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Master's in Molecular and Microbial Biology

Work carried out under the guidance of: Professor Doctor Inês Araújo and Professor Doctor Tiago Outeiro

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Direitos de Autor

Aknowledgements

Firstly, I would like to thank my family for their unconditional support and for accepting all my decisions. However, I would especially like to thank my parents, maternal grandparents, and Hugo for all their help and for being the essential pillars of my life. These are the people who always listen to me and who will always be here to help me with their patience, affection, love, and unrivalled support.

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Thank you all.

Abstract

Alpha-synuclein (α-Syn) is present in Lewy Bodies and its aggregation has been associated with Parkinson's disease (PD). However, the cause of α-Syn aggregation is unknown. An increase in reactive nitrogen species (RNS) in PD has been described, which can lead to oxidative posttranslational modifications such as S-nitrosylation. This chemical process involves the covalent attachment of a nitric oxide group (NO) to a cysteine thiol within a protein to generate an S-nitrosothiol (SNO). Because it does not have cysteine residues in its structure, α-Syn is not a direct target of S-nitrosylation. When S-nitrosylated, enzymes such as protein disulfide isomerase (PDI) and serine racemase (SR) can cause misfolded proteins to aggregate. Nonetheless, no research has been conducted to determine how S-nitrosylation of these proteins is involved in α-Syn aggregation. The current study aimed to determine how S-nitrosylation in the proteome, induced by CysNO treatment, affects α-Syn aggregation and cell viability by examining the presence of α-Syn aggregates and cleaved caspase-3 in SH-SY5Y cells expressing WT, A53T, or A30P α-Syn. The western blot results indicate that PDI is indeed S-nitrosylated. However, SR was not proven to be S-nitrosylated. These results indicate that only one protein (PDI) may contribute to the aggregation process of α-Syn. Using the immunocytochemistry technique, the percentage of transfected cells, the number of transfected cells with aggregates, the presence of cleaved caspase-3 (an indicator of cell death) and the presence of condensed/fragmented nuclei were quantified. These results showed that S-nitrosylation increases aggregation of α-Syn, depending on its form. And that the amount of cytotoxicity present in cells exposed to CysNO is not significant. These studies suggest that S-nitrosylation may increase the aggregation process of α-Syn. Future studies could elucidate a cause-effect between the nitrosylation of proteins such as PDI and the aggregation of α-Syn.

Keywords: Parkinson´s disease; Alpha-synuclein; S-nitrosylation; Aggregation

Resumo

A doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum, sendo a primeira a doença de Alzheimer. A idade é um dos fatores de risco mais importantes no desenvolvimento da DP, e os homens são mais suscetíveis do que as mulheres. Os sintomas principais da DP são a rigidez dos movimentos, bradicinesia, tremores em repouso e instabilidade postural. Existem duas formas da DP, a forma esporádica, que tem uma origem idiopática e que está associada ao envelhecimento, e a forma familiar, com uma origem genética e um início precoce. A DP está integrada no grupo de sinucleinopatias, que constituem um grupo de patologias que estão associadas à acumulação da proteína alfa-sinucleína (α-Syn). Esta proteína agrega-se no tecido neuronal, originando os corpos de Lewy. O processo de neurodegenerescência evolui lentamente e propaga-se pelas diferentes áreas do sistema nervoso, nomeadamente na *substantia nigra pars compacta*, com a consequente perda dos neurónios dopaminérgicos e respetivos sintomas motores da doença. No entanto, a causa da agregação da α-Syn é incerta. Foi demonstrado que os agregados da α-Syn se propagam através de um processo de célula a célula, como os priões. Existem diferentes causas para a agregação da α-Syn, incluindo mutações ou modificações traducionais. A α-Syn é uma proteína solúvel que está expressa de forma abundante nas áreas pré-sinápticas e peri-nucleares do sistema nervoso central. Está envolvida na regulação da libertação de neurotransmissores, função sináptica e plasticidade sináptica. Foram identificadas várias mutações pontuais no gene *SNCA* da α-Syn, incluindo A30P, A53E, A53T, E46K, H50Q, and G51D. Apesar de possuírem diferentes taxas de agregação, foi demonstrado que as mutações A30P e A53T podem causar oligomerização precoce, o que pode levar ao aparecimento precoce da doença. Estas mutações vão ser muito importantes ao longo deste estudo. Recentemente, verificou-se um aumento das espécies reativas de nitrogénio (RNS) na DP, o que pode levar a modificações pós-traducionais conhecidas como S-nitrosilação. A S-nitrosilação consiste na ligação covalente de um grupo de óxido nítrico (-NO) a um tiol de cisteína numa proteína para gerar um S-nitrosotiol (SNO). A S-nitrosilação é uma reação que tem um papel principal na regulação da função proteica e nas vias de sinalização de vários processos fisiológicos. Já foi demonstrado que a S-nitrosilação tem capacidade de regular diversos processos celulares, como o

metabolismo, apoptose e resposta imune. Esta reação pode causar mudanças conformacionais, estimular ou inibir a atividade proteica, modificar interações proteína-proteína, e afetar a formação de agregados proteicos ou a sua localização dentro de circunstâncias fisiológicas. Como resultado, estas modificações podem ter impacto nas vias de transdução de sinais e na função neuronal. Como não tem resíduos de cisteína na sua estrutura, a α-Syn não é um alvo direto desta modificação pós-traducional. No entanto, existem outras proteínas que ao serem S-nitrosiladas podem levar à sua agregação e consequentemente pode levar ao aumento da sua toxicidade. Como exemplo existe a proteína dissulfureto isomerase (PDI) e a serina racemase (SR), que podem contribuir para a agregação de proteínas malconformadas. Os membros da família PDI podem formar, quebrar ou reorganizar ligações dissulfureto, para além de atuarem como chaperones moleculares e dissulfureto oxidorredutases/isomerases. Foi demonstrado que a PDI tem capacidade de bloquear a agregação da α-Syn devido à sua função de chaperone e oxidorredutase. SR tem a capacidade de converter a L-serina em D-serina, que é um co-agonista de recetores glutamatérgicos. Se a SR for fisiologicamente S-nitrosilada, a sua atividade enzimática que controla as interações com o ATP é suprimida. Ainda não foram realizados estudos com o objetivo de determinar de que forma a S-nitrosilação destas proteínas está envolvida na agregação da α-Syn. Portanto, o objetivo deste estudo é avaliar a contribuição das proteínas S-nitrosiladas, como a PDI e a SR, na agregação e toxicidade da α-Syn, na linha celular neuronal humana, SH-SY5Y. Esta linha celular é isolada a partir de um neuroblastoma metastático da medula óssea. Esta linha celular apresenta um fenótipo catecolaminérgico, contém enzimas como a tirosina hidroxilase e a dopamina- β- hidroxilase, e tem a capacidade de sintetizar dopamina e noradrenalina. A tirosina hidroxilase é a enzima limitadora da taxa de síntese de catecolaminas e converte a tirosina em L-DOPA, o precursor da dopamina, que é convertida em noradrenalina pela dopamina- β- hidroxilase e posteriormente em adrenalina. Para além disso, esta linha celular tem a capacidade de expressar constitutivamente o gene da α-Syn. De forma a determinar como a S-nitrosilação afeta a agregação da α-Syn e a viabilidade celular, examinou-se a presença de agregados de α-Syn e caspase-3 clivada em células SH-SY5Y previamente transfetadas com a α-Syn sobreexpressa (WT) e com os genótipos mutantes da α-Syn (A53T e A30P), na presença e ausência de tratamento com CysNO. Através da utilização do ensaio de

biotin switch, técnica que permite a substituição do S-nitrosotiol pela biotina na deteção e identificação proteómica de proteínas S-nitrosiladas em células. Foi utilizado o ascorbato, como agente redutor dos nitrosotióis. As proteínas biotiniladas são purificadas através da sua captação com resina de avidina imobilizada. Neste caso, a incorporação da biotina através da via de ligações dissulfídicas é uma vantagem, porque permite uma eluição fácil através da incubação com o agente redutor, permitindo também eliminar as proteínas biotiniladas endógenas, que permanecem ligadas à avidina. De forma a detetar-se as proteínas especificas, realizou-se um western blot contra estas proteínas. Os resultados do western blot indicam que a PDI é de facto S-nitrosilada. No entanto, não se provou que a SR era S-nitrosilada. Estes resultados indicam que apenas uma proteína (PDI) poderá contribuir para o processo de agregação da α-Syn. Através da utilização da técnica de imunocitoquímica, técnica laboratorial utilizada para se visualizar a localização de uma proteína pela utilização de um anticorpo primário específico, quantificou-se a percentagem de células transfetadas, o número de células transfetadas com agregados, a presença de caspase-3 clivada (indicador de morte celular) e a presença de núcleos condensados/fragmentados. Estes resultados demonstraram que a S-nitrosilação aumenta agregação da α-Syn, dependendo da sua forma. E que a quantidade de citotoxicidade presente nas células expostas ao CysNO não é significativa. Estes estudos sugerem que a S-nitrosilação pode aumentar o processo de agregação da α-Syn. Estudos futuros puderam elucidar uma causa efeito entre a nitrosilação de proteínas como a PDI e a agregação da α-Syn.

Palavras-chave: Doença de Parkinson; Alfa-sinucleína; S-nitrosilação; Agregação

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Abbreviations

α-Syn	Alpha-Synuclein
aa	Amino acids
ADP	Adenosine-diphosphate
AK	Adenylate kinase
AKDR	AK detection reagent
ALP	Autophagy-lysosomal pathway
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ATF6	Activating transcription factor 6
ATP	Adenosine-triphosphate
BCA	Bicinchoninic acid
BH4	Tetrahydrobiopterin
BSA	Bovine serum albumin
Ca2+/ CaM	Calcium/calmodulin
CcO	cytochrome c oxidase
CTD	C-terminal domain
Cys	Catalytic cysteine
DAAO	D-amino acid oxidase
DMEM	Dulbecco's Minimum Essential Medium
DTT	dithiothreitol
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmatic Reticulum
ERAD	ER association degradation
FAD	Flavin adenine dinucleotide
FBS	Fetal Bovine Serum
GluN	glutamate-binding subunits
Golga3	Golgin subfamily A member 3
GRIP	Glutamate receptor interacting protein
GSH	Glutathione

GSNO	S-nitroso glutathione
ICC	Immunocytochemistry
iNOS	Inducible nitric oxide synthase
Ipsc	Induced pluripotent stem cells
IRE1	Inositol-requiring enzyme 1
KO	Knockout
L-Arg	L-arginine
L-Cys	L-cysteine
LRRK2	Leucine-rich repeat kinase 2
LTP	Long-term potentiation
MAM	Mitochondria-associated endoplasmic reticulum membranes
Meth	Methamphetamine
mGluR	Metabotropic glutamate receptors
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtNOS	Mitochondrial nitric oxide synthase
MTS	Mitochondrial targeting sequence
NAC	Non-amyloid component
NMDAPRs	N-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NO-	Nitric oxide
NOR-3	(E)-ethyl-2-(E) -hydroxyimino-5-nitro-3-hexeneamide
NOS	Nitric oxide synthase
NT	N-terminal regulatory domain
P/S	Penicillin-streptomycin
PA	Phosphatidic acid
PD	Parkinson´s disease
PDI	Protein disulfide isomerase
PERK	Protein kinase RNA-like ER kinase
PFA	Paraformaldehyde
PFFs	Preformed fibrils
PICK1	Protein interacting with C kinase 1
PINK1	PTEN-induced putative kinase 1
PIP2	Phosphatidylinositol (4,5) -biphosphate

PLP	Pyridoxal 5’- phosphate
P-Ser	Phosphorylation of serine
P-Ser65-Ub	Phosphorylated Ser65 of Ub
PTMs	Post-translational modifications
PTP	Osmotic transition pore
QC	Quality control
REP	Repressor Element of Parkin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SNO	S-nitrosothiol
SNO-PDI	PDI S-nitrosilated
SNO-proteins	S-nitrosylated proteins
SR	Serine racemase
TBS-T	Tris-buffered saline with tween-20
TFEB	Transcription factor EB
TH	Tyrosine Hydroxylase
TLR4	Toll-like receptor 4
TNTs	Tunelling nanotubes
Ubl	Ubiquitin-like
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VAMP2	Vesicle-associated membrane protein 2
VMAT2	Vesicular Monoamine Transporter 2
Zn	Zinc

Introduction

1. Parkinson's Disease

Parkinson's disease (PD) was first described in 1817 by James Parkinson. PD is one of the most common neurodegenerative diseases, after Alzheimer's disease. About 1% of the population above 60 years is affected (Tysnes and Storstein, 2017), but this raises to 3% after the age of 80 (Antony et al., 2013). The major symptoms of PD are progressive and can be divided into motor and non-motor symptoms. Movement stiffness, bradykinesia, rest tremor, and postural instability are examples of motor symptoms. In contrast, non-motor symptoms like as cognitive decline, depression, gastrointestinal symptoms, olfactory and gustatory deficiencies, mood swings, sleep disorders, and dementia are more frequent in later stages. There are two forms of PD, the sporadic form, which has an idiopathic origin and is associated with a conjunction of environmental factors and genetic ones. And the familial form, with a genetic origin and early onset (Jankovic, 2008).

The selective death of dopaminergic neurons, particularly in the *substantia nigra pars compacta* in the midbrain (Hirsch, 1994), the loss of dopamine in the striatum (Kish et al., 2010), microgliosis (Croisier et al., 2005), and the development of fibrous inclusion bodies on the surviving neurons (Gibb et al., 1987) are the hallmarks of this disease. These are named Lewy bodies, in the cell bodies (soma), and Lewy neurites, in axons and neurites (Braak et al., 2003; Spillantini et al., 1997). Lewy bodies are composed of protein clusters like ubiquitin (Kuzuhara et al., 1988), tyrosine hydroxylase (Nakashima and Ikuta, 1984), synaptophysin (Nishimura et al., 1994), and especially Alpha- Synuclein (α-Syn) (Galvin et al., 1999; Spillantini et al., 1998; Tofaris and Spillantini, 2005), the last one promotes aggregation when mutated or overexpressed. These inclusions were initially discovered in post-mortem examinations of the brains of PD patients (Tofaris and Spillantini, 2005) (**Figure 1**).

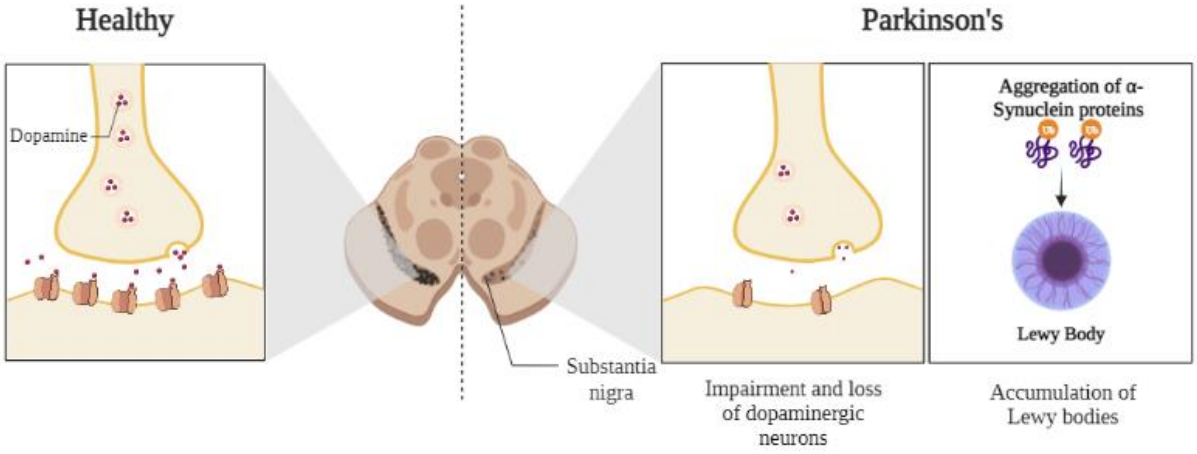

Figure 1 | Progression of PD in the Substantia Nigra. PD is a complex, progressive neurodegenerative disease characterized by the death of dopaminergic neurons and the formation of Lewy bodies, which are aggregations of α-Syn protein clusters coupled to ubiquitin. While the mechanism is not entirely understood, it is hypothesized that when Lewy bodies develop in the substantia nigra, they damage dopaminergic neurons and interfere with pathways essential for motor function and other cognitive processes. Created with BioRender.

Synucleinopathies are a group of diseases that are characterized by Lewy bodies. Dementia with Lewy bodies (Grazia Spillantini et al., 1998), multiple system atrophy (MSA) (Gai et al., 1998), Parkinsonism-dementia Complex of Guam (Yamazaki et al., 2000), pure autonomic failure (Arai et al., 2000), frontotemporal dementia (Bougea et al., 2017), and Krabbe disease are a few of these diseases (Smith et al., 2014).

The development of PD is more likely to occur due to a variety of environmental factors, including ageing, pesticide exposure, mitochondrial failure, and traumatic brain damage (Lill, 2016). Autosomal dominant, such as alpha-synuclein (*SNCA*) and Leucine-rich repeat kinase 2 (*LRRK2*), can also contribute to the development of PD. The *SNCA* gene, that generates the protein known as α-Syn and is the most significant gene in PD, enhances the mechanism of toxicity by expressing the protein that aggregates into Lewy bodies. Lewy bodies are also formed by the *LRRK2* gene, however other characteristics may occasionally be detected instead of Lewy bodies (Marín, 2006). Recessive mutations, such as those found in genes like PTEN-induced putative kinase 1 (*PINK1*), *Parkin* and *DJ-1* (*Park7*). Homozygous deletion of specific sites in these genes also results in parkinsonism symptoms including the formation of Lewy bodies, suggesting a relationship between the deletions and α-Syn expression. Some of these mutations lead to the appearance of reactive oxygen species (ROS), which play a central role in the progression of PD (Zhang et al., 2019).

Numerous studies are being performed to understand more about the molecular pathways that lead soluble unfolded proteins to aggregate and harm neuronal survival and function. Multiple studies have identified ROS and reactive nitrogen species (RNS) generation as significant pathogenic factors in neurodegenerative disorders (Nakamura and Lipton, 2011; Nakamura et al., 2013). Antioxidant dysregulation can be caused by environmental contaminants such as paraquat and maneb, as well as age-related low-grade chronic inflammation. This can result in an imbalance between the pro- and antioxidant systems, leading to protein misfolding and neurodegeneration (Nakamura et al., 2013).

1.1 Genetic forms PD

SNCA, also known as PARK1 and PARK4, was the first gene found in PD. PARK1 is caused by missense mutations, while PARK4 is generated by SNCA multiplications. The most frequent genetic cause of sporadic and inherited PD is mutation in the *LRRK2* gene, often known as *PARK8* (**Figure 2**). The product of this gene, also known as dardarin, is a 286 kDa highly conserved protein with numerous independent domains that are a member of the ROCO protein family (Funayama et al., 2002). Seven of the twenty mutations in this gene have been identified as pathogenic, and G2019S is the most frequent mutation in the *LRRK2* gene. As a result of this mutation occurs a substitution of an amino acid (glycine to serine) at position 2019 in the protein. An elevated risk of developing PD is linked to this mutation, which accounts for 5-6% of familial and 2% of sporadic cases of PD. R1441G/C, Y1699C, N1437H and I2020T are further documented mutations (Goldwurm et al., 2005). It is thought that mutant LRRK2 protein might cause an increase in kinase activity, which can disturb cellular functions and allow toxic substances to accumulate within neurons. These toxic substances may cause neuronal malfunction and ultimately cell death (Zimprich et al., 2004).

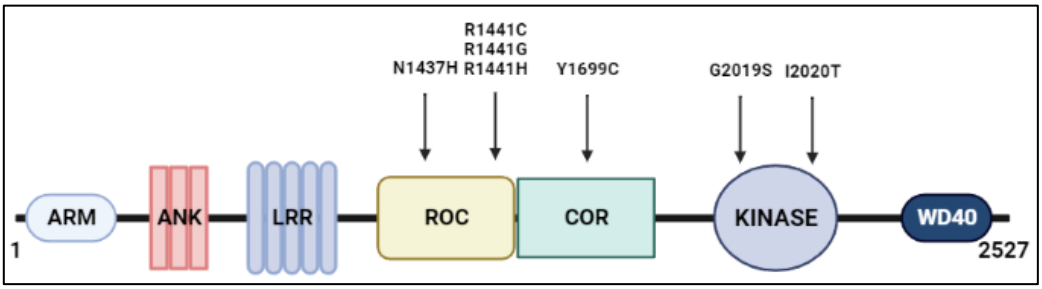

Figure 2| Schematic representation of the LRRK2 protein domains and pathogenic mutations associated with PD. Adapted from Bardien et al., 2011. Created with BioRender.

With 465 amino acids, *Parkin*, also known as the *PARK2* gene, is a ubiquitin ligase protein that has a variety of activities in cells, especially in neurons. Autosomal recessive mutations in this protein result in juvenile PD with early onset (Kitada et al., 1998). The ubiquitin-proteasome system (UPS), which is an intracellular protein degradation machinery, uses Parkin as an E2-dependent ubiquitin ligase. It was discovered that this protein has a role in the quality control (QC) of proteins associated with autophagy and UPS. E2 enzymes are initially recruited to the RING1 domain of Parkin-mediated ubiquitination, where the charged ubiquitin they carry is transferred to a catalytic cysteine (Cys431) in the RING2 domain. Finally, the charged ubiquitin is assigned to the primary amino group of the substrate via the formation of an iso-peptide bond (Wenzel et al., 2011). According to several research, the phosphorylation of ubiquitin (Kane et al., 2014; Koyano et al., 2014) and serine 65 (P-Ser65) (Kondapalli et al., 2012; Matsuda et al., 2010; Shiba-Fukushima et al., 2012) at the same site can both improve Parkin activity. Although, ubiquitin's Ser65 phosphorylation enhances its interaction with RING1 whereas phosphorylating Parkin's UbI domain promotes the separation of Parkin from RING1 (**Figure 3**). Although *Parkin* has cysteines in its structure, it may not be involved in the a-Syn aggregation process, since the forms of parkinsonism related to this mutation do not form Lewy bodies and are known as non-Lewy body disease.

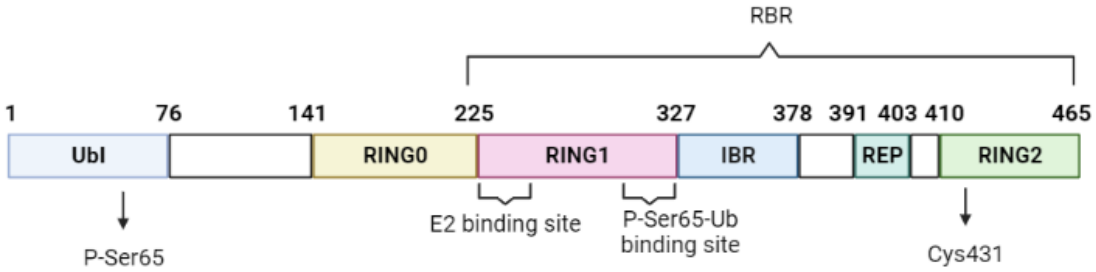

Figure 3 | The domain structure of Parkin. This protein has an N-terminal region that is ubiquitin-like (Ubl), a C-terminal region that is RING1-IBR-RING2 (IBR), and a middle segment that connects the two domains. The linker segment contains a zinc chelating RING0 domain that is situated adjacent to the RBR domains. Between the IBR and RING2 domains, there is an additional motif named the Repressor Element of Parkin (REP). Adapted from Wang et al., 2021. Created with BioRender.com

The protein PINK1, also referred to as BRPK and PARK6, protects cells against mitochondrial stress-induced malfunction and can activate Parkin in mitophagy, which helps the cell remove damaged mitochondria. *PINK1* gene has a 581 amino acid protein encoded by the 8 exons. The mitochondrial targeting sequence (MTS), transmembrane region (TM), N-terminal regulatory region (NT), N-lobe of the kinase domain, C-lobe of the kinase domain, and C-terminal domain (CTD) constitute PINK1.

PD caused by mutations in this protein is autosomal recessive and exhibits symptoms comparable to idiopathic PD without dementia (**Figure 4**) (Shimura et al., 2000; Spratt et al., 2014).

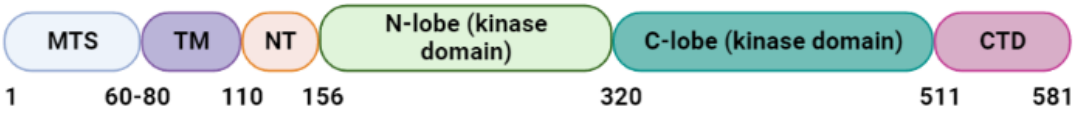

Figure 4 | Schematic representations of PINK1 domains. It has an N-terminal mitochondrial targeting sequence (MTS), a transmembrane domain (TM), an N-terminal regulatory domain (NT), a conserved protein kinase domain with an N- and C-lobe, and finally a C-terminal domain (CTD). Adapted from Quinn et al., 2020. Created with BioRender.com

Parkin overexpression may partially compensate for *PINK1* loss, whereas *PINK1* overexpression cannot, indicating that *PINK1* and *Parkin* share the same mechanism of activity (Gautier et al., 2008; Palacino et al., 2004). There is a model for Parkin activation that states that Parkin activity is suppressed in the normal state. By directly phosphorylating Ser65 in the Ubl domain and indirectly phosphorylating Ub Ser65 at the same location as Parkin, PINK1 can activate Parkin. To completely activate Parkin, these two mechanisms are necessary. When phosphorylated Ser65 of Ub (P-Ser65-Ub) attaches to the RING1 domain, Parkin's Ubl domain is completely released, which stimulates Ser65's phosphorylation by PINK1 in the UBL. The E2 binding site and Cys431 then become exposed when REP and RING2 are released, causing Parkin to switch to its fully active conformation (**Figure 5**) (Kumar et al., 2015).

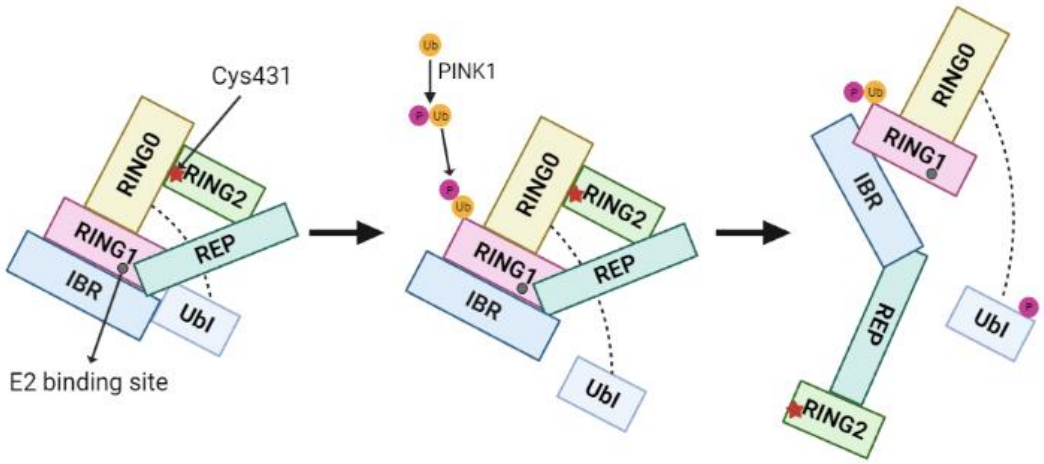

Figure 5 | Model of Parkin activation. Parkin activity is suppressed in the normal condition. PINK1 can activate Parkin by phosphorylating S65 in the Ubl domain directly and indirectly at the same location as Parkin. Phosphorylated S65 at the Ub binding site on the RING1 domain causes Parkin's Ubl domain to be completely released, facilitating PINK1-mediated phosphorylation of Ser65 in the UBL. As a result of the released REP and RING2, the E2 binding site and Cys431 are exposed, resulting in a fully active Parkin conformation. Adapted from Wang et al., 2021. Created with BioRender.com

Another gene associated with PD is identified as *DJ-1*, also known as *Park7*. Deletions in the *DJ-1* gene caused by mutations may trigger an uncommon type of autosomal recessive early-onset PD (Bonifati et al., 2003). 189 amino acid residues are encoded by the *DJ-1* gene (Moore et al., 2005, 2006). In the cytoplasm, mitochondria, and nucleus, the protein occurs as a homodimer (Zhang et al., 2005). DJ-1 is a protein sensor that responds to oxidative stress, protects cells from ROS, and assists to maintain proper dopaminergic function (Inden et al., 2006; Mitsumoto and Nakagawa, 2009; Taira et al., 2004). Since normal protein suppresses α-Syn aggregation and functions as a downstream mediator in PINK1/parkin-dependent mitophagy, it may potentially offer protection against PD (Imberechts et al., 2022; Shendelman et al., 2004).

Table 1 | Known genes associated with PD with respective proteins, position in chromosome, and disease type.

Gene	Protein	Chromosome	Inheritance
SNCA (PARK1/PARK4)	Alpha-synuclein	4q21	Autossomal Dominant
PARK2	Parkin	6q25·2–27	Autossomal Recessive
PARK3	Unknown	2p13	Autossomal Dominant
UCHL-1 (PARK5)	Ubiquitin c-terminal hydrolase	4p14	Autossomal Dominant
PINK1 (PARK6)	Pten-induced putative kinase 1	1p35–36	Autossomal Recessive
PARK7	DJ-1	1p36	Autossomal Recessive
LRRK2 (PARK8)	Leucine-rich repeat kinase 2	12p11·2– q13·1	Autossomal Dominant
ATP13A2 (PARK9)	Lysosomal type 5 ATPase	1p36.13	Autossomal Recessive
PARK10	Unknown	1p32	Unknown
GIGYF2 (PARK11)	GRB interacting GYF protein 2	2q37.1	Autosomal Dominant
PARK12	Unknown	Xq21-q25	X-linked
HTRA2 (PARK13)	HTRA serine	2p13.1	Autosomal Dominant
PLA2G6 (PARK14)	Phospholipase A2	22q13.1	Autosomal Recessive
FBXP7 (PARK15)	F-box only protein 7	22q12.3	Autosomal Recessive
PARK16	Unknown	1q32	Unknown
VPS35 (PARK17)	Vacuolar protein sorting 35	16q11.2	Autosomal Dominant

EIF4G1 (PARK18)	Eukaryotic translation initiation factor 4	3q27.1	Autosomal Dominant
DNAJC16 (PARK19)	DNAJ/HSP40 homolog subfamily C member 6	1p31.3	Autosomal Recessive

2. Alpha-synuclein

Maroteaux discovered α-Syn, a small intracellular protein, in 1988 (Maroteaux et al., 1988). Is continuously expressed in the brain (Jakes et al., 1994),but the neocortex, hippocampus, olfactory bulb, striatum, thalamus, and cerebellum contain the highest concentrations of α-Syn (Iwai et al., 1995). This protein is also expressed on red blood cells (Nakai et al., 2007), in the heart (Ueda et al., 1993; Uéda et al., 1994), pancreas (Ueda et al., 1993; Uéda et al., 1994), and skeletal muscles (Askanas et al., 2000; Uéda et al., 1994); Although, the concentration is much lower than in the brain (Ueda et al., 1993; Uéda et al., 1994).

2.1 α-Syn structure and conformation

α-Syn, which is encoded by the *SNCA* gene on chromosome 4q21–22 and has a molecular weight of 14.4 kDa and 140 amino acids (aa). It is a protein that is expressed in neurons and has three distinct domains in its primary aa sequence: i) The N-terminus, amphipathic, that is localized between the residue 1 and 60, has a structure of α-helix (Perrin et al., 2000); ii) a hydrophobic non-amyloid component (NAC) domain, between the residue 61 and 95, promotes the aggregation of α-Syn to form fibrils; iii) The last domain is C-terminus, between the residue 96 and 140, protects α-Syn from aggregation and is responsible for ion and polyamine binding. It has been demonstrated for the first two domains, N-terminus and NAC, to form an amphipathic α-helix that aids in binding to membrane lipids, and the NAC domain is also capable of conforming in a β-sheet shape that promotes aggregation (Sulzer and Edwards, 2019). The first two domains include seven repeats of the sequence KTKEGV, but the C-terminal region is rich in negatively charged amino acids and proline residues (**Figure 7**) (Mazzetti et al., 2023).

α-Syn is found on the cytosol as a soluble and unfolded monomer; nevertheless, may form a multimer when attached to the membrane of synaptic vesicles. The existence of a stable, insoluble tetrameric form in the cytoplasm is up for discussion

(Bartels et al., 2011a). This adherence to membranes stimulates the development of multimers and subsequently protects from the production of α-Syn fibrils (Davidson et al., 1998). This protein is mainly expressed in the presynaptic terminal of the brain (Withers et al., 1997), nucleus (Desplats et al., 2011; Kontopoulos et al., 2006), mitochondria (Li et al., 2007), endoplasmic reticulum (Calì et al., 2012) and in mitochondria-associated endoplasmic reticulum membranes (MAM) (Guardia-Laguarta et al., 2014).

2.2 α-Syn function

This protein was first isolated from a Torpedo electric organ, was used as a model system to study the protein because it has a high concentration of α-Syn, where is localized in presynaptic terminal of neurons (Maroteaux et al., 1988). Even though the specific function of α-syn remains unclear, it is thought to be essential for synaptic functions, which is consistent with subsequent research showing that α-Syn interacts with synaptobrevin-2, also known as VAMP2. VAMP2 has an essential role in the release of neurotransmitters from the presynaptic terminal. The interaction between α-Syn and VAMP2 is thought to be involved in the regulation of neurotransmitter release. Researchers found that the C-terminal of α-Syn can bind to the N-terminal of VAMP2 and modulate its conformation, which may affect the formation and stability of the SNARE complex. This complex it's a group of proteins that is involved in the fusion of synaptic vesicles with the presynaptic membrane and the release of neurotransmitters (Burré et al., 2010a). α-Syn has been shown to interact with the synapsin III, which is a member of the synapsin family of proteins that are involved in the regulation of neurotransmitter release and synaptic plasticity. The interaction between α-Syn and synapsin III is thought to be important for the regulation of synaptic vesicle dynamics and neurotransmitter release. Researchers has shown that α-Syn interacts with and modulates synapsin III and affect the distribution of both proteins within the presynaptic terminal (Zaltieri et al., 2015). α-Syn has been shown to interact with rab3A that is a member of the rab family of small GTPases that are involved in the regulation of synaptic vesicle trafficking and neurotransmitter release. The interaction between α-Syn and rab3A is thought to be involved in the regulation of synaptic vesicle dynamics and neurotransmitter release. Researchers found that α-Syn can bind to rab3A and modulate the activity of rab3A and its downstream effectors (Chen et al., 2013).

In addition to the interactions described above, there are others that suggest the function of α-Syn in the cell. Such as the interaction with Vesicular Monoamine Transporter 2 (VMAT2), where Knockout (KO) of α-Syn increases the levels of VMAT2 in the membrane vesicles, and consequently releases more dopamine, norepinephrine, and serotonin. In the absence of a-Syn, the reuptake of dopamine, norepinephrine, and serotonin decreases (Guo et al., 2008). Additionally, phospholipases D1 and D2, which hydrolyze membrane phospholipids to produce phosphatidic acid (PA), interact with α-Syn. These enzymes can be inhibited by α-Syn, indicating that α-Syn is involved in the cleavage of membrane lipids (Conde et al., 2018; Gorbatyuk et al., 2010; Payton et al., 2004). Another protein that has also been demonstrated to interact with α-Syn is Tyrosine Hydroxylase (TH), a rate-limiting enzyme involved in dopamine synthesis. The activity of this enzyme has been found to be inhibited by α-Syn (Yu et al., 2004), which decreases phosphorylation and stabilizes dephosphorylated tyrosine hydroxylase (Peng et al., 2005). This interaction may generate implications for the regulation of dopamine levels and dopaminergic neurotransmission. There is still debate over α-Syn's function in lipid metabolism (Ellis et al., 2005), membrane curvature (Varkey et al., 2010), and/or chaperone-like activity (Kim et al., 2000). The typical role of α-syn in the cell, however, is difficult to define. It is difficult to determine what the exact role of a-syn is since it may be offset by the actions of other family members, detrimental overexpression, or even the protein's unstructured structure, which can take on many conformations depending on the environment.

2.3 Aggregation of α-Syn

The α-syn aggregation process is divided into three stages. The lag phase occurs when monomers begin to combine. The elongation phase, during which fibrils increase exponentially, and the stationary phase, during which most soluble protein is transformed into amyloid fibrils and the number of monomers is equal to the number of fibrils. The oligomers degrade into protofibrils, which link together to produce mature amyloid fibrils with a cross-β-sheet structure (Mehra et al., 2019; Villar-Piqué et al., 2016). It has been demonstrated that α-syn aggregates propagate by a cell-to-cell process like prions (Butler et al., 2022). Different causes, including mutations or post-

translational modifications (PTMs), can cause α-syn aggregation (**Figure 6**) (Koga et al., 2021).

2.4 Mutations of SNCA gene

Several point mutations (including A30P, A53E, A53T, E46K, H50Q, and G51D) have been identified in the *SNCA* gene of α-Syn and are localized in first domain. Despite being in the N-terminal domain, these mutations have no effect on the structure of the monomeric form. While the A53T and H50Q mutations are more likely to interact with membranes, the A30P, G51D, and A53E variants are less likely to occur (Krüger et al., 1998; Lesage et al., 2013a; Pasanen et al., 2014; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004). The α-Syn fibrillation rate accelerates with the familial PD mutations A53T, E46K, and H50Q in vitro (Conway et al., 1998; Ghosh et al., 2013; Greenbaum et al., 2005), whereas A30P, A53E, and G51D cause a delay in the aggregation rate (Ghosh et al., 2014; Lesage et al., 2013b; Li et al., 2001). Despite having differing effects on α-Syn aggregation, it has been demonstrated that the A30P and A53T mutations both cause early oligomerization, which causes the disease to manifest earlier (Conway et al., 2000). Due to a higher rate of fibril formation and reduced accumulation of toxic substances, such as oligomers, because of the rapid conversion of monomers to fibrils, the H50Q mutation causes the disease to appear later in life (Ghosh et al., 2013). Recently, a new familial mutation has been discovered, A53V. This mutation enhances early oligomerization and accelerates α-Syn fibrillation. Each mutation demonstrates a distinct mechanism that is in responsible of the beginning and development of the disease (Figure 7) (Mohite et al., 2018).

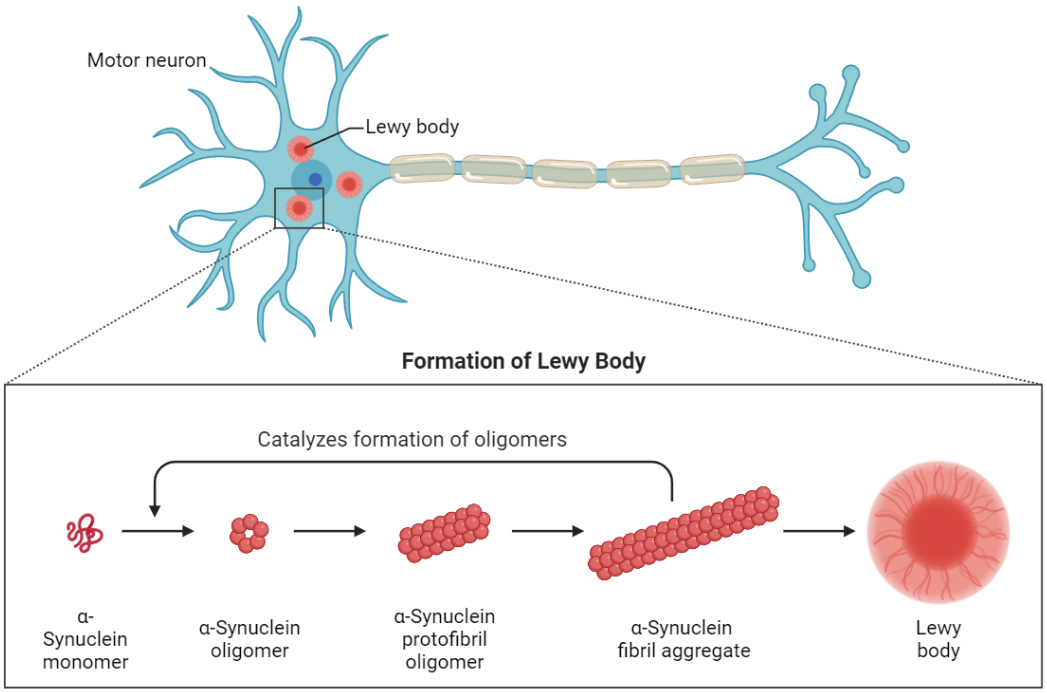

Figure 6| The formation of Lewy bodies because of α-Syn aggregation. Oligomers are formed when monomers combine. The oligomers gradually transform into protofibrils, which then develop into amyloid fibrils with a high beta cross shape. These produce the alpha-synuclein aggregates observed in Lewy bodies. Adapted from Villar-Piqué et al., 2016. Created by BioRender.com

2.5 Post-translational modifications of α-Syn

PTMs and changes in secondary structure are connected to the NAC region (Lashuel et al., 2012). PTMs include SUMOsylation, ubiquitination, phosphorylation, nitration, o-GlcNAcylation, acetylation, and N-terminal truncation. On α-Syn aggregation and toxicity, these PTMs can have either favorable or unfavorable effects (**Figure 7**) (Chen et al., 2019). PTMs can affect protein binding affinity to other proteins and lipids, as well as protein hydrophobicity (Burré et al., 2018). Phosphorylation is the most studied PTMs, with phosphorylation of serine 129 being the main alteration associated with PD. More than 90% of the α-syn found in Lewy bodies is phosphorylated under pathological environments, whereas only around 4% is phosphorylated under normal ones (Chen et al., 2019). Even though PTMs are usually associated with unfavorable effects like as aggregation induction and cytotoxicity, studies have shown that they can offer beneficial effects. Acetylation, for example, has been found to make α-syn more resistant to aggregation by enhancing the alpha-helix shape (Yoo et al., 2022). Nonetheless, further study is required to properly understand the significance of these changes (Burré et al., 2018).

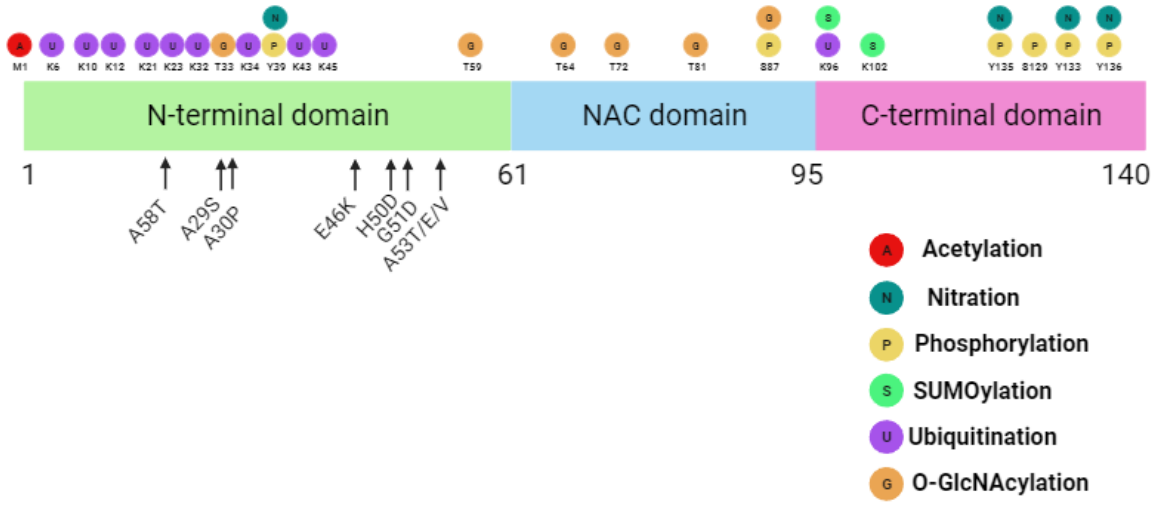

Figure 7|α-synuclein structure. The locations of missense mutations associated with familial PD and important PTM sites are shown. Adapted from Koga et al., 2021. Created by BioRender.com

3. Mechanism of α-Syn Toxicity

Cytotoxicity can be caused by α-Syn oligomers in a variety of ways, such as mitochondrial dysfunction, ER stress, loss of proteostasis, cell apoptosis, and neuroinflammation.

3.1 Mitochondrial Dysfunction

Recently, it has been discovered that α-Syn interacts with ATP synthase, regulates it, and increases its efficiency when it is in its unfolded monomeric state (Ludtmann et al., 2018). α-Syn could damage mitochondrial complex I and impair the respiration that depends on this complex. This enhances the opening of the osmotic transition pore (PTP), which causes the mitochondria to enlarge and ultimately causes cell death. It also causes the selective oxidation of ATP synthase and mitochondrial lipid peroxidation (Ludtmann et al., 2016). In addition to changing membrane potential and destroying calcium homeostasis, soluble prefibrillar α-Syn oligomers can also disrupt mitochondrial complex I activity and promote the release of cytochrome C (Luth et al., 2014).

There are several approaches that might cause α-Syn oligomers to trigger mitochondrial dysfunction. High-affinity binding α-Syn oligomers prevent TOM20 peptide receptors from attaching to TOM20's co-receptor TOM22, impairing mitochondrial respiration and increasing ROS (Di Maio et al., 2016). Overexpression of α-Syn in SH-SY5Y cells in culture results in the development of α-Syn oligomeric

species, whose presence is linked to mitochondrial fragmentation and the activation of the autophagic-lysosomal pathway in living cells (Plotegher et al., 2014). After reaching dopaminergic neurons, it has been hypothesized that α-Syn oligomers cause mitochondrial injury by activating cytochrome c oxidase 2 (Danyu et al., 2019). Human neurons generated from induced pluripotent stem cells (iPSC) of PD patients had reduced anterograde axonal transport of mitochondria because of α-Syn oligomerization (Prots et al., 2018). It has also been shown that α-Syn oligomers influence astrocytes' mitochondrial activity (Lindström et al., 2017).

3.2 Endoplasmic reticulum (ER) stress

The ER is involved in protein synthesis, folding, modification, and transport. Stress develops when the ER's capacity for protein folding exceeds saturation. Prior to the onset of PD, α-Syn accumulates in the mitochondria, where it forms toxic oligomers in vivo and cause ER stress (Colla et al., 2012a) . In the PD transgenic mouse model, treatment with the ER stress inhibitor salubrinal may significantly decrease the progression of the disease (Colla et al., 2012a, 2012b). The unfolded protein response (UPR), a cellular stress response mechanism, is activated when the ER is overwhelmed with misfolded and unfolded proteins. XBP1 is a transcription factor that is essential for this process. However, this mechanism is not activated by monomers or fibers, indicating that α-Syn oligomers are more toxic and can potentially interfere with cellular processes, including ER function (Castillo-Carranza et al., 2012). Caspases 3 and 9 can be activated and ER stress markers can be produced in cells with more α-Syn oligomers (Jiang et al., 2010). These findings collectively show that ER stress induced by α-Syn oligomers contributes to the pathophysiology of PD.

3.3 Loss of proteostasis

Two essential mechanisms in cells involved in the degradation and recycling of proteins are UPS and ALP. Both pathways are essential for the removal of aggregated or misfolded proteins such α-Syn. When levels of this protein increase, the ALP pathway is activated, although UPS is the primary pathway for a-Syn degradation under normal conditions (Ebrahimi-Fakhari et al., 2011a).

The 20S and 26S proteasomes' capacity to function can be inhibited by soluble, intermediate-sized oligomers of α-Syn by blocking the entrance of other proteasome substrates (Ebrahimi-Fakhari et al., 2011b; Emmanouilidou et al., 2010). The

overexpression of α-Syn in the A53T animal model has been shown to disrupt the 26S proteasome and cause UPS malfunction, which may accelerate neurodegeneration *in vivo* (McKinnon et al., 2020). Through S-nitrosylation and subsequent degradation, RNS triggered by exogenous α-Syn oligomers increases *Parkin* dysregulation (Wilkaniec et al., 2019).

The cells can remove preformed α-Syn aggregates, whether they are neuronal or not. This research has demonstrated that only the oligomeric intermediates, and not the final fibrillar inclusion bodies, are sensitive to the aggregate-clearing process. For the most part, the clearance of α-Syn oligomers is caused by the lysosomal degradation pathway (Lee et al., 2004). The ALP is initially activated in response to increase α-Syn concentrations provided by the transcription factor EB (TFEB) translocation to the nucleus. The amount of TFEB bound to α-Syn and trapped in cytoplasmic inclusions increases as α-Syn reaches toxic levels, preventing TFEB from migrating to the nucleus. The removal of α-Syn oligomers is the mechanism behind this protective effect (Decressac et al., 2013). All these findings demonstrate how α-Syn oligomers interact with degradation pathways: downregulation of UPS or ALP results in the accumulation of α-Syn oligomers, which in turn prevents the clearance process. Restoring proteostasis and becoming a possible therapeutic target for PD can be accomplished by removing α-Syn oligomers.

3.4 Synaptic impairment

As previously stated, α-Syn is prevalent in synapses, but its physiological role is unknown. By attaching to the vesicle-associated membrane protein 2 (VAMP2) and promoting the formation of the SNARE-complex, α-Syn normally maintains the physiological function of synapses (Burré et al., 2010b). According to other research, α-Syn often forms multimers with α-helical structure and can restrict the movement of vesicles (Adulla et al., 2023; Bartels et al., 2011b). These results collectively imply that α-Syn can have a variety of functions in sustaining synaptic homeostasis. However, certain conformations of α-Syn oligomers can potentially worsen synaptic efficiency.

Researchers created α-Syn variants with synthetic mutations (E35K and E57K) to analyze the protein's oligomeric forms and evaluate its toxic properties. These alterations produced α-Syn species that formed oligomers but not fibrils and, when

injected with lentiviral vectors into the substantia nigra, caused dopaminergic cell death (Winner et al., 2011). In an E57K mutant mouse model, synaptic and dendritic loss, decreased synapsin-1 and synaptic vesicle levels, and behavioral impairments were observed. But the WT α-Syn mouse showed less signs of these modifications (Rockenstein et al., 2014). Another study using human iPSC-derived E57K and E46K mutant neurons revealed a mechanism through which α-Syn oligomers result in axonal dysfunction. An increase in α-Syn oligomers causes a decreased in ATP levels, a pathological subcellular relocalization of proteins that control axonal transport, and what appears to be synaptic degeneration (Prots et al., 2018). This is in accordance with recent studies establishing that α-Syn oligomers reduce microtubule stability, kinesin-microtubule interaction, cellular cargo distribution, and neurite network morphology (Prots et al., 2013).

In the rat hippocampal region, prolonged exposure to α-Syn oligomers increases basal synaptic transmission by activating NMDA receptors, triggering the calcium-permeable AMPA receptors (Diógenes et al., 2012). The interaction of α-Syn oligomers with GluN2A-NMDA receptors impairs long-term potentiation (LTP) in striatal neurons and causes visual-spatial memory impairment (Durante et al., 2019). These findings imply that α-Syn oligomers contribute to the onset of PD within compromising synapses.

3.5 Apoptosis

In pathological concentrations, α-Syn can generate ROS, which is directly connected with redox metal ions. As a result, it caused oxidative stress and apoptosis (Deas et al., 2016). β-sheet-rich α-Syn oligomers interact with membrane lipids, causing aberrant calcium influx and lipid peroxidation in an iron-dependent manner (Angelova et al., 2020). In SH-SY5Y cells, extracellular α-Syn modifies membrane structure and generates nanopores. The disruption to the membrane structure changed the ionic homeostasis, activating the nitric oxide synthase (NOS) machinery and releasing nitric oxide (NO). S-Nitrosylation of regulatory proteins involved in neuronal function is induced by high NO levels. Abnormal S-Nitrosylation contributes to neuronal cell death and disease pathology because it alters the cytoskeletal network, protein folding machinery, and the UPS (Kumar et al., 2018). When monomers, fibrils, or stabilized forms of oligomeric α-Syn are introduced to

neuroblastoma cell lines, they do not cause significant cell death. However, exogenously added preformed fibrils (PFFs) with monomeric α-Syn bind to the plasma membrane and serve as nucleation sites for the formation of α-Syn fibrils, promoting the accumulation and internalization of these aggregates, which in turn activate both the extrinsic and intrinsic apoptotic cell death pathways (Mahul-Mellier et al., 2015). These findings suggest that α-Syn oligomer promotes apoptosis.

3.6 Neuroinflammation

α-Syn's aberrant neuronal aggregation affects neurons and glial cells such as astrocytes and microglia. In this study, it was shown that astrocytes exposed to neuronal α-Syn exhibited an inflammatory response (Lee et al., 2010a). Physiological concentrations of α-Syn oligomers sensitize toll-like receptor 4 (TLR4) production of proinflammatory cytokines in glial cells, and this response increases over time (Hughes et al., 2019). Another study has demonstrated that α-Syn's TLR2 ligand activity, which stimulates inflammatory responses in microglia, is conformation-sensitive. This suggests that only certain oligomers can interact with and activate TLR2 (Kim et al., 2013). Another research investigated the impairment caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the thymus and potential PD causes. MPTP induces ROS, followed by the formation of stable oligomers, and nitrated- α-Syn in the thymus. This activates caspase-8, NF-kB, NLRP3, and caspase-1 in the thymus (Wen et al., 2018).

These studies imply that PD does not have a single origin and that different oligomer conformations might result in distinct toxic mechanisms.

4. Pathophysiology of PD

To generate the insoluble Lewy bodies, aggregation starts with monomers bonding together to form dimers, trimers, oligomers, protofibrils, and ultimately fibrils (**Figure 6**). Even if for a while it was believed that Lewy bodies were the most toxic type; although, several investigations have shown that oligomers are indeed among the most neurotoxic forms (Karpinar et al., 2009; Pountney et al., 2005; Rockenstein et al., 2014; Winner et al., 2011). Oligomers can travel from cell to cell through exosomes and tunelling nanotubes (TNTs), which are membrane nanotubules or cytonemes that facilitate the selective transmission of cellular

components, pathogens, and electrical signals throughout cells (Danzer et al., 2012; Rostami et al., 2017).

In sporadic cases, the enteric nervous system or olfactory bulb may be regions where protein misfolding and easy aggregation first occur because these regions are more exposed to potential environmental factors and consequently experience high levels of oxidative stress (Christmann et al., 2022; Wi et al., 2018). α-Syn aggregates have the capacity to propagate across nerve cells. After an autopsy of PD patients who had received slices of fetal dopaminergic neurons as an experimental therapy several years before, their cell-to-cell spread was initially confirmed. Lewy bodies were present in the patient's surviving host dopaminergic cells in the *substantia nigra pars compacta*, which is normal, but aggregates were seen in the transferred cells, providing the first indication that this condition may spread to other cells. This discovery supports the possibility that PD is a prion disease since, in accordance with certain studies, misfolded proteins are the primary cause of prion diseases (Lindvall et al., 1994; Olanow et al., 2003).

Oligomers have a role in several of internal cellular processes that lead to neuronal apoptosis, which is responsible for the loss of neurons in PD. Normally, the cell would degrade these toxic aggregates, but the oligomers can interfere with the UPS and the autophagy-lysosomal pathway (ALP). As a result, α-Syn oligomers accumulate in the cell, increasing protein aggregation (McNaught et al., 2003; Olanow and McNaught, 2006; Rubinsztein, 2006).

5. Nitrosative stress in PD

Nitrosative stress is caused mostly by a high concentration of nitrogen-based free radicals, such as nitric oxide (NO^-) and nitrogen dioxide ($NO2^-$). Because these molecules have imbalanced valence electrons, they are extremely reactive and susceptible to filling their outer valence shell with electrons captured from other atoms or molecules. Secondary oxidant species such as peroxynitrite ($ONOO^-$) and hydroxyl anion (OH^-) can be produced as a result, in addition to toxic non-radicals like hydrogen peroxide (H_2O_2), nitrogen dioxide (N_2O_2), and nitrous acid (HNO_2). Although many of these are present in healthy neurons, a number of events can lead to their overproduction, which eventually destroys cellular components, resulting in neuronal

malfunction and increasing intensity and area of affliction in a time-dependent manner (Grisham et al., 1999; Kelm, 1999; Koppenol, 1998).

Molecular chaperones and antioxidant enzymes are two examples of the several cellular defense mechanisms that maintain the balance in ROS/RNS production. However, several events, like as aging, genetic mutations, and mitochondrial dysfunction or toxins, can compromise these defense mechanisms. Since neuronal antioxidant levels are insufficient, they are vulnerable to the stress that ROS/RNS generation causes. This results in the constitutive generation of ROS/RNS because neurons require a high rate of mitochondrial metabolism. Increased oxidative and nitrosative stress will develop if this production rises above a certain threshold (Kumar et al., 2018) .

5.1 Nitric Oxide (NO)

NO was discovered as a secretory product of mammalian cells considerably late. The recurrent search for the enigmatic nature of an endogenous vasodilator known as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980) came to an end when this labile mediator was discovered to be NO (Ignarro et al., 1987; Palmer et al., 1987). The earlier discovery that nitroglycerine, a previously known vasoactive medication used to treat angina pectoris, acts via the release of NO and activation of soluble guanylyl cyclase (sGC) contributed to the identification of EDRF as NO (Arnold et al., 1977; Katsuki et al., 1977). Mammalian cells produce NO by an enzymatic activity carried out by the NOS family, which oxidizes L-arginine (L-Arg) to produce citrulline and NO (Hibbs et al., 1988; Palmer et al., 1988). NOS can be expressed by neurons, glia, and vascular cells, making them potential NO power sources in the brain (Tajes et al., 2013). This process is catalyzed by particular oxido-reductases, including three isoforms of NO synthases (Bredt et al., 1991; Geller et al., 1993; Janssens et al., 1992). NO has a high reactivity, so its half-life is very short, about 1 to 10 seconds (Picón-Pagès et al., 2019).

NO is mainly produced by NOS, although additional sources of NO include non-enzymatic oxidation of L-Arg (Nagase et al., 1997), xanthine oxidase (Zhang et al., 1998), and other reductases that are able to convert nitrates to nitrites, which consequently produce NO (Jansson et al., 2008; Kozlov et al., 1999). NO's

biochemistry is challenging, and this free radical gas displays its actions either directly or by the formation of additional reactive nitrogen oxide species.

5.2 NO synthase

The NOS enzyme family produces the majority of NO: endothelial (eNOS), inducible (iNOS), neuronal (nNOS), and mitochondrial (mtNOS), with the last one being the isoform of nNOS expressed in the inner mitochondrial membrane (Tatoyan and Giulivi, 1998). All NOS are heterogeneous proteins that are coded for by various genes on multiple chromosomes and are also altered by alternative slicing (Guix et al., 2005).

NOS are proteins that require four cofactors to function properly. These are the heme (iron protoporphyrin IX), the flavin adenine dinucleotide (FAD), and the tetrahydrobiopterin (BH4) (Gorren and Mayer, 2007; Mayer et al., 1990). The latter is a redox-active pteridine that functions as an electron donor in NO production. NOS is divided into two domains, each with its own set of actions. The N-terminal domain functions as an oxygenase that binds BH4, heme, and L-Arg. The C-terminal domain is a reductase that binds FMN, FAD, NADPH, and calcium/calmodulin (Ca^{2+}/ CaM). Ca2+/ CaM is required to activate the gate that enables electron flow into the NOS's active center (Ghosh and Stuehr, 1995; Nishida et al., 1992; Xie et al., 1996).

To generate NO, oxygen and NADPH enter the reductase domain, allowing electron transport from the FAD to the FMN to the heme. This electron is required, together with oxygen, to convert L-Arg to N-hydroxyl-L-arginine (monooxygenase reaction I) (Stuehr, 2003). Following that, second monooxygenase process proceeds, requiring an electron, an oxygen molecule, and the cofactor BH4, to convert N-hydroxyl-L-arginine to L-citrulline and NO. BH4 is oxidized to produce BH2, which is subsequently reduced back to BH4 to balance NOS dimers in a couple/uncouple state. Zinc (Zn) ions that bind the oxygenase domains maintain NOS subunits together. NOS produces L-citrulline, water, NADP, and nitric oxide (**Figure 8**) (Stuehr and Haque, 2019).

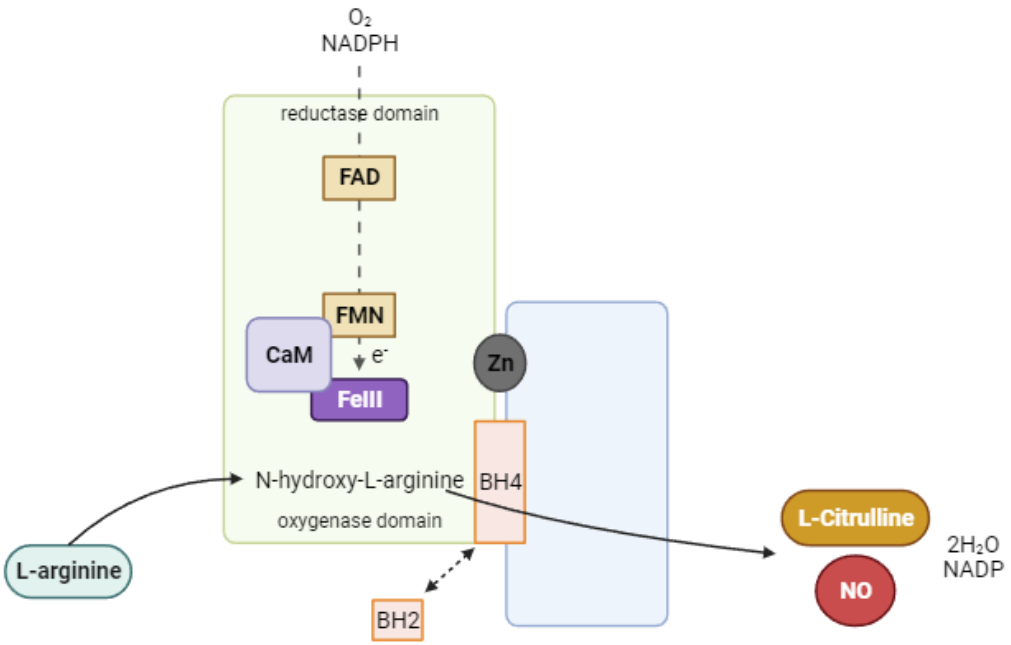

Figure 8| The nitric oxide synthase (NOS) enzyme is responsible for the first synthesis of NO. Oxygen and NADPH enter the reductase domain to create NO, permitting electron transfer from the FAD to the FMN to the heme. This electron, together with oxygen, is necessary for the conversion of L-arginine to N-hydroxyl-L-arginine (monooxygenase reaction I). The second monooxygenase process then begins, which requires an electron, an oxygen molecule, and the cofactor BH4 to convert N-hydroxyl-L-arginine to L-citrulline and NO. To balance NOS dimers in a couple/uncouple state, BH4 is oxidized to generate BH2, which is then reduced back to BH4. NOS subunits are bound together by zinc (Zn) ions that bind to the oxygenase domains. L-citrulline, water, NADP, and nitric oxide are all produced by NOS. Stykel et al., 2022. Created by BioRender.com

6. Mechanisms of NO signaling

The importance of NO-mediated PTMs (Stamler et al., 1992), NO control of mitochondrial activity (Bolaños et al., 1994; Cleeter et al., 1994), and the explosion of NO chemistry in biological contexts were revealed in the 1990s (Lancaster, 1994). NO promotes three mechanisms: I) NO signaling that is dependent on guanylate cyclase (sGC) and its dependent enzymes, known as "classical" NO signaling; II) NO binding to cytochrome c oxidase (CcO) in the mitochondria and its functional consequences, known as "less classical" NO signaling; and III) cGMP-independent NO signaling, known as "nonclassical" NO signaling.

6.1 "Classical" NO signaling

In the case of eNOS and nNOS, both of which are activated by increased calcium (Ca^{2+}) levels, NO is needed by sGC, an enzyme that converts GTP into cGMP. NO is bound together by a metal coordination connection with a strong affinity(Sayed et al., 2007). Once NO has been released from its association with the ferric or ferrous

ion inside the heme group of NOS, it may easily spread from its location of production and reach sGC in a variety of different cells, the most well-known of which being vascular smooth muscle cells and cerebellar neurons (Hall and Garthwaite, 2009; Rodríguez-Juárez et al., 2007; Russwurm and Koesling, 2004).

6.2 "Less classical" NO signaling

In the presence of oxygen, NO reversibly inhibits CcO (complex IV), the terminal enzyme of the mitochondrial electron transport chain. This interaction decreases the upstream components of the respiratory chain, promoting superoxide generation. MnSOD catalyzes the process that converts superoxide to H_2O_2. H_2O_2 can permeate into the cytosol and activate redox-sensitive signaling pathways. NO, and RNS can also alter cysteines and iron-sulfur centers, inhibiting complexes I and II (Mason et al., 2006).

6.3 "Nonclassical" NO signaling

Since EDRF was identified as NO, it has become evident that this free radical molecule may accomplish parts of its functions not just independently of cGMP, but also without the need for attachment to other metal centers, via target protein covalent PTM. This is known as nonclassical signaling, and it mostly impacts cysteine and tyrosine residues in proteins. S-nitrosylation, S-glutathionylation, and tyrosine nitration are the three most well-known NO-induced PTMs (Ischiropoulos, 2009; Lima et al., 2010; Mieyal et al., 2010).

Tyrosine nitration has been associated with the generation of $ONOO^-$ and NO_2 and is mostly regarded as an irreversible modification that can affect specific signaling pathways (Ischiropoulos, 2009; Pacher et al., 2007). $ONOO^-$ and nitrosothiol production both cause S-glutathionylation and additional forms of S-thiolation (Adachi et al., 2004; Radil et al., 1991). An important process in this study is S-nitrosylation, which involves the formation of a nitrosothiol (or thionitrite, R-S-N=O) at a cysteine residue (Lancaster and Gaston, 2004; Martínez-Ruiz and Lamas, 2004a).

7. S-Nitrosylation

The concepts "nitrosation" and "nitrosylation" need to be defined. The addition of a nitroso group, which is the diatomic group of NO, is referred to as "nitrosation" in chemistry. In contrast, "nitrosylation" refers to the addition of a nitrosyl group (like other

chemical additions containing the -yl- particle, such as acetylation, phosphorylation, and so on). Other researchers, however, distinguish between "nitrosation," which is the covalent insertion of a diatomic group of NO into another chemical group, and "nitrosylation," which is the integration of the radical •NO into metal via a complex connection. However, because the -yl- particle is widely used to characterize other PTMs (such as glycosylation and phosphorylation), and because of a growing understanding of the functional significance of this modification, pioneer researchers in the field have begun to refer to nitrosylation as a post-translational modification of thiols and metals (Myers et al., 1990; Stamler et al., 1992). The prefix "S-" refers to "S-nitrosation" or "S-nitrosylation," which is the incorporation of NO into a sulfur atom to form an S-NO bond, which can play essential regulatory roles in biological systems (for reviews, see Refs. (Martínez-Ruiz and Lamas, 2004b)).

S-nitrosylation is a PTM generated by NO that results in the production of S-nitrosylated proteins. S-nitrosylation is a covalent and reversible reaction in which a NO within a protein to a cysteine thiol to generate an S-nitrosothiol (SNO) of some proteins to control their activity (**Figure 9**) (Lipton et al., 1993; Nakamura et al., 2013). Even though many proteins contain several cysteine residues, this modification relies primarily on specific free thiols. S-nitrosylation can be accomplished by a variety of methods. The first mechanism is dependent on the target proteins' proximity to the source of NO generation. The linkage of nNOS with N-methyl-D-aspartate receptor (NMDARs) via mutual interactions with PSD-95 is an example of this; nNOS will enhance the S-nitrosylation of these two proteins. The compartmentalization of the target protein into a hydrophobic environment is the second biological pathway. The hydrophobicity will stabilize S-nitrosylating species, facilitating the synthesis of SNO proteins (Hess et al., 2005; Lei et al., 1992; Lipton et al., 1993; Nakamura et al., 2013; Stomberski et al., 2019).

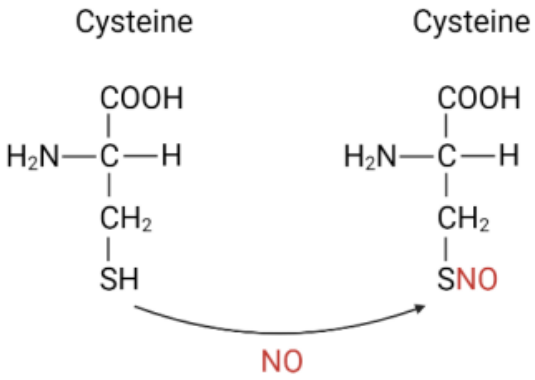

Figure 9| S-nitrosylation is a chemical reaction. S-nitrosylation is a chemical process in which a a nitric oxide group (NO) within a protein to a cysteine thiol to generate an S-nitrosothiol (SNO), resulting in the release of the hydrogen molecule and the creation of the SNO-group. Adapted from Sharma et al., 2021. Created by BioRender.com

S-nitrosylation is a critical PTM that plays a crucial role in the regulation of protein function and signaling pathways in many physiological processes. S-nitrosylation has been shown to regulate various cellular processes, such as metabolism (Chao et al., 2021; Chen et al., 2021; Shu et al., 2022), apoptosis (Hou et al., 2020; Kim et al., 2022b; Xie et al., 2022), and immune responses (Guo et al., 2019; Kaner et al., 2019; Nukui et al., 2020). Research has also shown that aberrant S-nitrosylation is implicated in several pathologies, including cancer (Firdaus et al., 2022; Fujiya et al., 2022; He et al., 2022), neurodegenerative diseases (Kim et al., 2022a; Oh et al., 2022; Yang et al., 2022), and cardiovascular disease (Zhang et al., 2021; Zhao et al., 2022; Zhou et al., 2021).

Furthermore, research suggests that S-nitrosylation may play a role in the progression of neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Understanding the mechanisms of S-nitrosylation and its effects on protein function may provide insights into potential therapeutic targets for treating these diseases and other conditions associated with the dysregulation of nitric oxide signaling. Recent studies have suggested that the process of S-nitrosylation, which involves adding a NO molecule to a protein, may play a role in the progression of PD (Zhong et al., 2020).

Both genetic mutations and exposure to environmental risk factors may trigger PD. Additionally, these elements enable the generation of ROS and RNS. The pathogenic and physiological processes that occur in the brain include these reactive molecules (Elbaz et al., 2007; Nakamura and Lipton, 2011; Ross and Smith, 2007) (**Figure 10**). Particularly, NO can cause PTMs that allow the formation of S-nitrosylated proteins (SNO-proteins). An additional nitrosyl group is added to the thiol group of a cysteine during the post-translational process of S-nitrosylation, resulting in the creation of S-nitrosothiol (SNO) (Wink et al., 2004). Thiol groups that are closer to the region that directly interacts with NOS are more likely to be S-nitrosylated, making this reaction selective (Doulias et al., 2010).

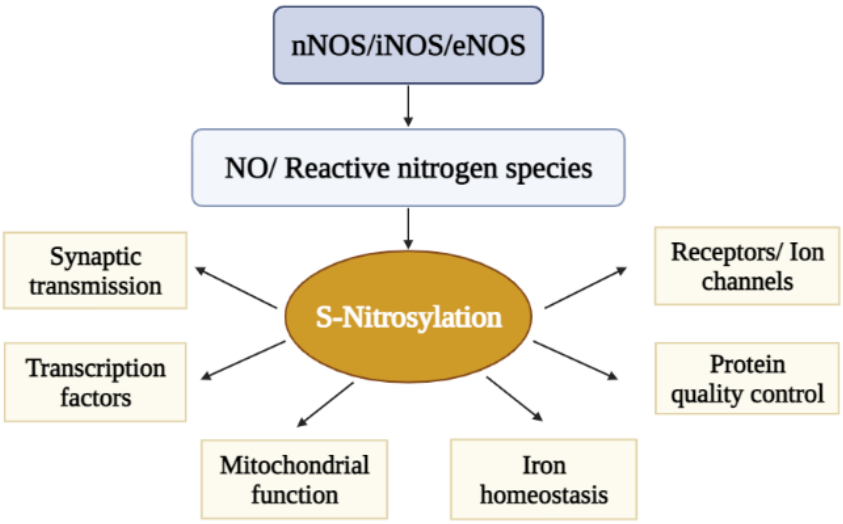

Figure 10 | Physiological and Pathophysiological functions of protein S-nitrosylation. Adapted from Nakamura et al., 2013. Created by BioRender.com

S-nitrosylation can cause conformational changes, stimulate, or inhibit protein activity, modify protein-protein interactions, and affect protein aggregate formation or localization under physiological circumstances. As a result, these modifications have an impact on signal transduction pathways and neuronal function. S-nitrosylation of specific proteins under pathological circumstances can result in protein misfolding, endoplasmic reticulum stress, mitochondrial malfunction, synaptic degeneration, and apoptosis (Nakamura et al., 2013). As a result, S-nitrosylation is an essential PTM that regulates protein activity and signaling pathways in many physiological processes, and its dysregulation might result in neurodegeneration (Hess and Stamler, 2012; Nakamura et al., 2013). Protein disulfide isomerase (PDI), for example, can be increased by increasing chaperone and isomerase activity in response to the accumulation of unfolded or misfolded proteins (Kabiraj et al., 2014). However, S-

nitrosylation can counteract this neuroprotective effect because SNO-PDI reduces its capacity to repair protein misfolding, resulting in the formation of protein aggregates, including those containing α-Syn (Nakamura et al., 2013). As a result, even though α-Syn does not have cysteines in its core sequence and so is not susceptible to S-nitrosylation, its aggregation may be impacted indirectly by it (Serrano et al., 2020).

PTMs may occur in some genes that cause sporadic cases of PD, like PARK2. Parkin is a member of the E3 ubiquitin ligase protein family and has two RING domains as well as an in-between-RING (IBR) domain. Specifically in the RING and IBR domains, this protein's cysteine residues are susceptible to S-nitrosylation. This causes the UPS activity dysfunction and the E3 ligase activity to be disrupted (Yao et al., 2004).

8. S-nitrosylated proteins that can be relevant for PD

8.1 Protein Disulfide Isomerase (PDI)

Members of the PDI family can form, break, or rearrange disulfide bonds in addition to acting as molecular chaperones and disulfide oxidoreductases/isomerases. Different proteins generate these disulfide bonds (S-S) between the -SH groups of cysteine residues. These bonds have advantageous characteristics like a structurally stable structure or a properly formed active site. Disulfide bonds that form between two protein chains play a crucial role in maintaining the cohesiveness of multimeric complexes (Alanen et al., 2004; Pirneskoski et al., 2004). The redox protein family of thioredoxins includes PDI as one of its members. This protein was found to be present, when decreased ribonuclease A was seen to reactivate in microsomal rat liver preparations in 1963 (Goldberger et al., 1963). The PDI enzyme was named as the one that catalyzes this process. The ER of eukaryotic cells and the periplasm of prokaryotic cells are the primary locations where disulfide bonds are formed, although it can be released to work on the extracellular matrix or cell surface. A well-known multifunctional protein, PDI has been extensively linked to numerous disorders, including cancer (Kim et al., 2018), infectious diseases (Wan et al., 2012), cardiovascular diseases (Woehlbier et al., 2016), osteogenesis imperfecta (El-Gazzar and Högler, 2021), and diseases of the metabolism (Montane et al., 2016) and the nervous system (Cheng et al., 2010a).

8.1.1 Structure of PDI

PDI is a soluble 55 kDa protein that is a member of the PDI family of proteins, all of which include the thioredoxin-like βαβαβαββα domain. (Kemmink et al., 1997). Thioredoxins are oxidoreductase enzymes with a dithiol-disulfide active site that contributes to redox signaling (Moran et al., 2001). In addition to PDI, 21 other family members have been described. However, the enzymatic characteristics of these proteins differ in terms of redox potential and hence substrate selectivity active site sequence (Jessop et al., 2009), and pKa of active site cysteine residues. They are mostly found in the ER, where they contribute to ER homeostasis by maintaining an oxidative environment (Anelli et al., 2002).

PDI contains 508 amino acids and consists in four domains ***a***, ***b***, ***b'***, ***a'***(Serrano et al., 2020). The domains **a** and **a'** are active sites in PDI, and they are separated by enzymatically inactive **b** and **b'** domains (**Figure 11**) (Alanen et al., 2004; Pirneskoski

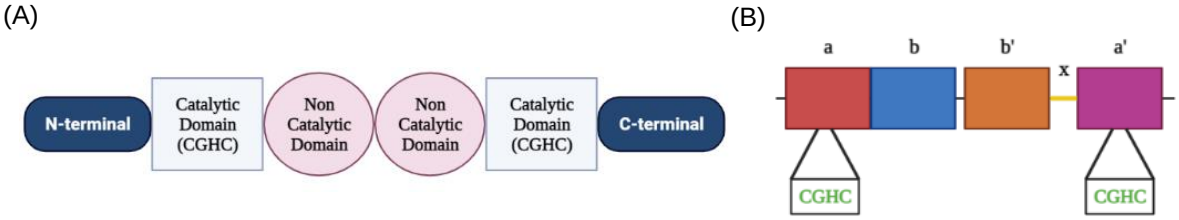

Figure 11 | Shemes about the structure of Protein disulfide isomerase (PDI). (A) Primary structure of the protein disulfide isomerase (PDI). (B) Scheme of PDI domains in the crystal structure. Khan et al., 2014. Created by BioRender.com

et al., 2004). The a and a' domains have a 47% similarity (Kemmink et al., 1996). Cysteine residues at the active site interact with the thiol group of a recently synthesized substrate, enabling protein disulfide bonds to form and isomerize. The **b'** domain of PDI forms the basis of the "U"-shaped structure and contributes to the binding of the misfolded proteins (Klappa et al., 1998).

A -CGHC- active site is present in domains **a** and **a'** and oversees the thiol-disulfide exchange process. The **b** and **b'** domains connect the active sites domains, **a** and **a'**. A small interdomain area on the protein's structure known as the *x*-linker can be found between the domains **b'** and **a'** (Alanen et al., 2004; Darby and Creighton, 1995; Freedman et al., 1998). The -CGHC- site regulates the total reduction potential of PDI, which in turn controls the active site cysteines' catalytic capacity to actively reduce or oxidize disulfide bonds (Chivers et al., 1997). The oxidizing capacity of the **a** and **a'** domains is similar, but their isomerase activity is lower (Darby et al., 1998).

Although PDI's chaperone-like activity and peptide binding capacity are deactivated when its C-terminal residues are deleted, this has no effect on the protein's catalytic activity in the production of disulfide bonds (Dai and Wang, 1997).

8.1.2 PDI chaperone activity

PDI can discriminate between partially folded, unfolded, and properly folded protein substrates, and it has a stronger propensity to attach to misfolded proteins rather than native proteins via hydrophobic interactions (Klappa et al., 1997). These characteristics, together with its structural flexibility, make PDI an extremely powerful chaperone (Irvine et al., 2014). Although PDI binds to an extensive variety of protein substrates in the ER, isolating and identifying the individual substrates *in vivo* is difficult. Several approaches are used to assess PDI chaperone activity *in vitro*. The rate of protein aggregation is measured using protein substrates that do not contain cysteine residues, such as GAPDH (Cai et al., 1994), rhodanese (Song and Wang, 1995), citrate synthase, alcohol dehydrogenase (Primm et al., 1996), or GFP, which increases fluorescent intensity as it interacts with PDI and folds into its native structure (Mares et al., 2011).

PDI is primarily involved in chaperone upregulation under ER stress. Misfolded proteins accumulate in the lumen because of a variety of physiological and pathological conditions that can affect the ER's function to fold proteins. This causes UPR to become active. In order to decrease the amount of unfolded proteins, the UPR curvatures the ER, decreases protein synthesis, and induces PDI and other chaperones to increase the capacity for protein folding (Hetz, 2012). Three major signaling branches—activating transcription factor 6 (ATF6) (Shoulders et al., 2013), protein kinase RNA-like ER kinase (PERK) (Harding et al., 2000), and inositol-requiring enzyme 1 (IRE1)—mediate the UPR (Hetz et al., 2011). Apoptosis results from extended UPR (Schröder and Kaufman, 2005).

With a high concentration of 0.4% of the total cellular protein in secretory tissues, PDI is widely expressed in numerous tissue and cell types of mammals. Some secretory cells' ER concentrations can approach 1 millimolar (mM) (Hillson et al., 1984; Lyles and Gilbert, 1991; Zapun et al., 1992). Although it can also be present in the mitochondria, the nucleus, the cytosol, the cell surface, and extracellular space, PDI is mostly concentrated in the ER lumen (Turano et al., 2002). PDI promotes misfolded

protein degradation via ER association degradation (ERAD) by translocating these proteins from the ER to the cytoplasm for degradation by the ubiquitin protease system (Lee et al., 2010b; Molinari et al., 2002).

PDI, interestingly, demonstrates both chaperone and anti-chaperone function depending on its starting concentration. When PDI's chaperone action is dominant, almost all the substrate protein is appropriately folded. At low concentrations, however, PDI promotes intermolecular disulfide crosslinking of substrates from massive inactive aggregates via anti-chaperone action (Puig and Gilbert, 1994).

As newly formed proteins are transported to the ER, their reduced forms of cysteine attack the CGHC electrophilic disulfides in the PDI to produce a transitory disulfide intermolecular of substrate. The next step results in the generation of an oxidized substrate (**Figure 12A**) and a reduced PDI when a second reduced thiol from the substrate attacks the intermolecular disulfide (**Figure 12B**). Using a single remaining cysteine nucleophilic residue inside the CGHC motive, the PDIs also isomerize the disulfides of the substrate in a reduced manner. The ER uses a flavin-dependent oxidase (Ero1) that only reduces oxygen (O_2) to H_2O_2 when PDI oxidation is occurring simultaneously to restore the oxidized PDI (Ellgaard and Ruddock, 2005).

8.1.3 PDI in PD

During neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction (Hu et al., 2000), but the upregulation of PDI represents an adaptive response to protect neuronal cells (Conn et al., 2004; Ko et al., 2002; Tanaka et al., 2000). Domain *a'* of PDI has an important function in inhibiting α-Syn fibril formation. PDI can also bind an intermediate species in the α-Syn fibril formation pathway, inhibiting fibril development (Cheng et al., 2010b). In PD patients, PDI is increased in dopaminergic neurons and Lewy bodies. In cultivated SH-SY5Y cells, co-expression of PDI and synphilin-1 significantly reduced discrete Lewy-body-like inclusions produced by synphilin-1 in the cytoplasm. The inhibitory effect of PDI was reduced by S-nitrosylation (Uehara et al., 2006).

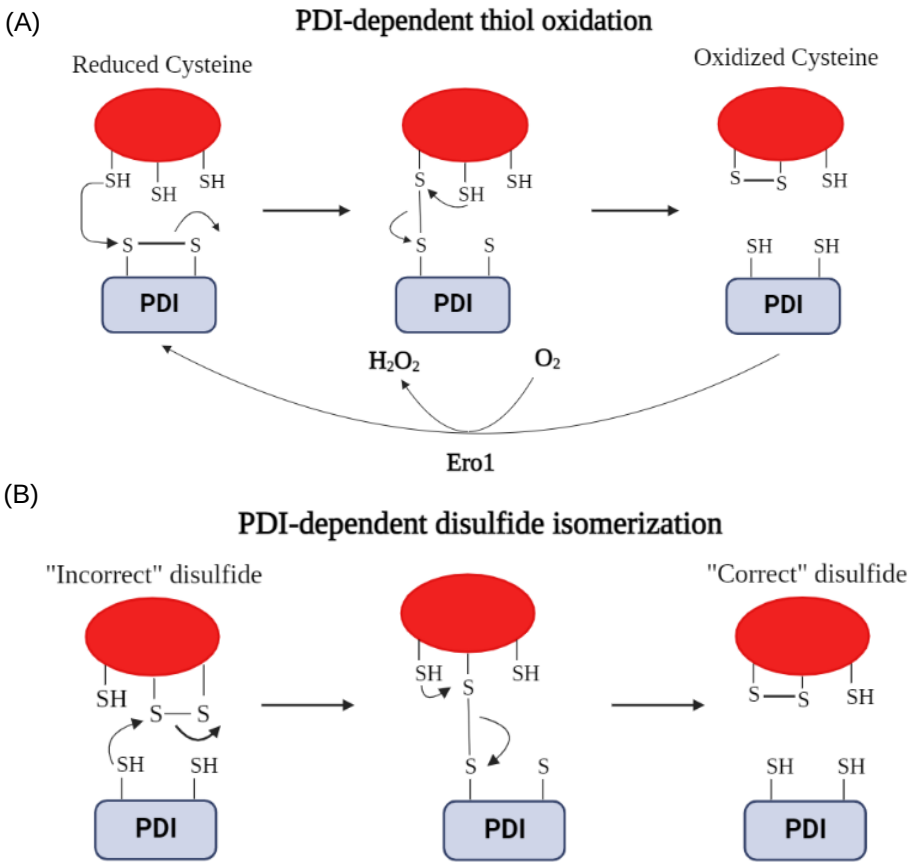

Figure 12 | PDI catalyzes protein folding in the ER. (A) Reduction of PDI. (B) Oxidation of PDI. The regeneration of this process depends on flavin-dependent oxidase (Ero1). Perri et al., 2016. Created by BioRender.com

8.1.4 PDI and S-nitrosylation

NO-induced S-nitrosylation of PDI reduces its enzymatic activity, causes polyubiquitinated proteins to accumulate, and activates the UPR. To identify the target site(s) of S-nitrosylation, HEK-293T cell lysates were transfected with WT or mutant PDI (cysteine-to-serine alterations in both active-site sequences) and a DAN assay was performed. The findings imply that S-nitrosylation targets thioredoxin-like domains (Uehara et al., 2006).

NMDA receptors are composed of tetrameric assemblies. There are three groups (GluN1, GluN2, and GluN3) and a total of seven different types of NMDA receptor subunits. Glycine is bound by GluN1 and GluN3 subunits, while glutamate is bound by GluN2 subunits. In most cases, two glutamate-binding subunits (GluN2) and two glycine-binding subunits comprise NMDA receptor tetramers (GluN1). An increased Ca^{2+} influx and subsequent NO generation from nNOS are caused by neurotoxic concentrations of NMDA. Under these circumstances, PDI exhibited NOS-dependent S-nitrosylation. Proteins that were polyubiquitinated or misfolded

accumulated because of SNO-PDI production, which also activated the UPR. A neuronal cell death caused on by ER stress, misfolded proteins, or proteasome suppression was also avoided by PDI's S-nitrosylation (Uehara et al., 2006) (**Figure 13**).

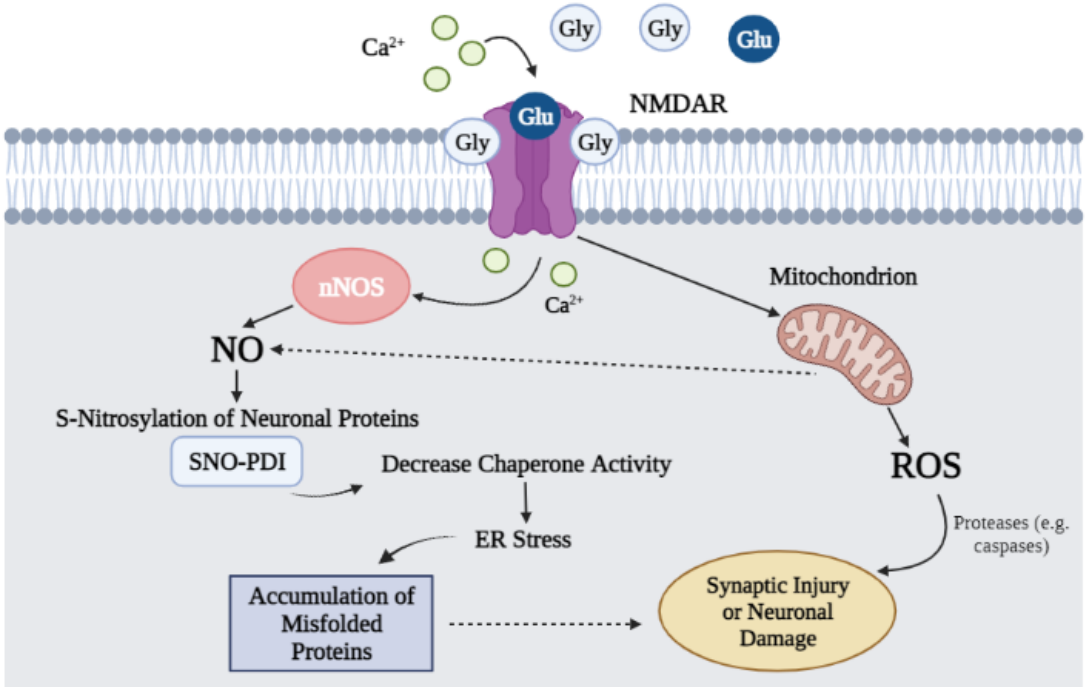

Figure 13 | Potential method by which S-nitrosylated species contribute to the accumulation of abnormal proteins and neuronal toxicity. Created by BioRender.com

PDI can block the aggregation of α-synuclein due to its chaperone and oxidoreductase characteristics. To stop the accumulation of misfolded proteins, PDI and members of its related family can act as chaperones. This includes proteins like α-Syn that do not have cysteine residues (Serrano et al., 2020). Protein aggregation, which is frequently linked to amyloid formation and neurodegeneration, cannot be prevented by PDI S-nitrosilated (SNO-PDI) (Andreu et al., 2012; Conway and Harris, 2015; Halloran et al., 2013). In organotypic brain slices and neuronal cell cultures, α-synuclein fibrils form due to the loss of PDI activity caused by its S-nitrosylation (Kabiraj et al., 2014; Wu et al., 2014; Xu et al., 2014).

Serrano and colleagues found in 2020 that PDI not only prevents α-Syn fibrillization - from starting, but also effectively stops the aggregation process once it has started (Serrano et al., 2020). Methamphetamine (Meth) consumers are at a significant risk of developing neurodegenerative disorders, including PD. To determine

whether oxidative stress promotes S-nitrosylation of PDI in Meth-treated cells, PDI was considerably S-nitrosylated in culture PC12 cells following Meth treatment. SNO-PDI causes dysfunction and the inability to reduce α-Syn aggregation (Wu et al., 2014).

The PDI S-nitrosylated site is C343 (Ogura et al., 2020), which is located directly below the substrate-binding site at the b' domain. Because the thiol group of C343 extends in the direction of the substrate binding site, S-nitrosylation at C343 may change the PDI binding affinity to the substrate protein and the conformation of the PDI-substrate complex (Wang et al., 2013). Glutathione (GSH) depletion by glutamate enhanced SNO-PDI at C343 and caused IRE1α phosphorylation in SH-SY5Y cells. Because SNO-PDI at C343 is stable in neurons, GSH deprivation causes neurodegeneration by inducing ER stress via phosphorylated IRE1α signaling from the early to late stages (Ono et al., 2023).

S-nitrosylation of PDI has been shown to impact α-Syn aggregation. It remains to be established in cellular models and preclinical models whether SNO-PDI contributes to the pathogenic aggregation of α-Syn.

8.2 Serine Racemase (SR)

Serine racemase (SR), is a pyridoxal 5'- phosphate (PLP)-dependent enzyme that was isolated from rodent brain (Wolosker et al., 1999). SR catalyzes the racemization of L-serine to D-serine, a co-agonist for glutamatergic NMDA receptors (Foltyn et al., 2005; Hashimoto et al., 1992; Marchetti et al., 2013; De Miranda et al., 2002). SR was subsequently found to catalyze the α,β-elimination of water from L-serine to D-serine, which produces pyruvate and ammonia (Foltyn et al., 2005; De Miranda et al., 2002).

The cells were transfected with the SR gene for the first time demonstrating that SR catalyzed the α,β-elimination process with L-serine. It was discovered that the reduction in L-serine was much higher than the overall synthesis of D-serine. This implies that L-serine has been converted to another molecule in the cells. The addition of L-serine to SR-transfected cells causes a significant rise in pyruvate concentration in the culture medium. To catalyze the deamination of L-serine to pyruvate, the enzyme requires extra cofactors. The addition of adenosine-triphosphate (ATP), and a lesser amount of adenosine-diphosphate (ADP), stimulates the formation of D-serine. Because compound Mg-ATP has a high affinity for several ATP-binding enzymes,

magnesium (Mg^{2+}) promotes both basal and ATP-stimulated D-serine synthesis (De Miranda et al., 2002).

Cook *et al.* demonstrated that Ca^{2+} binds to SR and proposed that the cation is a key regulator of the protein. Ca^{2+} stimulation of D-serine production was half maximum at 26 μM (Cook et al., 2002). Both Ca^{2+} and Mg^{2+} cations bind with identical affinity to a specific site. Mg^{2+} and ATP independently enhanced racemase activity: the nucleotide increased SR activity even in the presence of ethylenediaminetetraacetic acid (EDTA) (inhibited pyruvate synthesis), and the impact of divalent ion and ATP was additive (De Miranda et al., 2002).

SR could interact with α-Syn via regulation of NMDA receptor activation and negative feedback in NO generation, as well as indirectly via other proteins that are no longer nitrosylated. However, many questions remain unanswered. As a result, this study aims to discover what is the function of SR when it is S-nitrosylated and how it interacts with α-Syn.

8.2.1 Catalytic mechanism

SR belongs to the fold type II group of PLP-dependent enzymes (Goto et al., 2009). The enzyme is a homodimer, with each monomer (340 amino acids) composed of two domains, one small and one large, linked by a flexible loop. The large domain comprises most of the residues that interact with PLP as well as those involved in dimerization. The Mg-ATP binding site is located outside the catalytic site, at the interface between the domains (Goto et al., 2009; Smith et al., 2010). It has been suggested that L-serine binding to SR in conjunction with Mg-ATP changes the enzyme's conformation from open to closed (Goto et al., 2009).

Mechanistically, racemization and α,β-elimination processes share the same intermediate: this intermediate serves as an obstacle between the two pathways. L-serine binds to PLP, forming an exterior aldimine intermediate, which is then abstracted by Lys56, resulting in the production of a planar resonance-stabilized carbanion (Foltyn et al., 2005). Protonation on the other side of the carbanion intermediate (mediated by the Ser84-OH group) (Goto et al., 2009; Smith et al., 2010) produces D-serine (racemization reaction) at this point. Because the proton abstraction and protonation steps are done by separate residues (Lys56 and Ser84), which operate as acid/base catalysts. Protonation of the substrate's β-hydroxy group

removes water from the carbanion intermediate, forming an unstable aminoacrylate intermediate, followed by iminopyruvate release and spontaneous hydrolysis into pyruvate and ammonium ($NH4^+$). Mg-ATP controls the partition between β-hydroxyl group removal and racemization, favoring the former (Foltyn et al., 2005).

8.2.2 Physiological role of α,β-elimination

SR has previously been demonstrated to be a bifunctional enzyme: (i) synthesis of D-serine and (ii) α,β- elimination of water from L-serine in order to generate pyruvate and ammonia (De Miranda et al., 2002; Neidle and Dunlop, 2002). The α,β- elimination process catalyzed by SR provides a novel method for modulating intracellular D-serine levels in the brain. In an ATP-modulated manner, SR catalyzes the α,β-elimination of water from L-threonine and D-serine (Foltyn et al., 2005). Because α,β- elimination is a degradative process, it was proposed that it may be a mechanism for the downregulation of D-serine levels, particularly in brain regions lacking D-amino acid oxidase activity (Stříšovský et al., 2003). D-serine α,β- elimination occurs via an apparent futile process in which part of D-serine synthesized by SR is converted to pyruvate both *in vitro* and *in vivo* (Foltyn et al., 2005).

8.2.3 Physiological regulation of SR

SR and D-serine can be identified in protoplasmic astrocytes, which ensheath synapses and contain the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor (Komuro and Rakic, 1992). The researchers observed that SR binds to the AMPA receptor-binding protein to a glutamate receptor interacting protein (GRIP), usually coupled to the Glu2/3 subunits of AMPA receptor, resulting in significantly increased SR activity and D-serine release from glia, both of which are stimulated by AMPA receptor activation. GRIP's interaction protein has PDZ domains 4, 5, and 6, and SR binds to PDZ-6 but not PDZ-4 or PDZ-5. With a well-defined consensus sequence, PDZ domains interact with the three carboxyl-terminal amino acids of the protein. The carboxyl-terminal part of SR, -V-S-V, corresponds to the consensus PDZ domain binding ligand. GRIP only binds to the carboxyl-terminal of SR. Interactions with GRIP stop occurring when the carboxyl-terminal valine (V339) of SR is converted to glycine. Primary glial cultures were treated with AMPA to determine if GRIP's known interactions with AMPA receptors regulated SR. As a result, AMPA treatment triples D-serine release. The AMPA receptor antagonist NBQX significantly decreases these levels, demonstrating that endogenous glutamate contributes to

basal D-serine levels. Thus, AMPA receptor activation is required for D-serine release, while GRIP's binding to SR promotes D-serine synthesis physiologically (Kim et al., 2005). GRIP1's PDZ-6 domain is the location where SR binds to become active. The remaining portion of the C-terminal region of GRIP must bind for SR to fully activate (Baumgart et al., 2007).

Because of its interaction with lipid membranes, SR also binds to the protein interacting with C kinase 1 (PICK1), a PDZ domain-containing protein believed to affect subcellular localization and membrane expression of diverse binding partners. In the present study, genetic deletion of PICK1 induces a decrease in D-serine levels in the forebrains of newborn mice. The reduction in D-serine levels coincides with the level of PICK1. Transfected HEK293 cells with wild-type PICK1, SR, or both exhibited an increase in D-serine to SR in cells expressing both SR and PICK1 compared to SR alone (Hikida et al., 2008).

It has been demonstrated that SR interacts both in vitro and in vivo with the Golgin subfamily A member 3 (Golga3) proteins. Golga3 is linked to the Golgi apparatus's cytosolic surface, where it might play a role in vesicular trafficking. The 66 amino acids that compose the N-terminal region of SR are connected to Golga3. Golga3 and SR were found to be co-localized in the perinuclear area and cytoplasm, respectively. Golga3 inhibited the synthesis of SR's higher molecular weight conjugates. Golga3's decrease in SR ubiquitylation will lengthen the half-life of SR by preventing UPS degradation of SR (Dumin et al., 2006).

Glutamate receptors modulate SR interaction with membranes by multiple mechanisms. SR membrane binding regulates NMDAR activation and interaction with phosphatidylinositol lipids (Balan et al., 2009; Mustafa et al., 2009). After NMDAR stimulation, neuronal SR translocate to the plasma membrane at dendritic processes, inactivating the enzyme. This provides a method for NMDAR feedback inhibition by decreasing the production of the co-agonist D-serine after NMDA receptor activation. Palmitoylation of SR at serine/threonine residues and phosphorylation at Thr227 are required for this translocation (Balan et al., 2009). Phosphatidylinositol (4,5)-biphosphate (PIP2), which is found in membranes where SR has been identified, inhibits SR physiologically. Activation of metabotropic glutamate receptors (mGluR5)

on glia results in phospholipase C-mediated degradation of PIP2, which relieves SR inhibition (Mustafa et al., 2009).

Shoji and colleagues revealed that SR in human glioblastoma cells is suppressed by either endogenous NO generated by iNOS or exogenous NO released from (E)-ethyl-2-(E)-hydroxyimino-5-nitro-3-hexeneamide (NOR-3), a well-known NO donor. On the contrary, D-serine significantly activates SR. This suggests that the presence of D-serine inside cells may activate racemase activity via denitrosylation

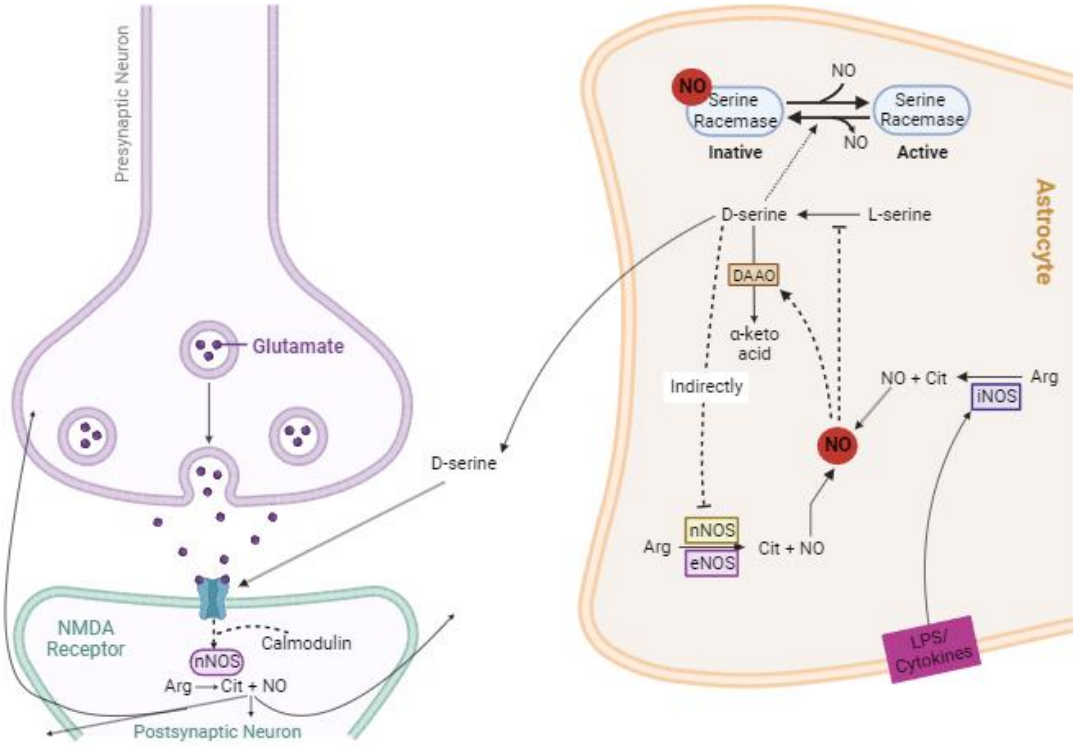

Figure 14| A regulatory loop exists between D-serine metabolism and NO metabolism. NO, generated either by n/eNOS under normal conditions or by LPS/cytokine-induced iNOS under inflammatory environments, may decrease serine racemase activity while increasing DAAO activity in astrocytes. D-serine synthesized by serine racemase, on the other hand, increases and reduces serine racemase and nNOS activity. As a result, D-serine's stimulation of serine racemase activity may be induced not only by a direct action on serine racemase but also indirectly by suppression of nNOS activity. Adapted from Shoji et al., 2006b. Created by BioRender.com

and inhibited by nitrosylation (Shoji et al., 2006a). In other study, Shoji, and colleagues, demonstrated that D-serine caused an inhibitory effect indirectly in nNOS activity. Furthermore, NO increased the activity of D-amino acid oxidase (DAAO), which catalyzes the oxidative deamination of D-amino acid, in a dose-dependent manner. This suggests that NO may accelerate D-serine elimination in astrocytes not only by inhibiting SR but also by increasing DAAO activity (**Figure 14**) (Shoji et al., 2006b).

If SR is physiologically S-nitrosylated, the enzyme activity that controls interactions with ATP is suppressed. The fact that NMDA transmission increases the S-nitrosylation of SR raises the possibility of a feedback loop that lowers the production of D-serine in the presynaptic membrane (Mustafa et al., 2007). SR S-nitrosylation is induced by NMDA transmission and is controlled by nNOS. Thus, NMDA receptor activation is necessary for NO production. Therefore, Mustafa and colleagues proposed that NMDA transmission controls SR in low basal conditions.

There are several SNO-sensitive cysteine residues in SR. One such residue, Cys113, is nitrosylated by S-nitroso glutathione (GSNO). This post-translational alteration permits the SR activity to decline. Since Cys 113 is situated between ATP binding sites, it is likely that S-nitrosylation regulates how ATP affects SR. Additionally, the adenosine base of ATP appears to be near the sulphur atom of Cys 113. The S-nitrosylation of SR is inhibited by ATP, which competes with ATP. By acting on the same region of the protein, ATP and NO reciprocally activate and inhibit the enzyme (Mustafa et al., 2007). In turn, this has an impact on ATP binding since ATP has the capacity to compete for nitrosylation (Mustafa et al., 2007).

8.2.4 Localization

Heart, skeletal muscle, kidney, and liver tissues also expressed the human SR mRNA. Through a Western blot analysis one major immunogen-specific band measuring approximately 62 kDa was present in the extracts of the kidney and heart, and one minor band measuring 80 kDa was present in the extract of the heart. In SR transfected cells, an approximately 40 kDa protein band was observed. The hippocampus, amygdala, thalamus, and cortex displayed the highest levels of staining in a primate brain with SR (Xia et al., 2004). D-serine was initially discovered in astrocytes, a class of glial cells that encases neurons and secretes many transmitters that control neurotransmission. However, new research has demonstrated that SR, the D-serine biosynthetic enzyme, is extensively expressed in brain neurons, implying that D-serine has a neuronal origin (Kartvelishvily et al., 2006; Wolosker et al., 2008).

During postnatal development, SR is mostly found in pyramidal neurons in the cerebral cortex and the hippocampus CA1 region of the brain. GABAergic Purkinje cells and their dendrites revealed reasonable staining in the cerebellum. This suggests that SR is expressed in the principal neurons regardless of whether they have an excitatory or inhibitory characteristic (Miya et al., 2008).

3- Phosphoglycerate dehydrogenase is an astrocytic enzyme that catalyzes the first committed step in the production of L-serine. L-serine transfers from astrocytes to neurons, where it is converted to D-serine by neuronal SR. This enzyme is prevalent in astrocytes, particularly in the corpus callosum and throughout the cerebral cortex (Ehmsen et al., 2013).

Objective

The function of proteins may be altered by post-translational changes such S-nitrosylation when NO release increases. α-Syn is not a direct target of S-nitrosylation because its core sequence lacks cysteine residues. However, several proteins, including protein-disulphide isomerase and serine racemase, are predisposed to S-nitrosylation, which can cause aggregation and misfolding. Nevertheless, no research has ever been done to determine how SNO proteins affect the aggregation, toxicity, and dissemination of α-Syn. Our aim is to evaluate the contribution of SNO proteins, such as PDI and serine racemase, to α-Syn aggregation and toxicity, in the human neuroblastoma cell line, SH-SY5Y.

Materials and methods

1. Cell culture

In this study, SH-SY5Y cells derived from a human neuroblastoma cell line were used, kindly obtained from Professor Tiago Outeiro (Department of Experimental Neurodegeneration, University Medical Center, Göttingen, Germany). SH-SY5Y cells were grown in a cell culture medium that consists of Dulbecco's Minimum Essential Medium (DMEM, Bioconcept, 1-26F09-I) with 10% Fetal Bovine Serum (FBS, Sigma, F7524) and 1% penicillin-streptomycin (P/S, Bioconcept, 4-01F00-H). To maintain the cells, they were kept in incubators at 37ºC in a humidified atmosphere with 5% CO_2 until 80-90% confluence was reached, which usually was achieved after 3-4 days.

2. Preparation of Cys-SNO

S-nitrosocysteine (CysNO) was prepared as previously described (Izquierdo-Álvarez et al., 2018; Martínez-Ruiz and Lamas, 2005). Thus, three solutions were required to prepare Cys.SNO: L-cysteine (L-Cys), sodium nitrite ($NaNO_2$) and potassium phosphate buffer. 200 mM L-Cys was prepared in 1 mL of 1 M HCl and added to a 200 mM solution of $NaNO_2$ diluted in 1 mL of water. Subsequently, 2 mL of 1M potassium phosphate buffer, pH 7.4, was added to the previous solutions after 30 minutes at RT in the dark. Several aliquots of the final solution have been divided and kept at -80 °C. The Cys-SNO concentration was determined using the Cys-SNO coefficient (ε_{338} = 900 $M^{-1}cm^{-1}$) and a spectrophotometric analysis at 338 nm using the NanoDrop 2000c (Thermo Fisher Scientific) (DeMaster et al., 1995). Since nitrosothiols are light-sensitive, the CysNO solutions were kept in the dark throughout all protocols that were used. It was obtained a concentration of CysNO between 30–40 mM with a 60–80% yield.

3. Treatment with CysNO

SH-SY5Y cells were grown in 100 cm^2 plates until 80-90% confluence and then treated with different CysNO concentrations. Briefly, the stock solution of CysNO was dissolved in culture medium to obtain a working solution of 10, 100 e 1000 μM of CysNO. Cells were washed 1x with PBS and incubated with the different concentrations of CysNO during 15 min at 37 ºC. Then, CysNO was removed, and cells were lysed as described subsequently.

4. Cell Lysates

To obtain the total cell lysates, SH-SY5Y cells were grown in 100 cm^2 plates until 80-90% confluence. Subsequently, cells were lysed in lysis buffer 1x (50 mM Tris-HCl, 10 mM EGTA, 1% Triton X-100 and 2 mM $MgCl_2$, supplemented with 1 mM sodium orthovanadate, 1 mM dithiothreitol (DTT, NZYtech) and 1 μg/mL Protease Inhibitor Cocktail Tablets (Roche), pH 7.4) at 4 °C. β-Mercaptoethanol (4x) and an appropriate volume of Lysis buffer 1x (in order to obtain 20 ug of protein to load on the SDS-PAGE gel) were added to the samples and subsequently heated in the Thermoblock at 95ºC, for 5 min. Samples were kept refrigerated until they were used.

To obtain the cell lysates for the biotin switch protocol, SH-SY5Y cells were grown in 100 cm^2 plates until 80-90% confluence. In the lysates for the Biotin switch assay, a lysis buffer composed of TENT (50 mM Tris pH 7.2, 1 mM EDTA, 100 μM Neocuproine and 1% Trinton X-100), Protease inhibitor Cocktail (Roche) and 50 mM N-Ethylmaleimide (NEM, SIGMA-ALDRICH) was used. Then, 500 μL of TENT was added to each plate to collect cells. The cells with the TENT were transferred to microcentrifuge tubes and subsequently centrifuged for 10 min at 12000 rpm at room temperature (RT). The supernatant was collected and 20% SDS was added to each sample (accordingly with the volume of each sample). Immediately, each sample was placed in a dry bath at 37°C for 30 min.

Finally, the proteins were quantified using the bicinchoninic acid (BCA) method and BSA was used to perform a standard curve. Samples were stored at -80°C, until Western Blot analysis.

5. Western Blot Analysis

Twenty μg of proteins (from total lysates) was separated on a 12% SDS-polyacrylamide gel and transferred to PVDF membranes. These membranes were blocked for 1 h at RT in 5% Non-fat dry milk or 3% Bovine Serum Albumin (BSA) Fraction V (NZYTech), depending on the primary antibody instructions. Primary antibodies were prepared (anti-PDI, 1:500, Invitrogen); anti-SR, 1:200 (Santa Cruz); anti-α-Synuclein (1:500, Biosciences) in tris-buffered saline with tween-20 (TBS-T) with 3% BSA and membranes were incubated with these antibodies overnight at 4°C. In the next day, the membranes were washed 3x with TBS-T during 5 min and then

incubated for 1h at RT with the secondary antibodies (anti-mouse, 1:5000 or anti-rabbit, 1:5000), which were prepared in TBS-T with 3% BSA. Subsequently, the membranes were washed again with TBS-T during 5 min and the Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) was prepared by combining ECL1 and ECL2 Solutions in a 1:1 proportion. Membranes were incubated with this reagent for 5 min and then images are acquired on the Invitrogen™ iBright™ Imaging System (ThermoFisher). Subsequently, after washing the membranes three times in TBS-T, they were incubated with a primary antibody for a housekeeping protein (α-Tubulin, 1:1000, Sigma), which was prepared in TBS-T with 5% Non-fat dry milk, during overnight at 4 ºC. Membranes were washed 3x with TBST and incubated with the respective secondary antibody for 1h at RT. After 3 washes with TBST, membranes were again incubated with the ECL solutions and images are acquired on the Invitrogen™ iBright™ Imaging System (ThermoFisher). The images were analysed with iBright Imaging Software version 5.1.0 (ThermoFisher).

6. Biotin switch assay and Western Blot analysis

To the biotin switch protocol 900 μg of protein sample, obtained from the SH-SY5Y lysates, was used. Each sample was combined with 3 volumes of cold acetone and mixed gently to homogenize. Subsequently these samples were kept at – 20 ºC for 10 minutes and then centrifuged for 10 minutes at 12000 rpm, 4 ºC (VWR, Himac). The supernatant was discarded, and the pellet was washed with 1 volume of cold acetone during 10 min at – 20 ºC. Samples were then centrifuged during 5 min at 12000 rpm, 4 ºC. Supernatants were discarded again. The pellets were resuspended in TENS (50 mM Tris-HCl pH 7.2, 1 mM EDTA, 0.1 mM neocuproine, 1% SDS) together with 100 mM ascorbate. Immediately, 1 mM Biotin-HPDP was added to each sample and incubated for 1h at RT without exposure to light. Following a second acetone precipitation, samples were re-suspended in HENS (250 mM Hepes pH 7.7, 1 mM EDTA, 100 mM NaCl, 0.5% Triton). Each sample was then incubated with the Jaffrey's Neutralization buffer (20 mM Hepes pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and centrifuged for 2 min at 13400 rpm. The remaining supernatant was added to the pre-equilibrated NeutrAvidin Plus UltraLick Resin (ThermoFisher, 53151) after 100 μL of it was set aside for the input fraction (total protein). The samples were kept at RT for 2h while being constantly rocked. Every sample was then centrifuged for 1 minute at RT 2000 rpm. The supernatants were transferred to a fresh eppendorf (non-

retained fraction, NR) at -20°C. The remaining resin was cleaned with wash buffer and centrifuged at 2000 rpm RT for 1 min. The cleaned resin was then mixed with equilibration buffer, centrifuged for 1 min at 2000 rpm, the supernatant was discarded, and 100 μL of elution buffer (20mM Hepes pH 7.7, 100 mM NaCl, 1 mM EDTA, 100 mM β- mercaptoethanol) was added. After that, the proteins that were bound to the resin were incubated for 20 minutes at 37°C in a dry bath. The mixture was then centrifuged for 1 minute at 13400 rpm, collecting the supernatant as Eluted Fraction. The eluted fractions could either be kept at −20 ° C or concentrated in the recommended concentrator (Concentrator 5301).

For the analysis of the Avidin Western Blot, which corresponds to the NR plus the respective input, Non-reducing Laemlli (NRL, 200 mM Tris pH 7.6, 8 mM EDTA, 4% SDS, 40% Glycerol, 0.08% Bromophenol blue) was added to each sample, while the Specific Western Blot corresponds to the Eluted Fractions plus the respective input with Reducing Laemilli (RL, NRL plus 20% β- Mercaptoethanol). The latter were denatured at 96 ºC for 10 min. Both were subjected to electrophoresis on a 10 or 12% SDS-polyacrylamide gel, which were transferred to PVDF membranes. Both membranes were blocked with 3% BSA in TBS-T for 1h at RT. The avidin membrane was incubated with Streptavidin HRP (1:2000 in 1% BSA with TBS-T) for 30 min at RT. The membrane was then washed three times with TBS-T for 5 min each. The membrane of the specific protein was incubated overnight at 4 °C with primary antibodies (anti-PDI and anti-SR in 3% BSA with TBS-T) and then the membrane was washed TBS-T three times for 5 min each. The secondary antibody (anti-mouse) was added for 1h at RT. The membrane was washed again with TBS-T. Then each membrane was incubated with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA) and the bands were visualized on the Invitrogen™ iBright™ Imaging System (ThermoFisher). The images were analysed with iBright Imaging Software version 5.1.0 (ThermoFisher).

7. Cell transfection

24h prior to transfection, approximately 50 000 SH-SY5Y cells were plated per well in a 12- well plate (inVitroCell) containing glass coverslips coated with Poly-L-Lysine (Sigma). SH-SY5Y cells were transfected with pcDNA (empty plasmid), WT and mutations (A53T and A30P) plasmids in non-supplemented DMEM/F12 using the Lipofectamine™ 3000 transfection reagent (ThermoFisher-Invitrogen). 48h after the

transfection, the medium was changed and the cells were treated with Cys-SNO (25 μM) during 24h, 48h and 72h. After these treatments an immunocytochemistry was performed.

8. Immunocytochemistry (ICC)

Transfected SH-SY5Y cells were washed with PBS1x and fixed with 4% paraformaldehyde (PFA) for 20 min at RT, followed by two more washed with PBS 1x. Following blocking for 1h in 2% BSA, 0,2% Triton X-100 and PBS1x, at RT. The primary antibodies used were: Cleaved caspase-3 (1:400, Cell Signaling Technology) and purified mouse anti-α-Syn (1:1000, Bd Biosciences), incubated for 1h at RT. After incubation with the primary antibody, cells were washed with 0,2% Triton X-100 and PBS 1x, then incubated with the secondary antibodies. Secondary antibodies used were: Alexa Fluor 594 donkey anti-rabbit (1:500, Life Technologies-Invitrogen, Carlsbad, CA, USA), Alexa Fluor 488 goat anti-mouse (1:500, Life Technologies-Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 647 Phalloidin (1:1000, Abcam) for 1h at RT. Afterwards, cells were stained with Hoechst (1:1000, Enzo) in PBS 1x for 10 min at RT. The coverslips are then cleaned with PBS 1x and mounted in microscopy slides using DAKO fluorescence mounting medium (DAKO, Agilent Technologies).

9. Confocal Imaging Experiments

Confocal fluorescence imaging experiments were performed by using a Zeiss laser scanning microscope 710 with 40x and 100x objective lenses and Zen 2009 software (Carl Zeiss). SH-SY5Y cells were stimulated with Argon lasers at 488 nm, 594 nm, and 647 nm. A 405-diode laser was used to stimulate the Hoechst 33342. Image J (National Institute of Health) or Zen 2009 (Carl Zeiss) software was used for image analysis. For each condition, twelve positions were assigned.

10. Cytotoxicity assay

To determine cell survival following Cys-SNO treatment, a non-destructive cytotoxicity assay was done using the ToxiLightTM bioassay kit (Lonza). The release of adenylate kinase (AK) from damaged cells is quantified using this kit. First, the AK detection reagent (AKDR) was reconstituted by adding the assay buffer to the lyophilized AKDR vial. The blue screw cap was reinstalled, and the mixture was gently mixed. The luminometer SpectraMax® iD3 (Molecular Devices) was designed to take an instantaneous 1-second integrated measurement of relevant wells after 15 minutes

at RT. Finally, the supernatant from the cells was transferred to a luminescent compatible 96-well plate, and the AKDR was applied to each well. After waiting 5 minutes, the plate was inserted into the luminometer, and the program was started.

11. Statistical analysis

The data is shown as mean + SEM. GraphPad Prism 8.0.1 was used for statistical analysis, which included two-way ANOVA followed by Sidak's multiple comparisons test. The number of nuclei was counted from Image J (National Institute of Health)

Results

1. PDI is S-nitrosylated at different concentrations of CysNO

SH-SY5Y cells derived from a neuroblastoma were treated with different concentrations of nitrosothiol CysNO, and an appropriate control, without treatment, was employed to evaluate reversible nitrosative alteration of proteins following NO exposure. A biotin switch assay is used to determine if PDI and SR are S-nitrosilated. The ascorbate reduction is specific for S-nitrosylation, which increases the method's sensitivity. Biotinylation of newly produced thiols using biotin-HPDP, which integrates into the thiol by producing a disulfide bridge. Biotinylated proteins are captured using immobilized avidin resins. The incorporation of biotin via the disulfide bond is beneficial because it allows for simple elution by incubation with a reducing agent, as well as the elimination of endogenously biotinylated proteins that remain linked to the avidin (**Figure 15A**). Following avidin capture, specific protein identification may be accomplished using a Western blot and an antibody directed against the protein. The results reveal that PDI is S-nitrosylated at all concentrations tested (10μM, 10mM, and 1mM). However, the highest concentration displays a more visible band when compared to the rest (**Figure 15B**). SR was not detected in SH-SY5Y cell lysates treated with CysNO, in contrast to PDI.

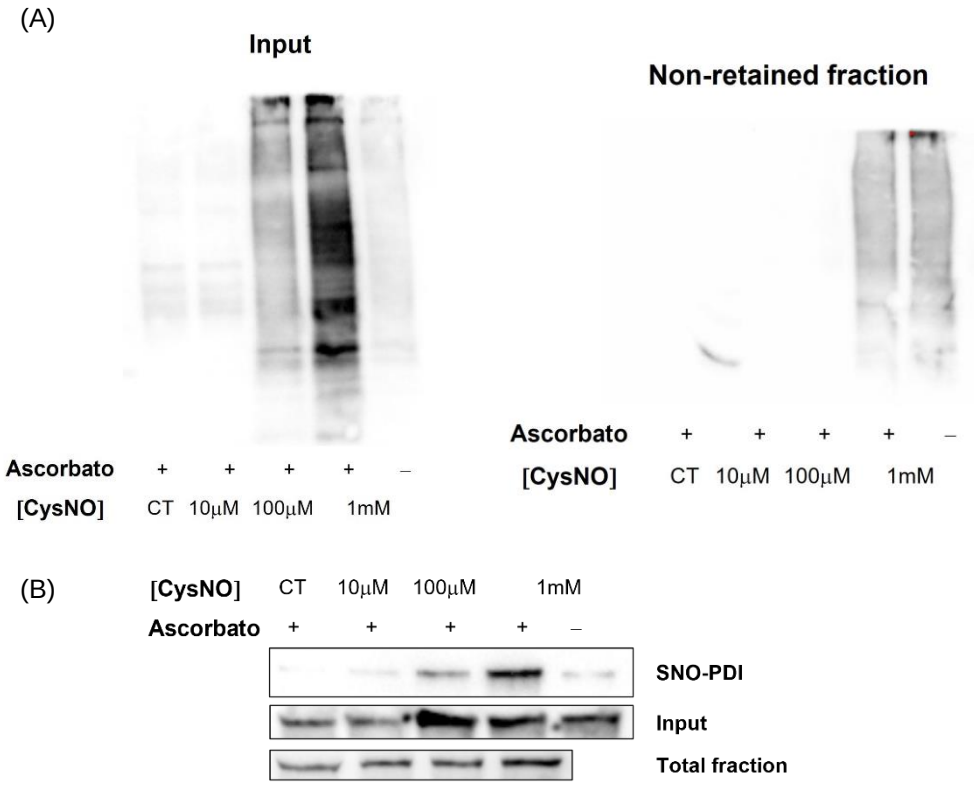

Figure 15| S-nitrosylation of PDI in cells treated with CysSNO. The cells were treated with different concentrations of CysSNO for 15 min. (A) Input and non-retained fraction of Avidin western blot. (B) S-nitrosilated PDI was detected by biotin switch assay and in total fraction, which represents a 59 KDa band. Based on n=3.

2. Transfected cells and presence of aggregates in SH-SY5Y cells

A western blot was performed to confirm the existence of α-Syn in SH-SY5Y cells, and the results showed that the cells contained the protein (**Figure 16**).

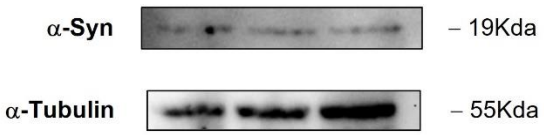

Figure 16| Representative image of the Western Blot protein detection of α-Syn in SH-SY5Y. Protein samples (20μg) from SH-SY5Y cells were loaded in a polyacrylamide gel. Blots were probed with an anti-α-Syn antibody (*Upper*) and an anti-α-Tubulin antibody (*Lower*) as loading control. Based on n=1

To investigate the potential impact of S-nitrosylation on the existence of α-syn and its development into aggregates, a quantitative analysis of transfected cells was conducted to determine the presence or absence of aggregates. SH-SY5Y cells were transfected with plasmids containing pcDNA (empty plasmid), WT, and mutations (A53T and A30P) (Figure 17A,18A, and 19A). After 48 hours, the medium was replaced, and the cells were treated with CysNO (25M) for 24 hours, 48 hours, and 72 hours. The confirmation of transfection was identified by using immunocytochemistry to visualize the presence of α-syn in the cells.

The graphs below (**Figure 17B,18B and 19B**) depict the quantification of the number of transfected cells. In the three time points, pcDNA plasmid present only basal levels of α-Syn. In general, the cells transfected with mutant α-syn displayed a comparable transfection rate at the three time points, yet this was not statistically significant. Overall, the control groups shown a higher rate of transfected cells compared with the groups treated with CysNO (25μM). At 24 hours upon the treatment, control groups contain a higher percentage of transfected cells than CysNO-treated groups, has shown in the figures above (**Figure 17B**).

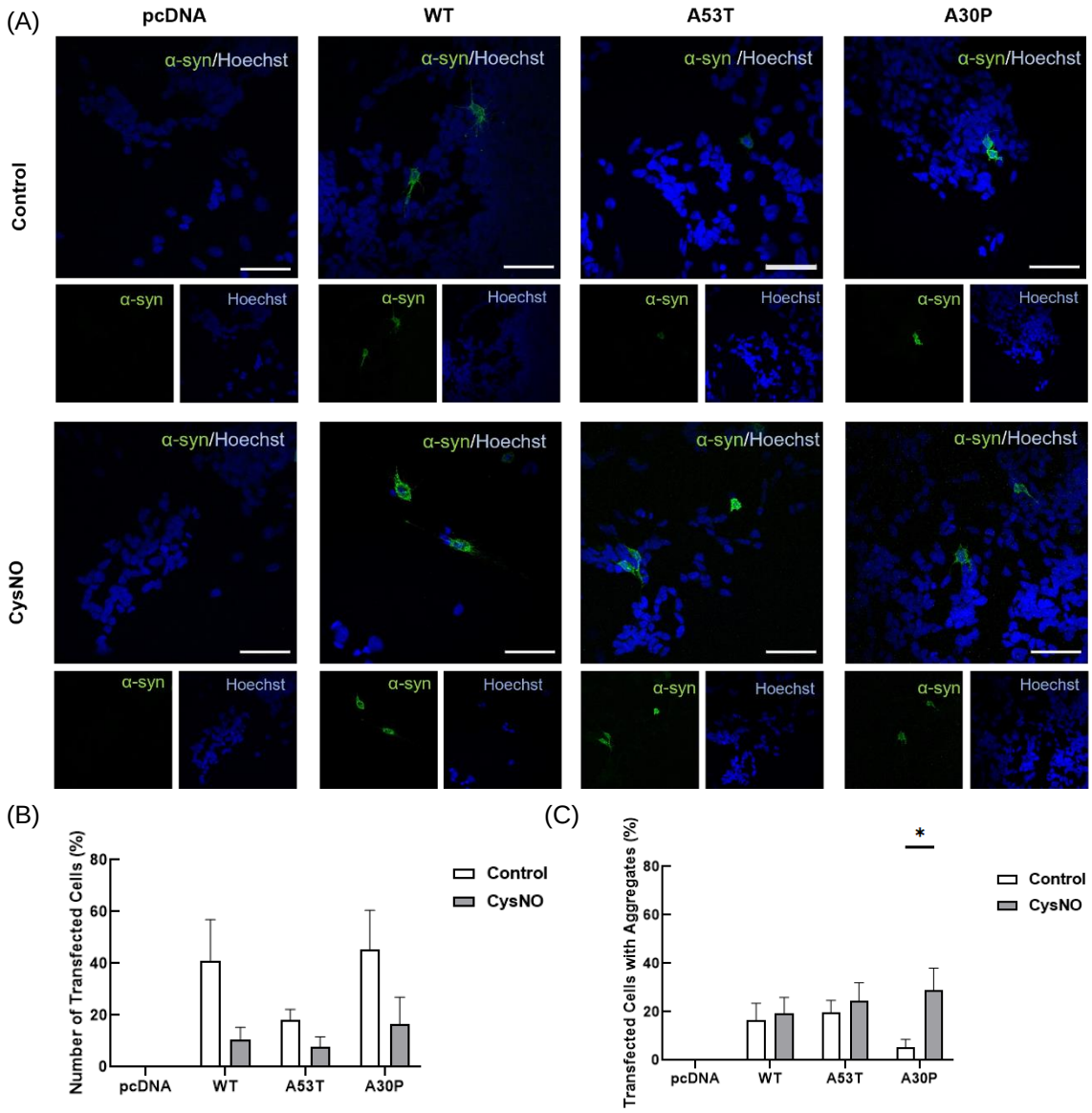

Figure 17| Analysis of transfected cells and aggregates of α-syn after 24 hours of with and without and CysNO treatment. (A) Confocal images of immunocytochemical staining for cultured SH-SY5Y cells of the control group and the group treated with CysNO for 24 hours. Cells were stained with anti-α-Syn antibody (488 nm) and Hoechst (358nm) for nuclei visualization. Scale bars represent 50 µm. (B) Histogram representing the number of transfected cells with pcDNA, WT α-syn, A53T and A30P α-syn mutant, and (C) the number of transfected cells containing aggregates in the control group and CysNO treated group. Values are in %. Data was analyzed through two-way ANOVA, 95% confidence, mean + SEM based on n=5. * P< 0.05 compared with control group.

The control groups had a higher percentage of transfected cells than the CysNO groups 48 hours after treatment, specifically in the WT and A30P genotypes, however, the difference was not as significant in the A53T genotype (**Figure 18B**).

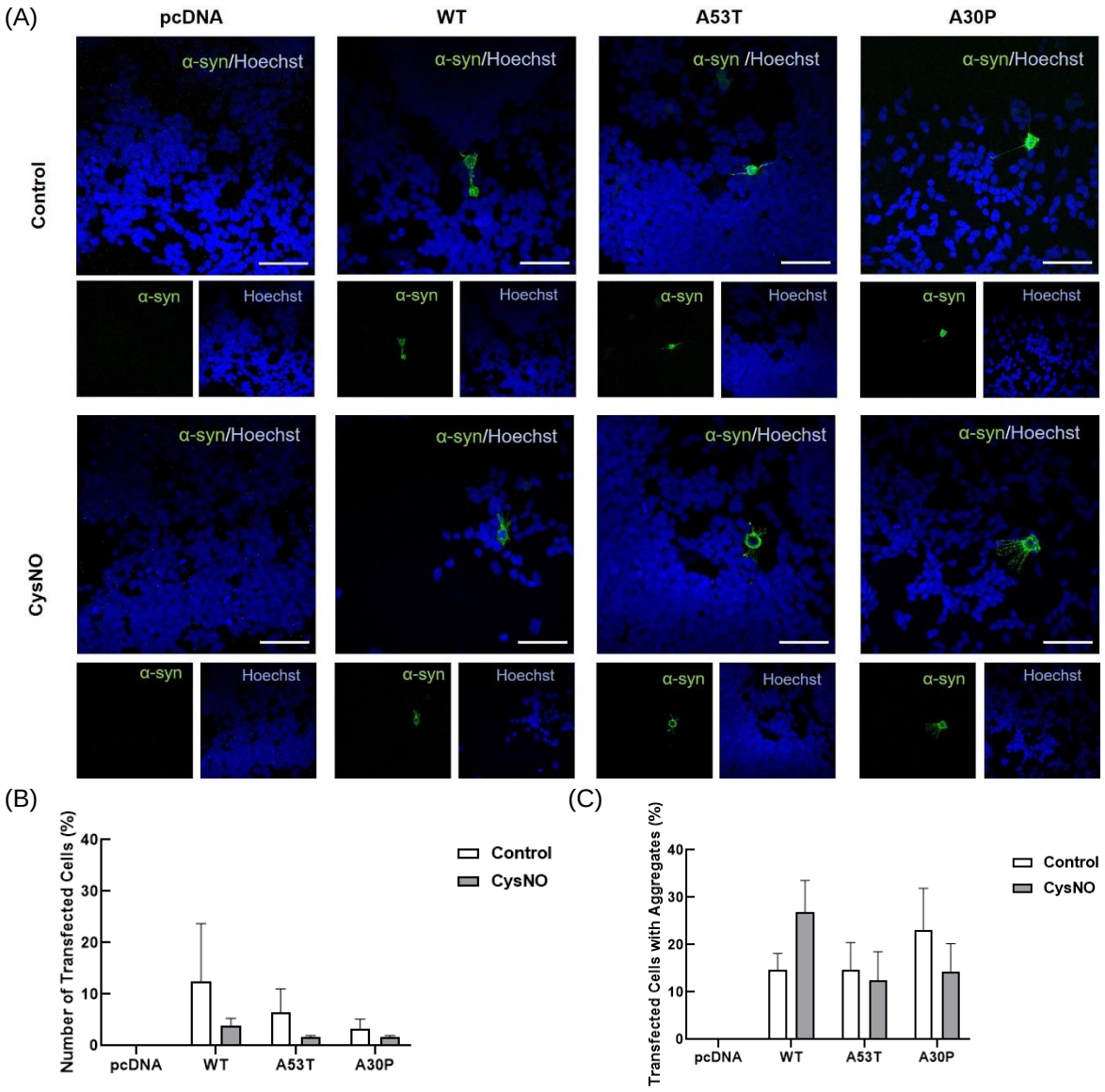

Figure 18| Analysis of transfected cells and aggregates of α-syn after 48 hours of with and without and CysNO treatment. (A) Confocal images of immunocytochemical staining for cultured SH-SY5Y cells of the control group and the group treated with CysNO for 48 hours. Cells were stained with anti-α-Syn antibody (488 nm) and Hoechst (358nm) for nuclei visualization. Scale bars represent 50 µm. (B) Histogram representing the number of transfected cells with pcDNA, WT α-syn, A53T and A30P α-syn mutant, and (C) the number of transfected cells containing aggregates in the control group and CysNO treated group. Values are in %. Data was analyzed through two-way ANOVA, 95% confidence, mean + SEM based on n=5.

The control groups had a higher percentage of transfected cells than the treated groups 72 hours after treatment, although the difference was not statistically significant (**Figure 19B**).

The results indicate that the control groups included more transfected cells than the CysNO groups at all three time points (24 hours, 48 hours, and 72 hours).

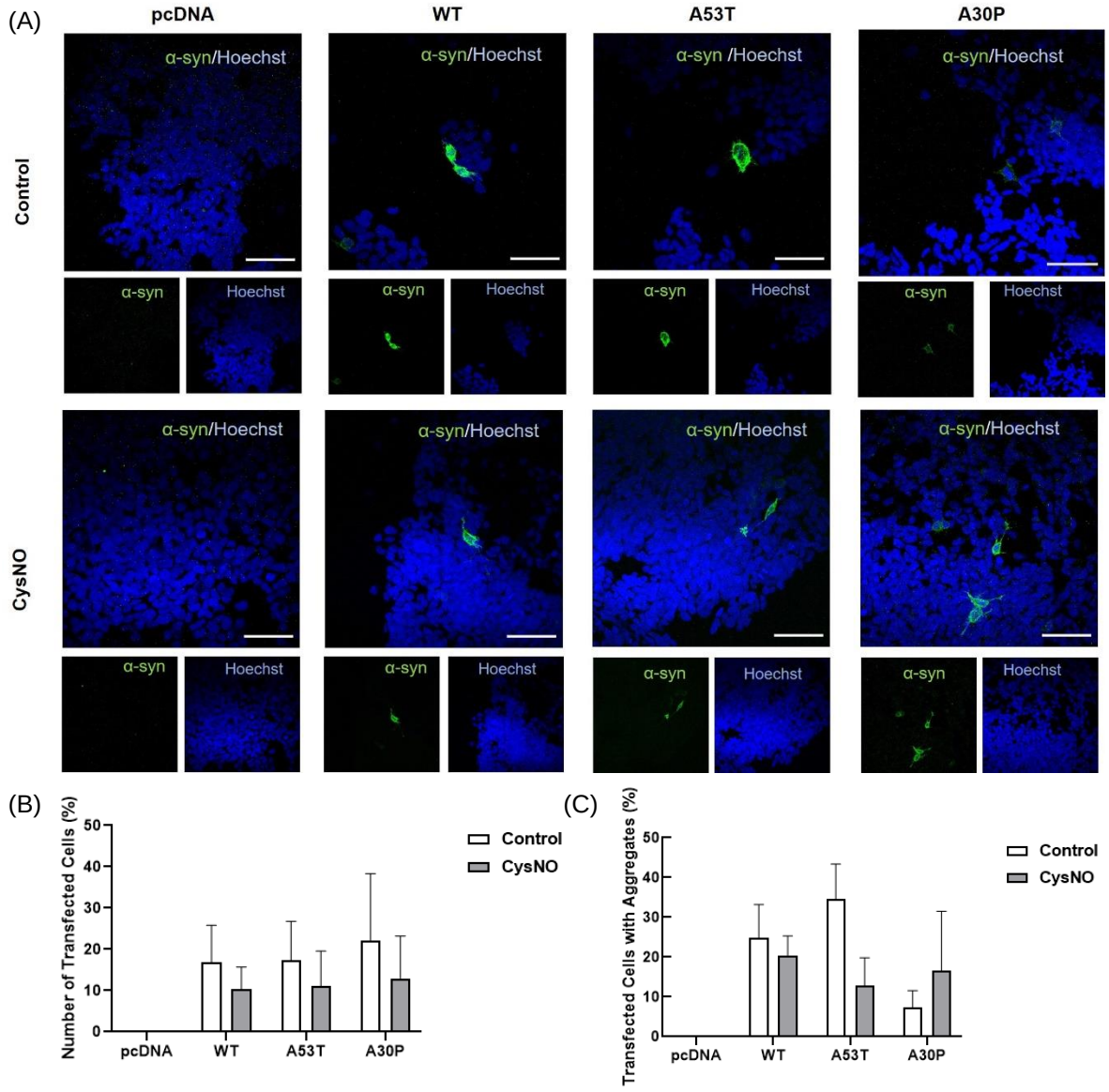

Figure 19| Analysis of transfected cells and aggregates of α-syn after 72 hours of with and without and CysNO treatment. (A) Confocal images of immunocytochemical staining for cultured SH-SY5Y cells of the control group and the group treated with CysNO for 72 hours. Cells were stained with anti-α-Syn antibody (488 nm) and Hoechst (358nm) for nuclei visualization. Scale bars represent 50 µm. (B) Histogram representing the number of transfected cells with pcDNA, WT α-syn, A53T and A30P α-syn mutant, and (C) the number of transfected cells containing aggregates in the control group and CysNO treated group. Values are in %. Data was analyzed through two-way ANOVA, 95% confidence, mean + SEM based on n=4.

The number of cells transfected with aggregates was counted to determine if treatment with CysNO increases the aggregation process of α-Syn. After 24 hours, the number of cells transfected with aggregates is higher when treated with CysNO, but there is only a statistical significance in the mutant A30P α-Syn. The WT condition shows a similar number of cells transfected with aggregates regardless the treatment with CysNO (**Figure 17C**). The WT condition exhibited an abundance of cells transfected with aggregates in the CysNO-treated groups after 48 hours. While there is a non-significant increase in the control groups in the α-Syn mutant conditions (A53T

and A30P) (**Figure 18C**). Additionally, 72 hours after CysNO treatment, the WT condition in both the control and treated groups exhibited similar values. In comparison to the control condition, the α-Syn mutant A53T condition had a higher percentage of cells transfected with aggregates. Unlike the A53T condition, A30P mutant α-Syn had a higher percentage in the CysNO-treated groups (**Figure 19C**). At both time points, it was not observed any aggregates in cells transfected with empty vector.

These data conclude that there are no significant differences between the conditions treated with CysNO and the control groups.

3. Analysis of cell viability in SH-SY5Y cells

A primary antibody, cleaved caspase-3, was utilized to determine if S-nitrosylation has an impact on cell survival. Caspase-3 is initially cleaved and thus activated once the apoptosis process initiates. This apoptotic marker was examined and quantified by immunocytochemistry.

The graphs below represent the percentage number of cells with cleaved caspase-3 in each condition at 24 hours, 48 hours, and 72 hours, respectively (Figure 20B, 21B and 22B). After 24 hours of treatment, in the WT and a-Syn mutant conditions (A53T and A30P) the groups with the treatment showed a higher percentage than the control groups. The control condition shows a low percentage of cleaved caspase-3 in both the control and CysNO groups (**Figure 20B**).

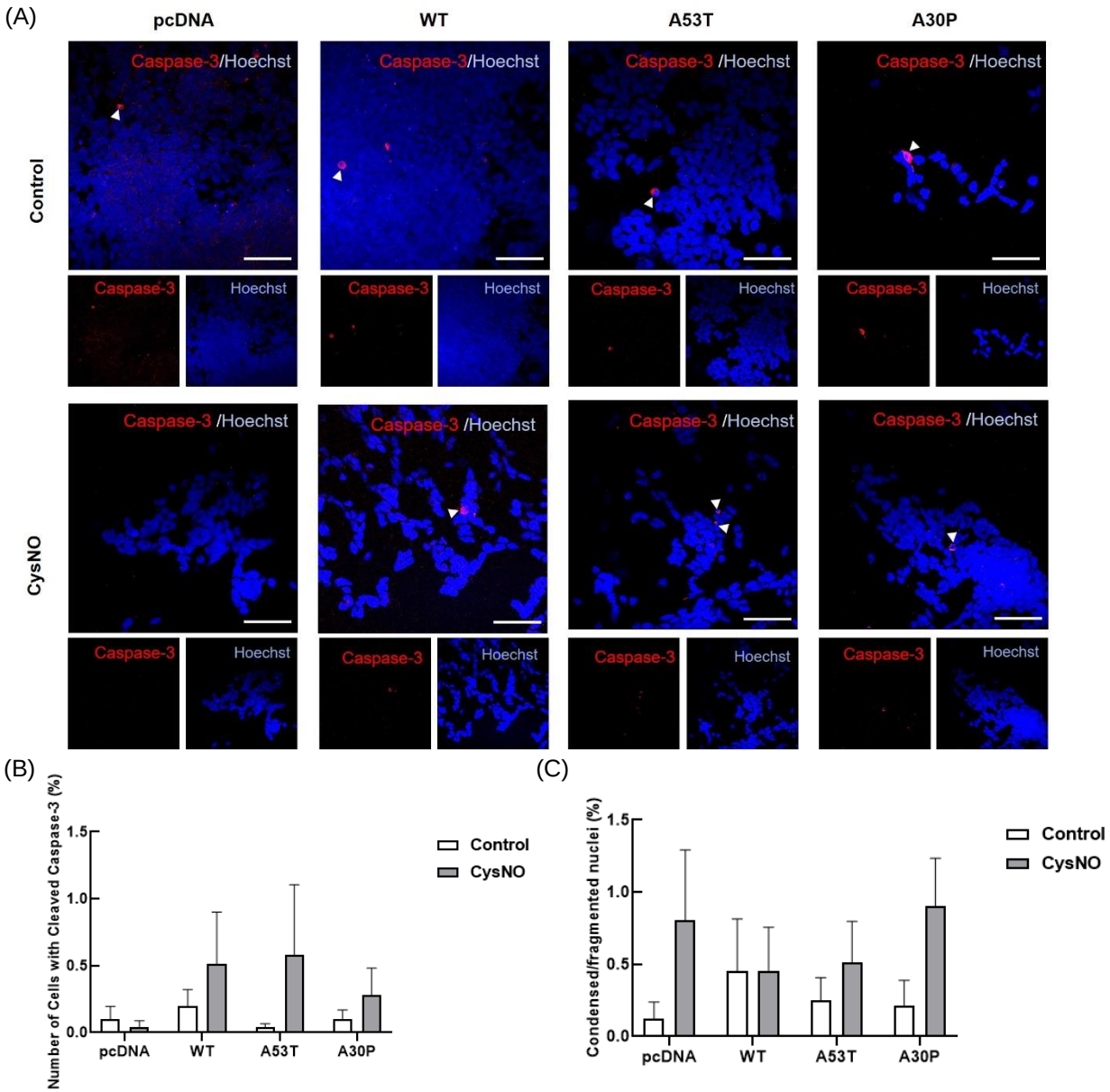

Figure 20| Analysis of the presence of cleaved caspase-3 and condensed/fragmented nuclei in cells after 24 hours with and without CysNO treatment. (A) Confocal images of immunocytochemical staining for cultured SH-SY5Y cells of the control group and the group treated with CysNO for 24 hours. Cells were stained with anti-cleaved caspase-3 antibody (594 nm) and Hoechst (358nm) for nuclei visualization. Scale bars represent 50 µm. The white arrows represent the presence of cleaved caspase-3. (B) Histogram representing the number of cells with cleaved caspase-3 that were transfected with pcDNA, WT α-syn, A30P and A53T α-syn mutant in the control group and CysNO treated group after 24 hours. (C) Histogram representing the number of condensed/fragmented nuclei present in cells transfected with pcDNA, wild-type α-syn, A30P α-syn mutant, A53T α-syn mutant in presence of DMEM (control) and CysNO. Values are in %. Data was analyzed through two-way ANOVA, 95% confidence, mean + SEM based on n=5.

The percentage of cells with cleaved caspase-3 was examined and quantified again after 48 hours, with the WT condition exhibiting a higher value in the control group. The remaining conditions (WT, A53T, and A30P) had substantially similar values in both the control and treatment conditions (**Figure 21B**).

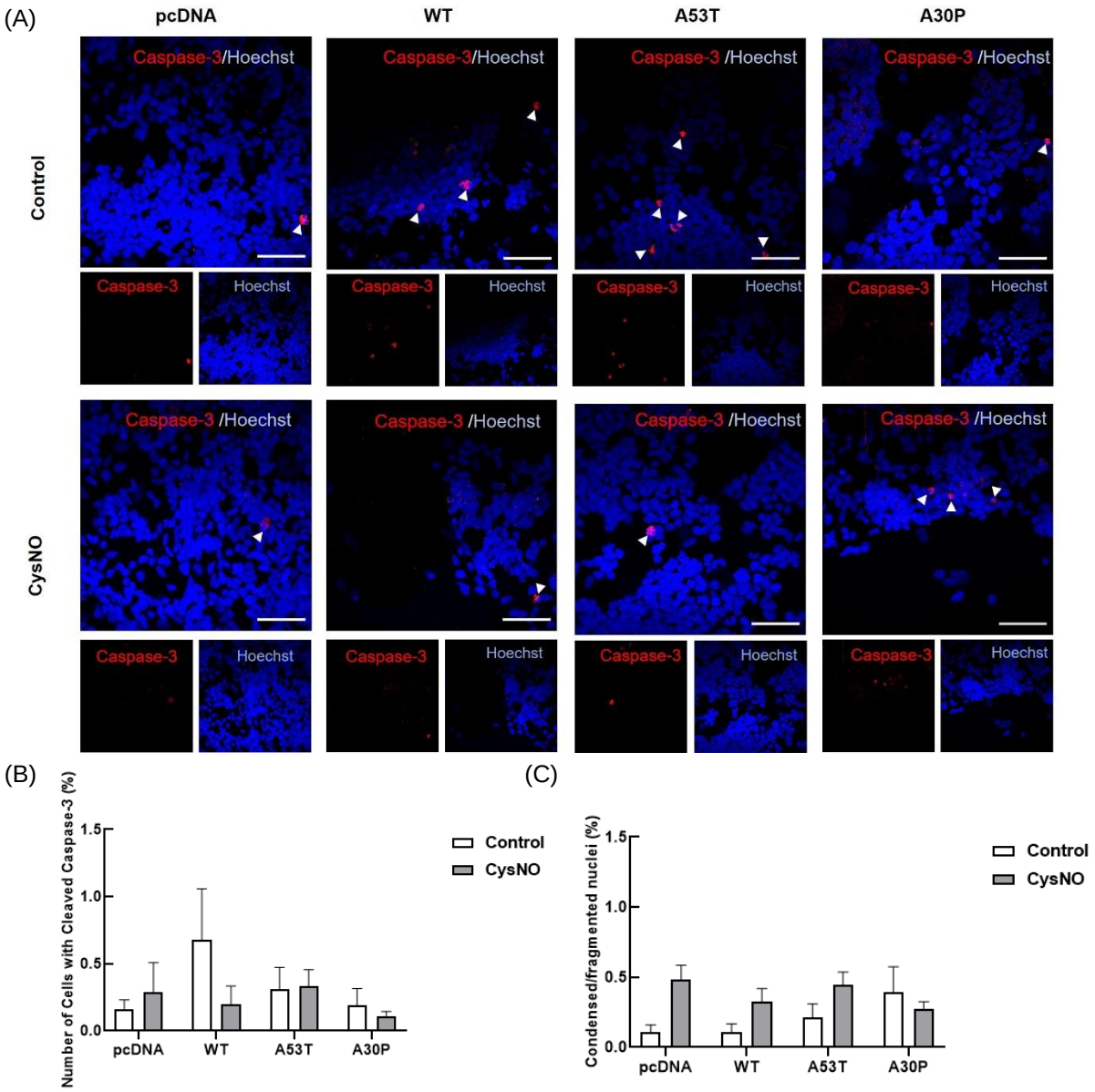

Figure 21| Analysis of the presence of cleaved caspase-3 and condensed/fragmented nuclei in cells after 48 hours with and without CysNO treatment. (A) Confocal images of immunocytochemical staining for cultured SH-SY5Y cells of the control group and the group treated with CysNO for 48 hours. Cells were stained with anti-cleaved caspase-3 antibody (594 nm) and Hoechst (358nm) for nuclei visualization. Scale bars represent 50 µm. The white arrows represent the presence of cleaved caspase-3. (B) Histogram representing the number of cells with cleaved caspase-3 that were transfected with pcDNA, WT α-syn, A30P and A53T α-syn mutant in the control group and CysNO treated group after 48 hours. (C) Histogram representing the number of condensed/fragmented nuclei present in cells transfected with pcDNA, wild-type α-syn, A30P α-syn mutant, A53T α-syn mutant in presence of DMEM (control) and CysNO. Values are in %. Data was analyzed through two-way ANOVA, 95% confidence, mean + SEM based on n=5.

The WT condition had the highest percentage in both the control and treatment groups after 72 hours. All the remaining conditions (pcDNA, A53T, and A30P) reveal no statistically significant differences between the control and treatment groups (**Figure 22B**). The results therefore reveal that the percentage of cells with cleaved caspase-3 is not statistically significant, implying that the cells exhibit low levels of this apoptotic marker. As a result, S-nitrosylation did not affect cell death in SH-SY5Y cells.

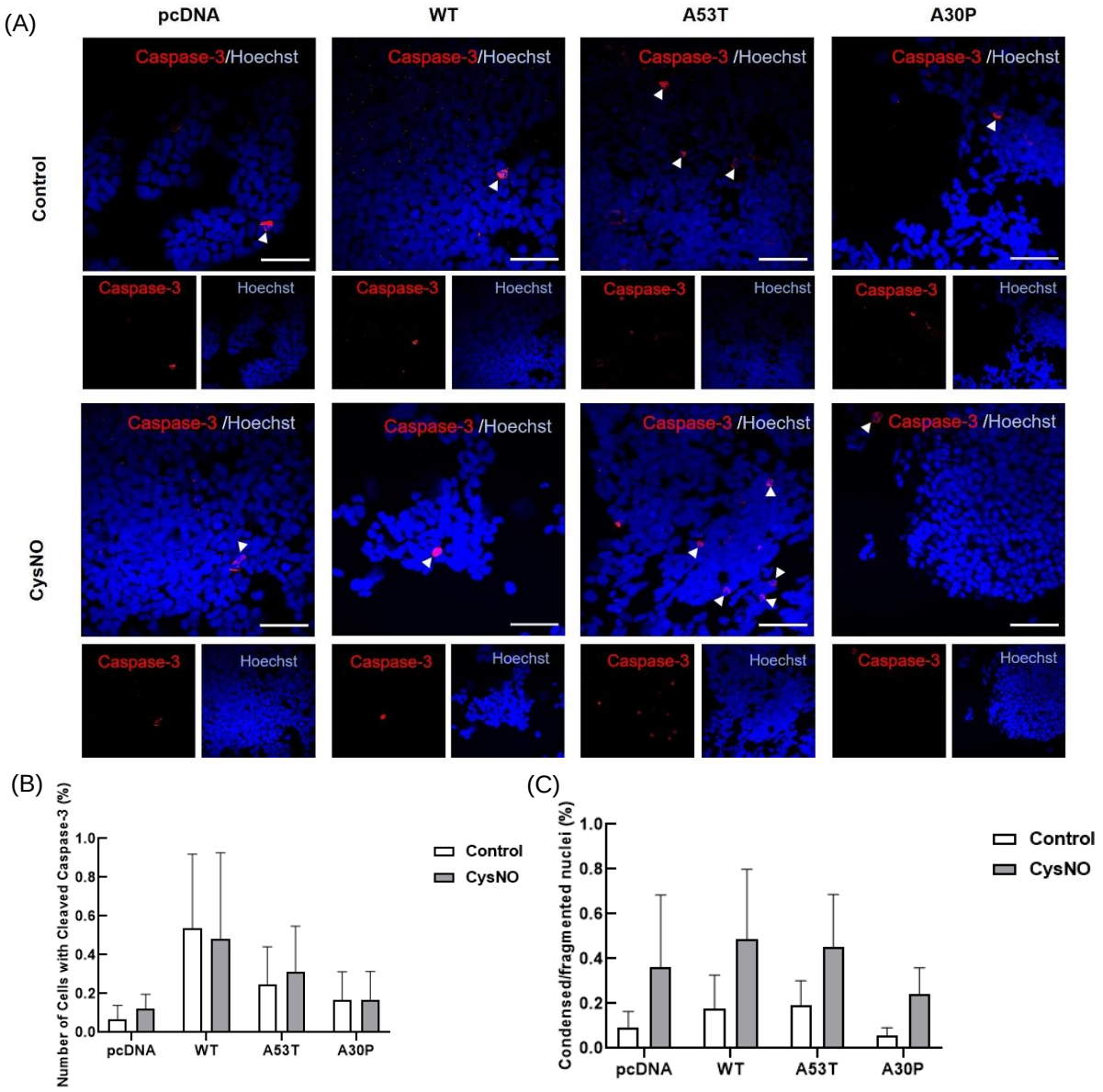

Figure 22| Analysis of the presence of cleaved caspase-3 and condensed/fragmented nuclei in cells after 72 hours with and without CysNO treatment. (A) Confocal images of immunocytochemical staining for cultured SH-SY5Y cells of the control group and the group treated with CysNO for 72 hours. Cells were stained with anti-cleaved caspase-3 antibody (594 nm) and Hoechst (358nm) for nuclei visualization. Scale bars represent 50 µm. The white arrows represent the presence of cleaved caspase-3. (B) Histogram representing the number of cells with cleaved caspase-3 that were transfected with pcDNA, WT α-syn, A30P and A53T α-syn mutant in the control group and CysNO treated group. (C) Histogram representing the number of condensed/fragmented nuclei present in cells transfected with pcDNA, wild-type α-syn, A30P α-syn mutant, A53T α-syn mutant in presence of DMEM (control) and CysNO after 72 hours. Values are in %. Data was analyzed through two-way ANOVA, 95% confidence, mean + SEM based on n=4.

To determine how S-nitrosylation affects nuclear morphology, the number of cells with condensed or fragmented nuclei was measured.

The three graphs in Figures 20C, 21C, and 22C compare the percentage number of condensed/fragmented nuclei identified in CysNO-exposed cellular groups with

control groups at different time points. After 24 hours, the WT α-Syn group had a similar value of condensed/fragmented cells, both with and without CysNO treatment. After CysNO treatment, the pcDNA, A53T, and 30P mutant of α-Syn had significantly more condensed/fragmented nuclei (**Figure 20C**). At 48 hours, the groups treated with CysNo in the pcDNA, WT α-Syn, and A53T α-Syn mutant conditions had higher percentages than the control groups. In contrast, the A30P α-Syn mutant had a higher percentage in the control groups (**Figure 21C**). Treatment with CysNO at 72 hours shows an increase in the percentage of condensed/fragmented nuclei in all the conditions present (pcDNA, WT, A53T and A30P) compared to the control group (**Figure 22C**). This data demonstrates that, overall, the treatment with CysNO increases the percentage of condensed/fragmented nuclei, even though it's not significant.

In morphological terms, cells transfected with pcDNA have a healthier nucleus, with a rounder and more oval structure compared to cells transfected with the WT condition and α-Syn mutant (A53T and A30P) (**Figure 20A, 21A and 22A**).

The ToxiLight bioassay was used to further investigate the effect of S-nitrosylation on the viability of SH-SY5Y cells, and the results are shown in the graphs below (**Figure 23**). This method was developed to assess toxicity in cultured mammalian cells and cell lines. The kit quantifies the release of AK from damaged cells. After 48 hours of transfection the medium with Lipofectamine was removed and replaced with fresh DMEM/F12. The supernatant of each condition (pcDNA, WT, A53T, and A30P) is withdrawn from the control and CysNo groups at three-time points (24 hours, 48 hours, and 72 hours). The percentages are normalised from pcDNA, corresponding to 100%. At 24 hours, the WT control and A30P α-Syn mutant groups showed a slightly higher percentage than the CysNO-treated groups. In contrast, the A53T α-Syn mutant condition had a control group with a lower percentage than the CysNO-treated group (**Figure 23A**). After 48 hours, the CysNO-treated groups showed a higher percentage of cytotoxicity in all genotypes compared to the control groups (**Figure 23B**). The percentage of groups treated with CysNO after 72 hours shows a small decrease in cytotoxicity compared to the control in all genotypes, which is not significant (**Figure 23C**). These findings revealed that there was no significant difference between the

control and CysNO. As a result, it can be inferred that CysNO does not affect the cytotoxicity of SH-SY5Y cells.

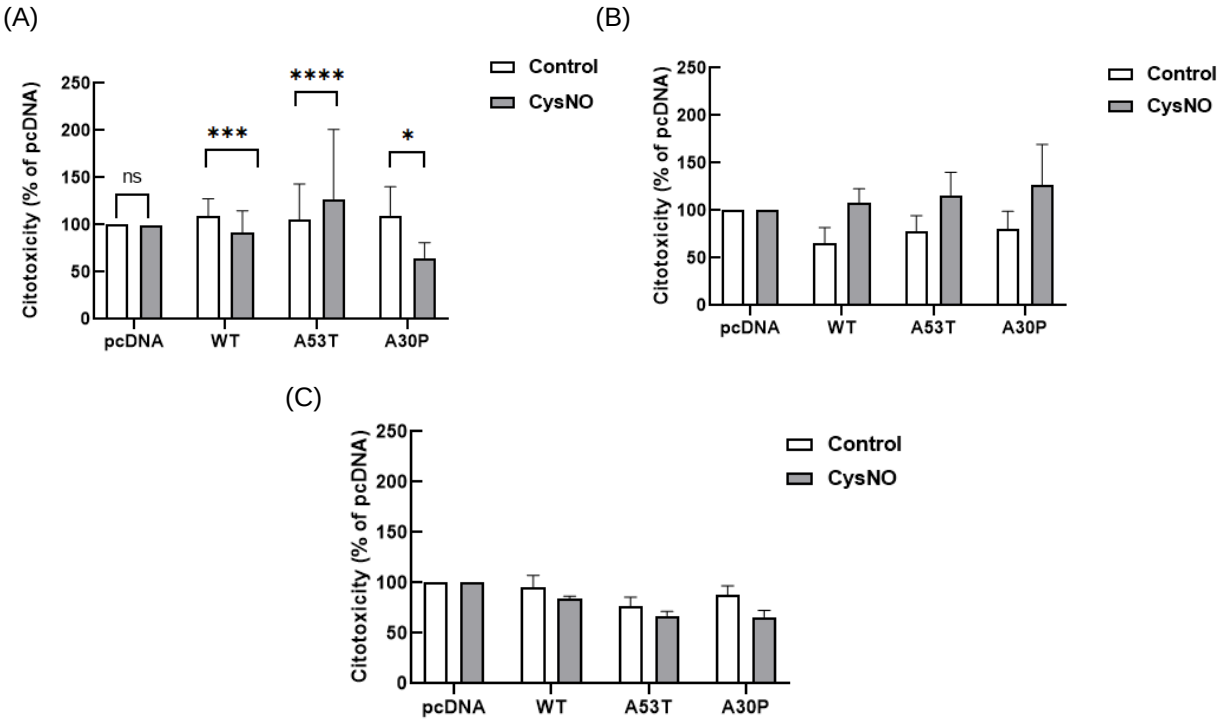

Figure 23| Percentages of cytotoxicity present in SH-SY5Y cells with and without CysNO treatment. (A) After 24 hours in the control group and the group exposed to CysNO. (B) After 48 hours in the control group and the group exposed to CysNO. (C) After 72 hours in the control group and the group exposed to CysNO. Values were measured by the non-destructive cytotoxicity assay and are presented in %. Data was analyzed through tow-way ANOVA, 95% confidence, mean + SEM based on n=2, n=5 and n=4, respectively. *, *** e **** P< 0.05 compared with control group.

Discussion

Parkinson's disease (PD) is a neurodegenerative disease and is characterized by nigrostriatal dopaminergic neuron loss. Genetic research enables the collection of clues to the etiology and pathology, even though the reason of the ongoing degeneration of these neurons is unknown. The characteristic of this disease, which causes synapse loss and neuronal death, is the accumulation of misfolded or aggregated proteins. There is indication that the excessive production of ROS and RNS can cause these misfolded proteins to accumulate in the brain. Mechanistically, this disease is associated not only with genetic mutations but also with risk factors like ageing and exposure to environmental toxins, which accelerate the generation of ROS and RNS and results in protein misfolding. S-nitrosylation is a process that involves NO-dependent signaling pathways that are mediated by PTMs of crucial cysteines. By changing protein activity, inducing conformational changes, altering protein folding, altering protein-protein interactions, and affecting protein subcellular localization, this response can have an impact on signaling cascades and neuronal functioning.

Previous studies have shown that nitrosative stress can cause PTM, such as S-nitrosylation, on PD-related proteins (Chung et al., 2004; Uehara et al., 2006). Because α-Syn does not have cysteine residues, it cannot be S-nitrosylated. Other proteins, including PDI and SR, lose their neuroprotective functions when S-nitrosylated, resulting in misfolded protein aggregation (Nakamura et al., 2013). Furthermore, recent research has shown that an increase in α-syn leads to an increase in NO production and S-nitrosylation of MAP1A scaffolds proteins (Hallam et al., 2022). As a result, an increase in α-syn aggregation or an increased rate of S-nitrosylation would be predicted in the presence of Cys-SNO.

The results of the biotin switch assay show that PDI is S-nitrosylated, as previously demonstrated (Uehara et al., 2006). If PDI is S-nitrosylated, its function as a chaperone is lost, and the α-Syn aggregation process continues. (Andreu et al., 2012). In contrast to PDI, no SR was found in SH-SY5Y cell lysates treated with CysNO. NMDA transmission causes SR S-nitrosylation, which is regulated by nNOS. Thus, NMDA receptor activation is required for NO generation. Therefore, in low basal conditions, NMDA transmission controls SR. The stimulation of SR activity by D-serine

may be caused not only by a direct impact on SR, but also indirectly by the inhibition of nNOS activity. (Mustafa et al., 2007).

SH-SY5Y cells have been modified to overexpress a-Syn (WT plasmid) and to have α-Syn mutants (A53T and A30P) present. The results show that S-nitrosylation does not affect aggregation or cytotoxicity in cells exposed to CysNO. The data demonstrate that there is no significant change in the three time points (24 hours, 48 hours, and 72 hours) following treatment, which can be explained by stress indicating the recovery from the stress caused by the transfection process as time passes. In terms of the impact on α-Syn aggregation, the results indicate that there are no significant changes in the three-time points between the conditions treated with CysNO and the control groups. However, to better understand how S-nitrosylation affects α-syn aggregation, the size of the aggregates and the number of aggregates present in the cells should be quantified and measured. Furthermore, to successfully demonstrate that the S-nitrosylation reaction occurred, a measurement of S-nitrosylated proteins in circumstances treated with CysNO would be beneficial. This might be accomplished by Western blot examination of cell lysates after transfection.

Apoptosis, also known as programmed cell death, is the regulated disintegration of organelles that does not cause inflammation or harm to surround cells. The presence of cleaved caspase-3 can indicate this process is active (Banfalvi, 2017). Caspase-3 is a protease that, when activated, is critical for maintaining homeostasis by regulating apoptosis and inflammation, as well as being required for neuronal cell death (Mcilwain et al., 2013). Since one of the objectives of this investigation was to determine how S-nitrosylation influenced the viability of cells expressing WT and mutant α-syn, apoptosis was measured by the presence of cleaved caspase-3 and cell morphology. The data indicate that S-nitrosylation has no effect on cell death; nevertheless, at 24 hours, the CysNO-treated groups had a higher percentage of cleaved caspase-3. Furthermore, the formation of condensed/fragmented nuclei, a morphologic hallmark of apoptosis (Mcilwain et al., 2013), was enhanced in cells treated with CysNO. The presence of cleaved caspase-3 was found to be lower in both groups transfected with A30P mutant, which is consistent with previous research showing that cells expressing α-syn A30P mutation have anti-apoptotic properties as evidenced by lower caspase-3 expression compared to A53T α-syn (Li and Lee, 2005).

S-nitrosylation seems to not affect the cytotoxicity of SH-SY5Y cells based on the results of the cytotoxicity experiment. This can be explained by S-nitrosylation's early protective function on SH-SY5Y cells expressing the various α-Syn genotypes investigated in this work. This might be explained by prior research, which indicated that S-nitrosylation inhibits caspase-3, impairing apoptosis (Rössig et al., 1999). To determine whether there is a link between S-nitrosylation and caspase-3, analysis might be performed to check whether caspase-3 is S-nitrosylated in the cells.

Conclusion

PDI is S-nitrosylated in the presence of CysNO. Which can lead to the aggregation of α-Syn and consequently to neurodegeneration. S-nitrosylation seems to enhance the presence of mutant aggregates at early time points, but extended exposure to CysNO may lead to an increase in aggregates in cells expressing A53P and A30P mutations. In terms of cell viability, the findings indicate that S-nitrosylation does not impair cell vitality. It is critical to determine whether the time frame chosen is more appropriate for this research.

More study is needed to completely understand how S-nitrosylation impacts α-syn aggregation and cell survival. It is important to explore the S-nitrosylation of PDI, which implies the aggregation of α-Syn. In the future, the quantity of α-syn aggregates and their size should be quantified following exposure to CysNO. It would also be necessary to assess the presence and quantity of S-nitrosylated proteins, as well as perform a Western Blot analysis and analyze by thiol redox proteomics to determine the amount of α-syn protein that is effectively S-nitrosylated and can increase the aggregation process of α-syn. To further understand how cytotoxicity is influenced and how S-nitrosylation affects cell viability, researchers must search for the colocalization of α-syn with cleaved caspase-3.

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