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Novel Insights into Neuroprotective Strategies Employing NQO1 and DCLK1 in Parkinson's Disease

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Authors' contributions

This work was carried out in collaboration among all authors. Authors KMS and GKK wrote the first draft of the manuscript. Author SNG managed the analyses of the study. Author KMS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Parkinson's disease (PD) is the most common movement disorder and is only second to Alzheimer's disease in most prevalent neurodegenerative diseases. Diverse causative factors lead to PD pathology among which oxidative stress and accumulation of α-Synuclein aggregates are considered to be key determinants for both sporadic and familial forms. In this review we focus on two novel research efforts to block elevated oxidative stress and α-Syn aggregates to provide neuroprotection to the dopaminergic neurons and thereby alleviating the motor symptoms displayed in PD animal models. A recently published effort from Luo and colleagues, discovered a pathway where the neuroprotective protein NQO1 is degraded upon phosphorylation by activated Akt and making the dopaminergic neurons susceptible to demise via elevated oxidative stress. Another recent report by Vázquez-Vélez and colleagues explored the regulatory relationship between the neuron expressed kinase DCLK1 and α-Syn in the context of human cellular and mouse models. These discoveries concentrate on different mechanisms of preventing the dopaminergic neurodegeneration in PD by reducing the oxidative stress and α-Syn aggregation via regulating key determinants of PD pathophysiology, NQO1 and DCLK1 respectively. This review emphasizes the possibility of employing both NQO1 and DCLK1 as promising therapeutic targets leading to future prospects of combinational therapies for devastating diseases like PD.

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Keywords: Parkinson's disease; neurodegeneration; α-synuclein; dopaminergic neurons.

ABBREVIATIONS

Parkinson 's disease	*(PD)*
α-Synuclein	*(α-Syn)*
Substantia Nigra pars compacta	*(SNpc)*
Dopamine	*(DA)*
Dopaminergic	*(DAergic)*
Oxidative stress	*(OS)*
Leucine rich repeat kinase 2	*(LRRK2)*
NAD(P)H:quinone reductase	*(DT-diaphorase;*
NAD(P)H-(quinone acceptor) oxidoreductase)	*(NQO1)*
Doublecortin like kinase 1	*(DCLK1)*
Reactive oxygen species	*(ROS)*
Dopamine quinone	*(DAQ)*
Nuclear factor erythroid 2 - related factor 2	*(Nrf2)*
Protein disulfide isomerase	*(PDI)*
Wild type	*(WT)*
Constitutively active	*(CA)*
Kinase dead	*(KD)*
Substantia Nigra	*(SN)*
Phospholipase D2	*(PLD2)*

1. INTRODUCTION

1.1 The Promise of Neuroprotective Therapies for Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is clinically characterized by four motor deficits namely; tremor, rigidity, bradykinesia and gait impairment [1]. These severe motor symptoms are attributed to the loss of dopamine (DA) producing neurons in the Substantia Nigra *pars compacta* (SNpc) and thus, causing a deficiency in DA in the striatum to which these dopaminergic (DAergic) neurons project [1]. In addition to the aforementioned motor symptoms, PD patients develop a variety of non-motor symptoms such as anosmia, sleep disorders, autonomic and cognitive impairment that are resistant to currently available treatments [2]. Therefore, developing neuroprotective strategies plays a pivotal role to impede the complications of this relentlessly progressive disorder.

The majority of the PD cases are sporadic with only a minor subset of the overall pool being familial incidences [1]. Examining the genetic components of familial PD forms allowed further apprehension of disease pathogenesis and the mechanistic details of the demise of neuronal populations involved in PD. Despite the profound research efforts to uncover the underlying genetic and cellular processes involved in PD, the etiology of the disease remains obscure to date. Both familial and sporadic forms of PD involve a growing list of monogenic and polygenic genetic risk factors [3]. However, certain other cellular processes such as mitochondrial dysfunction, neuroinflammation, alterations in catecholamine metabolism, oxidative stress (OS), environmental factors and impairment of basal cellular functions due to aging are also considered as key determinants of dopaminergic neuronal cell death in both familial and sporadic PD [4]. Accumulating evidence from the profound research carried out asserts the strong association of proteins such as PINK1, Parkin, DJ-1, Leucine rich repeat kinase 2 (LRRK2) and α-synuclein (α-Syn) to PD [1]. Among these mentioned factors, α-Syn and OS are considered to hold prominence for implications with PD pathogenesis.

The involvement of various molecular pathways has enabled more symptomatic treatments but no disease-modifying therapy to PD [5]. Existing PD therapy is geared towards replacing DA to alleviate the PD symptoms or using DA agonists to suppress DA release and thereby provide neuroprotection [2]. Furthermore, repurposing or re-formulating the previously approved drugs such as antioxidants have also gained significant attention among researchers to be used as neuroprotective agents for PD [6]. Nevertheless, neither the drugs approved show substantial efficacy nor they have adequate disease averting properties [2]. Furthermore, majority of the available symptomatic treatments have rendered further complications in PD patients due to side effects, short life span and permeability [6]. Hence, proving the dire necessity of elucidating novel medications aiming clearly established neuroprotective drug targets to slow or prevent disease progression once diagnosed. This review concisely summarizes the new insights gleaned from recent studies conducted to explore the possibility of employing two proteins namely NAD(P)H:quinone reductase (DT-diaphorase; NAD(P)H-(quinone acceptor) oxidoreductase; (NQO1) and Doublecortin like kinase 1 (DCLK1) to alleviate OS and α-Syn accumulation in SNpc respectively and thereby shield the demise of DAergic neurons in PD patients.

2. NQO1 AND DCLK1 – EMERGING THERAPEUTIC INTERVENTION FOR PD

OS stress is a deleterious condition that arises due to an imbalance between the production of reactive oxygen species (ROS) and the cell's capacity to scavenge the toxic ROS metabolites [7]. Under optimal conditions, these toxic species are eliminated via its self-defense antioxidant machinery. However, due to impaired antioxidant machinery or increased ROS production, the OS in the cell increases resulting in cell damage and ultimately cell death [7]. Nigral neurons are particularly vulnerable to OS due to the presence of DA and high Fe content associated with mitochondrial enzymes among which the presence of DA and its metabolism contributes predominantly towards the heightened OS [8]. DA molecule comprises an unstable catechol ring and thus it shows a high propensity to spontaneous oxidation in the presence of molecular oxygen yielding hydrogen peroxide, superoxide and DA-O-Quinone (DAQ) [9]. Under normal conditions, DA is readily taken up to synaptic vesicles by the vesicular monoamine transporter 2 and prevent DA breakdown by their low pH environment. However, when DA is released to the cytoplasm, it is prone to rapid spontaneous oxidation and subsequent degradation while causing detrimental effects by inhibiting complex I of the electric transport chain and enzymes essential for ATP synthesis [7].

A phase II detoxifying enzyme named NQO1 is expressed in the brain and it is capable of catalyzing the reduction of DAQ to more stable hydroquinone and thus, shields the DAergic neurons from destruction by DAQ [10]. NQO1 expression is suggested as a potential remedy for PD [11,12]. Nuclear factor erythroid 2 - related factor 2 (Nrf2), a transcription factor for *cis*-acting enhancer that regulates transcription of cytoprotective genes such as NQO1, is induced to ameliorate neuroprotection [13]. NQO1 has a strong correlation with the striatal DA system, in fact, the deficiency in NQO1 has been demonstrated to alter the behavioural outcomes in mouse models [14]. Furthermore, drastically increased NQO1 expression was observed in SNpc in active phase PD brains and it was virtually absent in the end-stage PD brains where the DAergic neurons were almost completely depleted [15]. The plausible evidence from the elegant experiments carried out by Luo and co-workers in The Journal of Neuroscience provides important insights into the underlying molecular mechanisms involved in the oscillating NQO1 expression in PD patients [16].

The authors initiated the study by performing *in vitro* phosphorylation assays using recombinant NQO1 and Akt, followed by proteomic analysis on the sites of phosphorylation. They observed phosphorylation of NQO1 by Akt in two sites, S40 and T128. This was further confirmed by constructing three NQO1 mutants, namely; S40A, T128A and the S40/T128 double mutant with Alanine substitutions resulting in the complete elimination of the phosphorylation of the double mutant. Furthermore, a novel polyclonal antibody against p-NQO1 T128 showed a curbed phosphorylation signal upon Akt knockdown in TrkB-stably transfected dopaminergic SH-SY5Y cells (BR6). In human α-SNCA transgenic PD mouse models, they observed a tight correlation between p-Akt and p-NQO1 T128 signals that showed a gradual decline with age. Moreover, parallel to NQO1 which showed peak expression levels at 7-11 months of age and gradually declined, FOXO3a, Nrf2 and PINK1 expression levels also showed comparable reduction in an age-dependent manner, while the OS escalated showing an inverse correlation. Overall, these results indicated the phosphorylation of NQO1 T128 by Akt in the PD mouse model.

Authors next assessed the association between Akt and NQO1 using a pull-down assay and observed that only WT-NQO1 (wild type) but not the phospho dead mutants, strongly interacted with Akt-CA (constitutively active) and Akt-WT but not with Akt-KD (kinase-dead) and hence confirmed that NQO1 binds with Akt in a phosphorylation-dependent manner, primarily at T-128 site. This result was confirmed by using Akt inhibitors and EGF treatment. Furthermore, Substantia Nigra (SN) of PD patients and age matched healthy controls showed strong co-localization of Akt/p-NQO1 and p-Akt/p-NQO1 staining. However, p-NQO1 and p-Akt levels were greatly diminished in PD brains and similar observations were made with wild type and human α-SNCA transgenic PD mice treated with MPTP. Remarkably, total NQO1 levels gradually declined while the total Akt levels did not change during these experiments. Furthermore, in an additional synucleinopathy, human dementia with Lewy Bodies, patient brains showed decreased BDNF, Trkb, p-Akt, p-NQO1 and NQO1 levels in contrast to the healthy control brains, indicating a phosphorylation dependent NQO1-Akt association.

In order to discover the underlying mechanism behind the inverse correlation between p-Akt and NQO1 levels in PD brains, the authors tested the levels of p-Akt and NQO1 upon BDNF and PI3K inhibitors using BR6 cells. They found that NQO1 level was increased in the presence of PI3K inhibitors and it was decreased upon BDNF treatment confirming the inverse correlation between the two species. Real-time PCR confirmed that Akt does not affect NQO1 in the transcriptional level, rather Akt poses the effect directly in the protein level indicating NQO1 protein is degraded upon Akt phosphorylation. To assess NQO1 degradation via ubiquitin proteasome pathway, the authors performed a pull-down assay using Akt-CA, Akt-KD and NQO1 and observed that NQO1 was strongly polyubiquitinated in the presence of Akt-CA but not with Akt-KD. This observation was further confirmed by treatment of BR6 cells with BDNF and Akt inhibitors in the presence of MG132, a proteasome inhibitor. The authors observed significant polyubiquitination of NQO1 with BDNF stimulation which was not observed with Akt inhibitors. They showed that NQO1 is polyubiquitinated in a phosphorylation-dependent manner, particularly T128 being the important site and thereby degrading the NQO1 via UPP.

Intuitively, the authors suggested that Parkin, which contains E3 ubiquitin ligase activity may be involved in ployubiquitination of NQO1 because Parkin mutations are strongly indicated in PD [17]. NQO1 strongly interacting with Parkin and p-NQO1 co-localizing with Parkin, compared to the mutants revealed the phosphorylation dependent ubiquitination of NQO1 with Parkin. Since Parkin is phosphorylated by PINK1 during mitophagy [8], the authors tested for a possible role for PINK1 in the phosphorylation of NQO1. However, the idea of PINK1 phosphorylating NQO1 was refuted upon showing that NQO1was polyubiquitinated in the presence of Akt but not with PINK1.

With cell survival assays, the authors showed the cytoprotective effect of phospho dead mutant NQO1 along with higher protein stability and TH activity compared to wild type. To confirm the *in vivo* efficacy of unphosphorylatable NQO1, the authors injected wild type and unphosphoryla table NQO1 into α-SNCA mice SN, followed by treatment with MPTP or saline after two months of viral delivery. One week after MPTP treatment, the DAergic neuronal cell death was monitored and elevated neuronal protection in the presence of wild type NQO1 was observed in comparison to the controls. The unphosphorylatable NQO1 incurred the maximum cytoprotection from MPTP. Similar observations were made when monitoring the motor functions after MPTP treatment, with unphosphorylatable NQO1 treated mice showing the least severe motor dysfunctions. The loss of TH is also repressed similarly by the treatment with WT and mutant NOQ1. Asparagine endopeptidase activation by MPTP imposed OS, leads to truncation of α-Syn and promotion of its aggregation in PD brains. Asparagine endopeptidase activity was partially hampered in the presence of WT NQO1 and was completely ceased with unphosphorylatable NQO1. Collectively, these results confirm that NQO1 phosphorylation and its subsequent degradation directly plays an important role in augmenting the OS and thereby increasing α-Syn aggregate formation.

PI3K/Akt pathway and Nrf2/NQO1 pathway are two independent cell survival pathways. PI3K/Akt pathway renders numerous functions such as neuronal growth, metabolism and cell survival [18]. Nrf2 is located in cell cytosol bound to a regulatory protein known as Keap1 which dissociates upon elevated OS inside the cell. The released Nrf2 is transported inside the nucleus and activates the transcription of antioxidant response element genes including NQO1 [19]. Similarly, the Akt signaling pathway is also activated to enhance cell survival via activating the NF-κB pathway [20]. Luo and colleagues [16] elucidate a crosstalk between PI3K/Akt pathway and Nrf2/NQO1 pathway, where activated Akt phosphorylate NQO1 in two different sites which makes the NQO1 protein susceptible to degradation via ubiquitination when the OS is higher. A recent study found that the protein disulfide isomerase (PDI), which was known for its dual function in protein folding and redox homeostasis affects the expression of NQO1. However, pieces of evidence that are seemingly counterintuitive for its function have been reported in the context of neurodegenerative diseases. For example, several studies have revealed that the inhibition of PDI prevents cell death. A recent work conducted using neuroblastoma cells found that the neuroprotective effect of PDI inhibition is driven by upregulating the expression of NQO1. This study provides novel insights into the findings of Luo and colleagues where further stabilization of NQO1 expression by inhibiting PDI as a better neuroprotective strategy compared to NQO1 stabilization against phosphorylation or degradation [21].

According to Luo and co-workers, it is evident that NQO1 stabilization could be a promising drug target [16]. A key determinant in the formation of α-Syn aggregates is the OS which is a consequence of NQO1 degradation. Thus, utilizing NQO1 as a potential drug target will provide more opportunities to address a wide range of issues linked with OS and PD. Although further research is required, it is evident that the treatment of PD with unphosphorylatable mutants or WT NQO1 or enhancing the NQO1 stability against phosphorylation and degradation could be beneficial. This is especially important for sporadic PD where the augmented OS is considered as the major causative factor. Prevention of DAergic neuronal death in SNpc and ameliorating the motor dysfunction associated with neurodegeneration could be achieved by the upregulation of NQO1 expression. This was successfully demonstrated in a recent study conducted using β-Caryophyllene that is able to augment the expression and the function of NQO1 [22].

Naturally occurring unstable NQO1 variants produced as a result of single nucleotide polymorphisms in *NQO1* have shown strong implications to cancers. A recent study was conducted using a small molecular chaperone demonstrated its potential to stabilize these unstable NQO1 variants while improving its enzymatic activity. This approach may be applicable in other adverse processes that result due to unstable NQO1. More specifically, this small molecular chaperone may be used to stabilize NQO1 to conserve its activity in DAergic neurons [23].

The key pathological hallmark of PD is Lewy bodies, which characteristically contain aggregates of α-Syn, a protein abundantly expressed throughout the central nervous system and is localized primarily in the cell soma, nucleus, and in presynaptic vesicles [15]. α-Syn is believed to facilitate synaptic activity by playing a major role in vesicle docking, fusion, and neurotransmitter release [16]. Phosphatidic acid (PA), which is produced with the aid of phospholipase D2 (PLD2) enzyme has shown commendable contribution in synaptic vesicle formation via recruitment of adapter complexes. Hence, it is essential to translocate the PLD2 enzyme to the sites of vesicle formation especially at times of high-frequency stimulation for vesicle recycling [7]. It has been reported that α-Syn is able to physically interact and regulate PLD2 via its N-terminal repeat region and thereby mediate binding to phospholipid membranes. Two mutations (A53T and A30P) in the N-terminal region of α-Syn has shown implications to autosomal dominant PD and alter the distinct role of α-Syn to associate and modulate phospholipids and thus, affect the vesicle recycling indirectly [7]. This could result in incomplete vesicle recycling causing a shortage of synaptic vesicles for neurotransmitter storage. In the context of DAergic neurons, this circumstance leads to the accumulation of DA in the cytoplasm and promotes OS through spontaneous oxidation of DA. Furthermore, mounting evidence has confirmed that DAQ is able to modify α-Syn by forming covalent adducts with α-Syn and thereby seeding the predilection of α-Syn to form toxic fibril species found in Lewy bodies. Recent studies indicate that it is also possible for the α-Syn fibrils to elevate cytoplasmic DA via binding and permeabilizing the synaptic vesicles by forming pores [9]. Fig. 1 depicts how a vicious cycle of escalated OS and aggregated α-Syn eventually results in progressive loss of DAergic neurons in PD.

Point mutations and multiplications in *SNCA*, the gene encoding α-Syn, are reported to cause the familial forms of PD, while data from GWAS and patient post-mortem samples have revealed risk variants of *SNCA* responsible for sporadic forms of PD [24]. In both familial and sporadic forms of the disease, α-Syn accumulation and aggregation occur due to impairments of oligomerization, folding and degradation of the protein [25]. Given the toxicity of these aggregates, multiple modalities of reducing the toxic intracellular accumulations could include, 1. Silencing *SNCA* gene (eg. siRNA/microRNA) 2. Increasing clearance of toxic aggregates (eg. Autophagy/Proteasome activation) and 3. Decreasing aggregate formation (eg. Post-translational modifications, modulators) [26]. To the latter end, a handful of regulators of α- Syn have been identified, of which the majority are post-translational regulators, together with a few transcriptional regulators, which are unlikely to become successful therapeutic candidates [27]. To address this void, the authors previously conducted a pooled RNAi screening and validation pipeline to probe the druggable genes capable of modifying α-Syn [28]. Out of the genes that were identified as significant influencers of α-Syn and potential therapeutic target candidates, DCLK1 stood out as a strong modulator of α-Syn. DCLK1 is a neuron-specific, microtubule binding kinase that can be targeted

effectively for the regulation of α-Syn. DCLK1 is a member of the Doublecortin (DCX) protein superfamily consisting of two N-terminal DCX domains and a C-terminal Ser/Thr kinase domain, with a Ser/Pro-rich PEST sequence in between. DCLK1 is known to be involved in microtubule polymerization, neurogenesis, and neuronal plasticity [29]. Recently, an extended study by Vázquez-Vélez and colleagues in the Journal of Neuroscience, elucidates the mechanism of α-Syn regulation by DCLK1 and its effects on synucleinopathy, predicting novel avenues for innovative pharmacological therapeutic targeting [30].

The authors began by confirming knockdown of DCLK1 via AAV8 mediated shRNA delivery to postnatal brain hippocampus and posterior cortex and observed a dramatic reduction in the α-Syn expression, but no reduction of *Snca* transcript levels, indicating post-transcriptional regulation of α-Syn by DCLK1. The majority of neurons expressed both α-Syn and DCLK1 and they co-localized in the neuronal soma of the cortex and SN. A strong interaction between DCLK1 and α-Syn was observed in HEK293T cells whereas a much weaker interaction was observed in the mouse brain. Co-localization with α-Syn and the known kinase activity of DCLK1, emphasized the importance to test whether DCLK1 could phosphorylate α-Syn. However, α-Syn lacks the phosphorylation motif for DCLK1, therefore, the *in vitro* kinase assay result of DCLK1 not phosphorylating α-Syn was anticipated, suggesting DCLK1 may not be directly involved in α-Syn phosphorylation. Furthermore, the authors tested whether the kinase activity of DCLK1 is necessary for its effect on α-Syn, using shRNA knockdown of DCLK1 in HEK293T cells and rescuing with wild type DCLK1 and a catalytically dead mutant of DCLK1. Both types were able to rescue the decrease of α-Syn levels regardless of the presence or absence of the Ser/Pro-rich PEST sequence, but the DCX domain alone was unable to show any effect. Collectively, these data suggest that the DCLK1 interacts with α-Syn via its kinase domain, but the effect of DCLK1 on α-Syn is not dependent on its kinase activity. Furthermore, using pharmacological inhibitors, they confirmed that DCLK1 protects α-Syn from lysosomal degradation.

To test the effect of DCLK1 on α-Syn, the authors carried out DCLK1 knockdown in Thy-1-α-Syn PD mouse model and a significant reduction was observed in pS129-α-Syn levels. However, this change was not observed when considering the 10X over expressed human or total α-Syn, even though all three types interacted with Dclk1. Nevertheless, they observed that pS129-α-Syn resides predominantly in the neuronal soma while human α-Syn is primarily seen in neuronal fibers. Further experiments demonstrated that the effect of DCLK1 is preferential only to the somatic α-Syn species. Next, with a viral model of α-Syn induced Parkinsonism, authors observed that the loss of DAergic neurons is rescued when shdclk1 was co-injected with a vector that contains *SNCA* cDNA but not with shLuciferase, indicating DCLK1 knockdown reduces phosphorylated α-Syn levels, thereby rescuing DAergic neuronal loss of SNpc in mice brains. The authors confirmed this finding with cortical neurons derived from iPSCs of patients with *SNCA* triplication, relating it to a human disease context. Taken together, these experiments strongly suggest that DCLK1 is a modulator of α-Syn levels, and interventions to reduce DCLK1 levels will in turn lower α-Syn accumulation and thereby exert neuroprotection.

In summary, the authors report a novel function for DCLK1, reducing α-Syn levels in the mature brain and genetic manipulations for knocking down DCLK1 shows great promise as a potential therapeutic strategy for PD. However, it is imperative to test this phenomenon with a knockout strategy to fully understand the therapeutic importance. The major caveat is the inefficiency of the knockout, given multiple unique transcripts and the effects of DCLK1 on microtubule stability. Therefore, a conditional knockout of DCLK1 in the adult mouse brain or designing efficient DCLK1 CRISPR/Cas9 knockout strategies on iPSC derived human neurons will facilitate the efforts of effective drug development against PD. As DCLK1 is a neuronally expressed kinase, it will be an attractive drug target, but given its role in neurodevelopment, careful analysis of DCLK1 targets, interactors and its mechanism of action on α-Syn regulation will further warrant its feasibility as a drug target.

Targeting synucleinopathies has been a challenge despite the different strategies employed to target stabilizing the physiological conformation, reducing the expression, inhibiting aggregation, enhancing intracellular and extracellular clearance, and interfering with the prion like cell to cell

spreading of α-Syn [31]. Diverse therapies including RNAi, immune therapy, inhibition of uptake of α-Syn by small molecules, application of engineered antibodies to prevent aggregation, and utilizing small molecule chaperones to rectify the misfolding proteins have reached different stages in pre-clinical and clinical studies [32]. However, many challenges including but not limited to, administration and penetration issues of antibodies, detrimental effects on neurons and peripheral tissues of RNAi therapy and identifying the most important toxic α-Syn species as the target are yet to be conquered [24]. Therefore, targeting α-Syn modulators could be a promising strategy, and DCLK1 with its capability to tightly regulate somatic α-Syn, could address some of the roadblocks with minimal effects on the regular function of α-Syn. Also, therapeutic targeting of kinases is a common approach, due to their involvement in different signaling pathways [33]. Interestingly, DCLK1 has been identified as a tumor specific stem cell marker and genetic inhibition and small molecule kinase inhibitors against DCLK1 have demonstrated suppressed DCLK1 activity, suggesting therapeutic potential for cancer [34]. Interestingly, the latter inhibitor, LRRK2-IN-1, targets LRRK2, which has been implicated in both familial and sporadic PD [34]. This raises the important question, does inhibiting DCLK1 via small molecule inhibitors reduce levels of α-Syn? Therefore, investigating the potential use of DCLK1 as a regulator of α-Syn would permit the use of existing beneficial Ser/Thr kinases inhibitors. Although targeting synucleinopathies through α-Syn regulators shows promise, in addition to these efforts, reducing α-Syn synthesis, increasing clearance and blocking α-Syn stabilization and the mechanism of neurodegeneration by α-Syn should be focused, which require multidisciplinary research for this multi-faceted disease [35]. Furthermore, discovering appropriate animal models and validated biomarkers reflecting disease progresssion will also assist the quest of PD therapy [36]. Therefore, discovering a novel function of DCLK1 in the mature brain to regulate .α-Syn, provides important insights to test promising disease-modifying therapies.

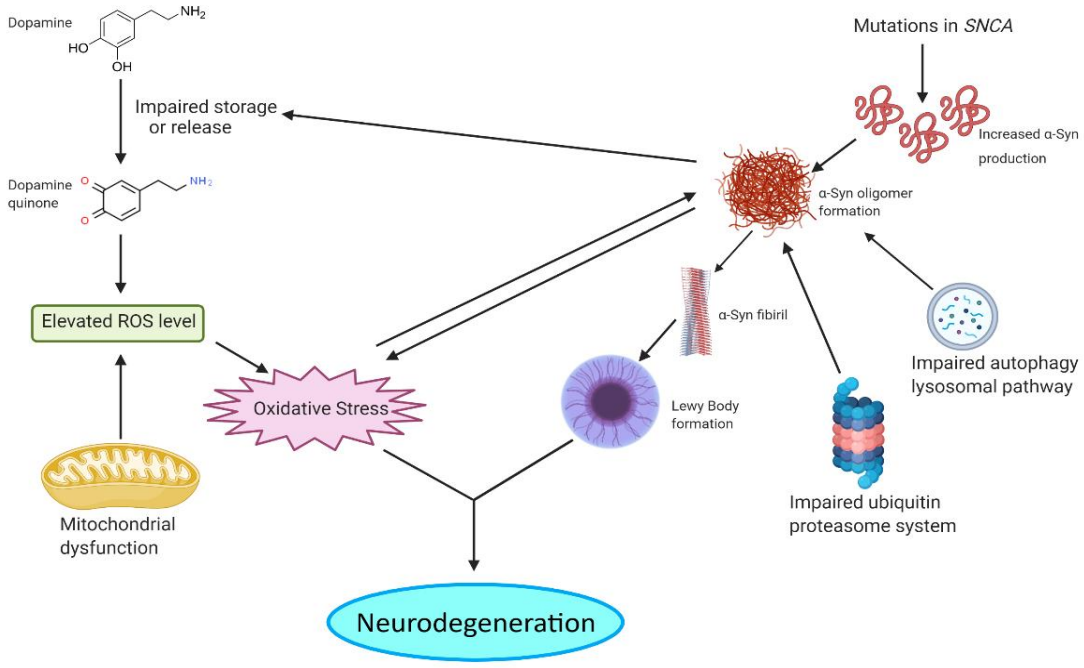

Fig. 1. Interplay between OS and α-Syn aggregates in the neurodegeneration process. The storage and release of DA are affected by the α-Syn oligomers, consequently elevating ROS levels inside the neurons. α-Syn oligomerization is supported by the heightened OS, mutations in *SNCA* and impairment in the protein degradation pathways

3. DISCUSSION

Despite the immense research efforts put forth on PD, the exact underlying neurodegeneration mechanism of the diseases remains elusive. However, information gleaned from recent studies on familial forms of PD allows deciphering the subtle and complex details of the disease pathogenesis. As per the mounting evidence from research conducted, it is crystal clear that PD is caused by a broad spectrum of factors that contributes to the overall landscape of disease trajectory and thus, a great deal of effort is required to understand the exact mechanistic details of the disease pathology. However, the notion that OS and α-Syn as the common denominator in PD pathogenesis will enable to explain the confounding intracellular events involved. This review explains the novel findings of employing two proteins to mitigate the demise of DAergic neurons in PD. Luo and colleagues elucidated a novel pathway involving NQO1 and Akt in which Akt phosphorylates NQO1 at two positions, S40 and T128 and making NQO1 susceptible to degradation through polyubiquitination. NQO1 being an antioxidant enzyme plays a crucial role in protecting the SN from toxic DA metabolites and it has been reported that NQO1 levels in PD brains decreased remarkably in an age dependent manner [15]. The authors confirmed a fundamental way of NQO1 degradation in PD brains by means of Akt phosphorylation. They further extended the research using mouse models coupled with several biochemical and cellular assays and demonstrated the possibility of using NQO1 stabilization as a potential remedy for PD via OS reduction. Similarly, Vázquez-Vélez and co-workers conducted an extended research to explore regulators of α-Syn and found that DCLK1 as a potent regulator. Further work of the authors confirmed post-transcriptional regulation of phosphorylated species of α-Syn by DCLK1 in synucleinopathy mouse models and established that α-Syn induced neurotoxicity in SN is alleviated upon DCLK1 knockdown. The authors explained the prospects of using *DCLK1* knockdown as a therapeutic target for PD in the context of humans using human patient derived iPSCs. Collectively, these results underpin the proposition that DCLK1 is a strong regulator of α-Syn and can be utilized as a drug target in the future owing to its druggability as a brain resident kinase. Furthermore, targeting a kinase imparts the benefit of repurposing the already approved kinase inhibitors to be tested against DCLK1 as well. Besides, utilizing the knowledge of well-established *in vitro* kinase assays for DCLK1, small molecule inhibitors can be tested effectively in the context of PD [37]. However, it is essential to carefully study the varying effect of kinase inhibitors displayed in a patient population and to study the unexpected toxicities associated with the inhibition of kinases [37]. In this context, investigating the precise role of DCLK1 in the human brain provides a better understanding of the effects of DCLK1 inhibition. This could be achieved by the development of a novel analytical technique to monitor the impact of DCLK1 inhibitors on its kinase activity [38]. Fig. 2 schematically represents the collaborative effect of the two pathways on neurodegeneration by blocking α-Syn degradation and phosphorylating NQO1 and eventually subjecting to degradation. The correlation of the two pathways highlights the importance of using a combined therapeutic approach for PD as it can help to address different pathophysiological conditions.

The emerging focus of PD therapeutics is non-DAergic strategies that can modify the disease condition rather than replacing the DA to reduce the motor symptoms. The pharmacological approaches for this include small molecule inhibitors, anti-inflammatory agents, iron chela tors and immunotherapies. α-Syn is considered an appealing drug target and thus, most of the pharmacological strategies are tailored that could either reduce its synthesis or enhance the clearance of α-Syn aggregates [39]. Short hairpin RNAs, antisense oligonucleotides have been proven to be effective in pre-clinical studies in reducing α-Syn synthesis [39]. However, there are concerns regarding this method due to the possibility of disrupting the normal physiological function of α-Syn. In addition, several compounds are in clinical studies that target inhibition of α-Syn misfolding such as glycerol phenylbutyrate, nilotinib and squalamine [39]. Nevertheless, the safety and tolerability of these drugs upon long term usage has to be examined carefully. The use of immunotherapies for PD against α-Syn oligomers is an attractive method to tackle this injurious process. Several synthetically produced monoclonal antibodies and intrabodies have demonstrated successful outcomes in phase I clinical trials and thus, further clinical studies are currently in progress. Furthermore, several non-DAergic neurotransmitters such as acetylcholine, serotonin, glutamate, adenosine and noradrenaline have shown suboptimal results in

positively improving the motor symptoms of PD patients during pre-clinical investigations. Therefore, further extensive research will require to show the efficacy and safety of these drugs for PD patients [39]. Even though the common clinical feature in PD is the nigrostriatal neurodegeneration, PD pathophysiology is heterogeneous with the involvement of several molecular and biological pathways. Majority of the disease-modifying therapies address only a single causative factor involved in PD pathology. However, in this review, we suggest a combined therapeutic approach using NQO1 and DCLK1 as targets to address two different but interconnected pathophysiological pathways that work sequentially towards neurodegeneration in PD (Fig. 2). As depicted in Fig. 2, DCLK1 inhibits the degradation of α-Syn leading to its accumulation and aggregation inside the neurons. Aggregated α-Syn disrupts the membranes of DA storage vesicles in nerve terminals, facilitating the release of DA freely into the cytoplasm. The free DA is then spontaneously oxidized resulting in elevated OS that leads to activation of NQO1 and Akt. Activated Akt phosphorylates NQO1 making it susceptible for degradation. Built on this, designing a combined therapeutic approach for simultaneous inhibition of NQO1 degradation and promoting excess α-Syn degradation may address several causative factors associated with PD including genetic mutations (eg., *SNCA* mutations), mitochondrial dysfunction, oxidative stress and protein aggregation. One of the frequently used PD drug targets is α-Syn and its gene *SNCA*, albeit blocking α-Syn alone may cause adverse effects to the normal function of the neurons. In contrast, inhibiting DCLK1 function may permit clearance of excess α-Syn, minimizing its aggregation. Similarly, stabilization of NQO1 also provides necessary antioxidant potential to the neurons to carry out the normal cellular functions involving the DA system. Unlike, most of the other drug targets that inhibit a cellular signaling pathway or enhance the production or uptake of DA, DCLK1 and NQO1 is focused on maintaining the normal activities inside cell involving DA and α-Syn.

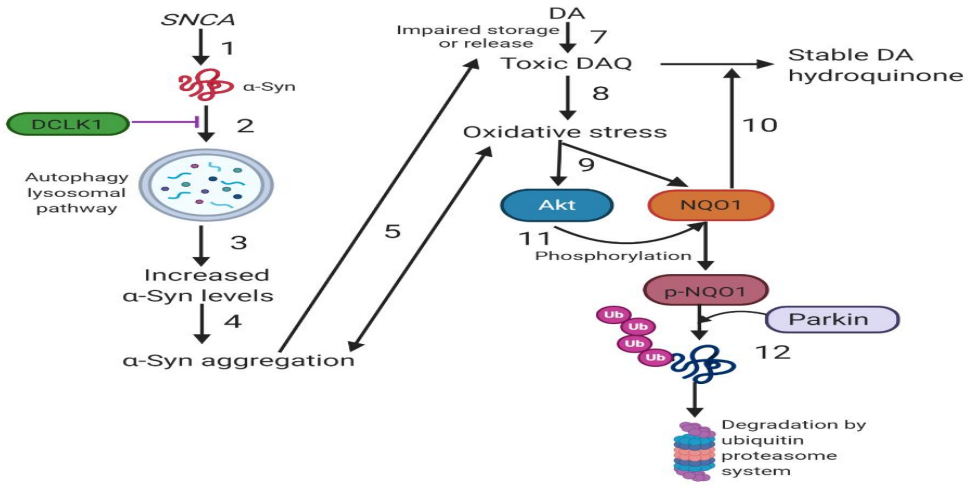

Fig. 2. Illustration of how DCLK1 and Akt/NQO1 hinder the α-Syn aggregate clearance pathway and DAQ stabilization mechanism inside DAergic neurons resulting in neurodegeneration. 1. α-Syn is produced by the transcription of SNCA. 2. DCLK1 inhibits the degradation of α-Syn by inhibiting the autophagy lysosomal pathway. 3. Consequently, α-Syn accumulate inside the cell. 4. Accumulated α-Syn form aggregates which then convert to toxic α-Syn oligomers and fibrils. 5. α-Syn aggregates affect the storage and release of DA. 7. When DA is freely available it undergoes autoxidation to form toxic DAQ. 8. Toxic DAQ increases OS inside the neuron. 9. Akt and NQO1 are activated as a response to cellular stress. 10. NQO1 catalyses the conversion of DAQ to a more stable hydroquinone form. 11. Activated Akt phosphorylates NQO1. 12. Phosphorylated NQO1 is polyubiquitinated with the involvement of parkin, which is subsequently degraded by the ubiquitin proteasome pathway

Although both NQO1 and DCLK1 explicitly manifested strong evidence as potential drug targets that can be manipulated to diminish DAergic neuronal loss in PD, further extensive research is required to validate the application of these proteins in the context of humans. It is imperative to check the side effects of complete or partial knockdown of DCLK1 as well as elongation of NQO1 stability against degradation in human-derived iPSCs and PD mouse models. Nevertheless, without the support of PD research models that mimic the exact biological phenomenon in human PD, the development of new drugs or discovering the mechanisms of PD pathology to identify potential drug target candidates seems unapproachable. Furthermore, discovering novel biomarkers is equally important to monitor the disease progression accurately as well as to escalate the research efforts by reducing the time frame required to show the successful engagement of the administered drugs in the targeted regions [40].

In addition, non-pharmacological and more ambitious approaches such as gene therapy, cell transplantation and deep brain stimulation techniques have gained notable attention among the other medications available [41]. However, drawbacks of these methods such as difficulty in regulating the amount of therapy delivered in gene therapy, ethical and safety considerations in cellular therapy and the inability to protect neurons from degeneration in both cell therapy and deep brain stimulations have raised hesitancies for clinical employment of these strategies as PD therapies [41]. Therefore, intensive research of the disease pathophysiology and genetics will allow the development of specific treatments tailored to alter the course of the disease. Equipped with the aforementioned advances, a combined therapeutic approach using both NQO1 and DCLK1 may open unprecedented avenues for a viable disease-modifying neuroprotective strategy for PD.

4. CONCLUSION

PD is a devastating neurodegenerative disorder that has affected many lives across the world. The intricacy and heterogeneity of PD pathophysiology along with the multifactorial causative factors involved makes PD a huge challenge for modern neurobiology to establish a specific and personalized drug for PD. In the search for PD therapeutics, identifying potential drug target candidates is a crucial aspect. In this review, we have highlighted the discovery of two crucial proteins that contribute to PD associated neurodegeneration namely; NQO1 and DCLK1 that have significant potential as drug targets. The interrelation of these two proteins in abating α-Syn aggregate formation and reducing OS opens up a new facet as a combinational therapeutic approach towards PD. Yet, it is essential to expand the research using novel techniques and biomarkers to monitor the precise effect of targeting NQO1 and DCLK1 in the context of humans. We strongly believe that the combined disease-modifying therapy targeting stabilization of NQO1 and inhibition of DCLK1 to reduce OS and α-Syn aggregates may provide enhanced long-term neuroprotection for PD patients over single-modality treatment.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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