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Protein S-nitrosylation and oxidation contribute to protein misfolding in neurodegeneration

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Abstract

Neurodegenerative disorders like Alzheimer's disease and Parkinson's disease are characterized by progressive degeneration of synapses and neurons. Accumulation of misfolded/aggregated proteins represents a pathological hallmark of most neurodegenerative diseases, potentially contributing to synapse loss and neuronal damage. Emerging evidence suggests that misfolded proteins accumulate in the diseased brain at least in part as a consequence of excessively generated reactive oxygen species (ROS) and reactive nitrogen species (RNS). Mechanistically, not only disease-linked genetic mutations but also known risk factors for neurodegenerative diseases, such as aging and exposure to environmental toxins, can accelerate production of ROS/RNS, which contribute to protein misfolding – in many cases mimicking the effect of rare genetic mutations known to be linked to the disease. This review will focus on the role of RNS-dependent post-translational modifications, such as S-nitrosylation and tyrosine nitration, in protein misfolding and aggregation. Specifically, we will discuss molecular mechanisms whereby RNS disrupt the activity of the cellular protein quality control machinery, including molecular chaperones, autophagy/lysosomal pathways, and the ubiquitin-proteasome system (UPS). Because chronic accumulation of misfolded proteins can trigger mitochondrial dysfunction, synaptic damage, and neuronal demise, further characterization of RNS-mediated protein misfolding may establish these molecular events as therapeutic targets for intervention in neurodegenerative diseases.

Graphical Abstract

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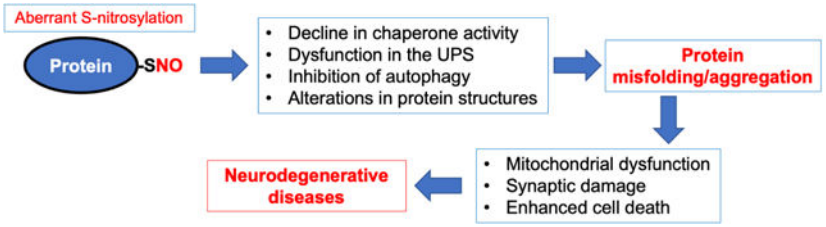
Declaration of competing interest

The authors declare that they have no conflicts of interest with the contents of this article.



Keywords

Protein S-nitrosylation; Tyrosine nitration; Protein misfolding; Autophagy; Molecular chaperones; Ubiquitin-proteasome system

1. Introduction

A key pathological event in the majority of neurodegenerative diseases involves excessive generation of misfolded proteins that can contribute to progressive loss of synapses and neurons [1, 2, 3]. For example, aggregated/misfolded α-synuclein (αSyn) is mainly found in patients with synucleinopathies such as Parkinson's disease (PD) and Lewy body dementia (LBD). Additionally, in Alzheimer's disease (AD), the most common form of dementia in the elderly, extracellular amyloid-β (Aβ) and intracellular phosphor-tau form amyloid plaques and neurofibrillary tangles, respectively; interestingly, however, over 50% of AD brains examined postmortem also exhibit αSyn deposits [4]. Accordingly, in the past few decades numerous studies have investigated the toxic effects of soluble misfolded proteins on neuronal function and survival as well as the molecular mechanisms leading to this protein aggregation. Emerging evidence suggests that increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), representing another key pathological feature of neurodegenerative diseases, is a critical driver of protein misfolding [5, 6]. In this review article, we provide a current overview of pathological events leading to excessive reactive nitrogen and oxygen species (RNS/ROS), including nitric oxide (NO)-related species, that trigger protein misfolding and aggregation, thus contributing to synaptic damage and neuronal injury. Here, we focus on NO-dependent signaling pathways mediated by post-translational modification (PTM) of critical cysteine thiols in a reaction known as S-nitros(yl)ation, which has been shown to be on a par with O-phosphorylation in terms of abundance and importance to cell physiology [7]. We will discuss specific examples showing that aberrant protein S-nitrosylation can compromise the quality control (QC) machinery of protein folding, including molecular chaperones, autophagy, and the ubiquitin-proteasome system (UPS) (Table 1). In addition, we delineate possible pathological roles of RNS-mediated nitration of tyrosine residues (see Section 2.3 for details).

1.1 Protein misfolding and aggregation in neurodegenerative diseases

Protein misfolding and aggregation serve as hallmarks of many neurodegenerative diseases including AD, PD, LBD, amyotrophic lateral sclerosis (ALS), Frontotemporal dementia (FTD), prion disease, and polyQ disorders (e.g., Huntington's disease [HD]) [8]. Protein misfolding often causes abnormal oligomers of soluble protein followed by sequestration into fibrils or aggregates. The histopathological appearance of large aggregates in these

neurodegenerative diseases has long been considered as pathological, leading to synaptic and neuronal malfunction. However, more recent data suggest that soluble oligomers are the most harmful species of misfolded protein [9]. Accordingly, the levels of large, aggregated Aβ plaques or tau tangles do not correlate with the severity of cognitive impairment in AD. Rather, soluble oligomeric species are thought to contribute to synaptic damage, representing the major pathological correlate of cognitive decline [10]. These oligomers often contain hydrophobic residues on their surface, facilitating binding to other cellular constituents such as proteins and lipid membranes. Through these aberrant interactions, soluble oligomers of misfolded proteins are thought to activate pathological signaling pathways, leading to synaptic and neuronal toxicity [11]. In contrast, the formation of large aggregates may be a protective response, at least in some cases, by sequestrating toxic oligomers into inclusion bodies [12, 13].

To counteract misfolded proteins, cells are equipped with QC machinery, including at least three components: Molecular chaperones, the UPS, and autophagy [1, 8, 14]. These QC systems not only assist proper folding of the proteins, thus preventing aggregation (e.g., via molecular chaperones), but target misfolded proteins for cellular degradation, eliminating toxicity associated with protein misfolding (via the UPS and autophagy mechanisms). Specifically, molecular chaperones (such as heat shock proteins [HSPs] and protein disulfide isomerase [PDI]) repair protein misfolding, and the UPS and autophagy are responsible for degradation of misfolded/aggregated proteins via the proteasome or lysosomal pathways. In general, the UPS participates in the degradation of short-lived, small-sized misfolded proteins that are tagged with polyubiquitin chains, whereas autophagy (generally meaning macroautophagy) is capable of removing larger insoluble substrates or even damaged organelles. Moreover, recent studies revealed extensive crosstalk between the UPS and autophagy-lysosomal degradation pathways, as exemplified by identification of key molecules common in both pathways, such as ubiquitin, parkin, p62, and HDAC6 [15]. There is also crosstalk between the macroautophagy pathway and the chaperone-mediated autophagy pathway (see section 6 for details) [16].

Supporting the view that protein aggregation represents an etiological feature of neurodegeneration, genetic mutations found in QC components (e.g., parkin, PINK1, Uch-L1, and ATP13A2) or aggregation-prone proteins (TDP-43 and αSyn) have been linked to rare forms of familial neurodegenerative diseases. Additionally, the activity of QC systems significantly declines with age or upon exposure to environmental risk factors, likely contributing to accumulation of misfolded proteins in sporadic neurodegenerative disorders. For example, increased oxidative and nitrosative stress in aged brains can facilitate accumulation of misfolded/aggreged proteins, in part via redox-based PTMs of key components of the QC machinery. In this review, we discuss signaling pathways in which excessive generation of NO and NO-related species contribute to protein misfolding and neurodegeneration via S-nitrosylation of critical cysteine thiols or nitration of tyrosine residues.

1.2 RNS in neurodegenerative diseases

There are three isoforms of NOsynthases (NOS) in mammalian cells, namely: NOS1 (or neuronal NOS [nNOS]), constitutively expressed in neural cells; NOS2 (or inducible NOS [iNOS]), expressed in various cell types upon inflammatory stimulation; and NOS3 (or endothelial NOS [eNOS]), mainly found in endothelium [17]. All three subtypes of NOS are homodimers, consume L-arginine and molecular oxygen as substrates, and utilize nicotinamide-adenine-dinucleotide phosphate (NADPH) as the electron donor. Each monomer of NOS has two domains, the C-terminal reductase domain and N-terminal oxygenase domain. The C-terminal domain contains the co-factor Flavin adenine dinucleotide (FAD) and Flavin mononucleotide (FMN), which can transfer electrons from NADPH to the heme site in the N-terminal domain, where the electrons are used to produce NO, probably in the form of $^{\bullet}NO$, with one free electron in its outer pi molecular orbital. The production of NO also requires calmodulin binding to NOS, which causes a conformational change that facilitates electron transfer from NADPH to heme. In the case of nNOS and eNOS, the binding of calmodulin relies on intracellular calcium/calmodulin levels. By contrast, the binding affinity of calmodulin to iNOS is much higher, allowing formation of the iNOS-calmodulin complex even in an environment with very low Ca^{2+} levels [17, 18]. When sufficient substrateL-arginine are not available, NOS has also been shown to generate ROS, specifically, superoxide anion ($O_2^{\bullet-}$) [19–21]. More recent studies have suggested that even at adequate L-arginine levels, the reduction of oxygen to superoxide anion ($O_2^{\bullet-}$) is an obligatory step in NO synthesis, but $O_2^{\bullet-}$ is not released under these conditions due to the action of co-factor tetrahydrobiopterin [21].

Endogenously produced RNS from the various NOS isoforms demonstrate Janus-faced effects, ranging from cytoprotective to neurotoxic effects. For example, when produced at low-to-moderate levels *in vivo*, RNS can activate signaling pathways maintaining physiological functions of the brain, including brain development, synaptic plasticity, and neuronal survival [22]. Initial studies on physiological functions of RNS revealed that the interaction of $^{\bullet}NO$ with the heme moiety of soluble guanylyl cyclase (GC) leads to formation of cyclic guanosine monophosphate (cGMP) [23]. cGMP and the downstream signaling molecule, cGMP-dependent protein kinase I (cGKI), participate in the regulation of not only neuronal transmission in the nervous system but also smooth muscle relaxation [18, 24]. However, ever increasing evidence suggests that cGMP-independent mechanisms for RNS bioactivity play even more critical roles in brain function. One critical mechanism, termed protein S-nitros(yl)ation, is a redox-mediated PTM resulting from a covalent reaction of an NO-related group (likely in the form of nitrosonium cation, NO^+) with a nucleophilic cysteine thiol group (more precisely, thiolate anion, RS^-) on the target protein [25, 26]. It should be noted that, because NO^+ is extremely unstable in solution, reacting immediately with water to generate nitrite (NO_2^-), transition metals, such as copper or iron, for example, in metalloproteins, are thought to catalyze transient oxidation of $^{\bullet}NO$ to NO^+, facilitating the *in vivo* formation of S-nitrosothiols (R-SNOs) [24, 27–29]. Alternative mechanisms of S-nitrosothiol formation have been proposed[29]. For instance, direct reaction between thiyl radical ($RS^{\bullet}$) and $^{\bullet}NO$ radical can lead to S-nitrosothiol formation [30]. Mechanism notwithstanding, protein S-nitrosylation participates in both physiological and pathophysiological actions of RNS [6, 31–38]. Another important pathological PTM

involving RNS is nitration of tyrosine residues, producing 3-nitrotyrosine. In the current review article, we will focus on the effects of these NO-dependent PTMs on protein misfolding and neuronal cell death [26].

2. RNS-mediated physiological and aberrant signaling in the CNS

In the central nervous system (CNS), nNOS is predominantly expressed in neuronal cells and is often associated with *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) through a mutual interaction with the postsynaptic density protein 95 (PSD-95) [39]. Activation of NMDARs by glutamate triggers Ca^{2+} influx into the neuron and subsequent nNOS activation. Under physiological condition, the activation of the synaptic NMDARs leads to moderate NO generation and can contribute to neuroprotective effects. For instance, S-nitrosylation of NMDARs themselves (as a negative-feedback reaction) and caspases inhibits their activity, providing a neuroprotective mechanism [26, 40, 41]. However, activation of extrasynaptic NMDARs due to Aβ oligomers or other stimulatory pathways, can generate excessive amounts of RNS that contribute to neurodegenerative phenotypes [42, 43]. Moreover, Aβ oligomers, αSyn, and toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can induce inflammatory iNOS expression in other types of brain cells, such as astrocytes, macrophages, and microglia, further increasing NO production in degenerating brains [44, 45]. Additionally, dysregulation of the antioxidative machinery, resulting from environmental toxins such as paraquat and maneb, or age-associated low-grade chronic inflammation (termed inflammaging), can also cause an imbalance of pro- and antioxidant systems, and eventually contribute to protein misfolding and neurodegeneration [6]. For example, signs of compromised antioxidative machineries, such as a decrease in reduced/oxidized glutathione ratio and diminished levels of nuclear factor erythroid 2-related factor 2 (Nrf2), are evident in brains with advanced aging or early AD, and these changes engender oxidative and nitrosative stress [46]. Notably, occurring particularly with nitrosative stress, protein S-nitrosylation is now a well-recognized mediator of the biological activity of RNS [6, 47, 48]. Mechanistically, protein S-nitrosylation can affect cell signaling pathways and neuronal function by altering protein activity, causing conformational changes, modifying protein folding, changing protein-protein interactions, and influencing protein subcellular localization [6, 27, 49]. In this review article, we focus on aberrant S-nitrosylation and nitration of proteins due to high levels of RNS that contribute to protein misfolding and aggregation, in part due to diminished cellular activity of the protein QC machinery.

2.1 Specificity of protein S-nitrosylation

Despite the fact that many proteins contain multiple cysteine residues, S-nitrosylation typically occurs on specific free thiols (or more properly thiolates) [6, 26, 50–52]. Multiple mechanisms of selective S-nitrosylation have been proposed [6, 28, 29]. The first determinant of selective S-nitrosylation entails the close proximity of the target proteins to the source of NO production, exposing the target proteins to a high concentration of S-nitrosylating NO-related groups [6, 29, 52]. The most well-characterized example of this paradigm is the presence of nNOS, NMDARs, and PSD-95 in the same protein complex. Localization of NMDARs and PSD-95 adjacent to nNOS facilitates S-nitrosylation of

these two proteins. The second biochemical mechanism that increases the selectivity of S-nitrosothiol formation is the compartmentalization of the target protein into a hydrophobic environment. In this case, hydrophobicity, as seen in lipid membranes or within a certain protein structure, stabilizes S-nitrosylating species, thereby promoting the efficiency of SNO-protein formation [52]. Thirdly, nucleophilic amino acid residues are often located near the target cysteine, potentially constituting a 'SNO motif' that facilitates reaction of the target cysteine residues with NO-related groups (particularly nitrosonium cation) [50, 53]. Along these lines, recent structural studies have revealed that the charged amino acid residue(s) is typically present within 8 Å of a SNO target site [54]. Fourthly, transnitrosylation between proteins is responsible for S-nitrosylation of one protein and removal of the SNO-group from the other (for a recent review, see [55] and section 2.2, below). Lastly, the stability of S-nitrosothiols can be counterbalanced by cellular de-nitrosylating enzymes, such as class III alcohol dehydrogenase (also known as ADH5 or GSNO reductase [GSNOR]), thioredoxin (Trx) and Trx-related proteins including PDI, and SNO-CoA reductase. These proteins catalyze reduction of a SNO group from the target cysteine thiol, removing the biological effects of S-nitrosylation [52]. S-Nitrosylation and denitrosylation are not necessarily enzymatically mediated [69]. Note that in most cases the proposed enzymatic reactions that catalyze S-nitrosylation (or transnitrosylation) and denitrosylation activity have not been deeply characterized. For example, apart from some studies by Marletta and colleagues on Trx-mediated transnitrosylation [29, 55–58], biochemical analyses to determine Michaelis-Menten kinetics as well as associated kcat/Km have not been conducted for the proposed S-nitrosylase/transnitrosylase or denitrosylase catalytic activities.

While S-nitrosylation is typically a labile modification, the same cysteine residue can often undergo further oxidation. For instance, an S-nitrosylated cysteine residue can facilitate formation of a stable disulfide bond with a nearby (vicinal) cysteine [59, 60]. Additionally, SNO-induced changes in the 3D structure of the target protein can precipitate further oxidation of the same thiol group [26, 61, 62]. Namely, such conformational alterations make the remaining thiol group more susceptible to reaction with ROS, generating sulfenic acid (-SOH) or even more stable oxidation products such as sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$) [59]. Hence, in this manner, pathological effects of SNO on the target protein can be prolonged through additional oxidation steps.

2.2 Protein-protein transnitrosylation

Low-molecular weight S-nitrosothiols, e.g., S-nitrosocysteine and S-nitrosoglutathione, serve as endogenous (S)NO donors, when kinetically and thermodynamically favorable, to produce S-nitrosylated proteins via transnitrosylation [29, 50]. In this reaction, mechanistically a cysteine thiol group (or, more precisely thiolate anion) most likely acts as a nucleophile, performing a reversible nucleophilic attack on the nitroso nitrogen to form an SNO-protein adduct. Additionally, proteins can transnitrosylate one another by a similar mechanism [47, 57, 63–69]. In protein-protein transnitrosylation, one protein effectively donates an NO-related group to another; one protein serving as the donor, while the other protein is the acceptor, forming a new S-nitrosothiol. Being in close contact during the reaction, the two proteins are typically found in a protein complex.

Redox potential, kinetic and thermodynamic considerations can drive the reaction in one direction over another, i.e., with one of the proteins becoming tranS-nitrosylated while the other is denitrosylated [69]. However, these reactions can also be reversible depending on the exact kinetic and thermodynamic details [29]. Mounting evidence suggests that entire networks of transnitrosylating proteins may represent the predominant mechanism mediating the biological function of protein S-nitrosylation [47, 63, 68]. For example, we recently identified a transnitrosylation network that involves transfer of 'SNO' from Uch-L1 to Cdk5 to Drp1, contributing to mitochondrial impairment and synaptic loss in models of neurodegenerative diseases [69]. Whether these transnitrosylation reactions can be catalytically triggered as an enzymatic process, however, will require further studies of their biochemical characteristics, including Michaelis-Menten kinetics, k_{cat}/Km, and increase in reaction rate over spontaneous reaction rate, as demonstrated in one example by Marletta and colleagues [29, 56–58].

2.3 Tyrosine nitration

Both *in vitro* and *in vivo*, $^{\bullet}NO$ is also known to react extremely rapidly with superoxide anion ($O_2^{\bullet-}$) to generate peroxynitrite ($ONOO^-$). Peroxynitrite can produce a pathophysiologically relevant PTM called tyrosine nitration [70, 71], although peroxynitrite-independent mechanisms (e.g., possibly involving H_2O_2-mediated oxidation of NO_2^-) for nitration may also exist [72]. Tyrosine nitration arises from the addition of a nitro group ($-NO_2$) to the 3' position of the tyrosine aromatic ring. Similar to protein S-nitrosylation, this type of NO-dependent PTM can also affect protein folding and function, thus ultimately negatively influencing neuronal survival as discussed in detail below.

3. Effects of RNS on protein aggregation, oligomer and fibril formation, and aggregated protein spread

Compelling evidence suggests that abnormal accumulation of protein aggregates caused by misfolding and fibrilization of proteins contributes to cellular dysfunction, neuronal damage, and synaptic loss in many neurodegenerative diseases, including AD, PD, and ALS/FTD. Moreover, misfolded proteins can often form self-templating, soluble oligomeric assemblies (which are likely the most toxic forms to the cells) that engage additional misfolded proteins or even recruit correctly folded proteins to induce their conversion to a misfolded form, yielding protofibrils and ultimately fibrillar aggregates. Aggregated proteins or misfolded oligomers can spread to adjacent cells and seed further aggregation, contributing to neuronal damage in widespread brain areas [2, 73]. Among aggregated proteins reported to propagate along neural networks are tau in AD, αSyn in PD or LBD, TDP-43 in ALS or FTD, and PrP^{sc} in prion disease. While genetic mutations in these genes can cause aggregation, aberrant PTMs (e.g., ubiquitination, phosphorylation, S-nitrosylation, or nitration) of non-mutant proteins can occur due to diverse environmental stimuli, thus triggering misfolding and aggregation, and hence simulating the more rare genetic mutations [74–76]. Here, we will discuss the evidence for RNS-mediated PTMs inducing aberrant protein aggregation and cell-to-cell spreading in neurodegenerative disorders.

3.1 S-Nitrosylation of TDP-43 contributes to its aggregation

FTD is the second most common form of pre-senile dementia after AD. FTD results in focal degeneration of the frontal and temporal lobes of the brain. ALS is a progressive neurodegenerative disorder characterized by loss of upper and lower motor neurons in the brain and spinal cord. These two neurodegenerative disorders are intrinsically linked by overlapping genetic, pathological, and clinical signatures, and may in fact represent two ends of a disease spectrum termed ALS/FTD spectrum disorder [77]. For example, mutations in the gene encoding *TAR DNA Binding Protein* (*TARDBP*, encoding the RNA-binding protein TDP-43) are associated with ALS/FTD spectrum disorder, although *TARDBP* mutations typically lead to ALS and represent rare familial cases (<1% of total ALS/FTD)[78]. Nonetheless, misfolded and aggregated TDP-43 has been identified as a major pathological protein accumulating in affected brain regions and motor neurons in 97% of sporadic ALS and 45% of FTD cases [79–82]. This high prevalence of TDP-43 proteinopathy in sporadic ALS/FTD prompted us to propose the hypothesis that the majority of TDP-43 aggregation in ALS/FTD spectrum disorder results from aging or various environmental factors associated with redox stress [83].

TDP-43 is a highly conserved and ubiquitously expressed DNA/RNA binding protein belonging to the heterogenous ribonucleoprotein family [84–86]. Under un-stressed conditions, TDP-43 is predominantly localized in the nucleus, regulating mRNA processing and stabilization, but it can form aggregates in the cytoplasm and possibly mitochondria under disease conditions [87, 88–91]. There are several key features that are commonly associated with aggregation of TDP-43, including TDP-43 ubiquitination, hyperphosphorylation, and S-nitrosylation [76, 81, 82, 92, 93].

In stressed cells, emerging evidence shows that TDP-43 is localized in cytoplasmic stress granules (SGs) and plays an important role in regulating the dynamics of SG formation and disassembly [94–97], although the biological function of SGs remain largely unknown. SGs are membraneless RNA-protein granules that form in the cytoplasm via liquid-liquid phase separation (LLPS) and often contain mRNA translation machineries [98]. Aberrant phase transition of cytoplasmic TDP-43 is associated with neurotoxicity, and cytoplasmic compartmentalization of the misfolded protein is thought to accumulate and result in the TDP-43 inclusions observed in ALS/FTD [99, 100]. Additionally, a number of studies have revealed that aggregated TDP-43 can spread to neighboring cells via cell-to-cell transmission and act as a seed to induce cytoplasmic aggregation of additional TDP-43 in the recipient cells, possibly contributing to the progression of TDP-43 proteinopathy [101, 102].

Advanced age and potential environmental toxins are thought to represent major risk factors for many neurodegenerative disorders, including ALS/FTD. Both of these risk factors can engender overproduction of intracellular RNS, likely contributing to protein misfolding and aggregation in ALS/FTD and other neurological diseases [6, 103]. Moreover, the FDA-approved drug edaravone (MCI-186), which scavenges RNS/ROS, delays progression of ALS if treatment begins at early stages [104–106]. This finding is consistent with the notion that oxidative and nitrosative stress represent contributors to ALS pathology.

Along these lines, in a recent study we reported that S-nitrosylation of TDP-43 fosters its localization in cytosolic SGs. S-Nitrosylation of TDP-43 was associated with its aggregation in *in vitro* ALS/FTD models (including human induced pluripotent stem cell (hiPSC)-derived motoneurons) and in postmortem human brain from FTD patient [76]. TDP-43 contains a total of six cysteine residues [53], among which Cys173 and Cys175 are surrounded by a partial SNO motif; these cysteine residues have been identified as SNO sites [76], and are located in the RRM1 (RNA recognition motif) domain. S-Nitrosylation of at least one these thiol groups facilitates the formation of an intra-molecular disulfide linkage, causing a conformational change in the TDP-43 structure and leading to aggregation (Fig. 1). Consistent with this notion, non-nitrosylatable mutant TDP-43 (C173A/C175A) shows a decrease in insoluble TDP-43 aggregation [76].

Moreover, S-nitrosylation-induced oligomerized or aggregated TDP-43 is able to spread not only to adjoining cells but also induce the generation of additional intracellular NO, promoting cell death of recipient cells [76, 107]. Moreover, in a critical *in vivo* experiment, we showed that the NOS inhibitor L-NAME prevented neuronal loss from TDP-43 toxicity, consistent with the premise that NO-related species contribute to pathological aggregation, cell-to-cell propagation, and neurotoxicity associated with TDP-43 proteinopathy. Taken together, these findings suggest that SNO-TDP-43 formation is an important factor that contributes to the development of ALS/FTD spectrum disorders and possibly other neurodegenerative diseases [76, 108].

3.2 Tyrosine nitration of αSyn leads to its aggregation

Evidence suggests that the formation of nitrated proteins in biological systems can involve free radical reactions. For example, peroxynitrite-derived radical (e.g., $^{\bullet}NO_2$) has been reported to react with a tyrosyl radical intermediate ($Tyr^{\bullet}$) to nitrate tyrosine residues, producing 3-nitrotyrosine [72]. Reminiscent of the effects of S-nitrosylation on protein aggregation, tyrosine nitration can precipitate protein misfolding [71]. Perhaps one of the most well characterized nitrated proteins associated with neurodegenerative disorders is αSyn [109, 110]. Using antibodies that recognize 3-nitrotyrosine, an early study found extensive accumulation of nitrated proteins in the core of LBs in human PD patient brains [111]; however, the identity of these nitrated proteins remain unknown. Subsequent studies by Ischiropoulos, Trojanowski, and Lee's groups identified αSyn as a major molecular target of tyrosine nitration in LBs and demonstrated that nitration promotes aberrant aggregation of αSyn [109, 110]. Note that αSyn does not contain any cysteine residue for S-nitrosylation, whereas all four of its tyrosine residues (i.e., Tyr39, Tyr125, Tyr133 and Tyr136) are susceptible to nitration [112]. These findings are consistent with the notion that, unlike many cases of protein S-nitrosylation, nitration of tyrosine residues can occur more indiscriminately. Interestingly, nitration of αSyn enhances is oligomerization either via inhibition of fibril formation or stabilization of oligomers [110, 113]. In addition, nitration at residue Tyr39 hinders the ability of αSyn to bind lipids [113]. These studies suggest that αSyn nitration affects lipid binding and protein aggregation, thus contributing to α-synucleinopathies. However, many unanswered questions regarding the exact pathological role of nitrated αSyn still exist. For example, additional studies are needed to determine if nitration of αSyn starts in the early stages (or even pre-symptomatic stage) of the disease,

thereby potentially also serving as a biomarker for PD or LBD [114]. Moreover, the impact of nitrated αSyn on neurotoxicity *in vivo* remains unclear.

3.3 Effects of tyrosine nitration on aggregation of Aβ, tau, and other proteins

Sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases results in production of Aβ peptides, in several forms including $Aβ_{1–40}$ and $Aβ_{1–42}$ [115]. Interestingly, human Aβ contains one tyrosine residue (Tyr10), which is replaced by a phenylalanine in mouse/rat Aβ. Additionally, RNS have been reported to trigger nitration of Aβ at Tyr10 in human AD [116]. An initial study demonstrated that nitration of Aβ accelerates its aggregation both *in vitro* and in transgenic mouse brains expressing human Aβ, possibly contributing to β-amyloidosis and cognitive impairment in AD. Moreover, a subsequent study revealed that Aβ nitration stabilizes toxic Aβ oligomers and impairs fibril formation, thus enhancing synaptotoxicity [117]. However, using a synthesized form of nitrated Aβ, other studies have shown that tyrosine nitration of Aβ prevents aggregation and enhances cell survival [118]. Thus, future studies are needed to resolve these discrepancies.

Tyrosine nitration of tau protein could alter its conformation, promoting tau aggregation. Along these lines, nitrated and aggregated tau was evident in human patient brains [119, 120]. In fact, tau nitration may be an early event in AD pathology, since nitrated tau appears before the mature neurofibrillary tangles [119]. Additionally, an S-nitrosoproteome analysis identified S-nitrosylated tau in a mouse model of AD [33]. Collectively, these findings are consistent with the notion that both S-nitrosylation and tyrosine nitration may modulate tau aggregation. Further studies will be required to determine possible molecular mechanisms of S-nitrosylation- and tyrosine nitration-induced tau aggregation.

Moreover, RNS may contribute to accumulation of misfolded proteins through tyrosine nitration of additional proteins. For example, tyrosine nitration of HSP90 and Trx1 inhibits their chaperone and oxidoreductase activities, respectively [121, 122]. Additionally, nitration triggers aggregation of human islet amyloid polypeptide (hIAPP) [123]. Using models of neurodegenerative disorders, it will be important to determine if tyrosine nitration of these proteins contributes to protein misfolding, synaptic damage, and neurotoxicity.

4. RNS and molecular chaperones

Many proteins are typically synthesized in cells in a non-native state, requiring chaperones and other regulators for proper three-dimensional folding in order to become functionally active. Additionally, molecular chaperones can assist in re-folding of aggregation-prone proteins, such as αSyn and tau, to diminish their potential for aggregation and neurotoxicity. Along these lines, decreased availability of molecular chaperones has been linked to numerous diseases, ranging from neurodegenerative disorders to cancer, diabetes, cystic fibrosis, and cardiovascular disease [1]. Moreover, enhanced expression of molecular chaperons has been shown to delay progression of abnormal phenotypes in models of PD and other neurodegenerative disorders [124]. Molecular chaperone pathways are present in various cellular compartments, including, the cytosol, ER, mitochondria, and nucleus. Various members of the molecular chaperone family or proteins exist in mammalian cells and are often known as heat-shock proteins (HSPs) because they are highly expressed

under conditions of temperature stress and other forms of cell stress. Additionally, classes of chaperones, such as protein disulfide isomerase (PDI) and glucose-regulated protein 78 (GRP78/BiP), are present in the endoplasmic reticulum (ER). However, the capacity of the molecular chaperones system, at least in model organisms, appears to decline with age, which represents a major risk factor for neurodegenerative diseases [125]. This decline in chaperone function likely contributes to the accumulation of misfolded proteins as we age. Here we discuss possible mechanisms of how aging-associated nitrosative stress disrupts molecular chaperone activity and contributes to protein aggregation.

4.1 S-Nitrosylation of PDI contributes to accumulation of misfolded proteins

Protein processing and folding occur in the ER, particularly for secreted proteins. In neurodegenerative disorders misfolded proteins often accumulate in the ER lumen and trigger ER stress. Cells utilize two critical processes to relieve damage derived from the accumulation of misfolded proteins: The unfolded protein response (UPR) and ER-associated degradation (ERAD) [5, 126]. The UPR includes three downstream pathways: 1) activation of pancreatic ER kinase (PERK), leading to phosphorylation of eukaryotic initiation factor 2 (eIF2), which subsequently inhibits global protein synthesis and decreases the influx of nascent proteins into the ER; 2) activation of ATF6 (activating transcription factor 6), and 3) activation of the inositol-requiring enzyme 1 (IRE1) pathway, which up-regulates mRNA splicing of X box-binding protein 1 (Xbp1) and then stimulates transcription of UPR target genes, including ER chaperones such as PDI and GRP78/BiP. The UPR usually acts transiently to regain protein homeostasis and promote cell survival, while prolonged UPR activation results in cell damage and death. Emerging evidence suggests that elevated levels of NO-related species can induce ER stress through the disruption of ER Ca^{2+} homeostasis, possibly via inhibition of the Ca^{2+}-ATPase activity of SERCA2a, contributing to prolonged activation of the UPR pathway [127–129]. Our group first reported that S-nitrosylation of PDI at the active site cysteines (Cys36 and Cys39) inhibits its protein isomerase and chaperone activities [130, 131]. Several PDI family members reside in the ER lumen and function as molecular chaperones as well as disulfide isomerases to assist protein folding. PDI belongs to the Trx-fold superfamily, featuring a common structural property named the Trx fold. All PDI isoforms possess at least one Trx domain with the conserved active site motif CXXC. Interestingly, we have found evidence for increased levels of SNO-PDI in the brains of human sporadic AD, PD and ALS. S-Nitrosylation-dependent inhibition of PDI activity results in increased accumulation of misfolded proteins in the ER, leading to prolonged activation of the UPR and subsequent neuronal cell death in models of AD, PD, and other neurological diseases (Fig. 2) [49, 130–134]. Moreover, we also observed that levels of S-nitrosylated PDI are increased in the spinal cord of the SOD1(G93A) mouse model of ALS, as well as in human patients with sporadic ALS [130, 135]. Because S-nitrosylation of PDI attenuates its ability to decrease aggregation of SOD1 and other aggregation-prone proteins in models of ALS [130], it is tempting to speculate that S-nitrosylation of PDI may be a critical reaction that contributes to accumulation of misfolded proteins in ALS as well as in AD, PD and possibly other neurodegenerative disorders associated with elevated levels of RNS. Interestingly, a more recent study found that PDI can act as a chaperone to physically interact with tau, thus preventing its hyperphosphorylation-mediated aggregation [133]. S-Nitrosylation of PDI

prevents this protective effect against tau aggregation, consistent with the notion that SNO-PDI contributes to tau misfolding and aggregation. In addition to PDI, other UPR-related proteins, such as IRE1α and PERK, have also been found to be aberrantly S-nitrosylated in neurodegenerative and other diseases, thus altering their function [126, 136]. These findings suggest that protein S-nitrosylation compromises the neuroprotective functions of the UPR at multiple levels. Further studies will be required to examine if SNO-IRE1α or SNO-PERK can lead to dysregulated protein folding in neurodegenerative diseases.

4.2 S-Nitrosylation of multiple molecular chaperones contributes to protein misfolding

In both neuronal and non-neuronal cells, S-nitrosylation appears to regulate the activity of other molecular chaperones and co-chaperones, including HSP90/70, mitochondrial chaperone TNF receptor–associated protein 1 (TRAP1), and valosin-containing protein (VCP). For example, HSP90 is a key regulator of protein homeostasis under both physiological and stress-induced damage with more than 200 substrate proteins identified to date [137–139]. HSP90 has three highly conserved domains, and each domain has its own function. The amino-terminal domain contains an ATP binding motif, the middle domain modulates ATPase activity and binding to co-chaperones and clients, and the carboxy-terminal domain (CTD) is important for both calmodulin binding and homodimerization. NO-related species can S-nitrosylate human HSP90 and thus inhibit its ATPase activity. Cys597 in the CTD, rather than in the ATPase domain, has been identified as the nitrosylation site, suggesting that a conformational change in the CTD inhibits the ATPase activity in an allosteric fashion at some distance from the active site [137, 140]. Intriguingly, SNO-HSP90 has been identified as a transnitrosylating agent towards the androgen receptor (AR), effectively transferring an NO group from HSP90 to AR(Cys601); S-nitrosylation of AR impairs its function as a transcription factor [141]. Further studies will be required to investigate if SNO-HSP90 contributes to protein aggregation in experimental models of neurodegenerative diseases.

Additionally, the mitochondrial-specific homolog of HSP90, TRAP1, has also been reported to be S-nitrosylated at Cys501 [142]. Acting as a molecular chaperone, TRAP1 has been reported to suppress the activity of mitochondrial electron transport chain complex II, which also represents succinate dehydrogenase (SDH) in the tricarboxylic acid (TCA) cycle. S-nitrosylation of TRAP1 accelerates its degradation, thus upregulating SDH, and sensitizing hepatocellular carcinoma cells to mitochondrially-targeted anti-cancer drugs such as SDH inhibitors. TRAP1 can also promote cell survival by decreasing ROS-dependent mitochondrial dysfunction [143, 144]. Additionally, the PD-linked kinase PINK1 (which has been shown to be S-nitrosylated in models of PD; see section 6.2 for details) opposes mitochondrial dysfunction and suppresses oxidative stress-mediated apoptosis, at least in part via phosphorylation of TRAP1 [145]. Further studies are needed to determine if aberrant S-nitrosylation modulates the PINK1-TRAP1 pathway to contribute to the etiology of PD and other neurological disorders.

Furthermore, S-nitrosylation of a co-chaperone of HSP70/HSP90, designated Hsp70/Hsp90 organizing protein (Hop; also known asstress-inducible phosphoprotein I [STI1 or STIP1]), causes the rapid degradation of Hop, and decreases its interaction with cystic fibrosis

(CF) transmembrane conductance regulator (CFTR) [146]. The CFTR is an apical plasma membrane chloride channel, and the HSP70-HSP90-Hop complex is an essential component for trafficking CFTR to the cell surface. Considering the disease CF, which is caused by genetic mutations in the gene encoding CFTR, the most common mutation involves loss of phenylalanine 508 (ΔF508). This deletion results in misfolding of the CFTR and consequent degradation of virtually all of the protein prior to its reaching the cell surface. This is the cause of abnormal Cl^- conductance in the lung and the resulting pulmonary disease phenotype. The decreased binding of SNO-Hop to ΔF508 CFTR assists maturation and cell surface expression of the mutant protein. Although ΔF508 CFTR is partly misfolded, it has the potential to be functional if expressed at the cell surface. Hence, an S-nitrosylating compound that promotes SNO-Hop formation has been proposed as an ancillary therapeutic approach for CF [146]. Moreover, because Hop has been shown to prevent Aβ and tau toxicity in models of AD[147, 148], it will be intriguing in future studies to determine if SNO-Hop affects Aβ or tau signaling.

Multiple proteomic studies have identified molecular chaperones as targets of S-nitrosylation in both neuronal and non-neuronal cell populations. For example, a proteomic study using neuroblastoma SH-SY5Y cells stimulated with rapamycin to challenge proteostasis identified more than 2,000 SNO-proteins, including a member of the Hsp70 family known as heat shock cognate (HSC70/HSPA8) [149]. In the case of HSC70/HSPA8, S-nitrosylation occurs at Cys17 located in the nucleotide binding region, presumably compromising the activity of HSC70/HSPA8 during protein folding, chaperone-mediated autophagy, and proteasomal degradation of substrate proteins. Other SNO-proteome analyses have also identified molecular chaperones in non-neuronal cells, including GRP78, Hsp70 protein 4, Hsc71, ORP150, calreticulin, and endoplasmin [150, 151]. In the future, additional S-nitroso-proteomic investigations using models of neurodegenerative disorders should help elucidate how S-nitrosylated chaperones may contribute to the pathophysiology of these diseases.

5. RNS and the UPS

The UPS is a key intracellular molecular machinery for degradation of ubiquitin(yl)ated proteins in eukaryotic cells. The sequential reaction of E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin-ligase enzymes) results in mono- or polyubiquitination of a substrate protein. In general, after polyubiquitinate, substrate proteins are selectively recognized by the proteasome for degradation into short peptides with subsequent reclamation of their amino acids [152]. In contrast, deubiquitinating enzymes in the UPS remove ubiquitin chains from substrates and prevent proteasomal degradation, reversing the effect of E3 ligases [153]. Under physiological conditions, misfolded proteins can be efficiently ubiquitinated and then degraded via the UPS. Accordingly, abnormalities in UPS function can result in the accumulation of misfolded proteins that subsequently form intra- and extracellular aggregates in the brain. As delineated below, protein S-nitrosylation has been shown to affect UPS function at multiple steps.

5.1 S-Nitrosylation of parkin results in protein misfolding and neurotoxicity

Rare mutations in the gene encoding parkin (*PARK2*) cause autosomal recessive juvenile PD and some very rare forms of adult PD. In contrast, dysfunctional parkin activity is thought to play an important role in virtually all cases of sporadic PD. Along these lines, we and others discovered that redox-mediated PTM of parkin largely account for its dysfunction in sporadic PD [154–157]. Parkin contains two RING domains as well as one in-between-RING (IBR) domain and belongs to the family of ubiquitin E3 ligase proteins. Our group and others demonstrated that S-nitrosylation occurs on multiple cysteine residues, mainly in the RING and IBR domains of parkin, disrupting its E3 ligase activity and leading to dysfunctional UPS activity (Fig. 3) [154–156, 158]. Moreover, S-nitrosylated parkin facilitates the formation of more stable oxidation products on the same cysteine residues, such as sulfinic acid (SO_2H) and sulfonic acid (SO_3H), via reaction with ROS, with the latter irreversibly inactivating the enzymatic activity of the protein [157]. Intriguingly, S-nitrosylation of parkin transiently stimulates its ubiquitin E3 ligase activity, followed by a decrease in activity [154, 155]. The initial increase in E3 ligase activity may reflect a neuroprotective aspect of SNO signaling, whereas downregulation of its activity results in UPS dysfunction, contributing to accumulation of misfolded proteins [154, 155, 159]. Additionally, parkin is an important mediator of mitophagy, a process by which the autophagy/lysosomal pathway removes damaged mitochondria (see also the section below on ‘RNS and autophagy’). Mitophagy is often impaired in many neurodegenerative diseases, including PD, AD, HD, ALS, and FTD [160]. Accordingly, recent studies have demonstrated that S-nitrosylation of parkin results in disruption of mitophagy activity, as described below [154–156, 161]. Additionally, our group and others found increased SNO-parkin levels in both animal models and human sporadic PD brains. Hence, dysfunctional parkin activity, occurring with either nitrosylation or rare genetic mutation, facilitates protein misfolding, defective mitophagy, and neuronal damage. These findings are consistent with the notion that aberrantly increased RNS/ROS production, as found in the vast majority of sporadic PD brains, phenocopies the effects of rare mutations in the gene encoding parkin via SNO-parkin-mediated protein misfolding, UPS dysfunction, and mitophagy impairment.

In addition to proteasomal cleavage of polyubiquitin chains, non-degradative ubiquitin signaling is known to influence neuronal survival via receptor trafficking, mitochondrial homeostasis, and inflammatory responses [162, 163]. For example, parkin promotes cell survival through the epidermal growth factor (EGF)-Akt signaling pathway [164]. In this scenario, parkin monoubiquitinates Eps15, an adaptor protein involved in EGF receptor (EGFR) endocytosis, interfering with binding of Eps15 to the EGFR. This delays EGFR internalization and thus promotes Akt signaling for neuronal survival. Additionally, parkin-mediated monoubiquitination of PICK1 (protein interacting with C-kinase 1) decreases current fluxed by the acid-sensing ion channel (ASIC) [165]. Since ASIC-mediated current is implicated in synaptic plasticity and neuronal injury, it is tempting to speculate that parkin-mediated PICK1 monoubiquitination contributes to neurodegeneration in diseases such as PD. Future studies will be required to determine if S-nitrosylation of parkin affects neuronal viability via monoubiquitination of Eps15 or PICK1.

5.2 S-Nitrosylated Uch-L1 and protein misfolding

RNS can also regulate activities of deubiquitinating enzymes such as Uch-L1 and USP9X [69, 166, 167]. Of these deubiquitinating enzymes, Uch-L1 is most abundantly expressed in the brain, constituting about 2% of total soluble protein [168]. Additionally, dysfunctional Uch-L1 activity has been linked to the pathogenesis of several neurodegenerative diseases, including PD and AD [166, 169, 170]. Along these lines, recent studies from our group and others have shown that S-nitrosylation of Uch-L1 at Cys152 inhibits its binding to ubiquitin, thereby decreasing its deubiquitinating activity (Fig. 3) [69, 166]. We further demonstrated that substitution of an Ala for Cys152 (producing a non-nitrosylatable Uch-L1 mutant) abrogated the effect of RNS on Uch-L1 catalytic activity and ubiquitin binding [69]. Moreover, similar levels of SNO-Uch-L1 were found to be present in human AD brains, mouse models of AD, and cell-based AD models [69], consistent with the premise that pathologically-relevant levels of SNOUch-L1 exist in AD. Additionally, S-nitrosylation leads to structural instability of Uch-L1, potentially serving as a seed to accelerate aggregation of αSyn and possibly other aggregation-prone proteins [166].

In addition to the canonical function of Uch-L1 as a deubiquitinating enzyme, in multiple cell-based and animal models of AD we recently reported that SNO-Uch-L1 can transnitrosylate Cdk5, a kinase that influences neuronal development and survival [171]. Dysregulation of Cdk5 activity is known to contribute to the pathogenesis of AD and other neurodegenerative disorders such as PD, HD, and ALS [172]. Cdk5 is S-nitrosylated at Cys83 and Cys157 [67, 171, 173], leading to an aberrant increases in Cdk5 kinase activity, thus triggering loss of synapses and neurons [67]. A unique, previously unknown biochemical pathway mediates this damage, involving transnitrosylation from Uch-L1 to Cdk5 to Drp1, culminating in the formation of SNO-Drp1 [67, 69, 174]. Drp1 is a dynamin-like GTPase that normally facilitates mitochondrial fission. Upon S-nitrosylation, however, enhanced dimerization/multimerization of Drp1 increases its GTPase activity, resulting in excessive mitochondrial fragmentation, bioenergetic compromise, and synaptic impairment and loss in models of AD [174, 175]. We found that the concerted transnitrosylation from Uch-L1 to Cdk5 to Drp1 proceeds in a kinetically and thermodynamically favorable fashion and is present at pathological levels in human AD brains [67, 69, 174]. Importantly, evidence strongly suggests that this non-canonical transnitrosylation network from SNO-Uch-L1 to SNO-Cdk5 to SNO-Drp1 contributes to synapse loss and cognitive decline *in vivo* in mouse models of AD because lentiviral expression of non-nitrosylatable mutant Uch-L1(C152A) significantly protects synapses from damage due to toxic Aβ oligomers [69].

5.3 S-Nitrosylation of other UPS-associated enzymes: Potential effects on protein misfolding

In addition to parkin, other ubiquitin E3 ligases, particularly those bearing RING domains, have been shown to undergo S-nitrosylation [64, 176–180]. The list includes X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis 1 (cIAP1), ubiquitin protein ligase E3A (UBE3A), product of the von Hippel-Lindau gene (pVHL), carboxy-terminus of Hsc70 interacting protein (CHIP), and Ring Finger Proteins (RNFs). For example, XIAP is a potent anti-apoptotic protein that not only directly inhibits caspase activity but facilitates proteasomal degradation of caspases. S-Nitrosylation of XIAP occurs in the RING domain

and inhibits its ubiquitin E3 ligase activity, thereby contributing to neuronal cell death. Additionally, S-nitrosylated caspase-3 can transnitrosylate XIAP. This reaction results in denitrosylation of caspase-3 (thus activating caspase activity) and S-nitrosylation of XIAP (consequently inhibiting its E3 ligase activity). The overall effect is to further activate the apoptotic pathway [64]. Concerning RNFs, Steve Tannenbaum's group reported that levels of SNO-RNF213 are aberrantly increased in a mouse model of tauopathy [178]. Further studies are needed to delineate the pathological role of these SNO-E3 ligases on protein misfolding and synaptic damage in models of neurodegenerative diseases.

Additionally, protein S-nitrosylation appears to affect multiple processes of UPSdependent protein degradation, including proteasome components, ubiquitin-conjugating E2 enzymes, and the substrates of E3 ligases. Firstly, RNS can regulate UPS function via S-nitrosylation of proteasome components. Specifically, the 20S catalytic core of the 26S proteasome includes at least 10 cysteine residues that can undergo S-nitrosylation, leading to inhibition of proteasomal catalytic activity [181]. Secondly, S-nitrosylation of ubiquitin-conjugating enzyme E2 D1 (UBE2D1), known to be implicated in the ERAD pathway, decreases its E2 activity, potentially contributing to accumulation of misfolded proteins in the ER [182]. In addition, S-nitrosylation inhibits the activity of other ubiquitin-conjugating enzymes, such as E2D3 and E2D4, thereby decreasing p53 ubiquitination in neuroblastoma SH-SY5Y cells [149]. Thirdly, S-nitrosylation of many ubiquitination target proteins affects their protein turnover and thereby modulates the half-lives of these cellular proteins [183–189]. Examples include phosphatase and tensin homolog (PTEN), iron regulatory protein 2 (IRP2), latent TGF-β binding protein 1 (LTBP1), B-cell lymphoma 2 (Bcl-2), and FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (FLIP). In the case of SNO-Bcl-2 and SNO-FLIP, S-nitrosylation inhibits their ubiquitination, thus increasing their cellular stability and suppressing apoptotic cell death. Given that S-nitrosylation regulates multiple UPS-related proteins and that transnitrosylation networks (e.g., SNO-Uch-L1/Cdk5/Drp1) have been shown to contribute to the pathogenesis of neurodegenerative disorders, it is anticipated that future studies will discover additional such networks that contribute to UPS dysfunction and protein misfolding.

6. RNS and autophagy

Autophagy is a lysosomal degradative process in which unfolded, aggregated proteins and damaged organelles are eliminated in order to maintain cellular homeostasis. Autophagic dysfunction disrupts the homeostatic state and influences cell survival, contributing to a variety of diseases including cancer, neurodegenerative disorders, cardiovascular disorders, and microbial infections [14]. Three major types of autophagy have been described: Macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy (hereafter referred to simply as autophagy) is the most prevalent form of autophagy and is associated with the formation of cytosolic double-membrane vesicles that sequester and degrade damaged cytosolic contents [190]. Macroautophagy can also selectively degrade damaged mitochondria through a process known as mitophagy. In microautophagy, lysosome takes up cytosolic materials via invagination of the lysosomal membrane. CMA is another form of selective (and constitutive) autophagy, involving direct uptake of substrate proteins with the consensus KFERQ pentapeptide motif or a similar

sequence into lysosomes; in this process, HSC70 recognizes and binds to the KFERQ motif and then delivers the substrate proteins to the lysosome. While excessive generation of ROS/RNS have been known to impair autophagy in many different disease states, including neurodegenerative disorders [191], mechanistic details for this phenomena have remained incompletely understood until recently [192, 193]. As summarized below, evidence now suggests that protein S-nitrosylation and tyrosine nitration of critical proteins compromise the autophagic machinery, thereby contributing to neurotoxicity associated with protein misfolding.

6.1 S-Nitrosylation of JNK and IKKβ inhibits autophagy

Many autophagy-related proteins are controlled by PTMs such as phosphorylation, O-GlcNAcylation, acetylation, and ubiquitination [194]. For example, starvation (a known inducer of autophagy) activates c-Jun N-terminal kinase 1 (JNK1), which in turn phosphorylates Bcl-2. Normally, Bcl-2 inhibits autophagy via interaction with the autophagy-related protein Beclin 1. Beclin 1 is a component of the class III phosphatidylinositol 3-kinase (PI3K)/hVps34 complex, which is critical for initiation of autophagosome formation [195]. Phosphorylation of Bcl-2, however, interrupts its binding to Beclin 1, allowing Beclin 1 to initiate autophagosome formation [196, 197]. Additionally, multiple stimuli favoring autophagy activate the IKK (IκB kinase) complex, containing IKKα and IKKβ [198]. Activated IKK stimulates autophagy signaling pathways through phosphorylation of AMP-activated protein kinase (AMPK). Activated AMPK induces autophagy through several signaling pathways. For example, AMPK stimulates autophagy by suppressing the inhibitory effect of mTORC1 [198].

Initial studies on the effect of NO on autophagy include the demonstration by Rubinsztein that S-nitrosylation of JNK and IKKβ impairs induction of autophagy at an early stage (Fig. 4) [192]. Specifically, S-nitrosylation of JNK at Cys116 inhibits its kinase activity [199], leading to a decrease in Bcl-2 phosphorylation. This results in an increase in Bcl-2/Beclin 1 complex formation, thus inhibiting autophagy. While S-nitrosylation of Bcl-2 itself enhances anti-apoptotic activity as discussed above, SNO-Bcl-2 does not appear to influence autophagy [192, 200]. Like SNO-JNK, S-nitrosylation of IKKβ (at Cys179) suppresses autophagy by inhibiting its kinase activity and thus activating mTOR [192, 198, 201, 202]. S-Nitrosylation of PTEN also activates the mTOR signaling pathway to downregulate autophagy [203]. Thus, S-nitrosylation inhibits autophagy via multiple signaling pathways. In line with these observations, the NOS inhibitor, L-NAME, facilitates clearance of mutant huntingtin and αSyn by increasing autophagy [192], consistent with the notion that decreasing NO production promotes autophagy to protect cells from protein misfolding.

Additionally, recent proteomic studies identified the lysosomal protease cathepsin D (CTSD) as a target of S-nitrosylation [204, 205]. S-Nitrosylation of CTSD at Cys329 was reported to inhibit processing of CTSD, which is required for its maturation, thus decreasing cellular CTSD activity [204]. However, the potential pathophysiological role of S-nitrosylated CTSD in neurodegenerative disorders remains unclear and requires future investigation. Further

studies will also be needed to determine if other endosome/lysosome-resident proteins are regulated via S-nitrosylation.

6.2 S-Nitrosylation of PINK1 inhibits parkin-mediated mitophagy

In contrast to the effect of RNS in inhibiting autophagy, as just discussed, RNS can also activate the autophagic process. For example, moderately low concentrations of RNS/ROS can stimulate autophagy in part via suppression of mTOR expression [206]. Notably, the dual actions of RNS on autophagy-related processes are even more evident during mitophagy.

Mitophagy represents a form of (macro)autophagy that selectively removes damaged, defective mitochondria. The process of mitophagy is tightly regulated by two PD-related proteins: parkin and PINK1 (phosphatase and tensin homolog-induced putative kinase protein 1) [207]. While PINK1 is unstable and rapidly degraded via the UPS under normal conditions, once mitochondria are damaged, PINK1 is stabilized on the outer mitochondrial membrane (OMM) where it recruits parkin. Additionally, at the damaged mitochondria membrane, PINK1 phosphorylates parkin, which activates its E3 ubiquitin ligase activity toinitiate mitophagy [208–210]. Moreover, as discussed above, at moderately low levels of RNS, S-nitrosylation of parkin itself in the early stages of PD may initially enhance its E3 ligase activity [154, 155]. Accordingly, activated parkin conjugates a polyubiquitin chain onto the OMM proteins VDAC1, MFN1 and MFN2, leading to the recruitment of autophagy adaptor proteins, including optineurin, nuclear dot protein 52 and p62/SQSTM1, among others, to stimulate mitophagy as a protective mechanism. Autophagy adaptor proteins bind directly to microtubule-associated protein 1A/1B-light chain 3 (LC3), sequestering defective mitochondria into autophagosomes for subsequent removal of the organelle [211].

Recent evidence suggests that depending on concentration, NO-related species can either upregulate or downregulate the mitophagic process [212]. For example, as just discussed, moderately low levels of NO, as occur under physiological conditions or in the early stages of neurodegeneration, can enhance parkin-dependent ubiquitination, mitophagy, and neuronal protection [154, 155, 213]. In contrast, we recently demonstrated that high levels of RNS, as seen under pathological conditions, can impair mitophagy via S-nitrosylation of PINK1 [193]. In this case, S-nitrosylation of PINK1 at Cys568, which is localized at the C-terminal region of the protein, allosterically decreases its kinase activity. Formation of SNO-PINK1 thus results in decreased phosphorylation of parkin as well as ubiquitin, with a consequent decrease in ubiquitin polymerization. This decrease in parkin phosphorylation inhibits its translocation to the OMM, leading to inhibition of mitophagy (Fig. 4) [193].

The mitochondrial uncoupler, valinomycin is known to stabilize PINK1 on the OMM and induce mitophagy. As expected, therefore, co-exposure of hiPSC-derived dopaminergic (DA) neurons to valinomycin and the NOS inhibitor L-NAME decreases SNO-PINK1 formation and increases mitophagy [193]. These findings are consistent with the notion that RNS negatively regulate mitophagy via SNO-PINK1-dependent pathways. As mentioned earlier, S-nitrosylation of parkin at Cys323 transiently increases its activity and promotes mitophagy, possibly reflecting a neuroprotective effect of lower concentrations of RNS at the early stages of a neurodegenerative disease [161]. However, under pathological

situations in which NO production is dramatically increased for prolonged periods, (i) S-nitrosylation of PINK1 can counteract the transient effects of SNO on parkin activity, and (ii) S-nitrosylation of parkin eventually diminishes its E3 ligase activity, as described above, further compromising mitophagy [155, 156, 193]. In the future, additional studies will undoubtedly elucidate additional SNO-proteins that influence autophagy in general and mitophagy in particular.

6.3 Tyrosine nitration of αSyn and autophagy

Misfolded αSyn can be degraded by both the UPS and autophagy, in particular CMA [214–216]. As delineated above, nitrated αSyn demonstrates an increased propensity to aggregate and phase separate into cytoplasmic inclusion bodies in PD and LBD brains [217]. Additionally nitrated αSyn appears to be neurotoxic to DA neurons in models of α-synucleinopathy [218]. Monomeric and dimerized forms of αSyn can be degraded via CMA. However, oligomerized or nitrated αSyn are not efficient substrates for CMA [113, 219]. Therefore, it has been postulated that nitration can cause a conformational change in αSyn that interferes with its targeting to the CMA machinery [113, 220]. Future studies are needed to support this premise.

7. Conclusions

Over the last few decades, numerous studies have identified protein misfolding and aggregation as key features in a myriad of neurodegenerative diseases. In this review, we highlighted pathological roles of RNS in accumulation of misfolded/aggregated proteins (Table 1). Specifically, we summarized recent evidence from both *in vitro* and *in vivo* studies that aberrant protein S-nitrosylation can compromise activity of the cell's protein QC machinery, thus contributing to abnormal protein accumulation and neurotoxicity. Further studies will be needed to uncover additional mechanisms underlying the proteotoxic effects of aberrant S-nitrosylation and should aid in development of new therapeutic approaches to neurodegenerative diseases. The utilization of novel S-nitrosoproteomic approaches with enhanced sensitivity (e.g., SNO-TRAP and organomercury enrichment [33, 221]) are expected to identify additional aberrantly S-nitrosylated proteins that disrupt molecular chaperones, the UPS and autophagy, and should provide new molecular insights into the role of NO-related species in neurodegenerative disorders.

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Abbreviations

ADH5	Alcohol dehydrogenase class III
ALS	Amyotrophic lateral sclerosis

AMPK	AMP-activated protein kinase
APP	Amyloid precursor protein
ATF6	Activating transcription factor 6
ATP13A2	ATPase cation transporting 13A2
Bcl-2	B-cell lymphoma 2
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cGKI	cGMP-dependent protein kinase I
cGMP	Cyclic guanosine monophosphate
CHIP	Carboxy-terminus of Hsc70 interacting protein
CMA	Chaperon-mediated autophagy
CNS	Central nervous system
cIAP1	Cellular inhibitor of apoptosis protein-1
CTD	Carboxy-terminal domain
DA	Dopaminergic
eIF2	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FAD	Flavin adenine dinucleotide
FLIP	FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
FMN	Flavin mononucleotide
FTD	Frontotemporal dementia
GC	Guanylyl cyclase
GRP78	Glucose-regulated protein 78
GSH	Glutathione
GSNO	S-Nitrosoglutathione
GSNOR	GSNO reductase
HD	Huntington’s disease
HDAC6	Histone deacetylase 6

HSC	Heat shock cognate
HSP	Heat shock protein
hIAPP	Human islet amyloid polypeptide
IBR	In-between-RING
IKK	IκB kinase
IRE1	Inositol-requiring enzyme 1
IRP2	Iron regulatory protein 2
JNK1	c-Jun N-terminal kinase 1
LBD	Lewy body dementia
LC3	Microtubule-associated protein 1A/1B-light chain 3
LIR	LC3 interacting region
LLPS	Liquid-liquid phase separation
LTBP1	Latent TGF-β binding protein 1
MFN	Mitofusin
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDAR	*N*-methyl-D-aspartate (NMDA)-type glutamate receptor
NOS	Nitric oxide synthase
OMM	Outer mitochondrial membrane
ORP150	Oxygen-regulated protein 150
PDI	Protein disulfide isomerase
PERK	Pancreatic ER kinase
PI3K	Class III phosphatidylinositol 3-kinase
PINK1	Phosphatase and tensin homolog-induced putative kinase protein 1
PSD-95	Postsynaptic density protein-95
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
pVHL	von Hippel–Lindau protein
QC	Quality control
RNF	RING finger protein

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRM1	RNA recognition motif 1
SDH	Succinate dehydrogenase
SG	Stress granule
SNOC	S-Nitrosocysteine
SOD1	Superoxide dismutase 1
SQSTM1	Sequestosome 1
αSyn	α-Synuclein
TARDBP	Transactivation response (TAR) DNA binding protein
TDP-43	TAR DNA binding protein-43
mTORC	Mammalian target of rapamycin complex
TRAP1	TNF receptor–associated protein 1
UBE2D1	Ubiquitin-conjugating enzyme E2 D1
UBE3A	Ubiquitin protein ligase E3A
Uch-L1	Ubiquitin C-terminal hydrolase L1
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
USP9X	Ubiquitin specific peptidase 9 X-linked
VCP	Valosin-containing protein
VDAC1	Voltage dependent anion channel 1
XIAP	X-linked inhibitor of apoptosis

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Highlights

- RNS contribute to protein misfolding and aggregation via aberrant S-nitrosylation
- Aberrant protein S-nitrosylation affects molecular chaperones
- Aberrant protein S-nitrosylation compromises the ubiquitin-proteasome system
- Aberrant protein S-nitrosylation impairs the autophagy-lysosomal pathway
- S-Nitrosylation is a potential therapeutic target for neurodegenerative disorders

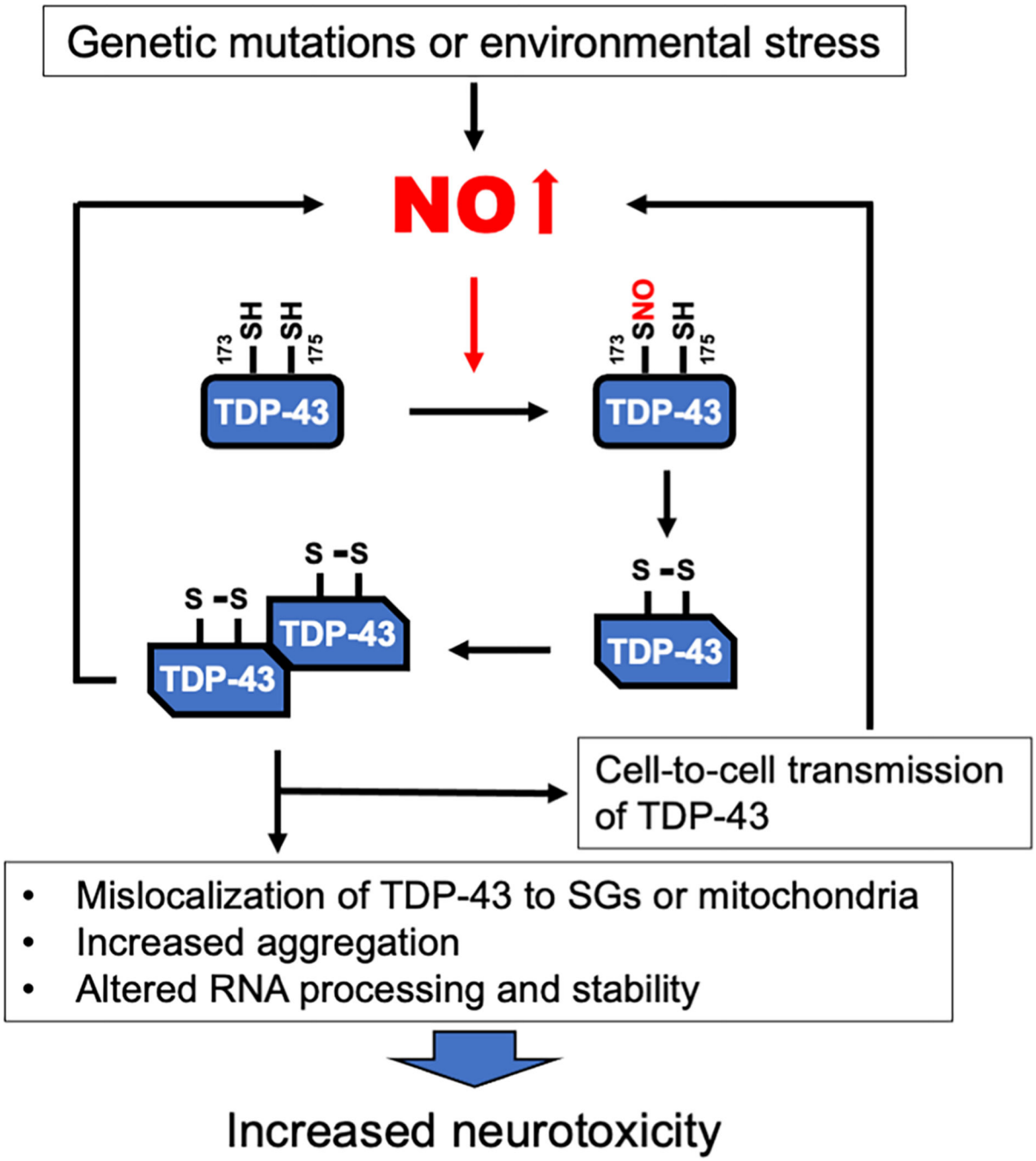

Fig. 1.

Proposed mechanisms of SNO-TDP-43-mediated neurodegeneration. Genetic mutations or various environmental stimuli can cause an elevation in intracellular NO levels that increase S-nitrosylated TDP-43. SNO-TDP43 triggers intra-molecular disulfide bond formation that alters its protein conformation, contributing to aggregation of TDP-43. SNO-TDP-43 can spread to adjoining cells, leading to neurotoxicity.

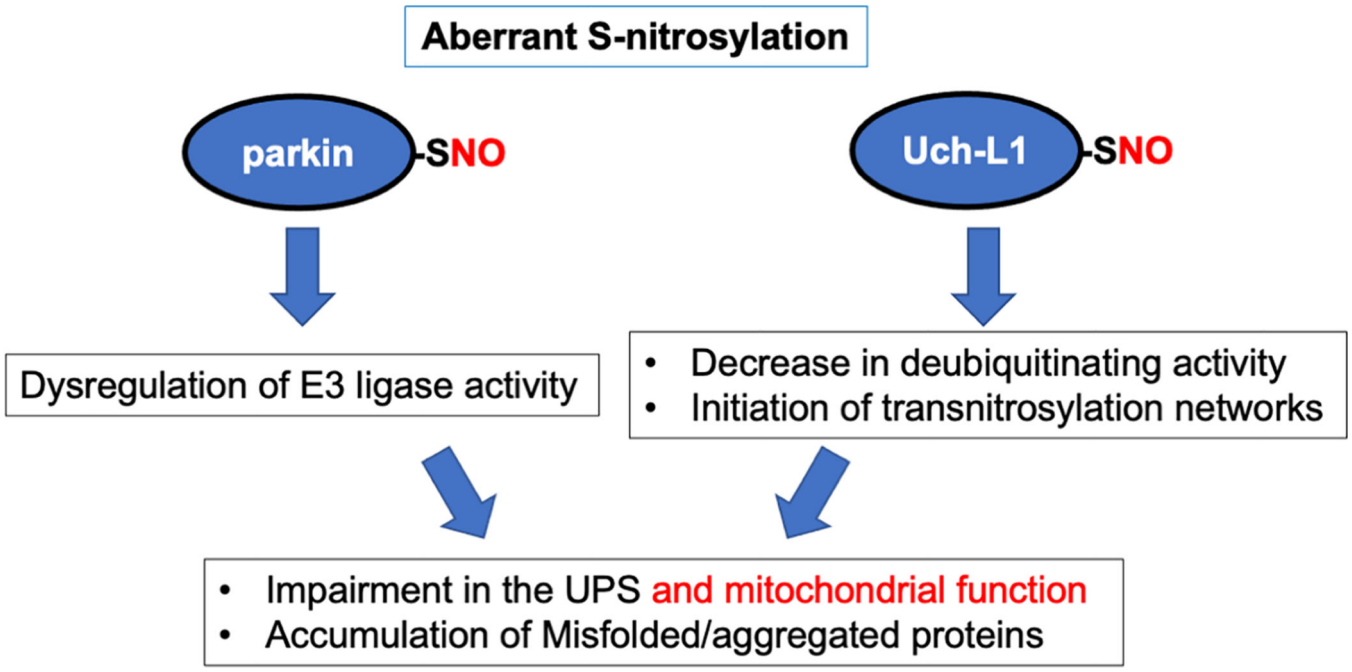

Fig. 2.

S-Nitrosylation of PDI impairs its chaperone and disulfide isomerase activity that modulates protein folding. When an excessive amount of NO is present under pathological conditions, PDI is S-nitrosylated at its active site cysteines, thus inhibiting its protein folding and chaperone activity, resulting in accumulation of misfolded proteins with increased cell damage and death.

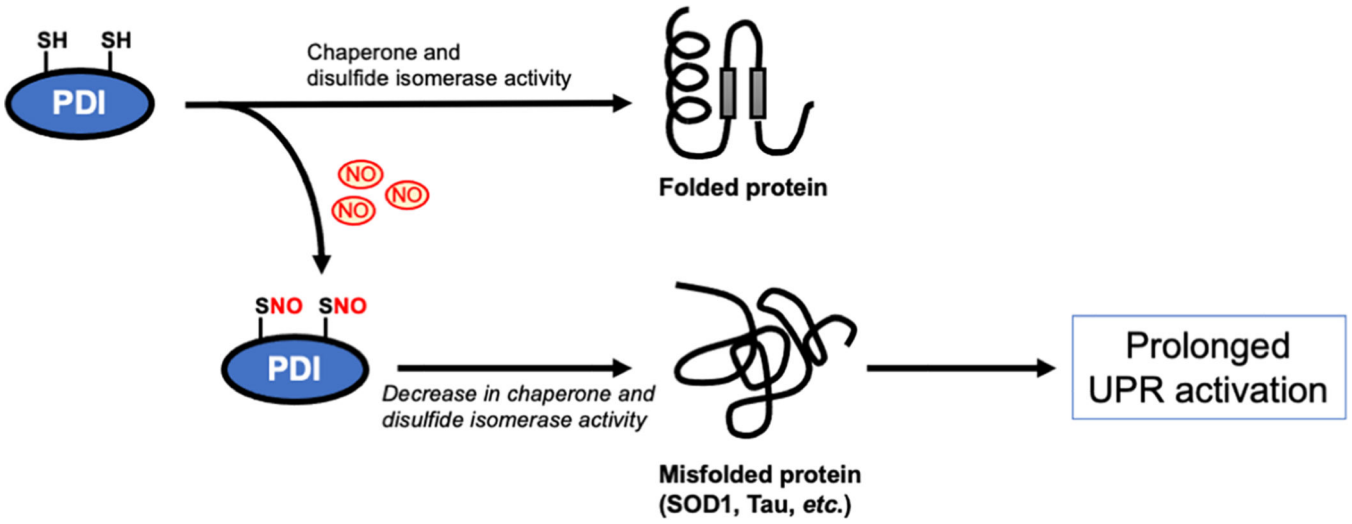

Fig. 3.

S-Nitrosylation of parkin and Uch-L1 impairs UPS activity. Aberrant S-nitrosylation of parkin disrupts its ubiquitin E3 ligase activity. S-Nitrosylated Uch-L1 manifests decreased deubiquitinase activity and increased transnitrosylation activity. Aberrant protein S-nitrosylation eventually impairs the function of the UPS, contributing to the accumulation of misfolded and aggregated proteins.

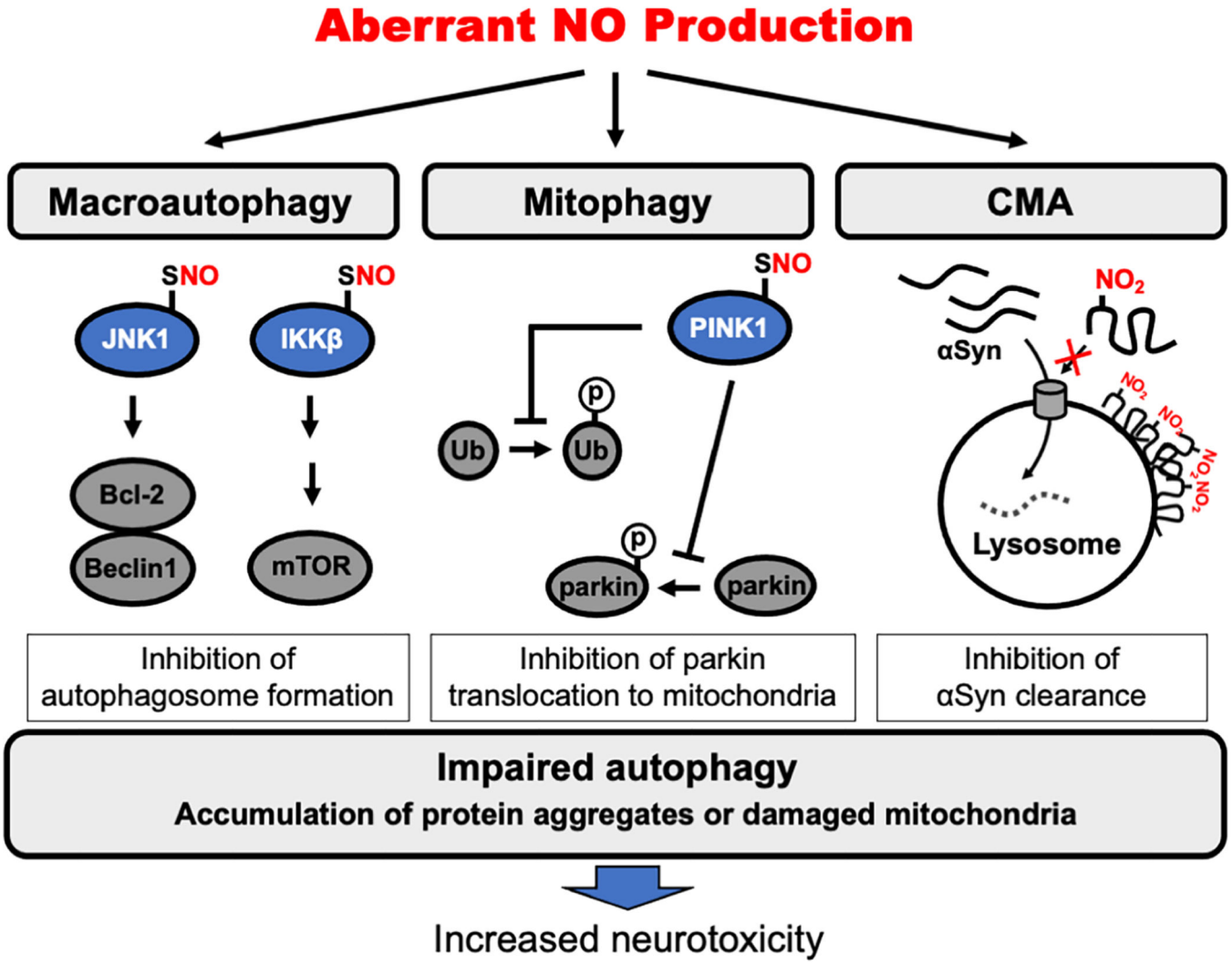

Fig. 4.

Regulation of autophagy by pathologically-elevated levels of NO-related species in neurodegenerative diseases. Pathologically increased levels of RNS inhibit autophagy under neuropathological conditions. Considering macroautophagy pathways, S-nitrosylation inhibits JNK1 and IKKβ kinase activity. As a consequence, downstream Bcl-2/Beclin 1 and mTOR pathways are altered, suppressing the initiation of autophagy. In mitophagy, S-nitrosylation of PINK1 decreases its kinase activity, thereby inhibiting phosphorylation of ubiquitin and parkin to suppress mitophagy. Considering CMA pathways, tyrosine nitration (denoted by the presence of NO_2 and detected as 3-nitrotyrosine) triggers a conformational change in αSyn that decreases its clearance through CMA. Overall, NO-mediated impairment in autophagy pathways contributes to accumulation of toxic oligomers/aggregates and damaged mitochondria. The inability to clear these proteins and damaged organelles leads to formation of inclusion bodies and contributes to neurodegeneration.

Table 1

List of major S-nitrosylated or nitrated proteins discussed in this review article

Category/Pathway	Target protein	Type of NO-dependent PTM	Consequence of NO-dependent PTM	Key Reference
Molecular chaperon	PDI	S-Nitrosylation	Decrease in chaperone and isomerase activity	[131]
	HSP90	S-Nitrosylation and nitration	Decrease in chaperone activity	[122, 140]
UPS	Parkin	S-Nitrosylation	Dysregulation in ubiquitination	[154–156]
	Uch-L1	S-Nitrosylation	Decrease in deubiquitinating activity; transnitrosylation	[69, 166]
Autophagy	JNK1/IKKβ	S-Nitrosylation	Inhibition of activity, leading to decreased autophagy flux	[192]
	PINK1/Parkin	S-Nitrosylation	inhibition of activity, leading to mitophagy dysfunction	[193]
Conformational change	TDP-43	S-Nitrosylation	Enhanced aggregation	[76]
	α-Synuclein	Nitration	Enhanced aggregation	[109]