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Hidden Networks of Aberrant Protein Transnitrosylation Contribute to Synapse Loss in Alzheimer's Disease

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Abstract

Emerging evidence indicates the importance of *S*-nitrosation in regulating protein function and activity. This chemical reaction has been termed protein *S*-nitrosylation to emphasize its biological importance as a posttranslational modification, in some ways reminiscent of phosphorylation. The reaction at cysteine thiols is distinct from other chemical reactions of nitric oxide (NO) that activate soluble guanylate cyclase via nitrosylation of heme or formation of peroxynitrite via reaction with superoxide anion to produce tyrosine nitration. Here, we review the importance of pathological, aberrant transnitrosylation reactions, i.e., transfer of the NO group from one protein to another, and its consequent effect on the pathogenesis of neurological disorders, to date on Alzheimer's disease (AD), but also expected to affect Parkinson's disease (PD)/Lewy body dementia (LBD), HIV-associated neurocognitive disorder (HAND), and other neurodegenerative and neurodevelopmental disorders.

1. Introduction

Regulatory *S*-nitrosothiol formation (generating RSNO), subsequently designated protein *S*-nitrosylation, modulates protein function, akin to *O*-phosphorylation [1]. The discovery of this reaction as a regulatory system for protein function was first reported on the *N*-methyl-D-aspartate (NMDA)-subtype of glutamate receptor in the brain [2, 3]. Subsequently, this field has exploded to demonstrate that ubiquitous protein *S*-nitrosylation on a multitude of targets plays important roles in both normal and aberrant cell signaling in health and disease (reviewed in [4–7]).

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Declaration of competing interest

The author discloses that he is an inventor on worldwide patents for the use of memantine and NitroSynapsin for neurodegenerative and neurodevelopmental disorders. Per Harvard University guidelines, he participates in a royalty-sharing agreement with his former institution, Boston Children's Hospital/Harvard Medical School, which licensed the FDA-approved drug memantine (Namenda®) to Forest Laboratories, Inc./Actavis/Allergan/AbbVie. He is a scientific founder of Adamas Pharmaceuticals, Inc. (now owned by Supernus Pharmaceuticals, Inc.), which developed or comarkets FDA-approved forms of memantine- or amantadine-containing drugs (NamendaXR®, Namzaric®, and GoCovri®), and of EuMentis Therapeutics, Inc., which has licensed NitroSynapsin and related aminoadamantane nitrates from the author's laboratory.

1.1 Chemistry of S-nitrosylation and transnitrosylation reactions

Chemically, to account for the electrons involved in the reaction, there are various proposed mechanisms for protein *S*-nitros(yl)ation (reviewed by [8]). These include oxidation of nitric oxide (NO·) to N_2O_3 (with NO^+-like character [which is NO· with one less electron in its outer pi molecular orbital]) followed by reaction with thiolate ($R\text{-}S^-$), radical reaction of NO· with thiyl radical, transition metal mediated transfer of NO^+-like species to thiolate anion, and transnitros(yl)ation (transfer of the NO group from one protein thiolate to another). In this latter case, the free thiolate anion (RS^-) of one protein initiates nucleophilic attack on the nitroso nitrogen of a second, *S*-nitrosylated protein (RSNO). Additionally, *S*-nitrosylation can lead to further oxidation reactions and more permanent changes of protein structure and function, for example to RSOH, RSO_2H, and RSO_3H [9].

1.2 Aberrant protein transnitrosylation in neurodegenerative disorders

In this review, we focus on aberrant protein transnitrosylation reactions that have been shown to be causal in the pathogenesis of neurological diseases. The prototypical example of a network of such reactions is represented by transfer of RSNO down the pathway from SNO-Uch-L1 to SNO-Cdk5 to SNO-Drp1, resulting in synaptic loss in AD [10–13]. This reaction stream connects mechanistically-distinct types of enzymes, i.e., a ubiquitin protein hydrolase (Uch-L1), a kinase (Cdk5), and a GTPase (Drp1), that are involved in disparate biochemical pathways, but can act in concert to mediate a completely different type of reaction activity, i.e., transnitrosylation. We have shown that each enzyme manifests this second, non-canonical function, triggering a pathological biochemical cascade in AD [13]. The resulting series of transnitrosylation reactions contributes to synaptic loss, the major pathological correlate to cognitive decline in AD. We conclude that enzymes with distinct primary reaction mechanisms can form a completely separate network for transnitrosylation. Given that this hidden or “dark” network of activity operates in the post-reproductive period in the shadow of neurodegenerative disorders associated with aging, natural selection pressure may be lessened on the aberrant alternative function. For this reason, these aberrant reactions may persist as humans evolved into living for a longer period of time. In fact, the term “ghost pathway” has been used to describe such actions since it operates in the unseen background of the canonical function of each enzyme, manifesting itself in this discreet form only during aging and neurodegeneration, particularly in Alzheimer’s disease brains (Figure 1) [13].

2. Kinetic and thermodynamic considerations for protein transnitrosylation

The processes of protein *S*-nitrosylation, denitrosylation, and transnitrosylation can either by non-enzymatic, e.g., influenced by mass action of abundant SNO-proteins like Uch-L1 [13], or enzymatic, with prime examples of *S*-nitroso glutathione reductase (GSNOR) acting as a denitrosylase [14, 15] and thioredoxin (Trx) as an *S*-nitrosylase under specified conditions [16]. To prove enzymatic behavior, kinetic studies are needed, showing formal derivation of rate constants and turnover number (kcat) to support an enzymatic process, i.e., showing that catalysis is kinetically sped up in the presence of the enzyme [13, 16]. To date, such data for

protein *S*-nitrosylation has only been obtained in a very limited number of cases (e.g., [16]). Similar to phosphorylation, possible motifs for *S*-nitrosylation have also been proposed, i.e., specific amino acid residues surrounding the reactive cysteine; nonetheless, the mechanism and the motifs involved are not yet completely understood [9, 17–19].

2.1 Kinetic considerations

As an example of an aberrant network of transnitrosylation reactions, our group recently described transfer of an NO group from SNO-Uch-L1 to Cdk5 and then to Drp1. We performed initial kinetic assays in cell-based systems using the biotin-switch assay to monitor *S*-nitrosylation of each protein. For example, we found evidence for formation of both SNO-Uch-L1 and SNO-Cdk5 in cell-based systems within 10 min of exposure to the physiological NO donor and transitrosylating agent, *S*-nitrosocysteine (SNOC). This was followed by increasing formation of SNO-Drp1 at 25 min, concurrently with a relative decrease in the amount of SNO-Cdk5 [13].

From this work, we propose that a cascade of transnitrosylation reactions, such as that represented by SNO-Uch-L1/SNO-Cdk5/SNO-Drp1, can possibly be comprised of a combination of enzymatic and non-enzymatic processes. For example, Uch-L1 is one of the most abundant neuronal proteins, and therefore high levels of SNO-Uch-L1 could possibly drive *S*-nitrosylation of Cdk5 by mass action alone. But the fact that in our kinetic experiments the presence of SNO-Cdk5 facilitated subsequent formation of *S*-nitrosylated Drp1 [13] could indicate that Cdk5 is acting as a catalyst for the overall reaction, transferring SNO from Uch-L1 to Drp1. In favor of this interpretation, the addition of SNO-Cdk5 to the reaction mixture of Uch-L1 and Drp1 always resulted in the transfer of SNO to Drp1 within minutes of incubation rather than to Uch-L1. Further study will be needed to clarify this possible alternative enzymatic action of Cdk5 as an *S*-nitrosylase in the transnitrosylation reaction mechanism rather than its canonical kinase action.

2.2 Thermodynamic considerations

With pathologically high levels of reactive nitrogen species (RNS), as may occur in degenerative diseases or possibly with aging, it should become more likely that aberrant *S*-nitrosylation of a particular protein might occur. The neurodegenerative disease process often goes on for many years. Under these conditions, it may be reasonable to assume that these redox reactions might go to steady state. This assumption is important as it allows us to quantitatively evaluate a single reaction pair or a cascade of transnitrosylation reactions among multiple proteins. We have developed such a quantitative method by adapting the principles of the Nernst equation to perform thermodynamic assessment of the reactions at steady state. We acknowledge, however, that these thermodynamic considerations assume steady state or at least approaching steady state, which may not hold in individual cells.

As we recently described [13], the Nernst equation quantifies the electromotive force for net electron movement between a redox pair. To calculate the difference in the electromotive force (ΔE) between two such redox pairs involved in transnitrosylation, for example, between Uch-L1 and Cdk5, we can calculate the difference in the values of their respective Nernst equations:

$$E^{0'}_{\mathrm{UchL1}} - E^{0'}_{\mathrm{Cdk5}} = -\frac{RT}{zF} \cdot \ln\left(\frac{[\mathrm{UchL1_{SNO}}][\mathrm{Cdk5_{red}}]}{[\mathrm{UchL1_{red}}][Cdk5_{SNO}]}\right) = 74.02 \quad mV \qquad \text{Eqn. 1}$$

$$\Delta G^{0'} = -RT \cdot \ln\left(\frac{[\mathrm{UchL1_{red}}][\mathrm{Cdk5_{SNO}}]}{[\mathrm{UchL1_{SNO}}][\mathrm{Cdk5_{red}}]}\right) = -18.35 \quad kJ/mol \qquad \text{Eqn. 2}$$

where 'SNO' represents the oxidized species (in this case, *S*-nitrosylated) and 'red' is the chemically reduced protein, as obtained from densitometric analysis of redox immunoblots. An important consideration in this quantification is that the Nernst equation uses log values, so the difference in Nernst equations represents the natural log of the ratio of effective concentrations of the reduced and oxidized forms of the protein. Thus, we do not need to know the absolute concentration of reduced and oxidized proteins, as only the ratio is required for the calculation. This fact allows us to quantify the relative redox potential for the reaction.

With this method, we found that transnitrosylation from SNO-Uch-L1 to Cdk5 is very energetically favorable in intact cells (from Eqn. 1, $\Delta E^{o'}$ = 74.02 ± 4.60 mV). Knowing the electromotive force allows us to calculate the associated Gibbs free energy (from Eqn. 2, $\Delta G^{o'}$ = −18.35 ± 1.14 kJ/mol). A Gibbs free energy approaching −20 kJ/mol indicates that the reaction is thermodynamically very favorable and, if kinetic considerations permit, will proceed spontaneously. Moreover, similar calculations for the subsequent transnitrosylation reaction from SNO-Cdk5 to Drp1 also showed it to be energetically favorable [12]. Taken together, these thermodynamic considerations show that the transnitrosylation reactions from SNO-Uch-L1 to SNO-Cdk5, and then to SNO- Drp1 are all energetically favorable [10, 12, 13].

3. Unbiased analysis of the *S*-nitrosoproteome

To discover additional transnitrosylation pathways, it will be critical to identify *S*-nitrosylated proteins in a more comprehensive manner. Various biochemical methods have been developed to detect *S*-nitrosylated proteins in such a systematic fashion. Here, we briefly review recent advances using mass spectrometry to detect and quantify these reactions in an unbiased manner. While no approach is completely unbiased, relatively unbiased analysis of the *S*-nitrosoproteome can be performed with specialized probes for *S*-nitrosothiols, such as triarylphosphine coupled with mass spectrometry (MS) of flash frozen human or mouse postmortem brain with a technique designated *SNO*TRAP [20, 21]. Another approach is chemoselective enrichment for *S*-nitrosothiols, e.g., with an organomercury resin, followed by MS analysis of peptides [22–24]. Other approaches using MS have been used, including resin-assisted capture known as *SNO*-RAC [25] and *SNO*SID [26], each of which builds on the biotin-switch technique to enrich for previously biotin-labeled SNO-cysteine sites [25] or using isobaric mass tag labeling with iodoTMT™ of these cysteines [27]. Because the triarylphosphine group of the *SNO*TRAP method reacts directly with S-nitrosothiols, as opposed to indirect detection, for example, by replacing SNO sites with biotin after ascorbate treatment, *SNO*TRAP may offer deeper coverage of SNO-sites, especially for complex tissues like the brain with many cell types.

To date, these studies have reported some ~2,000 proteins that are *S*-nitrosylated in either normal or diseased tissues, including brains with neurodegenerative disorders such as Alzheimer's disease (AD) and HIV-associated neurocognitive disorder (HAND) compared to controls [28]. However, not yet understood is the finding that *S*-nitrosylation is upregulated for some networks of proteins in diseased compared to normal conditions, while other proteins exhibit decreased *S*-nitrosylation. The former -- an increase in *S*-nitrosylation status -- can be envisaged to occur with the increased levels of NO found in many diseased tissues, but whether the latter – a decrease in the level of *S*-nitrosylated protein -- represents a compensatory/homeostatic change or a primary feature of the disease remains to be determined. Moreover, except in a few cases, it is not yet known if these *S*-nitrosylation events represent pathogenic changes in the disease process or just epiphenomena. Nonetheless, several examples show that aberrantly *S*-nitrosylated proteins can be causal in disease pathogenesis [6, 9, 10, 13, 29].

A recent and illustrative review comparing SNO-proteome techniques looked at the *S*-nitrosylation pattern of mouse heart. In that study, four laboratories compared their techniques and results in a meta-analysis for the concordance and discordance of the identified SNO-proteins and sites [30]. The 4 studies collectively identified 1,974 *S*-nitrosothiol cysteine residues in 761 proteins of which 75 (or ~4%) of the *S*-nitrosothiol cysteine residues in 44 proteins were identified by all four studies. A frequent occurrence was that the same protein was identified but at different cysteine sites, or at the same site with additional cysteine residues identified in one of the other studies. Given the differences in techniques, this is somewhat expected, and in fact, similar data have been obtained in comparing phosphorylation and other posttranslational modifications among different studies and techniques [30]. This kind of analysis would be beneficial for CNS tissues as well. We may well find not only detection of different peptides and proteins but also differences in SNO-sites for an individual protein when obtained by various techniques. Additionally, these MS studies can estimate the degree of *S*-nitrosylation of a given protein using various software programs such as MaxQuant (open source) and Spectrum Mill (Agilent), quantifying peak intensity profiles in the MS spectra. This can be useful in comparing diseased states versus normal brain protein *S*-nitrosylation status, e.g., in determining the AD vs. control *S*-nitrosoproteome.

Concerning the issue of detecting *S*-nitrosylated proteins by MS in the absence of labeling of *S*-nitrosothiols – the ephemeral nature of many (but not all) *S*-nitrosylated cysteine residues makes top-down detection of unlabeled and un-enriched *S*-nitrosylated proteins difficult. This has been accomplished, however, for some peptides such as XIAP. In this case, to preserve *S*-nitrosylation, we performed reactions in the dark at low pH, with transition metal chelators, and at low temperature. Additionally, for acquisition of MS/MS spectra, in collaboration with the proteomics lab at Scripps Research of John Yates III, we employed a top-down approach together with electron transfer dissociation (ETD), which, compared to conventional collision-induced dissociation (CID) methods allowed us to preserve the labile *S*-nitrosylation modification on larger peptides [31].

Importantly, however, many endogenous *S*-nitrosothiols persist for a sufficiently long period that they can be detected by MS. While it is true, that SNO formation on one of two

vicinal thiol groups can facilitate intra- or intermolecular disulfide formation between these groups [2, 32], we know from the existing *S*-nitrosoproteome MS database that potentially thousands of proteins are *S*-nitrosylated to affect their function, and disulfide formation is not obligatory to exert this action. Taken together, the field has shown that while cysteine residues were previously thought to be primarily structural in nature by forming disulfides for proper protein folding and conformational changes, clearly SNO and other oxidation reactions on free thiols can participate in important cell signaling pathways.

4. Mechanisms of action of aberrantly *S*-nitrosylated and transnitrosylated proteins in the nervous system

Cysteine thiols are often located in proteins at important locations for cell function, e.g., catalytic or allosteric sites that control enzymatic activity, ligand-binding or allosteric sites on ion channels, DNA binding regions or other functional sites of transcription factors, and binding sites between proteins or their chaperones. Hence, *S*-nitrosylation or other posttranslational modifications of these cysteines can regulate the activity of the given protein. Many examples of such proteins have now been published [6]. Additionally, aberrant *S*-nitrosylation can contribute to abnormal signaling pathways or protein misfolding, aggregation, and liquid-liquid phase transition [33–35].

4.1 Specific example of a transnitrosylation cascade.

As mentioned above, the prototypic example of a network of transnitrosylation reactions produces transfer of RSNO down the pathway from SNO-Uch-L1 to SNO-Cdk5 to SNO-Drp1. This cascade forms an alternative biochemical network to the canonical function of these enzymes, consisting of concerted transnitrosylation from one protein to another that contributes to synapse loss in AD. This aberrant transnitrosylation activity is critical to the pathogenesis of the disease, as synapse loss is arguably the best neuropathological correlate to cognitive decline in AD. In this newly uncovered pathway, we demonstrated that an enzyme known for deubiquitylation, Uch-L1, contributes to this transnitrosylation cascade, leading to transfer of an NO group from Uch-L1 to Cdk5. In turn, the canonical kinase enzyme Cdk5, rather than phosphorylating Drp1, transnitrosylates Drp1. We have shown that these reactions are kinetically and thermodynamically favored, as discussed above. Several groups have also demonstrated that the Drp1, as a GTPase, normally participates in mitochondrial fission, as observed during mitochondrial biogenesis. Critically, however, when excessively activated by *S*-nitrosylation, Drp1 causes mitochondrial fragmentation and extensive cristae damage to the mitochondrial matrix [10, 36]. This aberrant response results in bioenergetic compromise of the neuron, with consequent synaptic damage, as occurs in the context of AD (Figure 1) [10–13]. Collectively, this work shows that a series of enzymes heretofore known to be in unrelated pathways and with disparate catalytic properties, participate in a network of aberrant transnitrosylation reactions that contribute causally to the disease process [13].

4.2 Therapeutic approaches to prevent synaptic loss in AD due to the aberrant transnitrosylation network

One approach we have taken to ameliorating aberrant protein *S*-nitrosylation in neurons is to inhibit excessive activity of the NMDA-type of glutamate receptor. If unabated, overstimulation of NMDA receptors leads to increased Ca^{2+} influx and activation of neuronal NO synthase (nNOS), which is physically tethered to the NMDA receptor via a PDZ domain. We reasoned that by inhibiting excessive NMDA receptor activity, the generation of increased NO can be ameliorated (Figure 1), and thus aberrant transnitrosylation can be abated.

Building on our experience and success in developing the FDA-approved drug, memantine (in the form of Namenda®, NamendaXR®, and Namzaric®), which preferentially blocks NMDA-type glutamate receptor-operated channels when they are excessively open, our group more recently designed and synthesized a variety of new drugs with improved efficacy [37, 38]. One issue with memantine itself is that it is positively charged, and when neurons are ill and allow excessive influx of cations (in part via NMDA receptor-operated channels), the neurons become positively charge or depolarized and therefore repel memantine out of the channel, thus relieving its blockade. To get around this problem, we designed drugs like NitroSynapsin (a.k.a. NitroMemantine or EM-036), which offer dual allosteric inhibition of the NMDA receptor by first blocking the ion channel with a memantine-like moiety, but then targeting a nitro group 'warhead' to the protein *S*-nitrosylation sites on the NMDA receptor to offer additional inhibition that is not influenced by neuronal depolarization (Figure 1). Interestingly, the NMDA receptor was the first protein on which *S*-nitrosothiol formation was shown to regulate its activity, and thus the first example of protein *S*-nitrosylation [2, 3]. The dual allosteric mechanism of NitroSynapsin is necessary to improve the relatively modest effect of memantine itself in abating the excessive electrical activity, and thus NO formation observed in AD mouse models and human brain [38, 39]. Thus, drugs like NitroSynapsin may offer therapeutic benefit, in part by decreasing the amount of NO generated in neurons and hence transnitrosylation pathways. Such drugs are slated to enter human clinical trials within the next year or so.

5. Contentious areas and avenues for future study of transnitrosylation networks

We briefly review here evidence that transnitros(yl)ation networks between what were thought to be unrelated proteins can underlie not only transfer of NO species in the cell but also aberrant biochemical pathways that contribute to neurological diseases, with an example drawn for synaptic damage in AD. Directions for future study include the determination if transnitrosylation may be one of if not the major mechanism for transporting NO within a cell or between cells. One future type of experiment to look for additional networks of transnitrosylating proteins is the use of protein-protein affinity labels coupled with an *S*-nitrosoproteomic MS technique to detected protein complexes that may transnitrosylate one another. Bioinformatic approaches may complement this effort to delineate the potential interactome of transnitrosylated proteins (Table 1).

One issue in the field that remains an enigma concerns the level of *S*-nitrosylated protein in disease. It is not known why some proteins manifest increased but others decreased levels of *S*-nitrosylation in a diseased brain such as AD. While increases in the level of protein *S*-nitrosylation might be expected in the face of the increased levels of RNS that occur with aging and disease[34], the fact that the pattern of SNO-proteins changes, with some proteins showing less *S*-nitrosylation remains unexplained. One possible explanation for the decrease in *S*-nitrosylation status of a particular protein is that it has participated in increased transnitrosylation (or transfer of its NO group) to another protein under pathological conditions. Additionally, we have found that *S*-nitrosylation of a given protein in the brain can vary by sex, and these differences also remain to be explained and explored more deeply.

Finally, futuristic approaches to drug development may be aimed at combatting aberrantly *S*-nitrosylated proteins by target-specific denitrosylation. One method for drug development in this regard is NMR fragment-based screening. This method can be used, for example, to detect a peptide or other small molecule designed to bind to a *S*-nitrosylated (SNO)-protein target. The small molecule binding to the SNO-protein target would be designed to also bear a denitrosylating moiety adducted to the small molecule that gives the therapeutic compound specificity for the target. In this manner, it may be possible to design drugs directed toward a specific protein that has been aberrantly *S*-nitrosylated and to remove the SNO group. Similarly, drugs could be designed to provide directed *S*-nitrosylation of a target, and such a drug is illustrated in the example of NitroSynapsin discussed above, which binds to excessively activated NMDA receptor-operated channels and then donates its nitro warhead to further inhibit receptor activity. Interestingly, a similar approach using another nitro-aminoadamantane drug has recently been reported to prevent infection by SARS-CoV-2, preventing the spread of COVID-19 via coating the virus with a memantine-like moiety bound to its viroporin ion channel and then donating its nitro warhead to the viral target, ACE2, which prevents the virus from binding to ACE2 [40]. This type of designer drug strategy holds promise for the future of drug development to affect the level of SNO-proteins.

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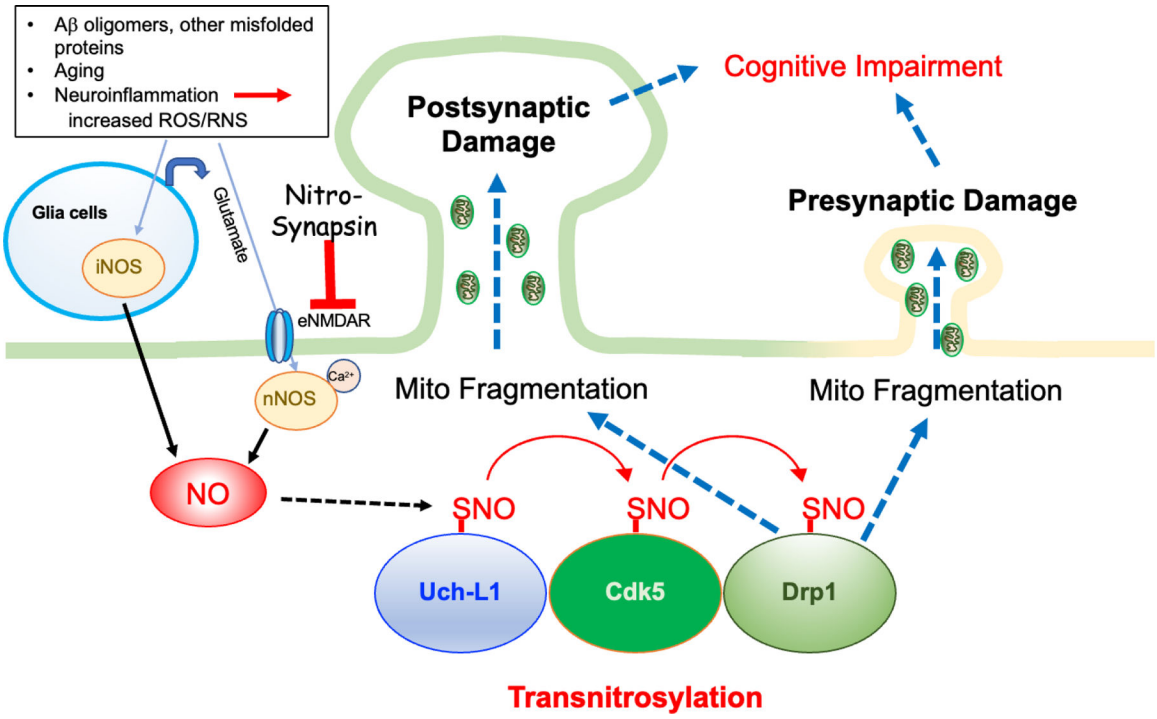

Figure 1. Concerted transnitrosylation reactions in the pathophysiology of synapse loss in Alzheimer's disease (AD).

Oligomerized amyloid-β peptide (Aβ), neuronal hyperexcitability, and aging-associated neuroinflammation trigger NO production via neuronal or inducible NO synthase (nNOS or iNOS), resulting in *S*-nitrosylated Uch-L1 (SNO-Uch-L1). Transnitrosylation from Uch-L1 to Cdk5 to Drp1 results in aberrant SNO-Drp1 formation, causing excessive stimulation of Drp1 and consequent mitochondrial fragmentation. The resulting bioenergetic failure contributes to synaptic damage underlying cognitive decline. NitroSynapsin is a novel, improved version of memantine with dual allosteric action of inhibiting excessive (mainly extrasynaptic) NMDAR activity, and therefore can abate, at least in part, damage to synapses by limiting excessive NMDAR activity and consequent RNS production via nNOS stimulation. The drug mechanism of action is (i) open-channel block of the NMDAR-associated ion channel when it is excessively open, mediated by the aminoadamantane moiety, and this facilitates (ii) targeted delivery of a nitroglycerin-like NOx moiety to *S*-nitrosylation sites(s) on the NMDAR that further inhibits excessive receptor activity [37, 38]. eNMDAR, extrasynaptic *N*-methyl-D-aspartate–type glutamate receptor; ROS, reactive oxygen species; RNS, reactive nitrogen species such as NO (adapted from [13]).

Table 1.

Controversies and Directions for Future Study

•Is transnitrosylation the major transport system for NO between proteins in the cell or among cells?
•Protein affinity labels coupled to MS detection of the S-nitrosoproteome to discover new transnitrosylation networks – are these hidden networks abundant?
•Bioinformatic approaches to uncover hidden transnitrosylation networks
•Why are some S-nitrosylated proteins upregulated while others are downregulated in disease states?
•Why are S-nitrosylated proteins differentially expressed in male and female human brain, particularly in disease states?
•Futuristic denitrosylating therapeutic drugs. Can such drugs be ‘directed’ or ‘targeted’ to a specific S-nitrosylated protein?