

# Redox Reactions Induced by Nitrosative Stress Mediate Protein Misfolding and Mitochondrial Dysfunction in Neurodegenerative Diseases

Zezong Gu · Tomohiro Nakamura · Stuart A. Lipton

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**Abstract** Overstimulation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors accounts, at least in part, for excitotoxic neuronal damage, potentially contributing to a wide range of acute and chronic neurologic diseases. Neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD), manifest deposits of misfolded or aggregated proteins, and result from synaptic injury and neuronal death. Recent studies have suggested that nitrosative stress due to generation of excessive nitric oxide (NO) can mediate excitotoxicity in part by triggering protein misfolding and aggregation, and mitochondrial fragmentation in the absence of genetic predisposition. S-Nitrosylation, or covalent reaction of NO with specific protein thiol groups, represents a convergent signal pathway contributing to NO-induced protein misfolding and aggregation, compromised dynamics of mitochondrial fission-fusion process, thus leading to neurotoxicity. Here, we review the effect of

S-nitrosylation on protein function under excitotoxic conditions, and present evidence suggesting that NO contributes to protein misfolding and aggregation via S-nitrosylating protein-disulfide isomerase or the E3 ubiquitin ligase parkin, and mitochondrial fragmentation through  $\beta$ -amyloid-related S-nitrosylation of dynamin-related protein-1. Moreover, we also discuss that inhibition of excessive NMDA receptor activity by memantine, an uncompetitive/fast off-rate (UFO) drug can ameliorate excessive production of NO, protein misfolding and aggregation, mitochondrial fragmentation, and neurodegeneration.

**Keywords** S-Nitrosylation · Molecular chaperone · Ubiquitin-proteasome system · Protein misfolding · Mitochondrial fission · Neurodegeneration

## Introduction

A convergent feature for most neurodegenerative disorders is excessive generation of reactive nitrogen and oxygen species (RNS/ROS), which can contribute to neuronal cell injury and death [1–5]. While many intra- and extracellular molecules may participate in neuronal injury, accumulation of nitrosative stress due to excessive generation of nitric oxide (NO) appears to be a potential factor contributing to neuronal cell damage and death [6, 7]. A well-established model for NO production entails a central role of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in nervous system. Excessive activation of NMDA receptors drives  $\text{Ca}^{2+}$  influx, which in turn activates neuronal NO synthase (nNOS) as well as the generation of ROS [8, 9]. Accumulating evidence suggests that NO can mediate both protective and neurotoxic effects by reacting with cysteine

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Z. Gu (✉)  
Department of Pathology and Anatomical Sciences,  
University of Missouri-Columbia School of Medicine,  
One Hospital Drive,  
Columbia, MO 65212, USA  
e-mail: guze@health.missouri.edu

Z. Gu · T. Nakamura · S. A. Lipton (✉)  
Center for Neuroscience, Aging and Stem Cell Research,  
Sanford-Burnham Medical Research Institute,  
10901 North Torrey Pines Road,  
La Jolla, CA 92037, USA  
e-mail: slipton@burnham.org

S. A. Lipton  
Department of Neurosciences,  
University of California at San Diego,  
9500 Gilman Drive,  
La Jolla, CA 92039, USA

residues of target proteins to form S-nitrosothiols (SNOs), a predominant process in cells termed S-nitrosylation because of its effects on the chemical biology of protein function [10–12].

Mitochondria are powerhouse in cells because of their vital role in producing energy. In the nerve system, the importance of bioenergetics of mitochondria for highly consuming ATP is that neurons can meet the high energy demands of proper neuronal function. Substantial findings indicating that mitochondria frequently undergo two opposing bioenergetics processes—fission and fusion that regulate their morphology, number, and facilitate the transmission of energy across long distances of axonal projections in neurons [13, 14]. The disruption of this dynamic equilibrium may herald cell injury or death and may contribute to developmental and neurodegenerative disorders. Though a longstanding concept suggesting that normal mitochondrial respiration may also generate free radicals, principally ROS, and one such molecule, superoxide anion ( $O_2^-$ ), reacts rapidly with free radical NO to form the very toxic product peroxynitrite ( $ONOO^-$ ) [15, 16]. Recent discovery from our group and others indicating that mitochondrial fragmentation triggered by the dysfunction of the fission-inducing protein dynamin-related protein 1 (Drp1), for example, contributes to synaptic damage and subsequent neuronal loss because of nitrosative/oxidative stress and impaired bioenergetics [14, 17–23].

Another hallmark for neurodegenerative diseases is by the accumulation of misfolded proteins and aggregation that adversely affect neuronal connectivity and plasticity and trigger cell death signaling pathways [24, 25]. For example, degenerating brain contains aberrant accumulations of misfolded, aggregated proteins, such as  $\alpha$ -synuclein and synphilin-1 in Parkinson's disease (PD), and  $\beta$ -amyloid ( $A\beta$ ) and tau in Alzheimer's disease (AD). The inclusions observed in PD are called Lewy bodies (LBs) and are mostly found in the cytoplasm. AD brains show intracellular neurofibrillary tangles, which contain hyperphosphorylated tau, and extracellular plaques, which contain  $A\beta$ . These aggregates may consist of oligomeric complexes of non-native secondary structures and demonstrate poor solubility in aqueous or detergent solvent. Other disorders manifesting protein aggregation include Huntington's disease (a polyQ disorder), amyotrophic lateral sclerosis (ALS), and prion disease [26]. The aforementioned disorders are also termed “conformational diseases” because of the emergence of protein aggregation in the brain [27].

Importantly, dysfunction on protein misfolding and/or mitochondrial fragmentation in neurodegenerative disorders can result from either (1) a rare mutation in the disease-related gene encoding the protein or (2) conformational changes by posttranslational modification to the protein engendered by nitrosative/oxidative stress, which may well

account for the more common sporadic cases of the disease [28]. Therefore, a key theme of this review article is the hypothesis that nitrosative/oxidative stress contribute to protein misfolding and mitochondrial fragmentation in the brains of the majority of neurodegenerative patients. Here, we discuss specific examples showing that S-nitrosylation of ubiquitin E3 ligases such as parkin or endoplasmic reticulum chaperones such as protein-disulfide isomerase (PDI) is critical for the accumulation of misfolded proteins, and that the mitochondrial fission-inducing protein Drp1 S-nitrosylation mediates  $A\beta$ -related mitochondrial fragmentation and synaptic injury in neurodegenerative diseases such as PD, AD, and other conditions [18, 29–32]. We also discuss the neuroprotective mechanism of action of NMDA open-channel blockers like memantine and NO-related drugs for the treatment of neurodegenerative disorders [7, 33].

### **NMDA Receptor-mediated Glutamatergic Signaling Pathways Induce $Ca^{2+}$ Influx and Generation of RNS/ROS**

It is well-known that the amino acid glutamate is the major excitatory neurotransmitter in the brain. Glutamate is present in high concentrations in the adult central nervous system and is released for milliseconds from nerve terminals in a  $Ca^{2+}$ -dependent manner. After glutamate enters synaptic cleft, it diffuses across the cleft to interact with its corresponding receptors on the postsynaptic face of an adjacent neuron. Excitatory neurotransmission is necessary for the normal development and plasticity of synapses and for some forms of learning or memory; however, excessive activation of glutamate receptors is implicated in neuronal damage in many neurological disorders ranging from acute hypoxic-ischemic brain injury to chronic neurodegenerative diseases. It is currently thought that overstimulation of extrasynaptic NMDA receptors mediate this neuronal damage, while, in contrast, synaptic activity predominantly activates survival pathways [34–37]. Intense hyperstimulation of excitatory receptors leads to necrotic cell death, but more mild or chronic overstimulation can result in apoptotic or other forms of cell death [38–40]. The NMDA receptor has attracted attention for a long period of time because it has several properties that set it apart from other ionotropic glutamate (AMPA and kainate) receptors. One such characteristic is that NMDA receptor-coupled channels are highly permeable to  $Ca^{2+}$ , thus permitting  $Ca^{2+}$  entry after ligand binding if the cell is depolarized in order to relieve block of the receptor-associated ion channel by  $Mg^{2+}$  [41, 42]. Subsequent binding of  $Ca^{2+}$  to various intracellular molecules can lead to many significant consequences. In particular, excessive activation of NMDA receptors leads to the production of

damaging free radicals (e.g., NO and ROS) and other enzymatic processes contributing to cell death [6, 16, 39, 40, 43, 44].

Excessive activation of glutamate receptors is implicated in neuronal damage in many neurological disorders. John Olney coined the term “excitotoxicity” to describe this phenomenon [45, 46]. This form of toxicity is mediated at least in part by excessive activation of NMDA-type receptors [6, 7, 47], resulting in excessive  $\text{Ca}^{2+}$  influx through a receptor’s associated ion channel. Increased levels of neuronal  $\text{Ca}^{2+}$ , in conjunction with the  $\text{Ca}^{2+}$ -binding protein calmodulin (CaM), trigger the activation of nNOS and subsequent generation of NO from the amino acid L-arginine [8, 48]. NO is a gaseous free radical (thus, highly diffusible) and a key molecule that plays a vital role in normal signal transduction but in excess can lead to neuronal cell damage and death. The discrepancy of NO effects on neuronal survival can also be caused by the formation of different NO species or intermediates: NO radical ( $\text{NO}^\bullet$ ), nitrosonium cation ( $\text{NO}^+$ ), nitroxyl anion ( $\text{NO}^-$ , with high energy singlet and lower energy triplet forms) [16]. Three subtypes of NOS have been identified; two constitutive forms of NOS—nNOS and endothelial NOS (eNOS)—take their names from the cell type in which they were first found. The name of the third subtype—inducible NOS (iNOS)—indicates that expression of the enzyme is induced by acute inflammatory stimuli. For example, activated microglia may produce neurotoxic amounts of NO via iNOS expression in various neurodegenerative diseases. All three isoforms are widely distributed in the brain. Each NOS isoform contains an oxidase domain at its amino-terminal end and a reductase domain at its carboxy-terminal end, separated by a  $\text{Ca}^{2+}$ /CaM binding site [8, 48–51]. Constitutive and inducible NOS are also further distinguished by CaM binding: nNOS and eNOS bind CaM in a reversible  $\text{Ca}^{2+}$ -dependent manner. In contrast, iNOS binds CaM so tightly at resting intracellular  $\text{Ca}^{2+}$  concentrations that its activity does not appear to be affected by transient variations in  $\text{Ca}^{2+}$  concentration. Interestingly, in order to terminate iNOS-mediated NO production, microglia may redistribute iNOS to the aggregate for inactivation [52].

Recent studies further pointed out the potential connection between RNS/ROS and mitochondrial dysfunction in neurodegenerative diseases, especially in PD [5, 53]. Pesticide and other environmental toxins that inhibit mitochondrial complex I result in oxidative and nitrosative stress, and consequent aberrant protein accumulation [29, 30, 32, 54, 55]. Administration to animal models of complex I inhibitors, such as MPTP, 6-hydroxydopamine, rotenone, and paraquat, which result in overproduction of RNS/ROS, reproduces many of the features of sporadic PD, such as dopaminergic neuron degeneration, up-regulation

and aggregation of  $\alpha$ -synuclein, LB-like intraneuronal inclusions, and behavioral impairment [5, 53]. In addition, it has recently been proposed that mitochondrial cytochrome oxidase can produce NO in a nitrite ( $\text{NO}_2^-$ ) and pH dependent but non- $\text{Ca}^{2+}$ -dependent manner [56].

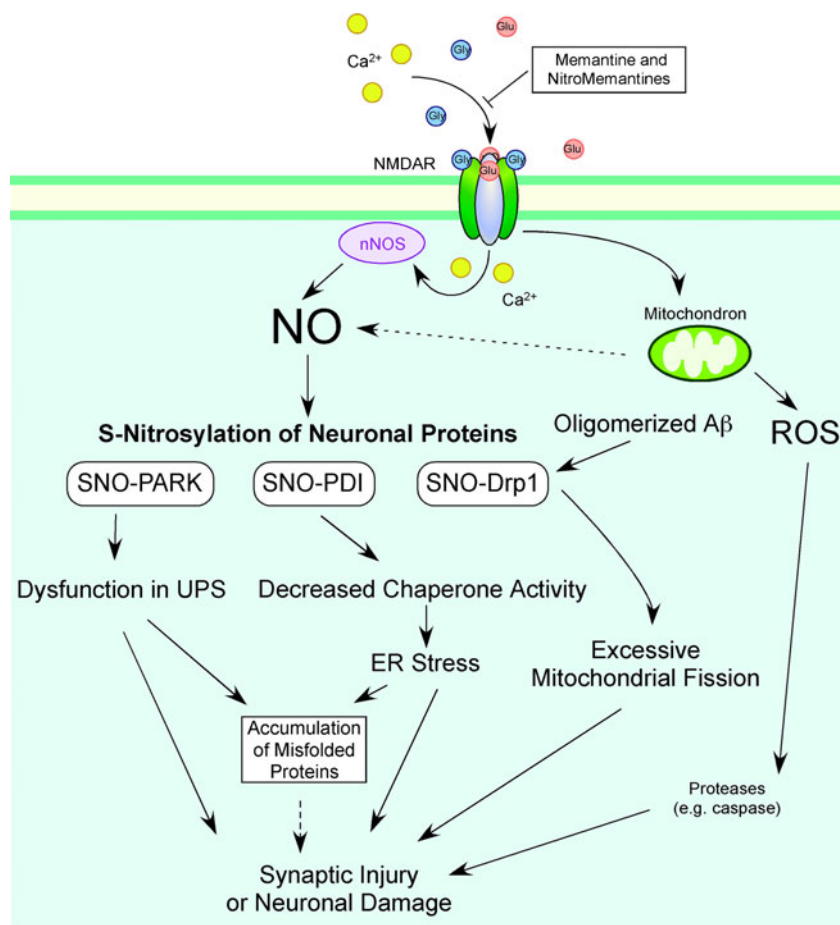
Increased nitrosative/oxidative stress is associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates [28, 57]. However, until recently, little was known regarding the molecular and pathogenic mechanisms underlying contribution of NO to the formation of inclusion bodies such as amyloid plaques in AD or LBs in PD.

### Dysfunction in UPS in Neurodegenerative Disorders

In general, protein aggregates do not accumulate in unstressed, healthy neurons due in part to the existence of cellular ‘quality control machineries.’ For example, molecular chaperones are believed to provide a defense mechanism against the toxicity of misfolded proteins because chaperones can prevent inappropriate interactions within and between polypeptides, and can promote refolding of proteins that have been misfolded because of cell stress. In addition to the quality control of proteins provided by molecular chaperones, the ubiquitin-proteasome system (UPS) and autophagy/lysosomal degradation are involved in the clearance of abnormal or aberrant proteins. When chaperones cannot repair misfolded proteins, they may be tagged via addition of polyubiquitin chains for degradation by the proteasome. In neurodegenerative conditions, intra- or extracellular protein aggregates are thought to accumulate in the brain as a result of a decrease in molecular chaperone activities or dysfunction in UPS (Fig. 1). In fact, several mutations that disturb the activity of molecular chaperones or UPS-associated enzymes can cause neurodegeneration [25, 58, 59]. Along these lines, postmortem samples from the substantia nigra of PD patients (versus non-PD controls) manifest a significant reduction in proteasome activity [60]. Moreover, overexpression of the molecular chaperone HSP70 can prevent neurodegeneration *in vivo* in models of PD [61].

Misfolded proteins are thought to form small toxic oligomers. Molecular chaperones can facilitate proper protein folding and thus prevent protein aggregation. Alternatively, if a higher molecular mass complex of oligomers forms, this may offer protection by avoiding the toxicity caused by soluble oligomers. UPS-mediated proteasome degradation can serve as a rapid and efficient pathway to remove misfolded proteins. Additionally, macroautophagy can enhance the clearance of misfolded proteins that are poor substrates for the proteasome, such as highly toxic oligomers and aggregates [62]. Under patho-

**Fig. 1** Possible mechanism whereby S-nitrosylated species contribute to the accumulation of aberrant proteins and mitochondrial fragmentation. NMDAR hyperactivation triggers generation of NO/ROS and cytochrome C release from mitochondria associated with subsequent activation of caspases, causing neuronal cell damage and death. S-nitrosylation of parkin (forming SNO-PARK) and PDI (forming SNO-PDI) can contribute to neuronal cell injury in part by triggering accumulation of misfolded proteins, whereas SNO-Drp1 mediates A $\beta$ -related excessive mitochondrial fission and synaptic injury. Memantine and NitroMemantine preferentially block overstimulated (pathological/extrasynaptic) NMDAR activity while relatively sparing normal (physiological/synaptic) activity



logical conditions, reduced activity of protein quality control systems, such as molecular chaperones, UPS, and autophagy leads to accumulation of toxic oligomers, which in turn contributes to the progression of ‘protein conformational diseases.’ Historically, lesions that contain aggregated proteins were considered to be pathogenic. Recently, several lines of evidence have suggested that aggregates are formed through a complex multi-step process by which misfolded proteins assemble into inclusion bodies; currently, soluble (micro-)oligomers of these aberrant proteins are thought to be the most toxic forms via interference with normal cell activities, while frank macroscopic aggregates may be an attempt by the cell to wall off potentially toxic material [8, 63]. Our newly published findings indicating that synaptic NMDA receptor activity induces mutant huntingtin (htt) inclusions via a T complex-1 ring complex-dependent mechanism rendering neurons more resistant to mutant htt-mediated cell death, whereas stimulation of extrasynaptic NMDA receptors increases the vulnerability of mutant htt-containing neurons to cell death by impairing the neuroprotective cyclic AMP response element-binding protein—peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) cascade and

increasing the level of the small guanine nucleotide-binding protein Rhes, which is known to sumoylate and disaggregate mutant htt [37]. Additionally, at least in yeast and cell culture models, highly toxic aggregates accumulate in a perivacuolar compartment where the autophagic pathway catalyzes clearance of aggresomes. Relatively less toxic misfolded proteins are sequestered in juxtanuclear inclusions, which often contain molecular chaperones and proteasomes as part of the quality control machinery [64].

### Nitrosative Stress Regulates Protein Misfolding and Neuronal Cell Death

Extreme nitrosative/oxidative stress can facilitate protein misfolding and aggregation—and very likely vice versa. This relationship between RNS/ROS and protein misfolding is thought to play a role as a pathogenic trigger of neurodegenerative diseases, although the exact mechanism underlying RNS/ROS-mediated aggregate formation has remained elusive. Recent scientific advances, however, have implied that NO-related species may significantly participate in the process of protein misfolding through



protein S-nitrosylation (and possibly nitration) under degenerative conditions.

Early investigations indicated that NO participates in cellular signaling pathways, which regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death [43, 65–67]. In general, NO exerts physiological and some pathophysiological effects via stimulation of guanylate cyclase to form cyclic guanosine-3',5'-monophosphate or through S-nitrosylation of regulatory protein thiol groups [9, 16, 57, 68–70]. S-Nitrosylation is the covalent addition of an NO group to a critical cysteine thiol/sulfhydryl (RSH or more properly, thiolate anion, RS<sup>−</sup>) to form an S-nitrosothiol derivative. Such modification modulates the function of a broad spectrum of mammalian, plant, and microbial proteins. In general, a consensus motif of amino acids comprised of nucleophilic residues (generally an acid and a base) surround a critical cysteine, which increases the cysteine sulfhydryl's susceptibility to S-nitrosylation [11, 12]. In contrast, denitrosylating enzymes and pathways, such as those mediated by thioredoxin/thioredoxin reductase, PDI, and intracellular glutathione, can decrease the lifespan of protein S-nitrosothiols [71–73]. Our group first identified the physiological relevance of S-nitrosylation by showing that NO and related RNS exert paradoxical effects via redox-based mechanisms—NO is neuroprotective via S-nitrosylation of NMDA receptors (as well as other subsequently discovered targets, including caspases), and yet can also be neurodestructive by formation of peroxynitrite (or, as later discovered, reaction with additional molecules such as MMP-9 and GAPDH) [16, 74–81]. Over the past decade, accumulating evidence has suggested that S-nitrosylation can regulate the biological activity of a great variety of proteins, in some ways akin to phosphorylation [11, 16, 29, 30, 32, 80–88]. Chemically, NO is often a good “leaving group,” facilitating further oxidation of critical thiol to disulfide bonds among neighboring (vicinal) cysteine residues or, via reaction with ROS, to sulfenic (SOH), sulfinic (SO<sub>2</sub>H), or sulfonic (SO<sub>3</sub>H) acid derivatization of the protein [30, 32, 80, 89]. Alternatively, S-nitrosylation may possibly produce a nitroxyl disulfide, in which the NO group is shared by close cysteine thiols [90].

Analyses of mice deficient in either nNOS or iNOS confirmed that NO is an important mediator of cell injury and death after excitotoxic stimulation; NO generated from nNOS or iNOS is detrimental to neuronal survival [91, 92]. In addition, inhibition of NOS activity ameliorates the progression of disease pathology in animal models of PD, AD, and ALS, suggesting that excess generation of NO plays a pivotal role in the pathogenesis of several neurodegenerative diseases [93–96]. Intriguingly, levels of glutathione diminish by approximately 30% in the aged brain [97], potentially assisting the accumulation of S-

nitrosothiols in elderly. Although the involvement of NO in neurodegeneration has been widely accepted, the chemical relationship between nitrosative stress and accumulation of misfolded proteins has remained obscure. Recent findings, however, have shed light on molecular events underlying this relationship. Specifically, we recently mounted physiological and chemical evidence that S-nitrosylation modulates the (1) ubiquitin E3 ligase activity of parkin [29–31], and (2) chaperone and isomerase activities of PDI [32], contributing to protein misfolding and neurotoxicity in models of neurodegenerative disorders.

Additionally, peroxynitrite-mediated nitration of tyrosine residue(s) may potentially contribute to dysfunctional protein folding and neuronal cell injury. For instance, nitration of  $\alpha$ -synuclein and tau effects oligomer formation in vitro. Furthermore, it has been reported that nitrated  $\alpha$ -synuclein and tau selectively accumulate in inclusion bodies in PD and neurofibrillary tangles in AD brains [98–101]. Collectively, these findings support the proposition that S-nitrosylation and possibly nitration can influence aggregate formation and neurotoxicity.

### Parkin and Ubiquitination

Recent studies on rare genetic forms of PD have found that mutations in the genes encoding parkin (*PARK2*), PINK1 (*PARK6*),  $\alpha$ -synuclein (*PARK1/4*), DJ-1 (*PARK7*), ubiquitin C-terminal hydrolase L1 (UCH-L1) (*PARK5*), leucine-rich repeat kinase-2 (LRRK2) (*PARK8*), or ATP13A2 (*PARK9*) are associated with PD pathology [102–109]. The discovery that mutations in these genes predispose patients to very rare familial forms of PD have allowed us to begin to understand the mechanism of protein aggregation and neuronal loss in the more common sporadic forms of PD. For instance, the identification of  $\alpha$ -synuclein as a familial PD gene led to the recognition that one of the major constituents of LBs in sporadic PD brains is  $\alpha$ -synuclein. In addition, identification of errors in the genes encoding parkin (a ubiquitin E3 ligase) and UCH-L1 in rare familial forms of PD has implicated possible dysfunction of the UPS in the pathogenesis of sporadic PD as well. The UPS represents an important mechanism for proteolysis in mammalian cells. Formation of polyubiquitin chains constitutes the signal for proteasomal attack and degradation. An isopeptide bond covalently attaches the C-terminus of the first ubiquitin in a polyubiquitin chain to a lysine residue in the target protein. The cascade of activating (E1), conjugating (E2), and ubiquitin-ligating (E3) type enzymes catalyzes the conjugation of the ubiquitin chain to proteins. In addition, individual E3 ubiquitin ligases play a key role in the recognition of specific substrates [110].

Mutations in the parkin gene can cause autosomal recessive juvenile Parkinsonism (ARJP), accounting for some cases of hereditary PD manifest in young patients with onset beginning anywhere from the teenage years through the 40s [58, 102, 111]. Parkin is a member of a large family of E3 ubiquitin ligases that are related to one another by the presence of RING finger domains. Parkin contains a total of 35 cysteine residues, the majority of which reside within its RING domains, which coordinate a structurally important zinc atom often involved in catalysis [112]. Parkin has two RING finger domains separated by an “in between RING” (IBR) domain. This motif allows parkin to recruit substrate proteins as well as an E2 enzyme (e.g., UbcH7, UbcH8, or UbcH13). Point mutations, stop mutations, truncations, and deletions in both alleles of the parkin gene will eventually cause dysfunction in its activity and are responsible for many cases of ARJP as well as rare adult forms of PD. Parkin mutations usually do not facilitate the formation of LBs, although there is at least one exception—familial PD patients with the R275W parkin mutant manifest LBs [113]. Biochemical characterization of parkin mutants show that not all parkin mutations result in loss of parkin E3 ligase activity; some of the familial-associated parkin mutants (e.g., the R275W mutant) have increased ubiquitination activity compared to wild type [114–116]. Additionally, parkin can mediate the formation of non-classical and “non-degradative” lysine 63-linked polyubiquitin chains [117, 118]. Likewise, parkin can mono-ubiquitinate Eps15, HSP70, and itself possibly at the multiple sites. This finding may explain how some parkin mutations induce formation of LBs and why proteins are stabilized within the inclusions.

Several putative target substrates have been identified for parkin E3 ligase activity. One group has reported that mutant parkin failed to bind glycosylated  $\alpha$ -synuclein for ubiquitination, leading to  $\alpha$ -synuclein accumulation [119], but most authorities do not feel that  $\alpha$ -synuclein is a direct substrate of parkin. Synphilin-1 ( $\alpha$ -synuclein interacting protein), on the other hand, is considered to be a substrate for parkin ubiquitination, and it is included in LB-like inclusions in cultured cells when co-expressed with  $\alpha$ -synuclein [120]. Other substrates for parkin include parkin-associated endothelin receptor-like receptor (Pael-R) [121], cell division control related protein [122], cyclin E [123], p38 tRNA synthase [124], and synaptotagmin XI [125],  $\alpha$ / $\beta$  tubulin heterodimers [126], as well as possibly parkin itself (auto-ubiquitination). It is generally accepted that accumulation of these substrates can lead to disastrous consequences for the survival of dopaminergic neurons in familial PD and possibly also in sporadic PD. Therefore, characterization of potential regulators that affect parkin E3 ligase activity may reveal important molecular mechanisms for the pathogenesis of PD. Heretofore, two cellular

components have been shown to regulate the substrate specificity and ubiquitin E3 ligase activity of parkin. The first represents posttranslational modification of parkin through S-nitrosylation (see below for details) or phosphorylation [127], and the second, binding partners of parkin, such as CHIP [128] and BAG5 [129]. CHIP enhances the ability of parkin to inhibit cell death through up-regulation of parkin-mediated ubiquitination, while BAG5-mediated inhibition of parkin E3 ligase activity facilitates neuronal cell death. In addition, several groups have recently reported that parkin-mediated mono-ubiquitination could contribute to neuronal survival via a proteasome-independent pathway [115, 116, 130, 131]. For example, parkin mono-ubiquitinates the epidermal growth factor receptor (EGFR)-associated protein, Eps15, leading to inhibition of EGFR endocytosis [130]. The resulting prolongation of EGFR signaling via the phosphoinositide-3 kinase /Akt (PKB) signaling pathway is postulated to enhance neuronal survival.

Another important molecule that links aberrant UPS activity and PD is the ubiquitin hydrolase Uch-L1, a deubiquitinating enzyme that recycles ubiquitin. Autosomal dominant mutations of Uch-L1 have been identified in two siblings with PD [106]. Interestingly, a recent study suggested that a novel ubiquitin-ubiquitin ligase activity of Uch-L1 might also be important in the pathogenesis of PD [132]. Additional mutations in  $\alpha$ -synuclein, DJ-1, PINK1, and LRRK2 may contribute to UPS dysfunction and subsequently lead to PD.

### S-Nitrosylation of Parkin Links Nitrosative Stress to Sporadic Parkinsonism

PD is the second most prevalent neurodegenerative disease and is characterized by the progressive loss of dopamine neurons in the substantia nigra pars compacta. Appearance of LBs that contain misfolded and ubiquitinated proteins generally accompany the loss of dopaminergic neurons in the PD brain. Such ubiquitinated inclusion bodies are the hallmark of many neurodegenerative disorders. Age-associated defects in intracellular proteolysis of misfolded or aberrant proteins might lead to accumulation and ultimately deposition of aggregates within neurons or glial cells. Although such aberrant protein accumulation had been observed in patients with genetically encoded mutant proteins, recent evidence from our laboratory suggests that nitrosative/oxidative stress are potential causal factors for protein accumulation in the much more common sporadic form of PD. As illustrated below, nitrosative/oxidative stress, commonly found during normal aging, can mimic rare genetic causes of disorders, such as PD, by promoting protein misfolding in the absence of a genetic mutation

[29–31]. For example, S-nitrosylation and further oxidation of parkin or Uch-L1 result in dysfunction of these enzymes and thus of the UPS [29, 30, 133–136]. We and others recently discovered that nitrosative stress triggers S-nitrosylation of parkin (forming SNO-parkin) not only in rodent models of PD but also in the brains of human patients with PD and the related  $\alpha$ -synucleinopathy, diffuse LB disease. SNO-parkin initially stimulates ubiquitin E3 ligase activity, resulting in enhanced ubiquitination as observed in LBs, followed by a decrease in enzyme activity, producing a futile cycle of dysfunction in UPS [30, 31, 117] (Fig. 1). We also found that rotenone led to the generation of SNO-parkin and thus dysfunctional ubiquitin E3 ligase activity. Moreover, S-nitrosylation appears to compromise the neuroprotective effect of parkin [29]. These mechanisms involve S-nitrosylation of critical cysteine residues in the first RING domain of parkin [30]. Our unpublished observation and other reports indicating that nitrosative/oxidative stress can also alter the solubility of parkin via posttranslational modification of cysteine residues, which may concomitantly compromise its protective function [137–139]. Additionally, it is likely that other ubiquitin E3 ligases with RING-finger thiol motifs are S-nitrosylated in a similar manner to parkin to affect their enzymatic function; hence, S-nitrosylation of E3 ligases may be involved in a number of degenerative conditions.

The neurotransmitter dopamine (DA) may also impair parkin activity and contribute to neuronal demise via the modification of cysteine residue(s) [140]. DA can be oxidized to DA quinone, which can react with and inactivate proteins through covalent modification of cysteine sulfhydryl groups; peroxyne has been reported to promote oxidation of DA to form dopamine quinone [141]. DA quinone can preferentially attack cysteine residues (C268 and C323) in the RING1 and IBR domains of parkin, forming a covalent adduct that abrogates its E3 ubiquitin ligase activity [138, 140]. DA quinone also reduces the solubility of parkin, possibly inducing parkin misfolding after disruption of the RING-IBR-RING motif. Therefore, nitrosative/oxidative species may either directly or indirectly contribute to altered parkin activity within the brain, and subsequent loss of parkin-dependent neuroprotection results in increased cell death.

### The Unfolded Protein Response and PDI

The endoplasmic reticulum (ER) normally participates in protein processing and folding but undergoes a stress response when immature or misfolded proteins accumulate [142–145]. ER stress stimulates two critical intracellular responses. The first stress response represents expression of chaperones that prevent protein aggregation via the unfold-

ed protein response (UPR), and is implicated in protein refolding, post-translational assembly of protein complexes, and protein degradation. This response is believed to contribute to adaptation during altered environmental conditions, promoting maintenance of cellular homeostasis. At least three ER transmembrane sensor proteins are involved in the UPR: PKR-like ER kinase, activating transcription factor 6, and inositol-requiring enzyme 1. The activation of all three proximal sensors results in the attenuation of protein synthesis via eukaryotic initiation factor-2 kinase and increased protein folding capacity of the ER [146–149]. The second ER stress response, termed ER-associated degradation (ERAD), specifically recognizes terminally misfolded proteins for retro-translocation across the ER membrane to the cytosol, where they can be degraded by the UPS. Additionally, although severe ER stress can induce apoptosis, the ER withstands relatively mild insults via expression of stress proteins such as glucose-regulated protein (GRP) and PDI. These proteins behave as molecular chaperones that assist in the maturation, transport, and folding of secretory proteins.

During protein folding in the ER, PDI can introduce disulfide bonds into proteins (oxidation), break disulfide bonds (reduction), and catalyze thiol/disulfide exchange (isomerization), thus facilitating disulfide bond formation, rearrangement reactions, and structural stability [150]. PDI has four domains that are homologous to thioredoxin (TRX; termed a, b, b', and a'). Only two of the four TRX-like domains (a and a') contain a characteristic redox-active CXXC motif, and these two-thiol/disulfide centers function as independent active sites [151–154]. These active-site cysteines can be found in two different redox states: oxidized (disulfide) or reduced (free sulfhydryls or thiols). During oxidation of a target protein, oxidized PDI catalyzes disulfide formation in the substrate protein, resulting in the reduction of PDI. In contrast, the reduced form of the active-site cysteines can initiate isomerization by attacking the disulfide of a substrate protein and forming a transient intermolecular disulfide bond. As a consequence, an intramolecular disulfide rearrangement occurs within the substrate itself, resulting in the generation of reduced PDI. The recently determined structure of yeast PDI revealed that the four TRX-like domains form a twisted "U" shape with the two active sites facing each other on opposite sides of the "U" [155]. Hydrophobic residues line the inside surface of the "U," facilitating interactions between PDI and misfolded proteins. Specifically, the b' domain of PDI constitutes a part of the base of the "U"-shaped structure and contributes to the efficient binding of misfolded proteins [156]. Several mammalian PDI homologues, such as ERp57 and PDIP, also localize to the ER and may manifest similar functions [157, 158]. Increased expressions of PDIP in neuronal cells under

conditions mimicking PD suggest the possible contribution of PDIp to neuronal survival [157]. Additionally, ERdj5, an ER reductase that contains four TRX-like domains, forms a functional ERAD complex with GRP, promoting the degradation of misfolded proteins via ERAD [159].

In many neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction [157, 160–162], but upregulation of PDI represents an adaptive response promoting protein refolding and may offer neuronal cell protection [157, 158, 163, 164]. In a recent study, we reported that the S-nitrosylation of PDI (to form SNO-PDI) disrupts its neuroprotective role [32].

### S-Nitrosylation of PDI Mediates Protein Misfolding and Neurotoxicity in Cell Models of PD or AD

Disturbance of  $\text{Ca}^{2+}$  homeostasis within the ER plays a critical role in the accumulation of misfolded proteins and ER stress because the function of several ER chaperones requires high concentrations of  $\text{Ca}^{2+}$ . In addition, it is generally accepted that excessive generation of NO can contribute to activation of the ER stress pathway, at least in some cell types [165, 166]. Molecular mechanisms by which NO induces protein misfolding and ER stress, however, have remained enigmatic until recently. The ER normally manifests a relatively positive redox potential in contrast to the highly reducing environment of the cytosol and mitochondria. This redox environment can influence the stability of protein S-nitrosylation and oxidation reactions [167]. S-Nitrosylation can enhance the activity of the ER  $\text{Ca}^{2+}$  channel-ryanodine receptor [168], which may provide a clue to how NO disrupts  $\text{Ca}^{2+}$  homeostasis in the ER and activates the cell death pathway. Interestingly, we have recently reported that excessive NO can also lead to S-nitrosylation of the active-site thiol groups of PDI, and this reaction inhibits both its isomerase and chaperone activities [32]. Mitochondrial complex I insult by rotenone can also result in S-nitrosylation of PDI in cell culture models. Moreover, we found that PDI is S-nitrosylated in the brains of virtually all cases examined of sporadic AD and PD. Under pathological conditions, it is possible that both cysteine sulfhydryl groups in the TRX-like domains of PDI form S-nitrosothiols. Unlike formation of a single S-nitrosothiol, which is commonly seen after de-nitrosylation reactions catalyzed by PDI [84], dual nitrosylation may be relatively more stable and prevent subsequent disulfide formation on PDI. Therefore, we speculate that these pathological S-nitrosylation reactions on PDI are more easily detected during neurodegenerative conditions. Additionally, it is possible that vicinal (nearby) cysteine thiols reacting with NO can form nitroxyl disulfide [90], and such

reaction may potentially occur in the catalytic side of PDI to inhibit enzymatic activity. In order to determine the consequences of S-nitrosylated PDI (SNO-PDI) formation in neurons, we exposed cultured cerebrocortical neurons to neurotoxic concentrations of NMDA, thus inducing excessive  $\text{Ca}^{2+}$  influx and consequent NO production from nNOS. Under these conditions, we found that PDI was S-nitrosylated in a NOS-dependent manner. SNO-PDI formation led to the accumulation of polyubiquitinated/misfolded proteins and activation of the UPR. Moreover, S-nitrosylation abrogated the inhibitory effect of PDI on aggregation of proteins observed in LB inclusions [32, 120]. S-Nitrosylation of PDI also prevented its attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition (Fig. 1). Further evidence suggested that SNO-PDI may in effect transport NO to the extracellular space, where it could conceivably exert additional adverse effects [84]. Additionally, NO can possibly mediate cell death or injury via S-nitrosylation or nitration reactions on other TRX-like proteins, such as TRX itself and glutaredoxin [83, 169, 170].

In addition to PDI, S-nitrosylation is likely to affect critical thiol groups on other chaperones, such as HSP90 in the cytoplasm [171] and possibly GRP in the ER. Normally, HSP90 stabilizes misfolded proteins and modulates the activity of cell signaling proteins including NOS and calcineurin [25]. In AD brains, levels of HSP90 are increased in both the cytosolic and membranous fractions, where HSP90 is thought to maintain tau and A $\beta$  in a soluble conformation, thereby averting their aggregation [172, 173]. Martínez-Ruiz et al. [171] recently demonstrated that S-nitrosylation of HSP90 can occur in endothelial cells, and this modification abolishes its ATPase activity, which is required for its function as a molecular chaperone. These studies imply that S-nitrosylation of HSP90 in neurons of AD brains may contribute to the accumulation of tau and A $\beta$  aggregates.

### Dynamins and Mitochondrial Fission

Another important finding for the pathogenesis of neurodegenerative disorders is from studies of mitochondrial function. Neurons rely heavily on mitochondria for their energy supply and specialized neuronal functions, including membrane ionic pumps, channel activity, and synaptic transmission. In most cell types, including neurons, mitochondria are found as thread-like, tubular organelles that often split apart and/or fuse together [174]. Genetic studies in yeast, *Drosophila* and *Caenorhabditis elegans* have identified several key factors including the large guanosine triphosphatase (GTPase) family dynamins and Drp1 that are essential for this process. Interestingly, these

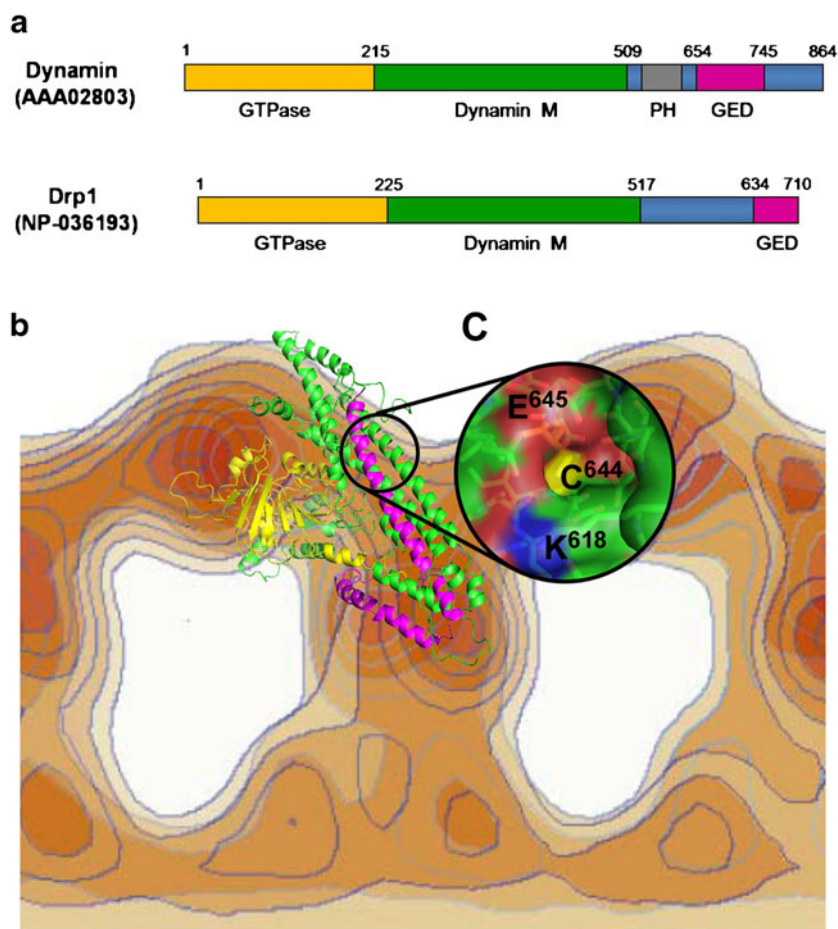


factors are highly conserved throughout evolution, and their homologues can be found in human and mouse. The domain structure of dynamins includes an N-terminal GTPase domain, a dynamin middle (M) domain, a pleckstrin homology (PH) domain for membrane targeting, a GTPase effector domain (GED) that regulates self-assembly and activation of GTP hydrolysis, and a proline-rich region in the C-terminal. Dynamins are large GTPases that regulate vesicular traffic and endocytosis [175, 176]. Studies in yeast favor a model in which dynamin-1 self-assembles into spirals and localizes to mitochondrial membrane constriction sites and show that the stalk region of dynamin undergoes a large conformational change that drives tube constriction [175]. The amino acid sequence of Drp1 shares significant similarity to that of dynamins (Fig. 2). Drp1 shows significant homology to dynamin in the GTPase, dynamin M, and GED domains. Interestingly, however, the C-terminal PH domain of dynamin is missing in Drp1. Instead, Drp1 contains a unique proline-rich domain with an SH3 binding motif that is different from the proline-rich region in dynamin and thus may represent a unique regulatory domain. Mitochondrial fission in mammalian cells is regulated by Drp1 for its proliferation and

distribution as well as cellular adaptation of the altered energy demands. Overexpression of Drp1 triggers mitochondrial fission in *C. elegans* [177]. Conversely, knock-down of Drp1 by RNAi silencing blocks mitochondrial outer-membrane scission, although inner-membrane scission still occurs. Similarly, expression of a dominant-negative Drp1 mutant, such as Drp1K38A, that is unable to bind GTP results in a fused, interconnected mitochondrial network [178].

Very little is known about the signal-transduction pathways that initiate mitochondrial fission. However, several observations point toward  $\text{Ca}^{2+}$ -mediated nitrosative/oxidative stress that regulating mitochondrial fission. First, dynamin activity appears to be regulated by the level of intracellular  $\text{Ca}^{2+}$  [14]. Second, physiological  $\text{Ca}^{2+}$  concentrations accelerate A $\beta$ -peptide aggregation [57]. Third,  $\text{Ca}^{2+}$  release from the ER promotes the translocation of Drp1 from the cytoplasm to the outer mitochondrial membrane [179]. Fourth, NO-induced nitrosative stress leads to mitochondrial fission upon induction with NO [17]. Thus, it is possible that  $\text{Ca}^{2+}$  may trigger nitrosative/oxidative stress and generate excessive NO and other ROS, thus activating Drp1 and related fission-inducing molecules.

**Fig. 2** Atomic-resolution model of Drp1 for its S-nitrosylation motif. **a** Domain comparison of GTPase family members—dynamin and Drp-1. *GTPase* GTPase domain, *Dynamin M* dynamin-like middle domain, *GED* GTPase effector domain, *PH* pleckstrin homology domain. **b** Atomic resolution model of Drp1 superimposed onto electron density map of homologous domains of dynamin dimer: GTPase (yellow), forming the head; Dynamin M (green), and GED (magenta) domains, forming the stalk. **c** Predicted ribbon model structure of human Drp1 showing putative acid/base S-nitrosylation motif around Cys644 (yellow). C Cys, E Glu (red for negatively charged surface), K Lys (blue for positively charged surface)



### S-Nitrosylation of Drp1 Mediates A $\beta$ -related Mitochondrial Fragmentation and Neuronal Injury

Disrupting the balance between mitochondrial fission and fusion can lead to excessive mitochondrial fragmentation. Evidence indicating that mitochondrial fragmentation links dysfunction of Drp1 to synaptic damage and neuronal loss due to nitrosative/oxidative stress and impaired bioenergetics [13, 14]. Excessive fission results in abnormally small mitochondria with fragmented cristae [17], as observed in electron microscopy studies of neurons in human Alzheimer's disease (AD). Drp1 homologs are S-nitrosylated, which regulates their activity [176, 180]. Furthermore, A $\beta$  is imported into mitochondria and locates to the inner membrane cristae [181]. A $\beta$  oligomers induce excessive mitochondrial fission and neuronal damage in an NO-mediated fashion [17, 21, 182]. Therefore, we determined whether Drp1 is S-nitrosylated and thereby contributes to synaptic damage and neuronal injury.

First, we collected evidence showing that NO induces mitochondrial fission and S-nitrosylation of Drp1. Fluorescent images show fragmented, smaller mitochondria in a dose-dependent manner in cortical neurons transfected with mitochondrial marker mito-DsRed2 after NO donor S-nitrosocysteine (SNOC) exposure, due to fission. Using the benchmark biotin-switch assay to detect nitrosylated proteins [82], we found that SNOC induced S-nitrosylation of Drp1 (forming SNO-Drp1) in neurons before inducing mitochondrial fission. To investigate whether endogenously generated NO can induce SNO-Drp1, we used HEK293 cells stably expressing nNOS after incubation with the calcium ionophore A23187 to activate nNOS. Drp1 was S-nitrosylated by endogenous NO. Using the same conditions under which A $\beta$  causes mitochondrial fragmentation and consequent neuronal damage [17], we found that A $\beta$  could induce SNO-Drp1 formation. Additionally, we tested the effect of endogenously produced A $\beta$ , generated from amyloid precursor protein (APP) in conditioned medium of N2a/APP695 stable neuronal cell lines or CHO cells stably expressing human APP with the Val<sup>717</sup> → Phe mutation (designated 7PA2 cells). Exposing N2a cells to SNOC or conditioned medium resulted in SNO-Drp1 formation. We also found elevated levels of SNO-Drp1 in vivo in brains of the AD transgenic mouse model Tg2576, which expresses high levels of the Swedish APP mutation (Lys<sup>670</sup> → Asn, Met<sup>671</sup> → Leu). To extend these findings to humans, we examined brains obtained shortly after death from patients manifesting AD. We found increased SNO-Drp1 levels in 17 of 17 AD brains studied, but not in brains of deceased Parkinson's disease patients or controls who died of non-CNS causes. Moreover, relatively similar amounts of SNO-Drp1 were detected in A $\beta$ -treated neurons in culture and in human Alzheimer's disease brains,

suggesting that this pathway might be pathophysiologically relevant. Thus, formation of SNO-Drp1 is a characteristic feature for AD progression. Therefore, SNO-Drp1 could potentially serve as a biomarker for AD.

We next identified the target cysteine residue for S-nitrosylation on Drp1. Sequence alignment of Drp1 revealed four distinct structural domains: an N-terminal GTPase domain, a dynamin-like M domain, and a C-terminal GED domain (Fig. 2a). The GED affects both GTPase activity and Drp1 dimer formation [183]. In search of S-nitrosylated cysteine residue(s), we mutated each of the nine Drp1 cysteines. To assess S-nitrosylation, we performed a chemical assay using 2,3-diaminonaphthalene (DAN) on immunoprecipitates from HEK293 cells transfected with wild-type or mutant Drp1. This assay monitors release of NO from thiol by conversion of DAN to fluorescent 2,3-naphthylthiazole. Among the cysteine mutants, only C644A (Cys<sup>644</sup> → Ala), located in the GED domain, decreased SNO-Drp1 formation. Cys<sup>644</sup> was confirmed as the S-nitrosylated residue by biotin-switch assay. A23187 exposure, to activate nNOS and produce endogenous NO, resulted in SNO-Drp1 formation, which was virtually eliminated in Drp1 (C644A) but not other cysteine mutant cells. We then constructed an atomic model of Drp1 based on comparative homology using the structure of bacterial dynamin-like protein (PDB ID: 2J68-A) [184]. We found that the Drp1-GED domain contains a consensus motif for S-nitrosylation flanking Cys<sup>644</sup> (Fig. 2c) [11, 12]. Homology modeling also predicted that Cys<sup>644</sup> is exposed to the protein surface, increasing its accessibility to NO and thus facilitating nitrosylation. Thus, Cys<sup>644</sup> was essential for SNO-Drp1 generation.

Dimer formation of the homologous protein dynamin, a process influenced by residues in the GED domain, increases dynamin GTPase activity [175]. The Drp1-GED domain is critical for inter and intramolecular interactions and GTPase activity [175]. We found that S-nitrosylation of Cys<sup>644</sup> in the GED domain led to dimerized SNO-Drp1 and increased GTPase activity. In contrast, NO failed to induce dimerization/oligomerization or to increase GTPase activity of the Drp1(C644A) mutant. The GTPase activity of Drp1 (C644A) protein was as reduced as that of dominant-negative Drp1 with a Lys<sup>38</sup> → Ala mutation [DN-Drp1 (K38A)] [17, 23], consistent with the notion that S-nitrosylation of Drp1 at residue Cys<sup>644</sup> increases its GTPase activity. We then superimposed the atomic-resolution model for Drp1 onto a lower-resolution 3D reconstruction representing dimerization of the homologous dynamin molecule (Fig. 2b) [175]. Superimposition allowed us to examine the potential effect of NO on Drp1 domain structure. This suggested that NO-induced dimerization triggered a conformation change in the stalk region, composed of the M

and GED domains, thereby regulating GTPase activity analogous to dynamin dimerization [175].

Next, we examined the effect of S-nitrosylation of Drp1 on mitochondrial fragmentation. An intact Drp1-GTPase domain is required for mitochondrial fission. RNAi knockdown of Drp1 or mutation producing DN-Drp1 (K38A), located in the GTPase domain, inhibits excessive mitochondrial fragmentation [17, 23]. Because nitrosylation of Drp1 in the GED domain activates GTPase activity, we reasoned that SNO-Drp1 should activate mitochondrial fission. In cortical neurons, NO induced mitochondrial fragmentation [17, 182], and overexpression of WT-Drp1 slightly enhanced this effect. In contrast, expression of Drp1(C644A) or DN-Drp1(K38A) abrogated NO-mediated mitochondrial fission. Among the nine possible Drp1-cysteine mutants, only C644A suppressed NO-induced mitochondrial fragmentation, consistent with the observation that C<sup>644</sup> was the predominant Drp1 nitrosylation site. As a control, Drp1(C644A) mutant did not act as a dominant negative to decrease Drp1-mediated mitochondrial fission that was not triggered by NO. Following, we investigated the effect of A $\beta$ /NO-mediated nitrosylation of Drp1 on neurotoxicity. Exposure to NO triggers neuronal damage or death in the presence of WT-Drp1 [17, 182]. However, expression of Drp1(C644A) largely protected the neurons, consistent with the notion that S-nitrosylation of Drp1(C<sup>644</sup>) is required for NO-mediated damage. During A $\beta$ -induced neurotoxicity, synaptic damage has been reported to represent one of its earliest manifestations [185]. Exposure to oligomerized A $\beta$  results in loss of dendritic spines, representing neuronal postsynaptic sites; this form of A $\beta$ -mediated injury is dependent on NMDA-type glutamate receptor activity [185]. Additionally, dendritic mitochondria localize to developing synaptic sites in a Drp1-dependent manner [19, 20]. Because NMDA receptor stimulation activates nNOS and thus generates NO, we asked if A $\beta$ -induced synaptic damage might be mediated at least in part by NO. Indeed, A $\beta$  decreased the number of dendritic spines in an NO-dependent manner. Importantly, transfection with mutant Drp1(C644A), which lacks the nitrosylation site, abrogated the effect of A $\beta$  on spine density, consistent with the notion that S-nitrosylation of Drp1 is involved in synaptic loss after exposure to A $\beta$ .

Overall, these results show that pathophysiologically relevant amounts of SNO-Drp1 form in human AD brains. Exposure of cerebrocortical neurons in culture to A $\beta$  results in the formation of relatively similar amounts of SNO-Drp1 as found in human AD brain. This S-nitrosylation of Drp1 causes dimer formation and increased GTPase activity, thus accelerating the process of mitochondrial fragmentation and contributing to neuronal synaptic damage or cell death. This relationship between nitrosative stress and mitochon-

drial fragmentation, suggests that Drp1 can be a target of S-nitrosylation in AD. Drp1 is nitrosylated via a redox-mediated pathway in response to A $\beta$  oligomers causing mitochondrial fission and synaptic damage. Thus, the redox consequences of nitrosative stress, resulting in Drp1 activation, may provide a mechanistic link between the actions of oligomeric A $\beta$ , mitochondrial fission and neuronal damage. Drp1 may represent a drug target to antagonize the progression of neurodegenerative disorders in which nitrosative stress and mitochondrial dysfunction play a key role.

Taken together, our recent findings suggest that aberrant nitrosylation events contribute to both protein misfolding and excessive mitochondrial fragmentation in neurodegenerative conditions, thus contributing to synaptic damage and neuronal cell death.

### Potential Treatment of Excessive NMDA-induced Ca<sup>2+</sup> Influx and Free Radical Generation

One mechanism that could potentially curtail excessive Ca<sup>2+</sup> influx and resultant overstimulation of nNOS activity would be inhibition of NMDA receptors. Until recently, however, drugs in this class blocked virtually all NMDA receptor activity, including physiological activity, and therefore manifest unacceptable side effects by inhibiting normal functions of the receptor. For this reason, many previous NMDA receptor antagonists have disappointingly failed in advanced clinical trials conducted for a number of neurodegenerative disorders. In contrast, studies in our laboratory first showed that the adamantane derivative, memantine, preferentially blocks excessive (pathological) NMDA receptor activity while relatively sparing normal (physiological) activity. Memantine does this in a surprising fashion because of its low (micromolar) affinity, even though its actions are quite selective for the NMDA receptor at that concentration. “Apparent” affinity of a drug is determined by the ratio of its “on-rate” to its “off-rate” for the target. The on-rate is not only a property of drug diffusion and interaction with the target, but also the drug’s concentration. In contrast, the off-rate is an intrinsic property of the drug-receptor complex, unaffected by drug concentration. A relatively fast off-rate is a major contributor to memantine’s low affinity for the NMDA receptor. The inhibitory activity of memantine involves blockade of the NMDA receptor-associated ion channel when it is excessively open (termed open-channel block). The unique and subtle difference of the memantine blocking sites in the channel pore may explain the advantageous properties of memantine action.

Also critical for the clinical tolerability of memantine is its uncompetitive mechanism of action. An uncompetitive

antagonist can be distinguished from a noncompetitive antagonist, which acts allosterically at a noncompetitive site, i.e., at a site other than the agonist-binding site. An uncompetitive antagonist is defined as an inhibitor whose action is contingent upon prior activation of the receptor by the agonist. Hence, the same amount of antagonist blocks higher concentrations of agonist relatively better than lower concentrations of agonist. Some open-channel blockers function as pure uncompetitive antagonists, depending on their exact properties of interaction with the ion channel. This uncompetitive mechanism of action coupled with a relatively fast off-rate from the channel yields a drug that preferentially blocks NMDA receptor-operated channels when they are excessively open while relatively sparing normal neurotransmission. In fact, the relatively fast off-rate is a major contributor to a drug like memantine's low affinity for the channel pore. While many factors determine a drug's clinical efficacy and tolerability, it appears that the relatively rapid off rate is a predominant factor in memantine's tolerability in contrast to other NMDA-type receptor antagonists [7, 47]. Thus, the critical features of memantine's mode of action are its uncompetitive mechanism and fast off-rate, or what we call a UFO drug—a drug that is present at its site of inhibitory action only when you need it and then quickly disappears.

Interestingly, memantine, which is chemically an adamantane, was first synthesized and patented in 1968 by Eli Lilly and Company, as described in the Merck Index. Memantine has been used for many years in Europe to treat PD, spasticity, convulsions, vascular dementia, and later AD [186, 187]. Interestingly, the efficacy of adamantane-type drugs in the brain was first discovered by serendipity in a patient taking amantadine for influenza (amantadine is chemically similar to memantine but lacks two side groups chains composed of methyl groups). This led scientists to believe that these drugs were dopaminergic or possibly anti-cholinergic, although, as stated above, we later discovered that memantine acts as an open-channel blocker of NMDA receptor (NMDAR)-coupled channel pore; memantine is more potent in this action than amantadine. A large number of studies with in vitro and in vivo animal models demonstrated that memantine protects cerebrocortical neurons, cerebellar neurons, and retinal neurons from NMDAR-mediated excitotoxic damage [188–192]. Importantly, in a rat stroke model, memantine, which was given up to 2 h after the ischemic event, reduced the amount of brain damage by approximately 50% [189, 190]. Furthermore, regulatory agencies in both Europe and the USA recently voted its approval as the first treatment for moderate-to-severe AD. It is currently under study for a number of other neurodegenerative disorders, including HIV-associated dementia, Huntington's disease, ALS, and also depression.

As promising as the results with memantine are, we are continuing to pursue ways to use additional modulatory sites on the NMDA receptor to block excitotoxicity even more effectively and safely than memantine alone. New approaches in this regard are explored below.

### Novel UFO Therapeutics: NitroMemantines

NitroMemantines are second-generation memantine derivatives that are designed to have enhanced neuroprotective efficacy without sacrificing clinical tolerability. S-Nitrosylation site(s) is located on the extracellular domain of the NMDA receptor, and S-nitrosylation of this site, i.e., NO reaction with the sulfhydryl group of a critical cysteine residue, down-regulates (but does not completely shut off) receptor activity [7, 33]. The drug nitroglycerin, which generates NO-related species, can act at this site to limit excessive NMDA receptor activity. In fact, in rodent models, nitroglycerin can limit ischemic damage [193], and there is some evidence that patients taking nitroglycerin for other medical reasons may be resistant to glaucomatous visual field loss [194]. Consequently, we carefully characterized the S-nitrosylation sites on the NMDA receptor in order to determine if we could design a nitroglycerin-like drug that could be more specifically targeted to the receptor. In brief, we found that five different cysteine residues on the NMDA receptor could interact with NO. One of these, located at Cys<sup>399</sup> on the NR2A subunit of the NMDA receptor, mediates  $\geq 90\%$  of the effect of NO under our experimental conditions [79]. From crystal structure models and electrophysiological experiments, we further found that NO binding to the NMDA receptor at Cys<sup>399</sup> may induce a conformational change in the receptor protein that makes glutamate and Zn<sup>2+</sup> bind more tightly to the receptor. The enhanced binding of glutamate and Zn<sup>2+</sup> in turn causes the receptor to desensitize and, consequently, the ion channel to close [88]. Electrophysiological studies have demonstrated this inhibitory effect of NO on the NMDA receptor-associated channel [16, 69, 79]. Moreover, as the oxygen tension is lowered (a pO<sub>2</sub> of 10–20 torr is found in normal brain, and even lower levels under hypoxic/ischemic conditions), the NMDA receptor becomes more sensitive to inhibition by S-nitrosylation [195].

Unfortunately, nitroglycerin itself is not very attractive as a neuroprotective agent. The same cardiovascular vasodilator effect that makes it useful in the treatment of angina could cause dangerously large drops in blood pressure in patients with dementia, stroke, traumatic injury, or glaucoma. However, the open-channel block mechanism of memantine not only leads to a higher degree of channel blockade in the presence of excessive levels of glutamate but also can be used as a homing signal for targeting drugs, e.g., the NO group, to



hyperactivated, open NMDA-gated channels. We have therefore been developing combinatorial drugs (NitroMemantines) that theoretically should be able to use memantine to target NO to the nitrosylation sites of the NMDAR in order to avoid the systemic side effects of NO. Two sites of modulation would be analogous to having two volume controls on your television set for fine-tuning the audio signal.

Preliminary studies have shown NitroMemantines to be highly neuroprotective in both in vitro and in vivo animal models [7, 33, 196]. In fact, they appear to be more effective than memantine at lower dosage. Moreover, because of the targeting effect of the memantine moiety, NitroMemantines appear to lack the blood pressure lowering effects typical of nitroglycerin. More research still needs to be performed on NitroMemantine drugs, but by combining two clinically tolerated drugs (memantine and nitroglycerin), we have created a new, improved class of UFO drugs that should be both clinically tolerated and neuroprotective.

### Conclusive Remarks

Excessive nitrosative/oxidative stress triggered by excessive NMDA receptor activation and/or mitochondrial dysfunction may result in malfunction of the UPS, molecular chaperones, or mitochondrial fission-fusion dynamics, thus contributing to abnormal protein accumulation, synaptic damage, and neuronal injury in sporadic forms of neurodegenerative diseases. Our elucidation of an NO-mediated pathway to dysfunction of parkin, PDI and Drp1 by S-nitrosylation provides mechanistic a link between free radical production, abnormal protein accumulation, mitochondrial fragmentation, and neuronal cell injury in neurodegenerative disorders such as AD and PD. Elucidation of this new pathway may lead to the development of additional new therapeutic approaches to prevent aberrant protein misfolding or mitochondrial fragmentation by targeted disruption or prevention of nitrosylation of specific proteins such as parkin, PDI or Drp1, respectively. This article also describes the action of memantine via uncompetitive antagonism of the NMDA receptor with a fast off-rate. NitroMemantines enhance the neuroprotective efficacy over memantine at a given dose owing to its additional ability to S-nitrosylate the NMDA receptor. These drugs preferentially inhibit pathologically-activated NMDA receptor while preserving its normal synaptic function; thus, they are clinically tolerated. In this review, we propose that the next generation of CNS drugs will interact with their target only during states of pathological activation and not interfere with the target if it is functioning properly. In the future, such perspectives should lead to additional novel, clinically tolerated neuroprotective therapeutics.

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