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Review

S-Nitrosylation and uncompetitive/fast off-rate (UFO) drug therapy in neurodegenerative disorders of protein misfolding

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Although activation of glutamate receptors is essential for normal brain function, excessive activity leads to a form of neurotoxicity known as excitotoxicity. Key mediators of excitotoxic damage include overactivation of *N*-methyl-D-aspartate (NMDA) receptors, resulting in excessive Ca^{2+} influx with production of free radicals and other injurious pathways. Overproduction of free radical nitric oxide (NO) contributes to acute and chronic neurodegenerative disorders. NO can react with cysteine thiol groups to form S-nitrosothiols and thus change protein function. S-nitrosylation can result in neuroprotective or neurodestructive consequences depending on the protein involved. Many neurodegenerative diseases manifest conformational changes in proteins that result in misfolding and aggregation. Our recent studies have linked nitrosative stress to protein misfolding and neuronal cell death. Molecular chaperones – such as protein-disulfide isomerase, glucose-regulated protein 78, and heat-shock proteins – can provide neuroprotection by facilitating proper protein folding. Here, we review the effect of S-nitrosylation on protein function under excitotoxic conditions, and present evidence that NO contributes to degenerative conditions by S-nitrosylating-specific chaperones that would otherwise prevent accumulation of misfolded proteins and neuronal cell death. In contrast, we also review therapeutics that can abrogate excitotoxic damage by preventing excessive NMDA receptor activity, in part via S-nitrosylation of this receptor to curtail excessive activity.
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Excitotoxic activation of glutamatergic signaling pathways that lead to neuronal cell injury and death is thought to be an important contributor to neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), polyglutamine (polyQ) diseases such as Huntington's disease (HD), glaucoma, human immunodeficiency virus-associated dementia, multiple sclerosis, and ischemic brain injury, to name but a few.[1,2] Excessive activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors and subsequent Ca^{2+} influx at least in part mediates the toxicity evoked by the glutamate.[3] The intracellular Ca^{2+} triggers the generation of nitric oxide (NO) by activating neuronal NO synthase (nNOS) in a Ca^{2+}/calmodulin (CaM)-dependent manner.[4,5] Although physiological levels of NO contribute to preserving cellular functions, intense accumulation of nitrosative stress due to excessive generation of reactive nitrogen species (RNS) like NO is thought to play a causal role in 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 ($NO\cdot$), nitrosonium cation (NO^+), nitroxyl anion (NO^-, with high-energy singlet and lower energy triplet forms).[6] Accumulating evidence suggests that NO can mediate both protective and neurotoxic effects by reacting with cysteine residues of target proteins to form S-nitrosothiols (SNOs), a process termed S-nitrosylation because of its effects on the chemical biology of protein function. In addition, normal mitochondrial respiration also generates free radicals, principally reactive oxygen species (ROS), and one such molecule, superoxide anion (O_2^-) reacts rapidly with free radical NO to form the very toxic product peroxynitrite ($ONOO^-$).[6,7]

An important theme of this article is the hypothesis that key regulators of excitotoxic damage, such as NO and ROS, control neurodegeneration in the brain via SNO formation. In this review, we discuss specific examples:

(a) S-nitrosylation of NMDA receptors can positively influence neuronal survival;[6,8–11]
(b) S-nitrosylation of cysteine protease caspase blocks apoptotic cell death;[12–16]

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Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AMPA, α-amino–3-hydroxy-5 methyl-4-isoxazole propionic acid; CNS, central nervous system; ER, endoplasmic reticulum; GAPDH, 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; PDI, protein disulfide isomerase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNO, S-nitrosothiol; PD, Parkinson's disease; UPS, ubiquitin proteasome system
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(c) S-nitrosylation of parkin (a ubiquitin E3 ligase) and protein-disulfide isomerase (PDI, an endoplasmic reticulum (ER) chaperone) can be injurious by effecting accumulation of misfolded proteins in neurodegenerative diseases such as PD, AD, and other conditions;[17–20]
(d) S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) formed in the cytoplasm transduces an apoptotic signal into the nucleus of neuronal cells; [21] and
(e) S-nitrosylation of matrix metalloproteinase (MMP)-2/9 leads to a unique extracellular pathway contributing to excitotoxic neuronal cell damage.[22]

NMDA Receptor-Mediated Glutamatergic Signaling Pathways Induce Ca^{2+} Influx

The amino acid glutamate is the major excitatory neurotransmitter in the brain and is present in high concentrations in the mammalian central nervous system (CNS). Once released from nerve terminals in a Ca^{2+}-dependent manner, glutamate enters the synaptic cleft and 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, decreased reuptake or increased release of glutamate from glial cells or other sources during CNS insult can lead to excessive activation of glutamate receptors, and this process has been implicated in neuronal damage in many neurological disorders ranging from acute hypoxic–ischemic brain injury to chronic neurodegenerative diseases. In fact, even in the absence of excessive levels of glutamate, NMDA receptors can be overstimulated if physiological block of their associated ion channels by Mg^{2+} is compromised.[3] It is currently thought that overstimulation of extrasynaptic NMDA receptors mediate this neuronal damage, whereas, in contrast, synaptic activity may activate survival pathways.[23,24] 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.[25–27]

Two large families of glutamate receptors exist in the nervous system, ionotropic receptors (representing ligand-gated ion channels) and metabotropic receptors (coupled to G-proteins). Ionotropic glutamate receptors are further divided into three broad classes, NMDA receptors, α-amino-3-hydroxy-5 methyl-4-isoxazole propionic acid (AMPA) receptors, and kainate receptors, which are each named after a synthetic ligand that can selectively activate its receptor. 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 receptors. One such characteristic, in contrast to most AMPA and kainate receptors, 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 to relieve block of the receptor-associated ion channel by Mg^{2+}.[28,29] Subsequent binding of Ca^{2+} to various intracellular molecules can lead to many significant consequences (Figure 1). 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.[3,6,26,27,30,31]

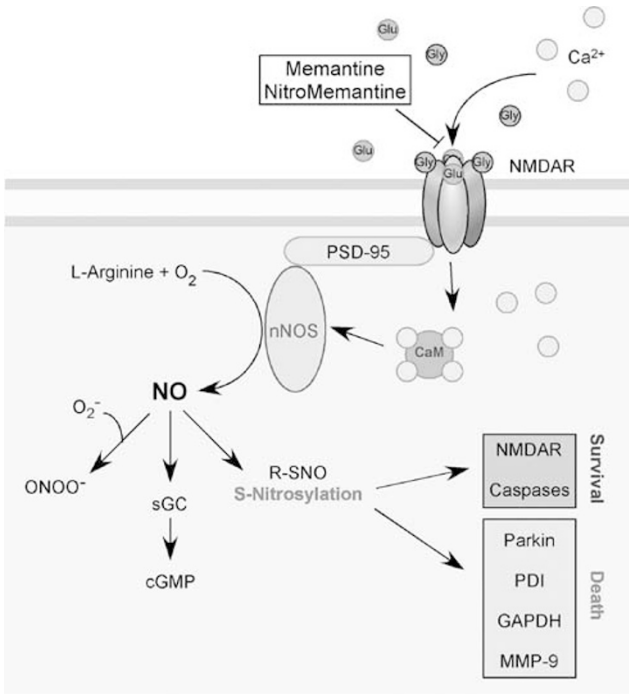

Figure 1 Activation of the NMDA receptor (NMDAR) by glutamate (Glu) and glycine (Gly) induces Ca^{2+} influx and consequent NO production via activation of nNOS. nNOS is part of a protein complex attached to the NR1 subunit of the NMDA receptor via binding of its PDZ domain to postsynaptic density protein (PSD-95). Many subsequent effects of NO are mediated by chemical, enzymatic, and redox reactions within neurons. NO activates soluble guanylate cyclase to produce cGMP, and cGMP can activate cGMP-dependent protein kinase. Excessive NMDA receptor activity, leading to the overproduction of NO can be neurotoxic. For example, S-nitrosylation of proteins such as parkin, PDI, GAPDH, and MMP-9 can contribute to neuronal cell damage and death. Neurotoxic effects of NO are also mediated by peroxynitrite ($ONOO^-$), a reaction product of NO and superoxide anion (O_2^-). In contrast, S-nitrosylation can mediate neuroprotective effects, for example, by inhibiting caspase activity and by preventing overactivation of NMDA receptors

Ca^{2+} Influx and Generation of NO

Increased levels of neuronal Ca^{2+}, in conjunction with the Ca^{2+}-binding protein CaM, trigger the activation of nNOS and subsequent generation of NO from the amino acid L-arginine (Figure 1).[4] 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. Three subtypes of NOS have been identified; two constitutive forms of NOS – nNOS and endothelial NOS – 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. All three isoforms are widely distributed in the brain. 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 can be detrimental to neuronal survival during various types of insult.[32,33] 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.[34–36]

Recent studies further pointed out the potential connection between ROS/RNS and mitochondrial dysfunction in neurodegenerative diseases, especially in PD.[37,38] Pesticide and other environmental toxins that inhibit mitochondrial complex I result in oxidative and nitrosative stress, and consequent aberrant protein accumulation.[17,19,20,39] Administration to animal models of complex I inhibitors, such as MPTP, 6-hydroxydopamine, rotenone, or paraquat, which result in overproduction of ROS/RNS, reproduces many of the features of sporadic PD, such as dopaminergic neuron degeneration, upregulation and aggregation of α-synuclein, Lewy body-like intraneuronal inclusions, and behavioral impairment.[37,38] Additionally, it has recently been proposed that mitochondrial cytochrome oxidase can produce NO in a nitrite (NO_2^-)- and pH-dependent but non-Ca^{2+}-dependent manner.[40]

Protein S-Nitrosylation Affects Neuronal Survival

Early investigations indicated that the NO group mediates cellular signaling pathways, which regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death.[30,41–43] In general, NO exerts physiological and some pathophysiological effects via stimulation of guanylate cyclase to form cyclic guanosine-3′, 5′-monophosphate (cGMP) or through S-nitros(yl)ation of regulatory protein thiol groups.[5,6,44,45] S-nitrosylation is the covalent addition of an NO group to a critical cysteine thiol/sulfhydryl (RSH or, more properly, thiolate anion, RS^-) to form an SNO derivative (R-SNO). Such modification modulates the function of a broad spectrum of extracellular, cytoplasmic, membrane, and nuclear 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.[46,47] 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 parkin, PDI, GAPDH, and MMP-9) (Figure 1).[6,8,9,12,14,16,17,19–22,48] 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.[10,49–54] 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 ($-SO_2H$), or sulfonic ($-SO_3H$) acid derivatization of the protein.[19,20,22,55] Alternatively, S-nitrosylation may possibly produce a nitroxyl disulfide, in which the NO group is shared by close cysteine thiols.[19,56]

Although the involvement of NO in neurodegeneration has been widely accepted, the chemical relationship between nitrosative stress and neuronal cell death has remained obscure. Recent findings, however, have shed light on molecular events underlying this relationship. Specifically, we and other groups have recently mounted physiological and chemical evidence that S-nitrosylation enhances (1) neuronal survival by inhibiting the activities of (a) NMDA receptors and (b) caspases, or (2) neuronal cell injury by regulating the (a) ubiquitin E3 ligase activity of parkin,[17,18,20] (b) chaperone and isomerase activities of PDI,[19] (c) nuclear translocation of GAPDH,[21] and (d) activity of MMP-9.[22] In particular, S-nitrosylation of PDI and parkin can regulate protein misfolding and neurotoxicity in models of neurodegenerative disorders, and SNO-PDI, or SNO-parkin has been found in human postmortem brain tissue from patients with neurodegenerative conditions.[17–20] In addition, NO controls survival of non-neuronal cells by S-nitrosylating thioredoxin (TRX), apoptosis signal-regulating kinase 1, JNK, Ras, NF-κB, IKKβ, Akt, FLICE inhibitory protein, and Bcl-2.[46,53,57] These findings lead to the question of whether S-nitrosylation of these proteins can also affect neuronal cell death.

Protein Misfolding and Neurodegeneration

A common sign of many neurodegenerative diseases is the accumulation of misfolded proteins that adversely affect neuronal function, connectivity and plasticity, and trigger cell death signaling pathways.[58,59] For example, degenerating brain contains aberrant accumulations of misfolded, aggregated proteins, such as α-synuclein and synphilin-1 in PD, and amyloid-β (Aβ) and tau in AD. Other diseases with aggregates or inclusion bodies include Huntington's (polyQ), ALS, and prion diseases.[60] 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) is 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 or proteasome activities. Currently, soluble oligomers of these aberrant proteins are thought to be the most toxic forms via interference with normal cell activities, whereas frank aggregates may be an attempt by the cell to wall off potentially toxic material.[61] Interestingly, neurotoxicity elicited by some aberrant proteins, such as Aβ_{1-42} peptide in AD, is thought to augment NMDA-type glutamate receptor activity and intracellular Ca^{2+} levels (reviewed in Lipton[1]). Furthermore, increased free Ca^{2+} levels and consequent nitrosative stress are associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates.[62,63] However, until recently, little was known regarding the molecular mechanism underlying the contribution of Ca^{2+}

and NO to formation of aggregates such as amyloid plaques in AD or Lewy bodies in PD.

S-Nitrosylation as a Potential Negative Regulator of Excitotoxic Signaling

S-Nitrosylation of the NMDA receptor downregulates its activity. nNOS binds to an adaptor protein PSD-95, which interacts with the C-terminus of the NMDA receptor, thus facilitating colocalization of these proteins and the local production of NO to S-nitrosylate the NMDA receptor. *In vitro* studies using recombinant NR1 and NR2A subunits of the NMDA receptor showed that S-nitrosylation of the receptor decreased the amplitude of its responses.[6,8,10,64] The NMDA receptor can be polynitrosylated at five cysteine residues of the NMDA receptor: Cys744 and Cys798 of NR1, and Cys87, Cys320, and Cys399 of the NR2A subunit.[8] Of these, Cys399 of NR2A mediates the predominant inhibitory effect of S-nitrosylation. S-nitrosylation of the other four cysteines facilitates the formation of disulfide bonds, also decreasing NMDA receptor function. Physiological levels of Zn^{2+} represent another inhibitory modulator of the NMDA receptor, and S-nitrosylation of the receptor can increase the effect of Zn^{2+} inhibition. Inhibition by Zn^{2+} involves enhanced glutamate affinity and receptor desensitization. Therefore, it has been postulated that S-nitrosylation of these thiol groups (particularly Cys399 on NR2A) allosterically regulates the quaternary organization of the receptor, thereby promoting ligand binding and desensitization of the NMDA receptor. Recently, we have found that relatively hypoxic conditions render the NMDA receptor exquisitely sensitive to S-nitrosylation of Cys744 and Cys798 on the NR1 subunit.[65] NO modification of these two thiols in NR1 further enhances S-nitrosylation of the Cys399 site on the NR2A, resulting in the inhibition of receptor activity by very low levels of NO. This finding highlights the physiological relevance of S-nitrosylation of the NMDA receptor because brain oxygen levels are normally low under physiological conditions (pO_2 of 10–20 mmHg) and even lower during hypoxia/ischemia (stroke). Moreover, it also suggests that these two cysteine residues on NR1 constitute a novel 'NO-reactive oxygen sensor motif' that controls the degree of nitrosylation-mediated inhibition of NMDA receptors in the hypoxic brain.

S-Nitrosylation of caspases inhibits apoptotic neuronal cell death. Caspases constitute a family of cysteine proteases, and many of them probably play an important role in the initiation or execution of apoptosis. Excitotoxic injury can be in part mediated by caspases.[16,27] It has been shown that NO can S-nitrosylate the catalytic cysteine of most or all caspases.[12–16] S-Nitrosylation inhibits protease activity of caspases and consequently prevents apoptotic death in many cell types. All caspases are expressed in cells as catalytically inactive zymogens and encounter proteolytic activation to form active enzymes during apoptosis. Mannick *et al*.[14] reported that NO constitutively S-nitrosylates the proform of caspases; however, following a variety of apoptotic stimuli, caspases are selectively de-nitrosylated. In some cases, de-nitrosylation can also trigger dissociation of protein-protein interactions if the interaction was S-nitrosylation-dependent.[66] Inhibition of caspase activity by S-nitrosylation accounts for one mechanism whereby S-nitrosylation prevents apoptotic cell death.

S-Nitrosylation as a Potential Positive Regulator of Excitotoxic Signaling

Nitrosative stress impairs protein ubiquitination in PD models via S-nitrosylation of parkin. 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 and oxidative stress are potential causal factors for protein accumulation in the much more frequent 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.[17,18,20]

Recently, Fallon *et al*.[67] reported that the protein parkin can mediate neuronal survival via a proteasome-independent pathway. In this model, parkin monoubiquitinates the epidermal growth factor receptor (EGFR)-associated protein, Eps15, leading to inhibition of EGFR endocytosis. The resulting prolongation of EGFR signaling via the phosphoinositide-3 kinase/Akt (PKB) signaling pathway is postulated to enhance neuronal survival. However, we and others have shown that S-nitrosylation and further oxidation of parkin, a ubiquitin E3 ligase, or UCH-L1, a deubiquitinating enzyme that recycles ubiquitin, results in dysfunction of these enzymes and thus of the UPS.[17,20,68–71] We found 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 Lewy bodies. SNO-parkin initially stimulates ubiquitin E3 ligase activity, resulting in enhanced ubiquitination, as observed in Lewy bodies, followed by a decrease in enzyme activity, producing a futile cycle of dysfunctional UPS (Figure 2).[17,18,20] Additionally, S-nitrosylation appears to compromise the neuroprotective effect of parkin.[17] It is likely that other ubiquitin E3 ligases with similar RING-finger thiol motifs are S-nitrosylated in a similar manner 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).[72] DA can be oxidized to DA quinone, which can react with and inactivate proteins through covalent modification of cysteine sulfhydryl groups; peroxynitrite has been reported to promote oxidation of DA to form DA quinone.[73] La Voie *et al*.[72] showed that DA quinone can attack one or more cysteine residues in the RING domain(s) of parkin, forming a covalent adduct that abrogates

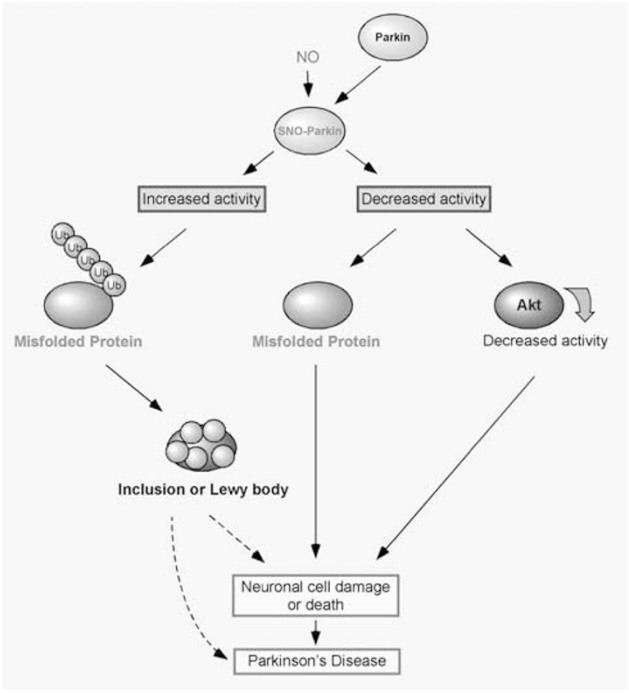

Figure 2 Possible mechanism of S-nitrosylated parkin (SNO-parkin) contributing to the accumulation of aberrant proteins and damage or death of dopaminergic neurons. Nitrosative stress leads to S-nitrosylation of parkin, and, initially, to a dramatic increase followed by a decrease in its E3 ubiquitin ligase activity.[17,18,20] The initial increase in this E3 ubiquitin ligase activity leads to enhanced ubiquitination of parkin substrates (e.g., synphilin-1, Pael-R, and parkin itself). Increased parkin E3 ubiquitin ligase activity may contribute to Lewy body formation and impair parkin function, as also suggested by Sriram *et al.*[112] The subsequent decrease in parkin activity may allow misfolded proteins to accumulate. Downregulation of parkin may also result in a decrease in the neuroprotective activity of Akt because of enhanced EGFR internalization and thus less Akt signaling.[67] DA quinone can also modify the cysteine thiols of parkin and reduce its activity.[72] Ub: ubiquitin

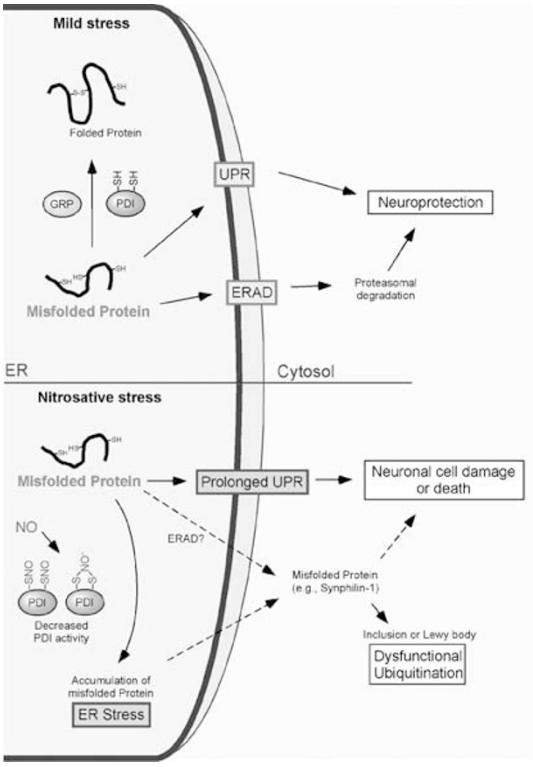

Figure 3 Possible mechanism of S-nitrosylated PDI (SNO-PDI) contributing to the accumulation of aberrant proteins and neuronal cell damage or death. ER stress is triggered when misfolded proteins accumulate within the ER lumen, inducing the UPR. The UPR is usually a transient homeostatic mechanism for cell survival, whereas a prolonged UPR elicits neuronal cell death. PDI modulates the activity of UPR sensors by mediating proper protein folding in the ER. Proteins that fail to attain their native folded state are eventually retrotranslocated across the ER membrane to be disposed of by cytosolic proteasomes. This process, known as ER-associated degradation, is essential in preventing protein accumulation and aggregation in the ER. Under conditions of severe nitrosative stress, S-nitrosylation of neuronal PDI inhibits normal protein folding in the ER, activates ER stress, and induces a prolonged UPR, thus contributing to aberrant protein accumulation and cell damage or death. For simplicity, S-nitrosylation of only one (of two) TRX domains of PDI is shown, resulting in formation of SNO-PDI or possibly nitroxyl-PDI, as described in Uehara *et al.*[19] and Forrester *et al.*[113]

its E3 ubiquitin ligase activity. DA quinone also reduced the solubility of parkin, possibly inducing parkin misfolding after disruption of the RING domain(s). Therefore, oxidative/nitrosative 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.

S-Nitrosylation of PDI mediates protein misfolding and neurotoxicity in cell models of PD or AD. The ER normally participates in protein processing and folding but undergoes a stress response when immature or misfolded proteins accumulate.[74–76] ER stress stimulates two critical intracellular responses (Figure 3). The first represents expression of chaperones that prevent protein aggregation via the unfolded 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. A second ER response involves attenuation of protein synthesis via eukaryotic initiation factor-2 kinase.[55,77–79] 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 catalyzes thiol/disulfide exchange, thus facilitating disulfide bond formation, rearrangement reactions, and structural stability.[80] PDI has two domains that are homologous to the small, redox-active protein TRX.[81] The two thiol/disulfide centers of these TRX-like domains function as independent active sites.[82] Accumulation of immature and denatured proteins results in ER dysfunction in brains of AD, PD, ALS, prion disease, cerebral ischemia, and possibly other neurodegenerative disorders, but upregulation of PDI represents an adaptive response promoting protein refolding and may offer neuronal cell protection.[83–90]

Recently, we reported that excessive NO can lead to S-nitrosylation of the active site thiol groups of PDI, and

this reaction inhibits both its isomerase and chaperone activities.[19] Moreover, we found that PDI is S-nitrosylated in the brains of AD and PD patients. 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 Ca^{2+} influx and NO production. 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 Lewy body inclusions.[19,91] S-nitrosylation of PDI also prevents its attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition (Figure 3). Further evidence suggests that PDI may transport NO to the extracellular space, where it could conceivably exert additional adverse effects.[51]

In addition to PDI, S-nitrosylation is likely to affect critical thiol groups on other chaperones, such as heat-shock protein (HSP) 90 in the cytoplasm[92] and possibly GRP in the ER. Normally, HSP90 stabilizes misfolded proteins and modulates the activity of cell signaling proteins, including NOS and calreticulin.[59] In AD brains, levels of HSP90 are increased in both the cytosolic and membranous fractions, where HSP90 is thought to maintain tau and Aβ in a soluble conformation, thereby averting their aggregation.[93,94] Martínez-Ruiz *et al*.[92] 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 suggest that S-nitrosylation of HSP90 in neurons of AD brains may possibly contribute to the accumulation of tau and Aβ aggregates.

The UPS is apparently impaired in the aging brain; additionally, inclusion bodies similar to those found in neurodegenerative disorders can appear in brains of normal aged individuals or those with subclinical manifestations of disease.[95] These findings suggest that the activity of molecular chaperones and the UPS may decline in an age-dependent manner.[96] Given that SNO-parkin and SNO-PDI do not exist in detectable quantities in normal aged brain,[17,19,20] we speculate that S-nitrosylation of these and similar proteins may represent a key event that contributes to susceptibility of the aging brain to neurodegenerative conditions.

S-Nitrosylation of GAPDH triggers binding to Siah, nuclear translocation, and neuronal apoptosis. In addition to its well-known role in glycolysis, GAPDH contributes to nuclear signaling pathways that initiate apoptotic cascades.[97,98] Translocation of GAPDH from the cytosol to the nucleus occurs in a number of cell systems during apoptosis.[97,98] Moreover, recent work reported a genetic association between the *GAPDH* locus on chromosome 12 and an elevated risk of late-onset AD, suggesting a possible contribution of GAPDH-mediated neurotoxicity to neurodegeneration.[99] However, the sequence encoding GAPDH lacks a nuclear localization signal, and therefore the molecular pathway to cell death has remained enigmatic. Hara *et al*.[21] recently found a regulated pathway in which NO generated in neurons S-nitrosylates the catalytic cysteine of GAPDH (Cys152 for human, and Cys150 for mouse and rat), enabling its cytotoxic activity (Figure 4). S-Nitrosylation affords GAPDH the ability to bind the ubiquitin E3 ligase Siah1, which harbors a nuclear localization signal, thereby escorting GAPDH into the nucleus.[21] In the nucleus, GAPDH stabilizes Siah1, enabling the degradation of nuclear proteins, such as nuclear receptor corepressor (N-CoR), via the ubiquitin E3 ligase activity of Siah1. Targeting nuclear proteins for degradation by Siah1 can contribute to cell death. Importantly, when GAPDH or Siah1 is depleted from primary cerebellar granule neurons by RNA interference, NMDA-induced neurotoxicity is markedly attenuated.

The SNO-GAPDH cascade may play a role in the pathophysiology of several neurodegenerative diseases. Recent studies have shown in a cell culture model of HD that mutant Huntingtin protein (mtHtt) can form a ternary complex with GAPDH and Siah1.[100] Expansion of the polyQ repeats in the N-terminal domain of mtHtt is the cause of autosomal dominant HD. Although mtHtt lacks a nuclear localization signal, mtHtt can translocate to the nucleus and produce neurotoxicity in cell culture models.[101–104] Reportedly, the mtHtt/SNO-GAPDH/Siah1 complex translocates to the nucleus, enabling mtHtt to contribute to toxicity.[100] Supporting

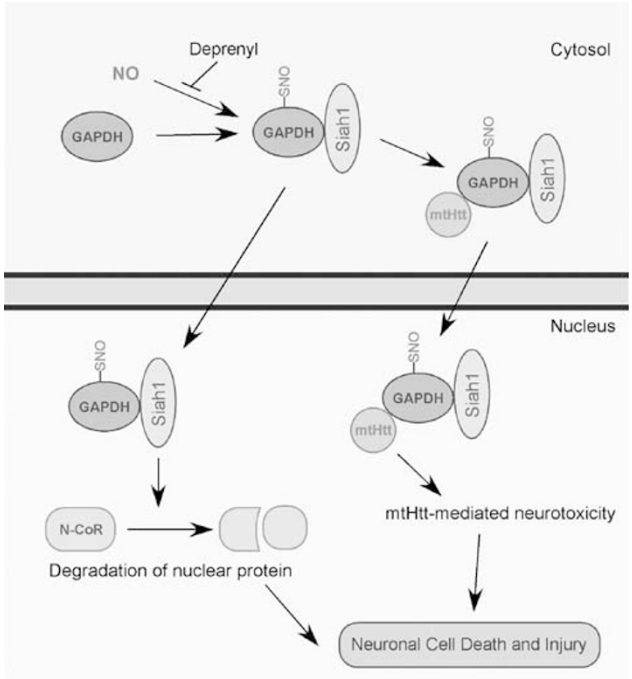

Figure 4 Possible mechanism of S-nitrosylated GAPDH (SNO-GAPDH) contributing to neuronal cell damage or death. Excess generation of NO caused by excitotoxic stress can S-nitrosylate GAPDH in the cytosol. S-nitrosylation of GAPDH promotes interaction between GAPDH and Siah, an E3 ubiquitin ligase. The nuclear localization signal of Siah1 allows nuclear translocation of the GAPDH/Siah complex. In the nucleus, GAPDH stabilizes Siah1, leading to the degradation of nuclear proteins such as nuclear receptor corepressor (NCo-R). GAPDH can also associate with mtHtt, and S-nitrosylated GAPDH thus brings mtHtt into the nucleus via Siah. As a consequence of the nuclear translocation of (mtHtt)/SNO-GAPDH/Siah1, neuronal cells undergo cell death. Deprenyl, a drug used for the treatment of early-stage PD, can help maintain dopaminergic neurons at least in part via binding to GAPDH and hence preventing its nitrosylation

these findings, Senatorov *et al*.[105] found nuclear accumulation of GAPDH in a transgenic model of HD. Additionally, SNO-GAPDH could also contribute to the pathogenesis of PD. *R*-(–)-deprenyl (Selegiline), which may ameliorate the progression of early stage PD, appears to prevent S-nitrosylation of GAPDH both in cellular and animal models of PD.[106] Initially, the effects of Deprenyl were thought to depend on inhibition of monoamine oxidase-B (MAO-B); however, recent research has suggested that Deprenyl increases survival of dopaminergic neurons independently of MAO-B inhibition, and GAPDH represents a possible target for the beneficial effect of Deprenyl.[107] It is postulated that the binding of Deprenyl to GAPDH interferes with the formation of SNO-GAPDH and its interaction with Siah1, thereby affording neuroprotection.

S-Nitrosylation of MMP-9 contributes to excitotoxicity in models of cerebral ischemia. Activation of specific MMPs is thought to play a deleterious role in brain injury during and after cerebral ischemia (stroke). In particular, after stroke human brain manifests augmented expression of MMP-9.[108] Our group has shown in animal models that MMP-9 colocalizes with nNOS during cerebral ischemia, leading to activation of MMP enzymatic activity via the formation of S-nitrosylated MMP-9 (SNO-MMP).[22] The critical cysteine residue that is S-nitrosylated in this reaction is located in the autoinhibitory region of MMPs that normally coordinates a zinc atom and thus inhibits enzymatic activity. Disturbance of the Cys-Zn^{2+} interaction via S-nitrosylation of the cysteine thiol activates the proform of MMPs by a mechanism known as the cysteine switch. Additionally, facile S-nitrosylation of this critical cysteine residue on MMP-9 can lead to further oxidation reactions, resulting in the formation of stable sulfinic ($-SO_2^-$) or sulfonic ($-SO_3^-$) acid derivatives and irreversible activation of the MMP. Thus, S-nitrosylation and subsequent oxidation can result in pathological activation of MMPs, contributing to neuronal injury and death during stroke.[22]

Potential Treatment of Excessive NMDA-Induced Ca^{2+} Influx and Free Radical Generation

One mechanism that could potentially curtail excessive Ca^{2+} 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, that is, 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.[1,109] 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. Memantine has been used for many years in Europe to treat PD, and 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.

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.

Future Therapeutics: NitroMemantines

NitroMemantines are second-generation memantine derivatives that are designed to have enhanced neuroprotective efficacy without sacrificing clinical tolerability. As mentioned earlier, a nitrosylation site(s) is located on the extracellular domain of the NMDA receptor, and S-nitrosylation of this site, that is, NO reaction with the sulfhydryl group of a critical cysteine residue, down-regulates (but does not completely shut off) receptor activity (Figure 1).[1] 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,[110] and there

is some evidence that patients taking nitroglycerin for other medical reasons may be resistant to glaucomatous visual field loss.[111] 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 cysteine residue #399 (Cys399) on the NR2A subunit of the NMDA receptor, mediates $\geqslant$90% of the effect of NO under our experimental conditions.[8] From crystal structure models and electrophysiological experiments, we further found that NO binding to the NMDA receptor at Cys399 may induce a conformational change in the receptor protein that makes glutamate and Zn^{2+} bind more tightly to the receptor. The enhanced binding of glutamate and Zn^{2+} in turn causes the receptor to desensitize and, consequently, the ion channel to close.[10] Electrophysiological studies have demonstrated this inhibitory effect of NO on the NMDA receptor-associated channel.[6,8,44] Moreover, as mentioned earlier, as the oxygen tension is lowered (a pO_2 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.[65]

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, for example, 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 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.[1] 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.

Conclusions

Free radical production, including ROS and RNS, may increase during the aging process, rendering the brain more vulnerable to neurodegenerative conditions. However, S-nitrosylation has both positive and negative effects on excitotoxic signaling pathways that contribute to the pathophysiology of many neurological disorders. For example, S-nitrosylation of NMDA receptors and caspases by physiological levels of NO can promote neuronal survival and therefore may represent antineurodegenerative mechanisms. In contrast, excessive nitrosative stress triggered by excitotoxic stimuli may result in dysfunction of ubiquitination and molecular chaperones, thus contributing to abnormal protein accumulation and neuronal damage in sporadic forms of several neurodegenerative diseases. For instance, our elucidation of an NO-mediated pathway to dysfunctional parkin and PDI by S-nitrosylation provides a mechanistic link between free radical production, abnormal protein accumulation, and neuronal cell injury in neurodegenerative disorders such as PD. Increasing NO levels during excitotoxic insult also result in S-nitrosylation of GAPDH and MMP-9, contributing to neuronal cell death and injury. Elucidation of these new NO-mediated degenerative pathways may lead to the development of new therapeutic approaches to prevent aberrant protein misfolding. For example, targeted prevention of nitrosylation of specific proteins such as parkin or PDI would be expected to be neuroprotective. Additionally, selective inhibition of hyperactivated NMDA receptors by memantine or the newer NitroMemantine drugs can exert neuroprotective effects without disrupting normal synaptic function. Memantine is thus clinically tolerated, is currently approved for moderate to severe AD, and is being tested for other neurological diseases. The second-generation drugs, termed NitroMemantines, offer promise for improved efficacy.

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