn released from neurons can elicit an innate immune response as a damage-associated molecular pattern, leading to activation of the NF-kB pathway and NLRP3

inflammasome priming and ultimately the release of pro-inflammatory mediators [35,36]. Chronically activated microglia can facilitate the spreading of α-syn aggregates via exosomal release, further exacerbating the PD pathology [37]. In a prion-like manner, aggregated α-syn spreads from cell to cell and promotes neuroinflammation and neurodegeneration [38]. Developing strategies that can reduce α- levels and aggregation into toxic fibrils is of importance for PD therapies.

Many other PD-related mutations can predispose neurons to adopt pathobiological processes that further facilitate the development and spreading of α-syn pathology. The next section of this review will describe the role of *GBA1* in PD pathogenesis.

2.3 Lysosomal function and its role in diseases

Lysosomes are membrane-bound organelles that serve as a digestive system of the cell and play essential roles in cellular homeostasis, immune defense, energy metabolism, repairing, synaptic function, and plasticity [39–42]. Formation of lysosomes involves the fusion of late endosomes with transport vesicles from the *trans*-Golgi carrying acid hydrolyses via mannose-6-phosphate receptor [43,44]. During maturation into lysosomes, late endosomes undergo a process of acidification via ATP-dependent transport of protons into the lysosomal lumen via proton pumps [45]. Acid hydrolyses dissociate from manosse-6-receptors in a pH-dependent manner, and the lysosomes finally mature as they acquire all functional components secreted from the Golgi [44]. Lysosomes contain over 50 different hydrolytic enzymes and other microbicidal substances that can degrade different macromolecules including proteins, nucleic acids, complex carbohydrates, and lipids [44,46]. The acidic pH of 5 within lysosomes ensures that the activity of enzymes is spatially restricted to lysosomes, thus protecting the rest of the cell from potential damage [43–45].

Lysosomes are crucial for maintaining cellular homeostasis by breaking down and recycling damaged or misfolded proteins, old organelles, and other cellular debris via autophagy-lysosomal pathways [42–44]. In autophagy, old or damaged organelle such as mitochondrion is enclosed by the ER membrane, forming vesicle autophagosome which fuses with lysosomes for digestion [41,43,44]. The byproducts of degradation, such as amino acids and sugars, are transported out of lysosomes to the cytosol for re-use by the cell. Moreover, lysosomes also play pivotal role in phagocytosis, wherein immune cells including macrophages and neutrophils engulf and

degrade pathogens, cellular debris, and apoptotic cells [40,44]. This process is essential for innate defenses, tissue remodeling, and cellular homeostasis. Phagocytosis starts with the engulfment of foreign particles into a phagocytic vesicle, or phagosome, followed by its fusion with a lysosome to form a phagolysosome [40,44]. Inside of phagolysosome, lysosomal enzymes such as proteases, lipases, nucleases, glycosidases and other microbicidal substances degrade the engulfed material, with the resulting byproducts exported to the cytosol for recycling.

Given the biological importance of lysosomes in cellular health, it is not surprising that deficiency in a single lysosomal enzyme can have a negative impact on its digestive abilities, resulting in the accumulation of ungraded substrates, lysosomal dysfunction, cellular stress, inflammation, and apoptosis [47,48]. Lysosomal storage diseases are a heterogeneous group of about 50 genetic diseases characterized by lysosomal dysfunction due to mutations in genes encoding lysosomal enzymes, proteins, receptors, or transporters [44,47]. For example, GD arises from a deficiency in GCase, leading to the accumulation of glucocerebrosides in macrophages, causing organomegaly, bone disease, and, in a severe form of the disease, neurological involvement [47]. Neurons, being long-lived and metabolically demanding cells, heavily rely on lysosomes to process large amounts of metabolic wastes and thus are particularly sensitive to defects in lysosomal function [45,49]. In the context of neurodegenerative diseases, lysosomal dysfunction, accumulation of misfolded proteins, damaged organelles and cellular substrates may further exacerbate cellular stress and disease pathology. GBA1-associated PD is a well-characterized example of the role of lysosomal dysfunction in PD that will be explored next.

2.4 *GBA1* in Gaucher disease and Parkinson's disease

The *GBA1* gene, located on chromosome 1q21, encodes the lysosomal enzyme GCase that is ubiquitously expressed throughout the body. GCase is responsible for the hydrolysis of glycosphingolipids such as glucocerebrosides into glucose and ceramide [50]. Synthesis and processing of GCase occurs in the rough endoplasmic reticulum (ER), where it binds to lysosomal integral membrane protein-2 (*LIMP-2/SCARB2*) for transit through the Golgi for further processing. Once delivered to the lysosome, GCase is released from LIMP-2 due to low pH and associates with its co-factor Saposin C for effective hydrolysis of glucosylceramide and glucosylsphingosine into ceramide and sugar [51].

The proper expression of *GBA1* is essential for development with double knockout of *GBA1* results in neonatal lethality in mice due to severe systemic and neural abnormalities [52]. Over 300 mutations ranging from missense to translocation events have been found in the *GBA1* genes with key phenotypical characteristics of lysosomal dysfunction due to substrate accumulation, inflammatory response, and neurological symptoms [53]. Deficiencies in GCase due to homozygous *GBA1* mutations result in the accumulation of glucosylceramide substrates within the lysosomes and the development of the most common lysosomal storage disease, GD. The disease is characterized by lipid-laden macrophages that accumulate in the spleen (hepatosplenomegaly), liver, and bone marrow, causing organ enlargement and inflammation [1]. Phenotypically, the disease is classified into three broad types based on the involvement and severity of neurological symptoms [55]. Type 1 non-neuronopathic GD (caused by mild mutations) is the most common form of the disease wherein patients may range from asymptomatic to those who develop hepatosplenomegaly, anemia, and thrombocytopenia (low platelets count). Type 2 acute neuronopathic GD (severe) is a fetal/neonatal form of the disease wherein infants are born with collodion skin and severe swelling. Due to rapidly progressive neurodegeneration, this form of the disease is usually fatal within the first three years of life. Lastly, type 3 (severe) chronic neuronopathic GD is characterized by hepatosplenomegaly, anemia, thrombocytopenia, and bone involvement although CNS involvement may develop later in life with less aggressive progression [55]. Although GD is considered a simple monogenic disorder, it is a highly heterogeneous disease wherein the relationship between the genotype and clinical phenotype is not straightforward [56]. Patients with identical genotypes can have different clinical phenotypes, whereas those with different genotypes can exhibit the same symptoms [57]. This suggests that additional elements and/or modifier genes are likely to be involved in the modulation and regulation of lysosomal GCase (e.g., *SNCA*, *TMEM175*, *SCARB2*, *CTSB*, *PSAP*).

Moreover, the classification of Type 1 forms of GD as non-neuronopathic has been challenged with cases where patients developed peripheral neuropathies as well as parkinsonism. Clinical cases of GD patients with PD promoted an investigation into *GBA1* after noticing an occurrence of PD in direct relatives of GD patients carrying heterozygous *GBA1* mutations [50]. Heterozygous mutations in *GBA1* represent the most common risk factor for PD. Approximately, 5-10% of sPD patients worldwide have *GBA1* mutations with increased prevalence in the Ashkenazi Jewish population up to 20% [3,58]. Approximately 300 *GBA1* mutations have been

identified in GD which can be categorized as mild (e.g., N370S) or severe (e.g., L444P). Mild and severe *GBA1* mutations confer differential risk of PD with odds ratios among *GBA1* mutation carriers ranging from 3.0-4.7, and 14.6-19.3, respectively [58]. Moreover, severe *GBA1* mutations are associated with a 5-year earlier age of onset and a higher risk for dementia compared to mild mutations. GBA1-PD patients have reduced GCase enzyme activity, with the greatest reduction observed in SN [59]. Interestingly, GCase activity is also reduced in PD patients without *GBA1* mutations and a reduction in GCase has been associated with normal aging [4].

Several mechanisms by which *GBA1* mutations impact GCase and promote α-syn pathology have been proposed [60]. There is strong evidence for a reciprocal relationship between GCase activity and α-syn levels wherein GCase dysfunction causes α-syn accumulation [5]. It is hypothesized that loss of GCase activity leads to the accumulation of undegraded substrates, leading to lysosomal dysfunction and impairment in autophagy-mediated α-syn turnover [6,8]. This results in α-syn accumulation and aggregation in the cytoplasm, subsequently inhibiting the trafficking of GCase to the lysosome. Accumulation of lipid substrates due to loss of GCase is also speculated to stabilize α-syn, promoting its oligomerization [61]. In addition to the bidirectional feedback loop between GCase and α-syn, the gain-of-function hypothesis proposes that misfolded mutant GCase are retained in the ER, promoting ER stress, activation of unfolded protein response (UPR), and α-syn accumulation [62].

Interestingly, enhancing GCase activity has been found to restore lysosomal function and reduce α-syn levels and aggregation [8]. Small molecule modulation of GCase enhanced lysosomal enzyme activity in iPSC-derived dopaminergic neurons with *GBA1*, *SNCA*, or *PARK9* mutations, which resulted in the reduction of lysosomal substrates and clearance of α-syn regardless of the disease-causing mutation. Given the reciprocal relationship between GCase activity and α-syn levels, enhancing GCase activity to decrease α-syn aggregates levels offers a potential therapeutic target for therapeutic development in PD.

2.5 GCase enhancing treatments

Traditional treatments for enhancing GCase function include enzyme-replacement therapy or substrate-reduction therapy [63]. Although recombinant enzyme has been used to elevate GCase levels in GD since the early 1990s, its inability to cross the BBB limits its application for

the treatment of PD [11]. Substrate-reduction therapy involves inhibition of glucosylceramide synthase, which catalyzes glucocerebroside synthesis, but its use for the treatment of GBA1-PD has shown limited success due to off-target side effects and poor permeability [63].

Pharmacological chaperones have been designed to stabilize and refold mutant misfolded GCase, thus promoting its trafficking through the ER and lysosome [11]. Small molecular chaperones such as ambroxol have shown some success at targeting the *GBA1* pathway. Numerous *in vitro* and animal studies report that ambroxol treatment increased brain GCase activity and reduced α-syn protein levels [9,64,65]. Moreover, high-dose ambroxol treatment was shown to cross the BBB and increase GCase levels in PD patients with or without *GBA1* mutation [66]. Lastly, gene therapy has shown promise in enhancing GCase levels and activity. Studies have shown that virally overexpressed *GBA1* increased GCase levels and reduced α-syn accumulation in mice overexpressing *SNCA* or with A53T mutation [67,68]. With the growing success of nucleic acid therapies, multiple clinical tests of *GBA1* gene therapy in PD patients have been initiated and show early promise as a potential targeted PD therapy. However, given the high failure rate of target candidates, once they enter the clinical PD trials, it is paramount to continue exploring new therapeutic avenues that can enhance *GBA1* expression. The strategic choice of potential targets requires a good understanding of the *GBA1* pathway and its regulatory agents.

2.6 Overview of microRNAs

miRs are a small (18-25 nucleotides) class of endogenous, highly conserved, non-coding RNAs that function as master regulators of target gene expression in plants and animals at post-transcriptional level [69]. Genomic studies identified over a thousand genes encoding miRs, accounting for about 3% of the total human genome [70,71]. Moreover, it has been estimated that over a third of protein-coding genes in the human genome are regulated by miRs either directly or indirectly, with an average of about 200 mRNAs targeted by a single miR [72–74]. miRs have been found to play a role in almost all biological functions including are cell growth, proliferation, apoptosis, immune response, tumorigenesis and suppression, autophagy, metabolic regulation, and neurodevelopment [69,75–81].

miR biogenesis starts with transcription into primary miRNA transcripts, followed by processing into precursor miRNAs, and maturation into guide strands [82]. Once a mature miRNA guide strand is loaded onto the RNA-induced silencing complex (RISC), it directs the silencing machinery to its target mRNA which contains miR binding sites called miR recognition elements (MREs) in the 3' untranslated region (3'UTR). The extent of complementarity between the seed regions of RISC-loaded miR and mRNA's MREs influences the mechanism of gene silencing with a near-perfect match resulting in mRNA degradation and imperfect match typically leading to translational repression [82]. In addition to negative regulation, miRs can also function as activators, enhancing transcript and protein levels via a variety of proposed mechanisms including interactions with promoter regions, stabilization of mRNA by blocking destabilizing proteins, via downregulation of proteins that negatively regulate transcription factors [13].

In addition to their impact at inter and intracellular levels, miRs can function in an interspecies manner. Due to their highly conserved nature, miRs can regulate gene expression in mammals, insects, plants, and even microbes [83]. In mammals, exogenous miRs from plant-based food are absorbed via the digestive tract and delivered into cells to exert a regulatory function on gene expression and/or biological processes [84]. For example, miRs within exosome-like nanoparticles from ginger were found to inhibit the expression of SARS-CoV-2 genes, induce IL-22 production, and ameliorate mouse colitis [85–87]. In addition to plant-derived miRs, mammalian miRs might also constitute a human diet [84]. For example, milk contains both plant-derived and endogenous mammalian miRs that function as a nutritional component [88,89]. While some studies suggest that certain miRs can resist degradation during cooking and digestion, others disagree with the research on food-derived miRs still ongoing with impact on human health [84,90,91].

2.7 miRs role in diseases

Given the high conservation of miR and their involvement in nearly all biological functions, it is no surprise that dysregulation in miR expression is associated with human diseases including viral infections, cancers, metabolic, and neurodegenerative diseases [79,92–96]. For example, *miR-122* is a liver-specific miR involved in hepatic development and regeneration, lipid, and glucose metabolism [97]. Interestingly, *miR-122* is also crucial for the viral life cycle of HCV, wherein binding of *miR-122* to the 5' UTR of the viral genome results in the stabilization

of viral RNA and enhanced replication. Miravirsen is a LNA inhibitor of *miR-122* developed for the treatment of chronic hepatitis C [97]. Due to sequence complementarity, miravirsen binds to miR, preventing its interaction with HCV RNA. The inhibitor-induced knockdown of *miR-122* has the consequence of destabilizing viral RNA, thus inhibiting viral replication. Miravirsen is the first miR-targeting therapeutic reaching clinical trials, with phase I trials demonstrating significantly reduced viral load [97]. As such development of miravirsen provided strong proof-of-concept for targeting miRs in therapeutic applications.

miRs are also involved in the development, progression, and metastasis of various cancers [79–81]. For example, *miR-21* acts as an oncogene, downregulating tumor suppressor genes such as PTEN and contributing to the development of hepatocellular carcinoma [98]. In addition to acting as oncogenes, miRs can also function as tumor suppressors (e.g., *miR-34a* whose levels are often downregulated in cancers) [99]. Many metabolic diseases are also associated with aberrant miR expression. For example, *miR-122* is the most abundant liver miR playing a critical role in lipid metabolism by regulating cholesterol levels [100]. Reduction in *miR-122* is associated with disrupted lipid homeostasis, fat accumulation in the liver, and non-alcoholic fatty liver disease (NAFLD) [101].

The reports of dysregulated miRs in CNS diseases are growing with many miRs identified to affect inflammatory, neuronal survival, or other intra- and intercellular properties in the context of AD, PD, and ALS[102]. For example, *miR-21* is upregulated in the serum of AD patients and associated with neuroinflammation. Finally, miRs have been found to be differentially expressed in prion disease, affecting the expression of prion-related proteins [103–105].

2.8 GBA1-regulatory miRs

The research on miRs has prompted screening for miR candidates that could modulate GCase activity. In 2014 Siebert et al. identified three miRs (*miR-127-5p*, *miR-19a-5p*, and *miR-1262*) in healthy HEK cells that downregulated GCase activity indirectly by acting on the expression of *GBA1* gene modifiers such as *SCARB2* and *PSAP* [13]. In addition to miRs that negatively regulate GCase activity, positive regulators were also identified (*miR-16-5p* and *miR-195-5p*) and were shown to up-regulate GCase activity more than 40% [13].

In working to elucidate the mechanisms regulating *GBA1* expression, Straniero et al. identified and functionally validated *miR-22-3p* as a negative regulator of *GBA1* and pseudogene *GBAP1* [12]. They found that *miR-22-3p* overexpression in HEK cells resulted in up to 70% reduction in *GBA1* and *GBAP* mRNA levels with a substantial decrease in GCase protein levels after 48 and 96 hrs. The transcriptional repression by miR-22-3p results from miR binding to the miRNA responsive element in the 3' UTR of the *GBA1* and *GBAP* mRNAs [12]. Furthermore, it was confirmed that the genes of *miR-22-3*/*GBA1*/*GBAP* regulatory network are expressed in iPSCs and iPSC-derived DNs from control and PD patients with heterozygous *GBA1* mutations (L444P and N370S) [12].

Although the study identified *miR-22-3p* as GBA1-regulating miR, it has not been yet investigated if a knockdown of *miR-22-3p* would have an opposing effect on enhancing GCase expression. *miR-22-3p* has been reported to be involved in numerous biological processes including cell proliferation, differentiation, and apoptosis and has been implicated in different types of cancers including breast, liver, and colorectal [75–77,80,94,106] . Moreover, transcriptomics studies investigating differentially expressed miRs in blood samples from PD patients report upregulated levels of *miR-22-3p*, although some report down-regulation [107–114]. As such, there is a further need to investigate the role of *miR-22-3p* and its expression profiling in the context of healthy cell and disease pathology. Given the role of *miR-22-3p* in repressing *GBA1* expression, this project is focused on investigating the impact of *miR-22-3p* knockdown on GCase enzyme activity in healthy and GBA1-PD iPSC-derived models. Strategies for targeting miRs will be investigated further.

2.9 Overview of antisense oligonucleotide

ASOs are single-stranded RNA or DNA molecules between 12 and 30 nucleotides in length that have emerged as a potent tool for targeting RNAs, offering treatment for a wide range of diseases [115]. They selectively bind to the target RNA through complementarity and Watson-Crick base pairing, forming stable duplexes with the target RNA. ASOs can modulate gene expression through several mechanisms of action. One mechanism is RNA degradation by endogenous nucleases such as ribonuclease H1 (RNase H1) which recognize any DNA/RNA heteroduplex [115]. This leads to cleavage and degradation of the RNA target while freeing the

ASO to bind to a different copy of RNA. Another mechanism by which ASO can target gene expression is through RNA steric hindrance by blocking sequences involved in splicing, translation, or binding sites for gene regulators [116]. ASOs are commonly used to alter splicing patterns by promoting the inclusion or exclusion of specific exon sequences which leads to different protein isoforms. At the pre-mRNA stage, ASO can destabilize the precursor RNA by preventing capping or polyadenylation at 3' and 5', respectively [4]. ASOs can also inhibit translation by binding the start codon or other critical regions on the mRNA, sterically hindering ribosome from binding to the mRNA [117]. In addition to translational silencing, ASOs can enhance the translation of mRNA by binding to upstream open reading frames or masking MRE of the mRNA, thereby preventing regulatory agents such as miRNAs from repressing the RNA [118].

Advancements in drug design, chemical modifications, and therapeutic applications have led to three generations of ASOs with increasingly improved pharmacokinetic (e.g., increased stability and half-life) and pharmacodynamic properties (e.g., target engagement and reduced off-target effects), leading to more effective and safer therapies[14,115,119]. The first-generation ASOs, synthetic deoxyribonucleotides, provided the proof-of-principle for the use of ASOs for translational silencing via activation of RNase H1-dependent degradation of RNA-DNA duplexes [115]. The second generation of ASOs involved the substitution of the non-bridging oxygen atoms with methyl, sulfur, and amine groups to improve stability and solubility [115,120]. These chemical modifications led to ASOs with methyl-phosphonates, phosphorothioates (PS), and phosphoramide backbones with increased resistance to nuclease degradation, thus improving its stability in a biological environment. In addition to the backbones, the second-generation ASOs can be endorsed with 2'-O-methyl (2'-OMe) or 2'-O-methoxyethul (2'-MOE) at the ribose sugar which significantly reduces toxicity, enhances nuclease resistance, and increases target engagement [115,121]. Interestingly, ASO's mechanism of action can change depending on the chemical modification. For example, additions of 2'-MOE are known to inhibit translation by sterically hindering of ribosomal machinery, while PS-based ASOs can promote an RNase H1-mediated degradation of the target RNA[115,122]. The third generation of ASOs has improved pharmacokinetic properties due to backbone modifications, including changes in the furanose ring, and conjugation with lipids, peptides, and sugars to facilitate targeted delivery to specific tissues or cells [122]. Locked nucleic acid (LNA) is one of the third-generation ASOs with a

methylene bridge connecting the 2'-oxygen and 4'-carbone of the ribose ring [123]. The resulting locked conformation improves binding affinity to the target RNA and enhances its resistance to nucleases. LNA mediates their function by tightly binding to the target RNA, sterically blocking mRNA translation or miR function. Compared to the second-generation, LNAs, and other third-generation ASOs, results in greater inhibition of target RNA at lower doses [124]. Despite their high specificity and effectiveness, LNAs have shown a potential to induce hepatotoxicity [125]. As such it is important to optimize the ASOs to minimize the risks of toxicity while retaining specificity to ensure patient safety.

2.10 Antisense oligonucleotide therapies for neurological diseases

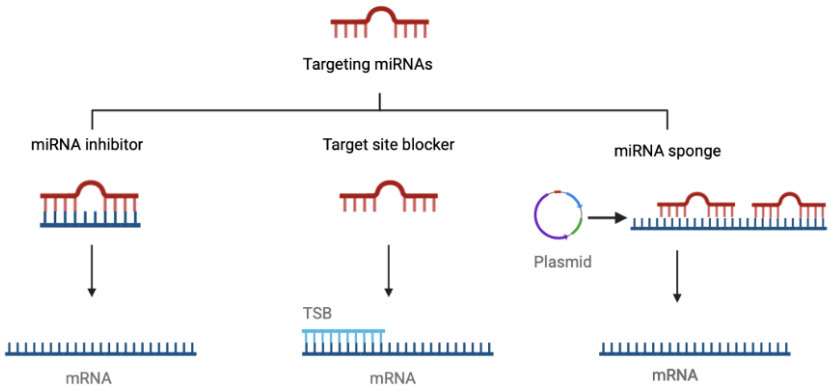
ASO technology has proved to be a targeted approach to address the underlying genetic defect, offering hope for patients with devastating conditions [14]. SMA is a pediatric neuromuscular disease that was considered fatal until the development of nusinersen (Spinraza) [116]. SMA arises due to mutations affecting the survival motor neuron 1 (*SMN1*). In addition to *SMN1*, humans also have a nearly identical copy of *SMN1*, and *SMN2*. Both genes are located on chromosome 5 with the difference of a single nucleotide change within exon 7 resulting in the skipping of exon 7 [116]. The consequence is skipping of exon 7 which is required for production of functional SMN protein hence, producing a truncated non-functional SMN2 protein. The inclusion of exon 7 in SMN2 pre-mRNA can generate functional SMN protein and offer a potential therapeutic avenue [116]. Nusinersen is a 2'-MOE and PS ASO designed to leverage this mechanism. By selectively modifying the splicing of SMN2 pre-mRNA, Nusinersen promotes the inclusion of exon 7, thereby enhancing the production of functional SMN protein [126]. Intrathecal administration of nusinersen in a cohort of SMA patients, predominantly comprising pediatric population resulted in a safe and dose-dependent increase of SMN protein in the spinal cord of SMA patients with the best clinical outcomes observed when nusinersen is administered early in the disease course [126].

The FDA approval of nusinersen in 2016 demonstrated the potential of ASOs to target genetic causes of diseases and opened doors for exploring similar approaches for other conditions. ALS is a fatal neurodegenerative disorder characterized by a progressive loss of motor neurons in the lower spinal column. The etiology of ALS is still unclear, but mutations in

genes such as the *SOD1* were identified in patients with the familial forms of ALS [127]. Tofersen (Qalsody) is a 2'-MOE and PS ASO that targets SOD1 mRNA via RNase H1-mediated degradation. Intrathecal administration of the drug was found to reduce SOD1 protein and neurofilament light chain, a biomarker of injured axons [128]. Tofersen was recently granted accelerated approval for the treatment of ALS patients with *SOD1* mutations. Overall, ASO technologies have shown a proof-of-principle and encouraged a spurge of ongoing clinical trials for other neurodegenerative diseases such as multiple sclerosis (ATL1102 targeting SNCA mRNA), PD (BIIB094 and BIIB101 targeting LRRK2 mRNA), and Alzheimer's disease (IONIS MAPTRx targeting MAPT mRNA) [116]. The ASO-based therapies offer long-lasting effects with minimized side effects and precision, enabling a personalized approach to treatment.

2.11 ASO-based strategies for targeting miRs

Current ASO-based methods for targeting miRs include miR inhibitors, Target Site Blockers (TSBs), and miR sponges (**Figure 1**). miR inhibitors, also called antagomiRs depending on the chemical modification, have a sequence complementary to the miRs of interest which results in the formation of a stable ASO:miR heteroduplex [124]. Depending on the extent of complementarity, binding between the inhibitor and miR can lead to either degradation or sequestration of the miR into P bodies [119]. An indirect approach to targeting miR is the use of TSB, also called miR masks [129]. These ASOs bind to the MRE of the mRNA, thereby sterically hindering the miR from accessing and inhibiting the mRNA. With a proper ASO design, the interaction between TSB and mRNA should not affect its translation into protein. Additionally, the miRNA sponge functions as endogenous competitive RNA that sequesters miRs [130]. This is a plasmid-based method where transgene is transcribed to have multiple MRE sites, effectively binding several miRs at once. Each of these strategies has advantages and limitations with the choice and design of ASO varying depending on the experimental purpose, practical limitations, and therapeutic applications (**Figure 1**). For this project, we chose to use an ASO-based inhibitor to investigate the consequences of direct knockdown of *miR-22-3p* on *GBA1* expression.



	miRNA inhibitor	Target site blocker	miRNA sponge
Advantages	• Specific inhibition of target miR • Easy design and delivery	• Blocks binding site on mRNA • Customization • Lower risk of off-target effects	• Function as a decoy to sequester multiple miRs • Versatility
Disadvantages	• Limited customization • Potential off-target effects	• Design complexity • Potential for unintended interactions with endogenous RNA	• Design complexity • Delivery challenges • Potential off-target effects

Figure 1. Advantages and disadvantages of ASO-based strategies for targeting miRs. Created with BioRender.

3. Project Objective and Aims

Given the evidence of a strong relationship between GCase deficiency and α-syn aggregation, enhancing GCase offers potential avenues to alleviate the lysosomal burden and α-syn pathology in PD [5,6,8]. As such. identifying and targeting potential players of the *GBA1* pathway can offer new strategies for GCase-enhancing therapy for PD.

miR-22-3p, a confirmed negative regulator of *GBA1* expression, presents an attractive target whose levels can be manipulated by ASO-based approach to modulate GCase and subsequently α-syn levels (**Figure 2**). We hypothesize that an ASO-based *miR-22-3p* mimic would artificially increase levels of *miR-22-3p* (***miR-22-3p* overexpression**), thereby repressing *GBA1* expression and further exacerbating α-syn accumulation and aggregation [12]. On the other hand, we expect that *miR-22-3p* inhibitor would form a stable ASO-miRNA heteroduplex, thereby preventing the interaction with MRE at the *GBA1* mRNA and inhibiting miRNA function (***miR-22-3p* knockdown**). Derepressing *GBA1* transcript is expected to cause upregulation of GCase levels and activity and rescue α-syn pathology.

Based on this idea, we propose that modulating *miR-22-3p* levels via an ASO-based mimic and inhibitor in iPSC-derived dopaminergic progenitors and neurons from GBA1-PD patients can: (1) modulate *GBA1* levels and GCase activity, (2) regulate α-syn levels by modulating GCase activity. Enhancing GCase expression is expected to ameliorate α- syn pathology in the context of PD.

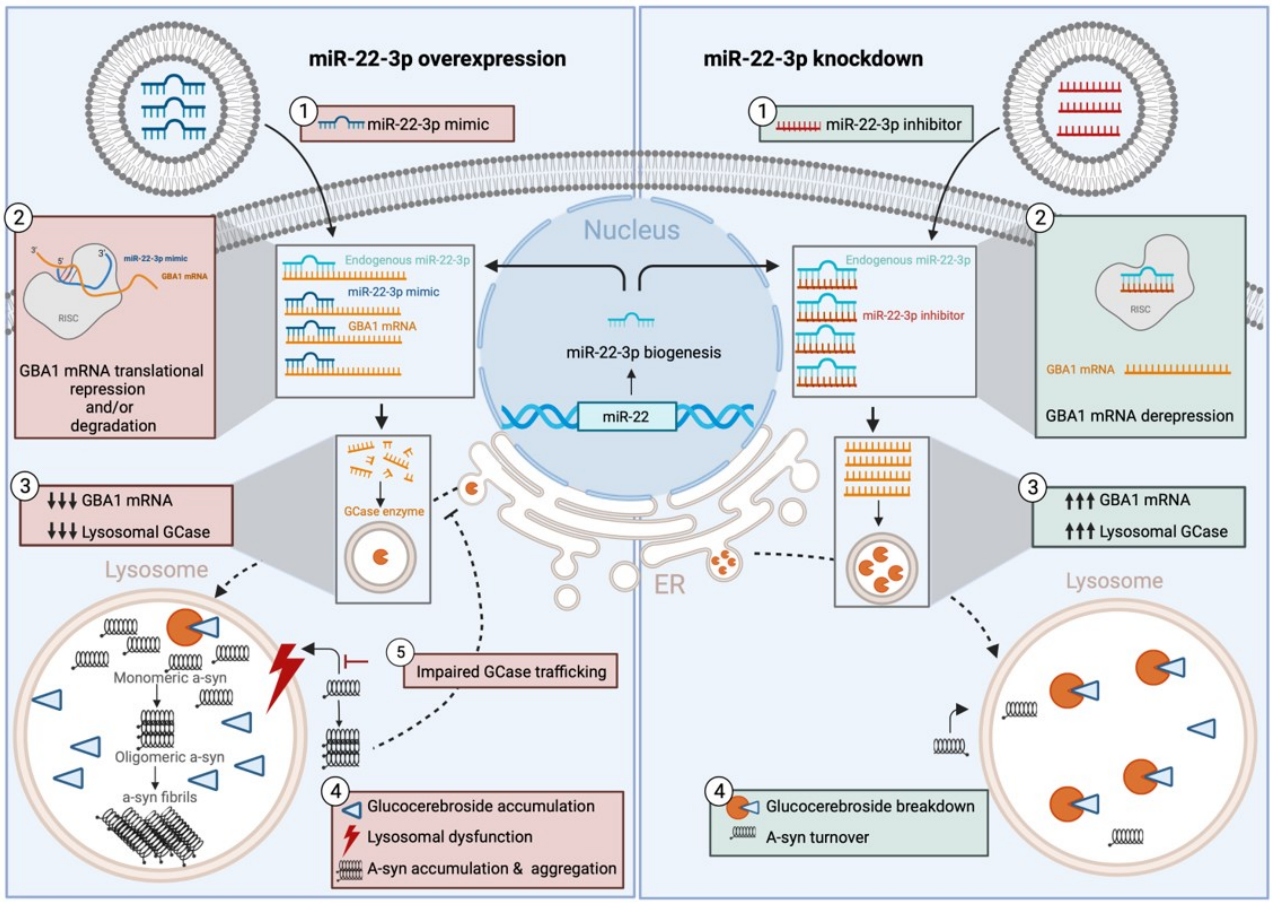


Figure 2. Proposed effect of miR-22-3p modulation (overexpression and knockdown) on GCase activity and α-syn levels. Created with BioRender.

The specific aims of this research project were as follows:

Aim 1: Establish whether modulating miR-22-3p directly influences GCase expression and activity in control iPSC-derived progenitors and neurons.

Aim 2: Investigate whether modulating miR-22-3p modulates GCase expression and activity in iPSC-derived dopaminergic neurons from PD patients with GBA1-L444P mutation.

Aim 3: Determine whether targeting miR-22-3p impacts α-syn levels and aggregation in GBA1-PD iPSC-derived dopaminergic neurons.

4. Methods

4.1 Induction and differentiation of iPSC-derived progenitors and neurons

The iPSC protocols were adapted from EDDU methods [131–133]. Briefly, iPSCs were cultured on mTeSR (STEMCELL) for at least two passages and seeded as a single cell suspension in a microfabricated embryoid body (EB) disk device (eNUVIO) to generate EBs (**Figure 3**). Following a week of culture in dopaminergic EB media, the EBs were replated and induced into neural rosettes using dopaminergic induction media (**Table 1**). Dopaminergic progenitors were then harvested from neural rosettes and cultured in dopaminergic progenitor media. Finally, progenitors were cultured for one week in differentiation media to obtain 1 week-old dopaminergic neurons.

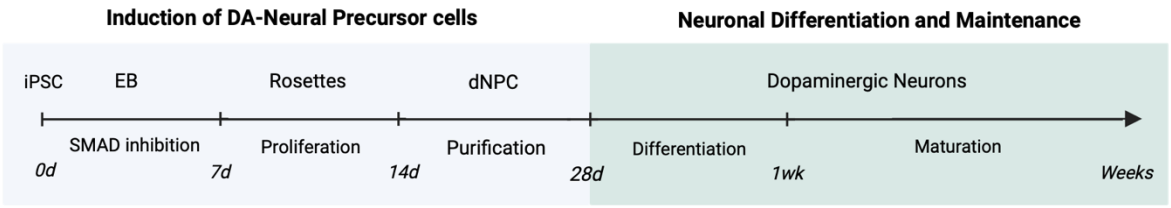


Figure 3. Workflow for iPSC induction into dopaminergic neural precursor cells (dNPCs) and differentiation into DNs. Created with BioRender.

Table 1 of cell culture media for induction and differentiation of dopaminergic progenitors and neurons from iPSCs.

Media	Components
Dopaminergic EB media	• DMEM/F12 • 1 x N-2 • 1 x B-27 • 1 x MEM NEA solution

	• 200 ng/mL Noggin • 200 ng/mL SHH • 1 uM CHIR-99021 • 10 uM SB431542 • 100 ng/mL FGF-8
Dopaminergic induction media	• DMEM/F12 • 1 x N-2 • 1 x B-27 • 1 x MEM NEA solution • 200 ng/mL Noggin • 200 ng/mL SHH • 3 uM CHIR-99021 • 10 uM SB431542 • 100 ng/mL FGF-8
Dopaminergic progenitor culture media	• 1 x STEMdiff Neural Basal Medium • 1 x STEMdiff Supplement A (50X) • 1 x STEMdiff Supplement B (1000X) • 1:1000 Purmorphamine
Differentiation media	• Neurobasal • 1 x N-2 • 1 x B-27 • 20 ng/mL BDNF • 20 ng/mL GDNF • 200 uM AA • 0.5 mM db-cAMP • 0.1 uM Compound E • 1 ug/mL laminin (Invitrogen)

4.2 miR-22-3p LNA mimic and inhibitor

miR-22-3p miRCURY mimic (LNA), Power inhibitor (DNA/LNA mixmer), and their respective scrambled negative controls were purchased from QIAGEN and resuspended per the manufacture's recommendations (**Table 2**). For the delivery method, we used RNAiMax Lipofectamine (Life Sciences) as a transfection reagent in conjunction with Opti-MEM Reduced-Serum Medium (Gibco). The workflow for transfection was adapted from Lipofectamine RNAiMax guidelines (**Figure 4**). Briefly, the day before transfection (Day 0), iPSC-derived progenitors were seeded at a density of 20,000 cells/cm^2 and 50,000 cells/cm^2 for progenitors and neurons, respectively. On Day 1 of transfection, miRCURY ASOs and Lipofectamine RNAiMax were diluted with Opti-MEM media, vortexed for 15 seconds, and then combined.

The ASO-lipid complex was left undisturbed for 20 min at room temperature, and then dispensed as seeds in a spiraling motion. Complete media change was performed the following day (Day 2). Following 48- or 72-hours post-transfection, progenitors or neurons, respectively, were collected for RNA and protein extraction. To estimate the transfection efficiency, ASOs can be ordered labeled with FAM, enabling visualization under the microscope.

Table 2 lists miRCURY LNA products (standard desalted) purchased from QIAGEN.

miRCURY LNA product	Product sequence 5' – 3'
Hsa-miR-22-3p miRNA Mimic (5)	AAGCUGCCAGUUGAAGAACUGU
Negative Control 5 miRNA Mimic (5)	GAUGCUACGGUCAAUGUCUAAG
Hsa-miR-22-3p Power Inhibitor (5)	C*A*G*T*T*C*T*T*C*A*A*C*T*G*G*C*A*G*C*T
Power Inhibitor Negative Control B	A*G*A*G*C*T*C*C*C*T*T*C*A*A*T*C*C*A*A

NB: miRCURY LNA miRNA Power Inhibitor are synthesized with alternating phosphorothioate bonds indicated by "*" in the product sequence.

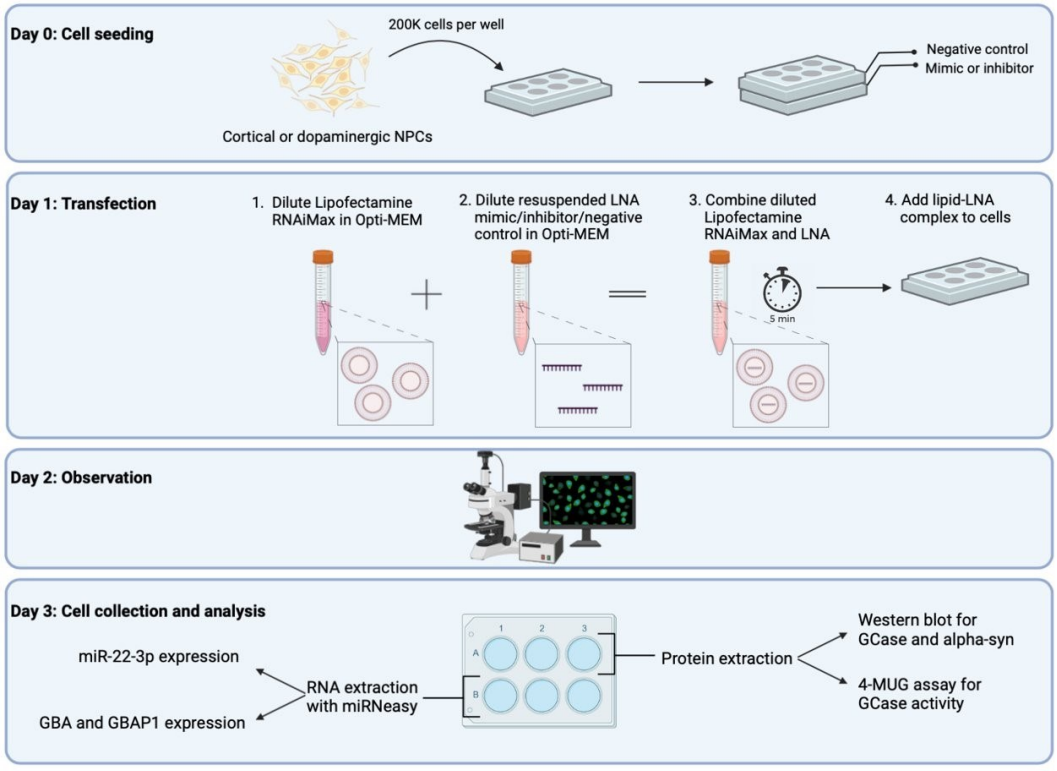


Figure 4. Schematic of the transfection experiment. Created with BioRender.

4.3 RNA isolation, cDNA synthesis and qPCR

RNA extraction of iPSC-derived progenitors and neurons was performed with miRNeasy RNA extraction kit (QIAGEN). For cDNA synthesis, *GBA1* and *miR-22* RNA were synthesized using iScript Reverse Transcription Supermix (BioRad) and TaqMan MicroRNA assay (Applied Biosystems), respectively. qRT-PCR for *GBA1* and *miR-22* was performed on QuantStudio5 Real-Time PCR System (Applied Biosystems) using PowerUp SYBR Green Master Mix (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied Biosystems), respectively. The final qPCR reaction volume was 10 uL with 5 uL of master mix, 0.5 uL primer mix, 1 uL of cDNA, and 3.5 uL of RNase-free water. The list of primers for the *GBA1* (IDT) and *miR-22* (ThermoFisher) with endogenous controls are listed in **Table 3** and **Table 4**, respectively. Relative gene expression was analyzed by Comparative CT Method (RQ ddCT) [134]. The *GBA1* expression was normalized to *GAPDH* and *ACTB*, whereas the *miR-22* expression was normalized to *RNU6B*. The Power inhibitor condition was further normalized to negative control for relative quantification. **Table 5** lists primers used for qPCR profiling for cell type markers.

Table 3 lists qPCR primers purchased from IDT.

Gene name	Exon location	Primer sequence 5'-3'	Assay ID
GBA (NM_001005741)	1 – 2c	TTCGTTTTGCCTCCGGTT	Hs.PT.58.40746061
		AGAGTCTCTGAAGGATAGAGGAT	
ACTB (NM_001101)	1 - 2	ACAGAGCCTCGCCTTTG	Hs.PT.39a.22214847
		CCTTGCACATGCCGGAG	
GAPDH (NM_002046)	2 - 3	ACATCGCTCAGACACCATG	Hs.PT.39a.22214836
		TGTAGTTGAGGTCAATGAAGGG	

Table 4 lists of TaqMan microRNA Assays purchased from ThermoFisher.

Gene name	miRNA sequence	Assay ID
Hsa-miR-22 (MIMAT0000077)	AAGCUGCCAGUUGAAGAACUGU	000398

RNU6B (NR_002752	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT	001093

Table 5 lists of TaqMan microRNA Assays purchased from Applied Biosystems.

Gene name	Assay ID
Nanog	Hs02387400_g1
Nestin	Hs04187831_g1
TH	Hs00165941_m1
MAP2	Hs00258900_m1
FOXP1	Hs00212860_m1
FOXG1	Hs01850784_s1
LHX2	Hs00180351_m1
GAPDH	Hs02786624_g1
ACTB	Hs01060665_g1

4.4 Western blot

Cells were rinsed with cold PBS and lysed by resuspension in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Roche). After 20 min on ice, the lysates we centrifuged at 12,000 xg for 15 min. The lysates were transferred to a fresh tube and stored at -20°C. Proteins were quantified using the Bio-Rad DC Protein assay (Bio-Rad). The lysates were mixed with 4x Laemmli buffer and boiled for 5 min at 95 °C. Equal amounts of protein (5-15 ug) were loaded for each sample and electrophoresed on a 12% polyacrylamide gel at 100 mV. Following SDS-PAGE electrophoresis, proteins were transferred onto nitrocellulose membranes via a Trans-Blot Turbo Transfer system (BioRad) at 2.5 mV for 10 min. To increase the sensitivity of GCase and α-syn detection, membranes were fixed for 30 min at RT in 4% paraformaldehyde (PFA) with 0.1% glutaraldehyde. The GCase and α-syn membrane fractions were then incubated in 5% BSA and 5% milk solution, respectively, diluted in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C (**Table 6**). Following washing in 0.1% TBST, the membranes were incubated with peroxidase secondary antibodies for 1h at room temperature. Membranes were washed in 0.1% TBST and revealed by Clarity Western ECL Substrate (Bio-Rad). Image acquisition and densitometry were performed using ChemiDoc MP System (Bio-Rad) and ImageJ.

Table 6 lists of antibodies used for immunoblotting.

Gene name	Catalog	Dilution
GBA1	Abcam ab55080	1:2,000
SNCA	Abcam138501	1:2,000
GAPDH	Proteintech 60004-1-lg	1:50,000

4.5 GCase enzyme activity assay

The GCase activity was measured using 4-methylumbelliferyl- β-D-glucopyranoside (4-MUG), an artificial substrate of GCase, that when is catalyzed by the enzyme releases fluorogenic byproduct 4-methylumbelliferone measured by a microplate reader. The GCase assay was performed according to the published methods [135]. The protein lysates (5-10 ug) were diluted with RIPA lysis buffer to a final volume of 20uL and loaded to 96-well black plate with the clear bottom (Corning) containing 40uL of reaction master mix (GCase assay buffer, 10% BSA, 2.5 mM 4MUD, and 25 mM CBE or distilled water). Using SpectraMax iD5, the fluorescence intensity was measured every 2 min with 30s shaking between cycles for 2.5 hrs at 37 °C. For analysis of enzyme kinetics, Vmax values for each sample were normalized to the protein concentration.

4.6 Immunocytochemistry

Cells were fixed with 4% PFA/PBS for 15 minutes, permeabilized with 0.2% Triton X-100/PBS for 15 minutes and blocked in 0.05% Triton X-100/2% BSA/5% Normal Donkey Serum/PBS for 1h at room temperature or overnight at 4 °C on a shaker. Cells were incubated in primary antibodies diluted in a blocking solution overnight at 4 °C on a shaker (**Table 7**). Next, secondary antibodies were applied for 2 hr at room temperature on a shaker followed by a 15-minute staining with Hoechst (ThermoFisher, H3570). Imaging was performed under the ImageXpress confocal microscope using a 40x objective with appropriate channels.

Table 7 lists of antibodies used for immunocytochemistry.

Gene name	Catalog	Dilution
Nestin	Abcam ab21624	1:500
SOX1	R&D system AF3369	1:500
FOXA2	R&D system AF2400	1:500
Tuj3	Millipore AB9354	1:2000
MAP2	EnCor Biotech CPCA-MAP2	1:1000

4.7 Statistical analysis

Statistical analyses and figures were generated using GraphPad Prism 10 software (La Jolla, CA). Unpaired t-test was used to compare two groups, while one- and two-way ANOVA followed by Bonferroni test were used for comparison between multiple groups. Normality was assessed for each experiment using tests such as Shapiro-Wilk and Kolmogorov-Smirnov to validate the use of parametric tests. Values are presented as mean±SEM. Asterisks in the figures denote statistical significance as follows: *p < 0.05, **p < 0.01, ***p< 0.001, ****p < 0.0001, ns p> 05.

5. Results

5.1 Cell type characterization of control iPSC-derived cells and target validation

The healthy AIW002-02 iPSCs, previously characterized for pluripotency and genomic integrity, were used as a control line for preliminary experiments [133]. We adapted published methods to induce control iPSCs into dNPCs that were then differentiated into 1-week-old DNs [131–133].

To confirm the phenotype of control iPSC-derived dNPCs and DNs, we performed immunocytochemistry (ICC) and qPCR for marker expression as part of the quality control process (**Figure 5**). ICC revealed positive staining for progenitor Nestin, FOXA2, and SOX1 in iPSC-derived dNPCs (**Figure 5A**). Following differentiation, iPSC-derived 1-week-old DNs stained positive for pan-neuronal marker MAP2, and mature dopaminergic marker TH. (**Figure 5B**). Moreover, a one-way ANOVA and a subsequent Bonferroni's test performed on the RT-qPCR results revealed that AIW002-02 iPSCs expressed pluripotent marker *Nanog*, whereas iPSC-derived progenitors expressed *Nestin*, Fs (2, 6) > 58.44, ps < 0.001 (**Figure 5C**). The AIW002-02 iPSC-derived 1-week-old DNs expressed *MAP2* and *TH* markers, Fs (2, 6) > 1152, ps < 0.0001 (**Figure 5C**). In addition to dopaminergic markers, we confirmed that AIW002-02 iPSC-derived cortical NPCs (cNPCs) expressed *Nestin* as well as cortex-specific markers *FOXP1*, *FOXG1*, and *LHX2* (**Figure 5D**).

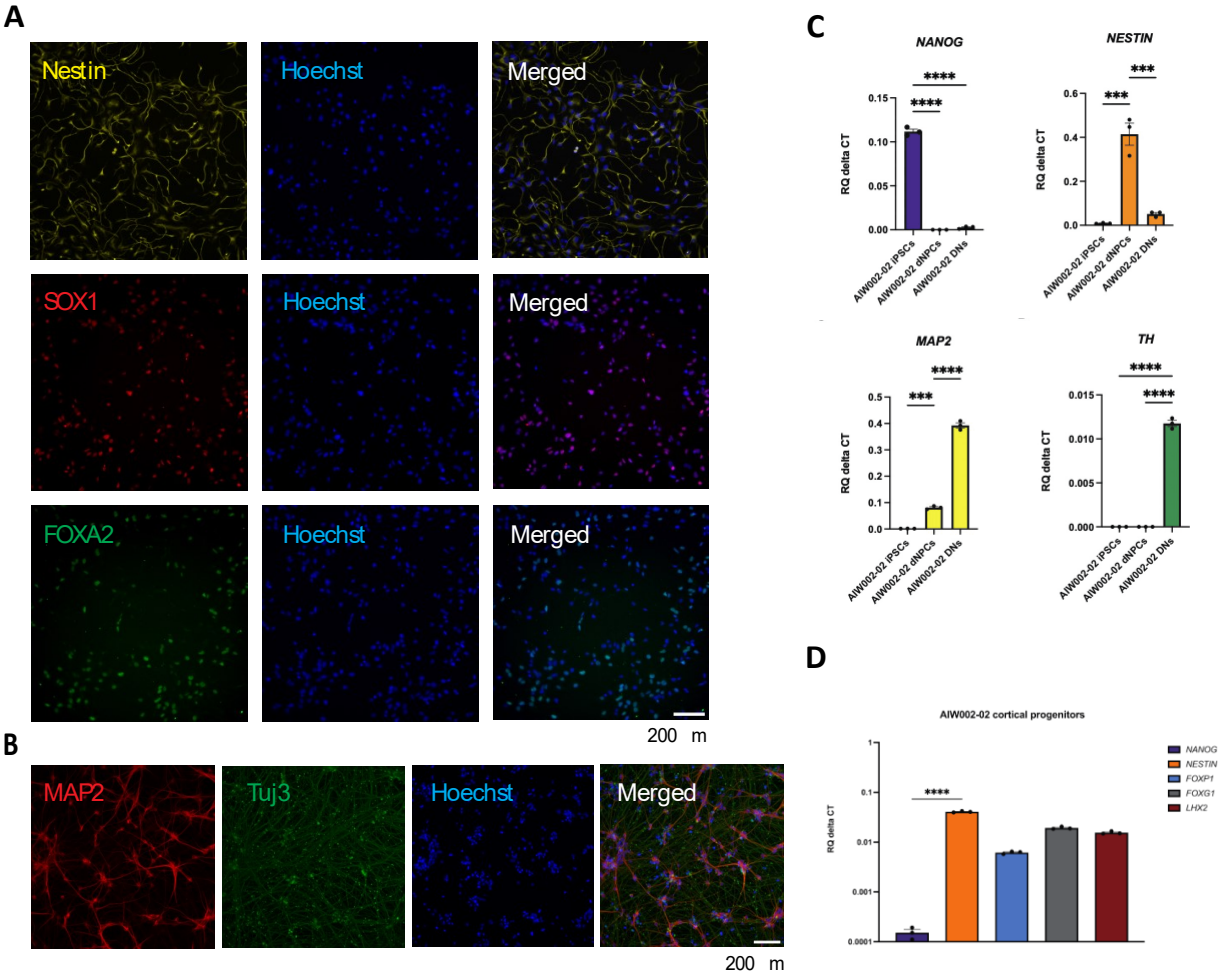


Figure 5. Cell type characterization of control iPSC-derived dopaminergic progenitors and neurons. (A) Immunocytochemistry revealed positive staining for Nestin, SOX1, and FOXA1 in control AIW00202 iPSC-derived dNPCs and (B) 1-week-old DNs. (C) qPCR for mRNA expression of pluripotency marker Nanog, progenitor marker Nestin, neuronal marker MAP2, and dopaminergic marker TH in DNs. (D) qPCR for mRNA expression of Nanog, Nestin, and cortex-specific markers FOXP1, FOXG1, and LHX2 in cNPCs. One-way ANOVA test. n=1, N=3 per conditions. Each marker is normalized to GAPDH. Error bars are mean±SEM. p*** < .001, **** < .0001.

Next, we measured endogenous expression of target genes in the *GBA1* pathway including *miR-22*, and *GBA1*, as well as *SNCA* in control iPSCs and iPSC-derived dNPCs and DNs (**Figure 6**). A one-way ANOVA and a subsequent Bonferroni's test performed on the preliminary qPCR results confirmed previous findings that *miR-22* and *GBA1* expression increases with differentiation towards neurons, Fs (2, 6) > 38, ps < 0.0004 [12] (**Figure 6A** and

6B). Although marginally, the expression of *SNCA* was also higher in the 1-week-old DNs compared to iPSCs and progenitors, F (2, 6) = 4.379, p = 0.0672 (**Figure 6C**). In addition to the dopaminergic system, we measured the target expression in AIW002-02 cortical progenitors. An unpaired t-test revealed that *miR-22* levels are significantly higher in cNPCs than in dNPCs, t (4) = 8.972, p = 0.0009 (**Figure 6D**). Contrarily, the *GBA1* expression is much higher in dopaminergic progenitors than in cortical, t (4) = 6.638, p = 0.0027 (**Figure 6E**).

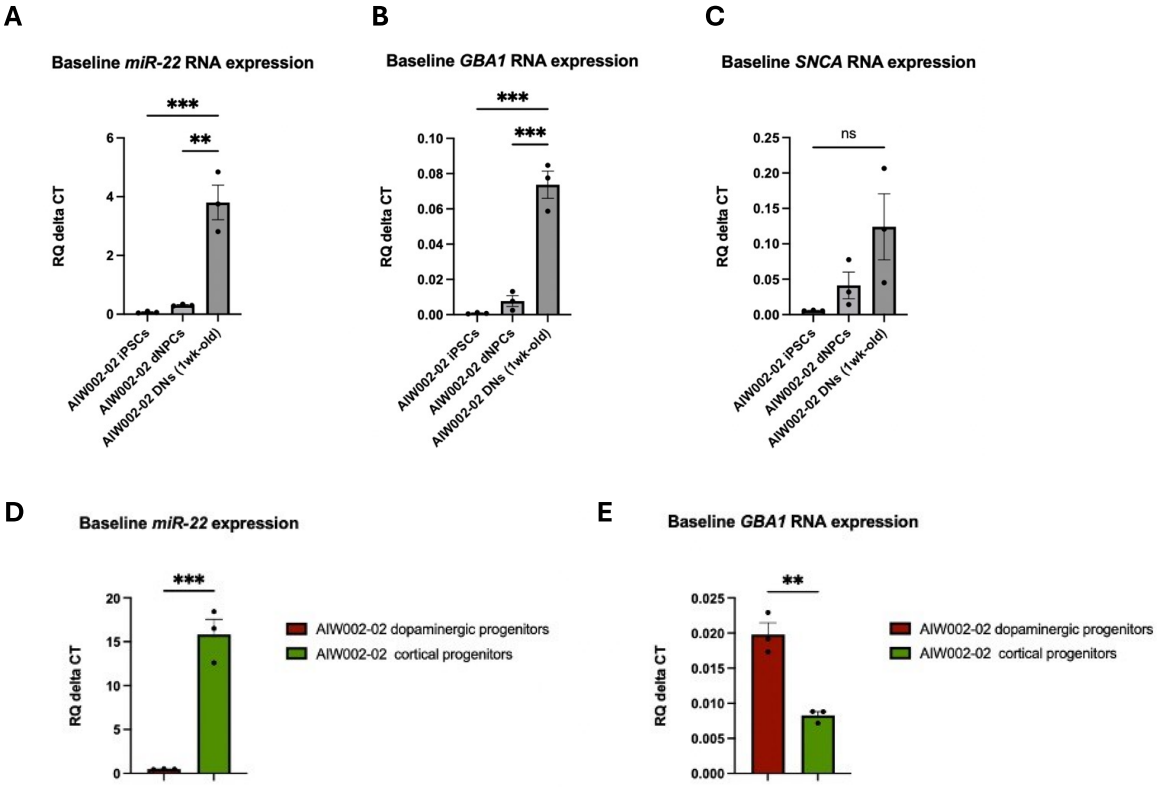


Figure 6. Baseline expression of target genes. qPCR for RNA expression of miR-22 in AIW002-02 iPSC-derived cell types. (A), GBA1 (B), and SNCA (C) in control AIW002-02 iPSCs, iPSC-derived dNPCs, and 1-week-old DNs. One-way ANOVA test followed by Bonferroni test. n=1, N=3 per conditions. miR-22 levels were normalized to RNU6B, and GBA1 and SNCA levels were normalized to GAPDH. Error bars are mean±SEM. ** p < .01, *** p < .001. (D) Baseline miR-22 and (E) GBA1 expression in control dNPCs and cNPCs. n=6, N=3 per conditions. n=6, N=3 per conditions. miR-22 levels were normalized to RNU6B and GBA1 levels were normalized to GAPDH. Error bars are mean±SEM. ** p < .01, *** p < .001.

5.2 Modulating *miR-22-3p* levels directly via ASOs influences *GBA1* mRNA expression in control iPSC-derived dopaminergic progenitors and neurons

After confirming detectable baseline levels of *miR-22* and *GBA1*, we tested whether 24-hour-long transfection with ASO-based mimic and inhibitor would overexpress and knockdown *miR-22-3p*, respectively, in control dNPCs. An unpaired t-test revealed that transfection with 5 nM *miR-22-3p* mimic led to a significant increase in *miR-22* levels compared to the scrambled negative control in healthy dNPCs, $t(4) = 14.85$, $p < 0.0001$ (**Figure 7A**). Contrarily, 50 nM *miR-22-3p* Power inhibitor elicited a significant reduction in *miR-22* levels, $t(4) = 7.365$, $p = 0.0018$ (**Figure 7B**). Furthermore, mimic-induced *miR-22-3p* overexpression led to downregulation in *GBA1* mRNA levels 48 hours post-transfection consistent with previous findings [12], $t(4) = 2.170$, $p = 0.0479$ (**Figure 7C**). Contrarily and more importantly, Power inhibitor-induced knockdown of *miR-22-3p* upregulated *GBA1* mRNA levels more than 2-fold compared to negative control, $t(4) = 3.486$, p 0.0252 (**Figure 7D**).

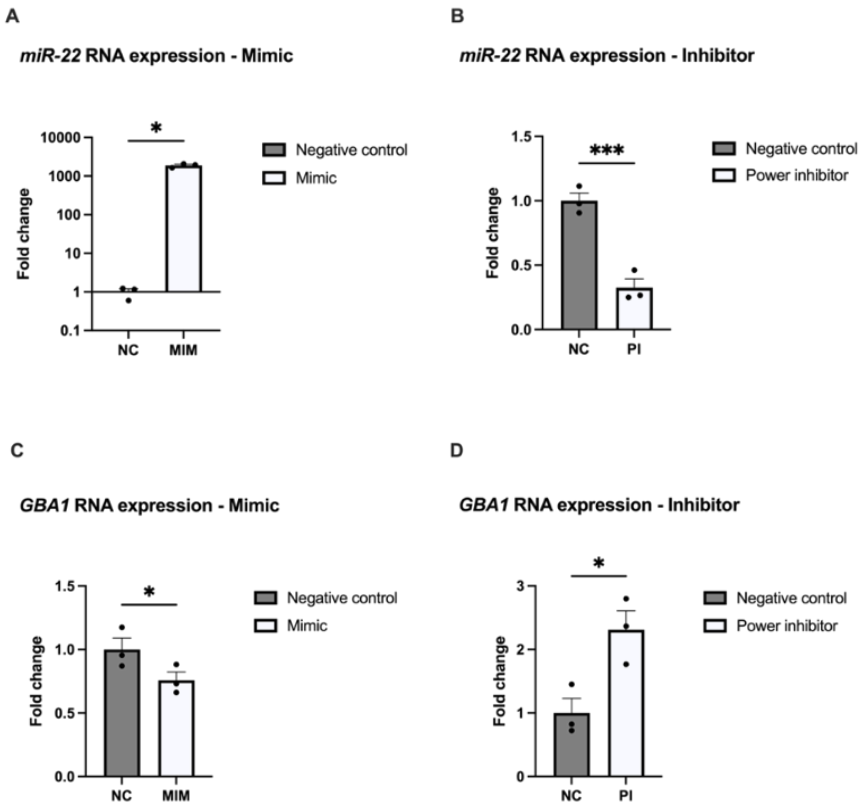


Figure 7. The effect of *miR-22-3p* overexpression and knockdown with mimic and Power inhibitor, respectively, in control dNPCs. (A) 5 nM *miR-22-3p* mimic upregulated *miR-22*

levels, whereas (B) 50 nM *miR-22-3p* Power inhibitor downregulated *miR-22* levels 48hr post-transfection in control dNPCs. (C) 5 nM *miR-22-3p* mimic downregulated *GBA1* mRNA levels, whereas (D) *miR-22-3p* Power inhibitor led to increased *GBA1* mRNA levels 48hr post-transfection in control dNPCs. Unpaired t-test. Mimic experiments were performed n=3, N=3, while Power inhibitor experiments n=5, N=3. *miR-22* levels were normalized to *RNU6B*, and *GBA1* levels were normalized *GAPDH*. Error bars are mean±SEM. * p < .05, *** < .001

Similar results were observed in AIW002-02 iPSC-derived cortical progenitors although to a greater extent. This can be attributed to the higher baseline expression of *miR-22* and the lower endogenous expression of *miR-22* and *GBA1* in the cortical system (**Figure 8A-D**).

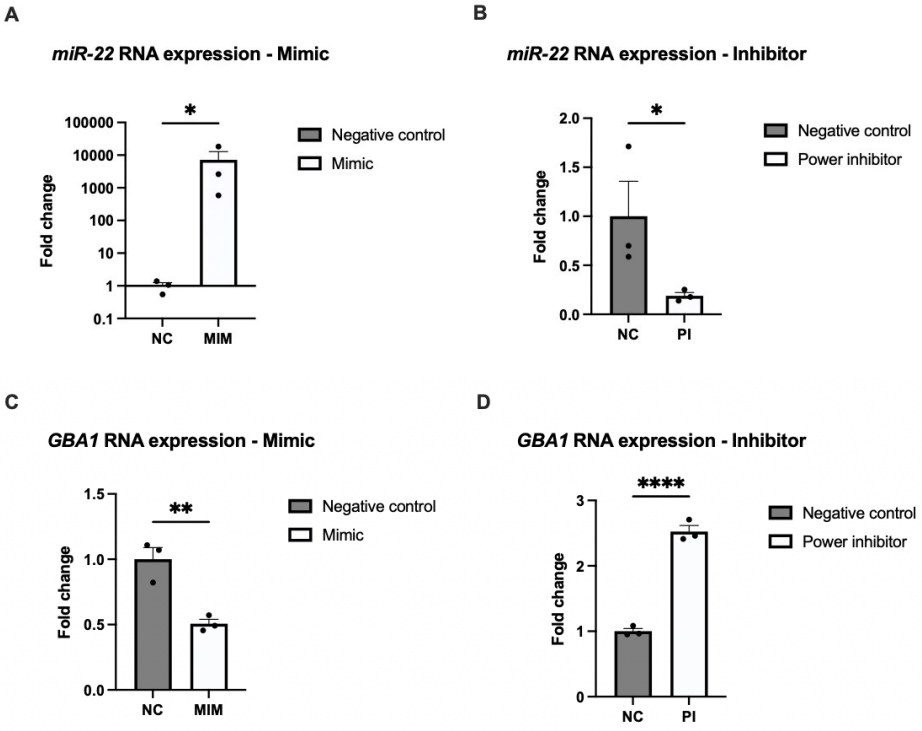


Figure 8.The effect of *miR-22-3p* overexpression and knockdown with mimic and Power inhibitor, respectively, in control cNPCs. (A) 5 nM *miR-22-3p* mimic upregulated *miR-22* levels, whereas (B) 50 nM *miR-22-3p* Power inhibitor downregulated *miR-22* levels 48hr post-transfection in control cNPCs. (C) 5 nM *miR-22-3p* mimic downregulated *GBA1* mRNA levels, whereas (D) *miR-22-3p* Power inhibitor led to increased *GBA1* mRNA levels 48hr post-transfection in control cNPCs. Unpaired t-test. Mimic experiments were performed n=3, N=3, while Power inhibitor experiments n=5, N=3. *miR-22* levels were normalized to *RNU6B*, and *GBA1* levels were normalized *GAPDH*. Error bars are mean±SEM. * p < .05, *** < .001.

After validating transfection conditions in iPSC-derived dNPCs, we focused on examining whether *miR-22-3p* Power inhibitor can enhance *GBA1* transcript levels in control iPSC-derived 1-week-old DNs. We performed a dose-range experiment to determine the optimal dose of Power inhibitor that maximizes *GBA1* mRNA levels with minimal toxicity (**Figure 9**). A one-factor between-subject ANOVA performed on the *miR-22* expression data revealed a significant effect for the group, $F(5, 12) = 43.20$, $p < 0.000$ (**Figure 9A**). Subsequent Bonferroni's test indicated that transfection with 80 nM, 120 nM, and 160 nM Power inhibitor decreased *miR-22* levels 72hr post-transfection, ps < 0.0001. Furthermore, a one-way ANOVA analysis on *GBA1* expression revealed a group effect, $F(5, 12) = 6.399$, $p = 0.0041$ (**Figure 9B**). Subsequent Bonferroni's test showed that 80 nM and 120 nM *miR-22-3p* Power inhibitor increased *GBA1* mRNA levels compared to negative control (ps < 0.0297).

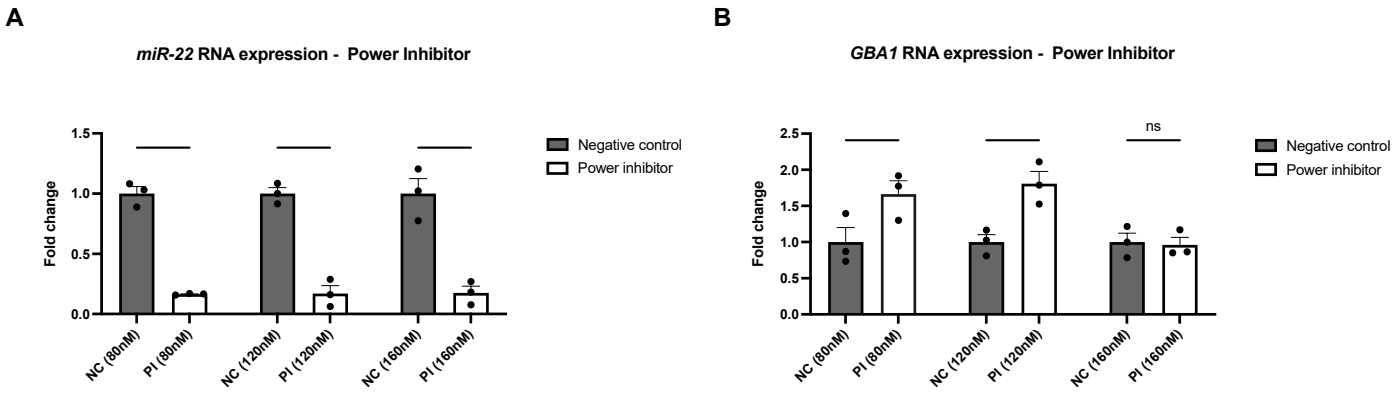


Figure 9 Dose optimization of miR-22-3p Power inhibitor in control iPSC-derived 1-week-old DNs. (A) 80 nM, 120 nM and 160 nM miR-22-3p Power inhibitor downregulated miR-22 RNA levels 72 hr post-transfection in control DNs. (B) 80 nM, and 120 nM miR-22-3p Power inhibitor upregulated GBA1 levels 72 hr post-transfection in control DNs. One-way ANOVA followed by Bonferroni's test. n=3, N=3 per conditions. miR-22 levels were normalized to RNU6B, and GBA1 levels were normalized GAPDH. Error bars are mean±SEM. * p < .05; ** < .01, **** < .0001.

Next, we compared the effects of 80 nM and 120 nM of *miR-22*-3p Power inhibitor on GCase protein levels and activity in 1-week-old control iPSC-derived DNs (**Figure 10**). The immunoblotting experiment showed that 80 nM *miR-22-3p* Power inhibitor increased GCase protein levels 72 hr post-transfection in control DNs, $t(4) = 3.052$, $p = 0.0380$ (**Figure 10A** and **10B**). Moreover, an unpaired t-test on 4-MUG data reported that 80 nM and 120 nM *miR-22-3p*

Power inhibitor increased GCase enzyme activity 72 hr-post transfection, ts (4) < 6.604, ps < 0.0027 (**Figure 10C** and **10D**, respectively).

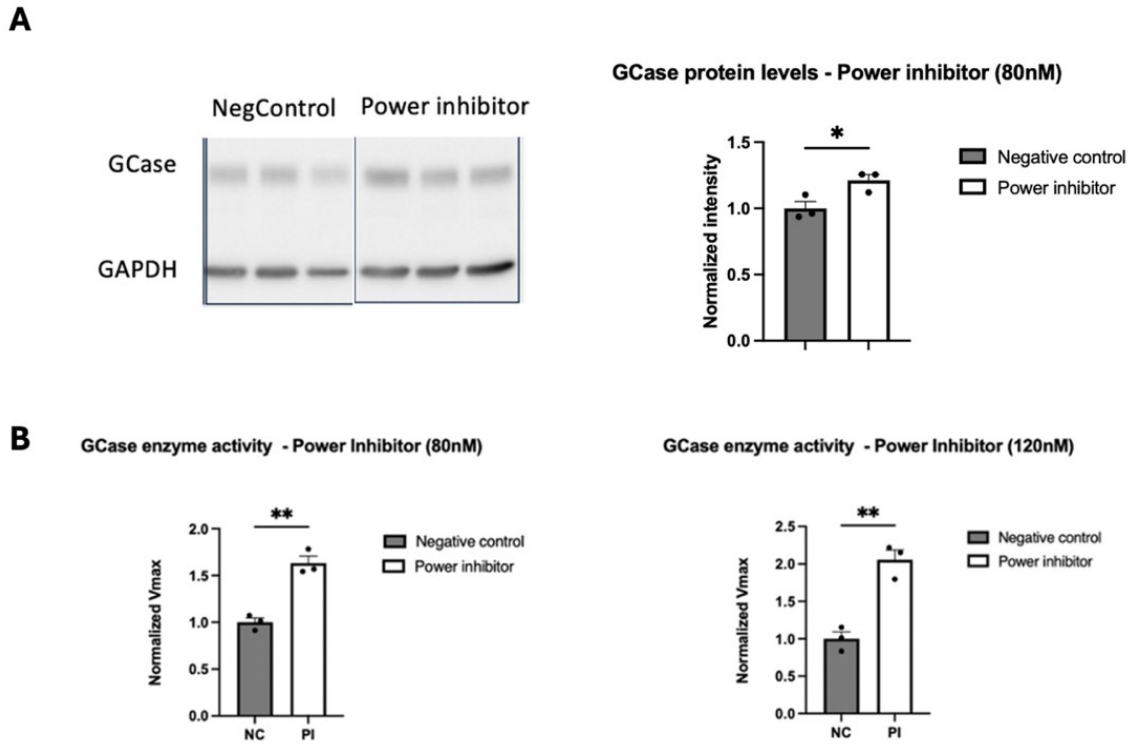


Figure 10. The effects of 80 nM and 120 nM miR-22-3p Power inhibitor on GCase activity and protein levels 72 hr post-transfection in control 1-week-old iPSC-derived DNs. (A) Immunoblot for GCase and GAPDH levels following transfection with 80 nM miR-22-3p Power inhibitor. 5 ug protein loaded. Unpaired t-test. n=3, N=3 per conditions. Normalized to GAPDH. Error bars are mean±SEM. * p < .05. (B) 4-MUG analysis shows 80 nM and 120 nM miR-22-3p Power inhibitor upregulated GCase activity. 5 ug protein loaded. Unpaired t-test. Experiments with 80 nM were performed n=3, N=3, while 120 nM Power inhibitor experiments n=2, N=3. Normalized to total protein and negative control. ** p < .01.

Considering efficient transfection in control iPSC-derived 1-week-old DNs, we tested the effect of 80 nM and 120 nM *miR-22-3p* Power inhibitor on GCase enzyme activity in older DNs 72 hr post-transfection (**Figure 11**). An unpaired t-test revealed that either dose failed to significantly upregulate GCase enzyme activity in control 3-weeks-old DNs, ts (4) < 1.456, ps > 0.2192 (**Figure 11A**). We attributed poor transfection efficiency to neuronal clumping prevalent in DNs with maturation. As such, we performed transfection with 80 nM *miR-22-3p* inhibitor in control XCL-1 iPSC-derived DNs with a less clumping propensity, and AIW002-02 and at 2.5

weeks of age. Unfortunately, the 80 nM Power inhibitor did not affect GCase activity in either line, ts (4) < 0.7116, ps > 0.5160 (**Figure 11B**). Although we did not test 120 nM *miR-22-3p* Power inhibitor, we suspect that further optimization of transfection conditions is required for older neuronal models.

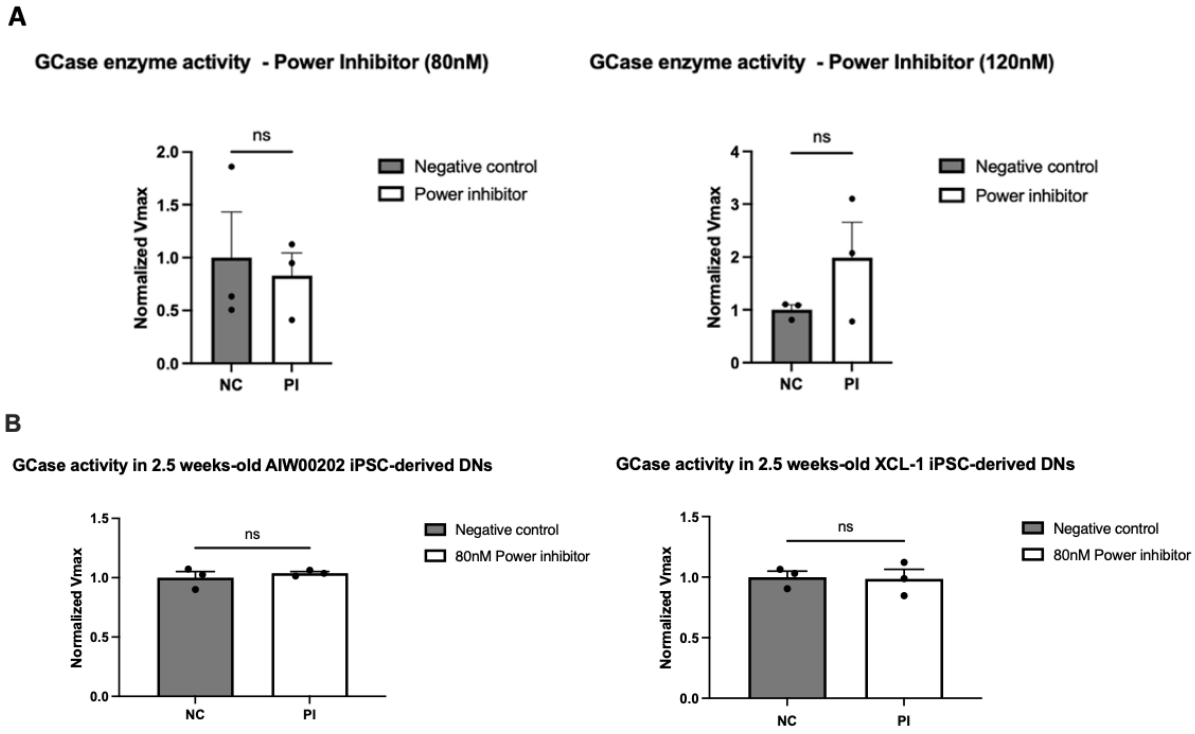


Figure 11. The effects of 80 nM and 120 nM miR-22-3p Power inhibitor on GCase activity and protein levels 72 hr post-transfection in control 3- and 2.5-weeks-old iPSC-derived DNs. (A) 4-MUG analysis shows 80 nM and 120 nM miR-22-3p Power inhibitor failed to upregulate GCase activity in control 3-weeks-old DNs. 5 ug protein loaded. Unpaired t-test. n=2, N=3. Normalized to total protein and negative control. (B) GCase enzyme activity in 2.5-weeks-old AIW002-02 and XCL-1 iPSC-derived DNs following 80 nM miR-22-3p Power inhibitor. 5 ug protein loaded. Unpaired t-test. n=2, N=3. Normalized to total protein and negative control.

5.3 Characterization of mutant *GBA1*-L444P iPSC-derived DNs and target validation

For the mutant lines, we used iPSCs derived from PD patient with a severe *GBA1*-L444P mutation, as well as isogenic corrected iPSCs. To characterize mutant iPSC-derived 1-week-old DNs, we performed immunoblotting for GCase and α-syn protein levels as well as 4-MUG assay for GCase enzyme activity (Figure 12). A two-way ANOVA on immunoblotting data assessing the effects of genotype (mutant vs isogenic) and age (1- and 2-weeks-old) on GCase protein

levels revealed a marginal significant main effect of genotype, F (1, 9) = 4.845, p = 0.0552, suggesting that mutant DNs have reduced GCase protein levels (**Figure 12A** and **12B**). For α-syn levels, a two-way ANOVA failed to detect a significant interaction or main effects, Fs (1, 9) < 3.87, ps > 0.0806, although visual inspection suggests a noticeable difference between mutant and isogenic counterparts, especially at 1-week stage (**Figure 12A** and **12C**). Finally, we assessed the effect of genotype and age on GCase enzyme activity (**Figure 12D**). A two-way ANOVA followed by Bonferroni's test revealed a significant main effect of genotype, F (1, 8) = 117.9, p < 0.0001, across two weeks, ps = 0.0001, confirming that mutant GBA1-L444P DNs have <50% reduced GCase enzyme activity compared to corrected line. These results collaborate reported inverse relationship between the *GBA1* and *SNCA* proteins in mutant GBA1 system [5–7,7].

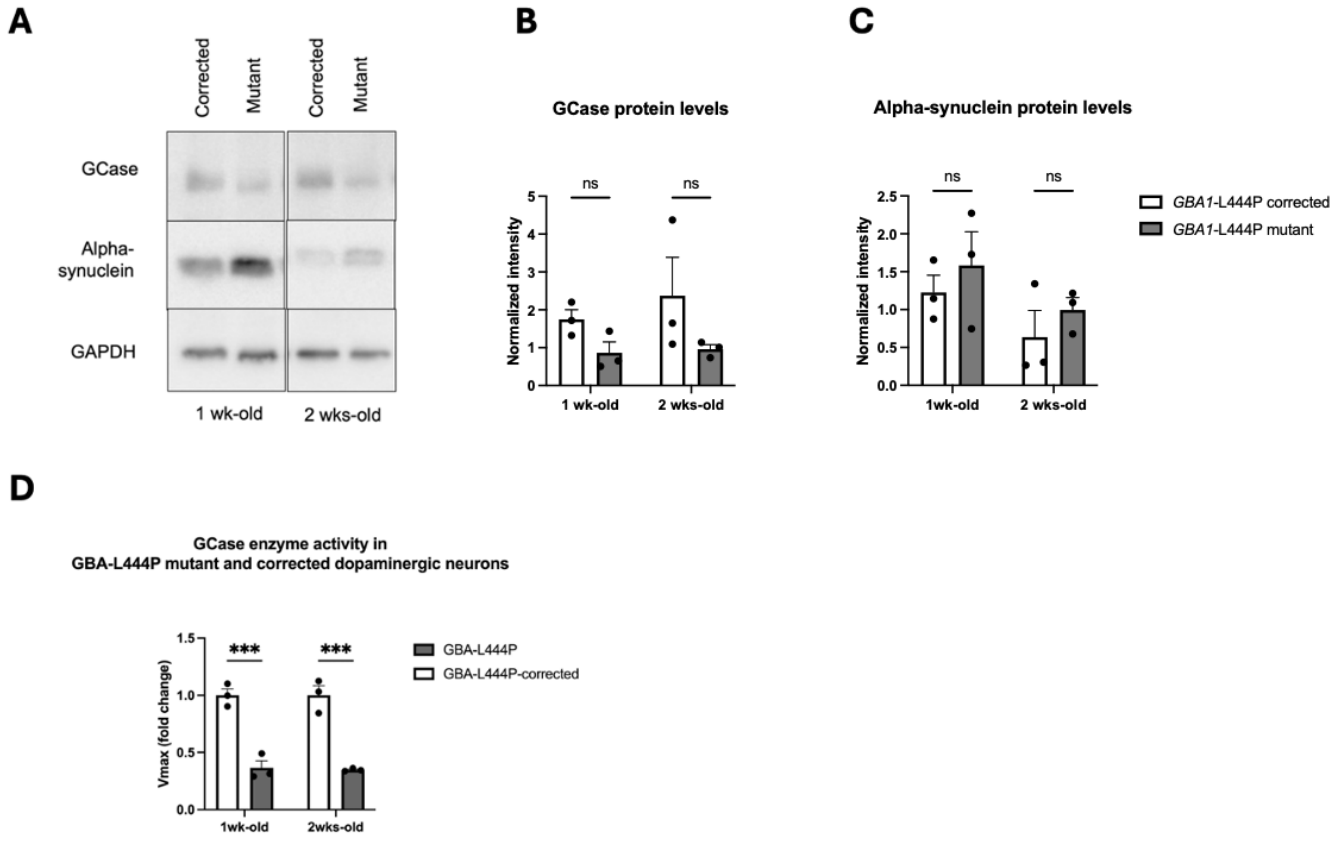


Figure 12. Characterization of mutant GBA1-L444P DNs. (A) Immunoblot for GCase (B), α-syn (C), and GAPDH in corrected and mutant 1- and 2-weeks-old GBA1-L444P DNs. 5 ug protein loaded. Two-way ANOVA. n=1, N=3 per conditions. Normalized to total protein and corrected genotype. Error bars are mean±SEM. ns non-significant. (D) 4-MUG analysis shows that in corrected and mutant GBA1-L444P DNs at two weeks. 5 ug protein loaded. Two-way

ANOVA followed by Bonferroni's test. n=1, N=3 per conditions. Normalized to total protein and corrected genotype. Error bars are mean±SEM. p*** < .001.

5.4 *miR-22-3p* Power inhibitor rescues GCase activity in mutant GBA1-L444P DNs

After confirming the mutant phenotype in the *GBA1*-L444P DN model, we tested whether 80 nM and 120 nM *miR-22-3p* Power inhibitor would rescue GCase enzyme activity in *GBA1*-L444P 1-week-old DNs 72 hr post-transfection (**Figure 13**). A one-way ANOVA conducted on the 4-MUG data assessing the effect of 80 nM *miR-22-3p* Power inhibitor on GCase activity revealed no significant difference between conditions, F (2, 6) = 0.3709, p = 0.7049 (**Figure 13A**). For 120 nM dose, a one-way ANOVA showed a significant effect for the group, F (2, 6) = 3.499, p = 0.0984 (**Figure 13B**). Subsequent Bonferroni's test indicated that 120 nM *miR-22-3p* inhibitor upregulated GCase enzyme activity compared to scrambled control (p = 0.0388). As such, we demonstrated that 120 nM *miR-22-3p* Power inhibitor caused knockdown of *miR-22*, consequently rescuing GCase enzyme activity in L444P mutant DNs to the levels comparable to the isogenic counterpart 72 hr post-transfection.

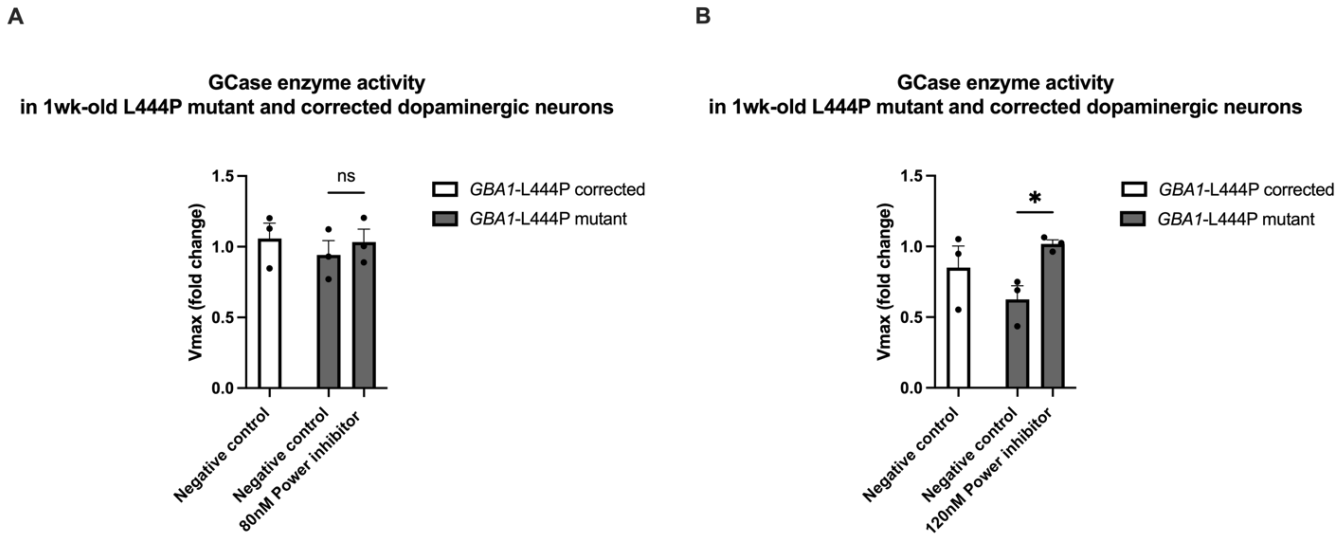


Figure 13. miR-22-3 Power inhibitor rescues GCase enzyme activity in 1-week-old GBA1-L444P DNs. (A) Transfection with 80 nM miR-22-3p Power inhibitor. 5 ug protein loaded. Two-way ANOVA. n=1, N=3 per conditions. Normalized to total protein and corrected genotype. Error bars are mean±SEM. ns non-significant. (B) 120 nM miR-22-3p Power inhibitor upregulated GCase enzyme activity in 1-week-old mutant DNs 72 hr post-transfection. 5 ug

protein loaded. Two-way ANOVA. n=1, N=3 per conditions. Normalized to total protein and corrected genotype. Error bars are mean±SEM. * $p < .05$.

6. Discussion

Heterozygous mutations in the *GBA1* confer about 5-10 times higher risk of developing PD with 5-10 years earlier onset of symptoms compared to idiopathic PD patients and a more severe and rapidly progressing disease phenotype [3,136,137]. Interestingly, decreased GCase activity is also observed in PD patients without *GBA1* mutations, suggesting deficiency in GCase plays a role in PD pathology [4,138,139]. Indeed, multiple studies report a strong inverse relationship between Case deficiency and α-syn levels in PD pathology [5–8]. *miR-22-3p* has been identified as a negative regulator of GBA1 expression, causing transcript degradation and/or translational suppression [12]. However, whether the knockdown of *miR-22-3p* would result in the opposite effect of enhancing *GBA1* expression has not been investigated until now

In this study, we leveraged ASO technology to modulate *miR-22-3p* levels and investigate the effect of *miR-22-p* knockdown on *GBA1* expression and GCase activity in healthy and mutant *GBA1* neuronal systems. To optimize the transfection experiments, we first validated *miR-22-3p* mimic and inhibitor in healthy iPSC-derived dNPCs and cNPCs before post-mitotic neurons given the additional challenges that come with transfecting older DNs [131–133]. We confirmed sufficient expression of *miR-22-3p*, *GBA1*, and *SNCA* expression in iPSC-derived cells for targeted manipulation by ASOs (**Figure 6**)[12]. Interestingly, dNPCs expressed less *miR-22* but more *GBA1* levels than cNPCs. We showed that 5 nM *miR-22-3p* mimic overexpressed *miR-22* levels which resulted in reduced *GBA1* mRNA levels 48 hr post-transfection in control dNPCs (**Figure 7**) and cNPCs (**Figure 8**), supporting the repressing ability of *miR-22-3p* [12]. Importantly, 50 nM *miR-22-3p* Power inhibitor significantly reduced *miR-22* and upregulated *GBA1* mRNA levels in dNPCs and cNPCs, with a greater effect observed in the cortical system which can be attributed to higher and lower expression of *miR-22* and *GBA1* levels in cNPCs, respectively.

After establishing transfection conditions in the progenitor system, our efforts were focused on control iPSC-derived DNs. We demonstrated that 80 nM and 120 nM *miR-22-3p* Power inhibitor downregulated and upregulated *miR-22* and *GBA1* levels, respectively, in 1-

week-old DNs 72 hr post-transfection (**Figure 9**). Moreover, an 80 nM dose increased GCase protein levels and enzyme activity by over 1.5-fold, while a 120 nM dose doubled GCase activity in healthy DNs (**Figure 10**). Finally, we tested the effect of *miR-22-3p* Power inhibitor on GCase enzyme activity in heterozygous GBA1-L444P iPSC derived 1-week-old DNs that exhibited the expected phenotype of reduced GCase levels and activity with an observable increase in α-syn levels (**Figure 12**) [5–7,139]. Transfection with 120 nM *miR-22-3p* Power inhibitor increased GCase activity more than 1.5-fold in 1-week-old mutant DNs 72 hr after transfection (**Figure 13**). We are yet to investigate the consequences of GCase enhancement by *miR-22-3p* knockdown on α-syn pathology. The next experiments will focus on determining whether targeting *miR-22-3p* impacts α-syn turnover by measuring levels of soluble and phosphorylated fibril forms of α-syn. To evaluate α-syn aggregation and spreading, we would take advantage of a pre-formed fibril update assay.

Nevertheless, we further extended the findings of Straniero et al. 2017 by demonstrating that knockdown of *miR-22-3p* via ASO-based inhibitor rescued GCase activity in iPSC-derived models of PD. It is important to note that the ASO-based strategy explored in this project is designed to rescue GCase activity by depressing and increasing *GBA1* expression, this primarily relying on the expression from the normal *GBA1* allele that compensates for GCase deficiency caused by the mutated allele. Considering that heterozygous GBA1-L444P mutations confer 50%-70% residual GCase activity, the 1.5-fold increase following 120 nM miR-22-3p Power inhibitor is expected to shift GCase activity from 50% to 75% of normal activity [140]. However, this treatment is not designed to address misfolding of mutated GCase nor ER stress, thus providing minimal therapeutic value alone in rescuing GCase activity in individuals with homozygous *GBA1* mutations affecting protein folding. Combination therapy with pharmacological chaperones such as ambroxol could facilitate the folding of GCase while increasing its levels. Such dual action might result in improved trafficking of GCase and alleviation of α-syn pathology by breaking the bidirectional feedback loop between GCase and α-syn. This strategy could be promising for treating a variety of neurological diseases affected by GCase deficiency such as severe forms of GD, familial PD with mutations in the *GBA1* or other lysosome-associated genes such as *LRRK2*, sporadic PD, as well as other synucleopathies such as dementia with Lewy bodies.

This project has several limitations and poses open questions that should be considered in future studies. For example, it is important to address the small sample size of n=1 as well as low protein loaded in several experiments. Due to experimental design, we have experienced low protein yield (<20 ug) in studies with DNs. More replications and scaling of conducted experiments would ensure the robustness of our results and increase statistical power. Moreover, for the evaluation of toxicity associated with transfection, we merely compared cell confluency and RNA expression between treated, untreated, and mock-transfection conditions. In the future, performing cell viability tests such as CellTiter-Glow should be used to provide more insight into toxicity associated with the dose range of ASOs. Furthermore, although iPSC-derived models are powerful tools for testing ASOs, contamination with concomitant cells such as fibroblasts, NPCs, or glial cells is a common issue in iPSC-derived cell culture. Considering the differential expression of target genes between cortical and dopaminergic systems, heterogeneous cell populations can contribute to variability. Among different strategies to minimize concomitant cells is FACS technology which allows for the isolation and purification of desired cell types. We also experienced challenges in modulating GCase activity in older DNs (**Figure 11**). We initially attributed this to poor transfection efficiency due to neuronal clumping. However, we did not measure changes in the expression of target genes, such as *miR-22-3p* and *GBA1,* following transfection with ASOs. This would have helped us determine whether the transfection failed completely or if nodulation is simply taking longer to occur, among other possibilities. Moreover, we are yet to confirm detectable expression of target genes in older DNs. Nevertheless, we expect that further optimization of ASO chemistry, transfection dose, and treatment exposure would improve the delivery efficiency in older neuronal model.

Once we succeed in optimizing transfection in older neurons, scaling up to 3D organoids would provide a great tool for the next phase of investigating the effects of *miR-22-3p* KD in a multi-cellular brain model. In addition to neuronal models, it is of interest to examine other cell types in terms of *miR-22-3p* and *GBA1* expression. Given that microglia rely on GCase activity for their phagocytic function and their implication in the neuroinflammation of many neurodegenerative diseases, understanding the effect of *miR-22-3p* knockdown in glial cells can offer a better understanding of how modulating GCase activity might influence microglial function, neuroinflammation, and the PD pathology.

Moreover, given the multifunctional and understudied role of *miR-22-3p* in healthy and disease states, future studies should investigate potential off-target effects and the global impact of *miR-22-3p* KD at the transcriptional level via RNA-sequencing. We also propose a target site blocker as a valuable alternative that could mitigate off-target effects. Specifically, the target site blocker is designed to protect the MRE on *GBA1* mRNA, thereby preventing *miR-22-3p* regulation on *GBA1* without affecting its other cellular functions (**Figure 1**). The interaction between TSB and *GBA1* mRNAs should not affect the translation of mRNA into protein.

7. Summary & conclusion

This project aimed to explore the therapeutic potential of targeting *miR-22-3p* with an ASO-based Power inhibitor in enhancing GCase enzyme activity in iPSC-derived DNs with the severe GBA1-L444P mutation. Given the inverse relationship between GCase deficiency and α-syn aggregation, enhancing GCase activity offers potential avenues to alleviate the lysosomal burden and α-syn pathology in PD. We hypothesized that modulating *miR-22-3p* levels with ASO-based *miR-22-3p* inhibitor would directly influence *GBA1* levels which consequently would ameliorate α-syn pathology in the context of PD. We first confirmed that transfection with *miR-22-3p* mimics reduced *GBA1* transcript levels, confirming its regulatory role. Furthermore, we demonstrated that *miR-22-3p* Power inhibitor caused a significant reduction in *miR-22* levels and an increase in *GBA1* mRNAs in control cNPCs and dNPCs 48 hr post-transfection. In control 1-week-old DNs, 80 nM and 120 nM *miR-22-3p* Power inhibitor upregulated GCase activity by more than 1.5-fold. Finally, we tested the GBA1-enhancing effect of *miR-22-3p* Power inhibitor in mutant GBA1-L444P iPSC-derived DNs and demonstrated that 120 nM dose increased GCase activity by 1.5-fold, which suggests a shift in activity from 50% to 75% of normal levels. In summary, we have accomplished aims 1 and 2 by demonstrating that 1) modulating *miR-22-3p* directly with ASOs influences *GBA1* transcript, protein levels, and GCase activity in healthy iPSC-derived progenitors and DNs, and 2) *miR-22-3p* KD with ASO-based inhibitor rescued GCase activity in iPSC-derived DNs from PD patient with a heterozygous L444P mutation. The next studies will focus on determining whether targeting *miR-22-3p* impacts α-syn levels and aggregation in GBA1-PD.

In conclusion, we have extended the study by Straniero et al. 2017 and validated the GCase-enhancing effect of targeting *miR-22-3p*. For this project, we utilized *miR-22-3p* Power inhibitor to knockdown its expression and de-repress *GBA1* mRNAs. We have shown that targeting *miR-22-3p* with Power inhibitor upregulated *GBA1* expression, GCase levels, and activity in both healthy and mutant GBA1-L444P systems. The results of this project provide proof-of-principle for ASO-based miR inhibitors against miR found to be dysregulated in neurodegenerative diseases.

Next experiments are directed towards confirming that 120 nM *miR-22-3p* Power inhibitor modulates *miR-22* and *GBA1* expression at the transcriptional level in mutant DNs. Moreover, although we have demonstrated the ability of *miR-22-3p* Power inhibitor to enhance GCase enzyme activity, we are yet to investigate the impact of *miR-22-3p* KD on α-syn levels and aggregation in the young and old mutant GBA1-L444P DNs.

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