The Roles of S-nitrosylation and S-glutathionylation in Alzheimer's Disease

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Introduction

Alzheimer's disease (AD) is the most prevalent form of human dementia, affecting over one third of persons aged 85 years or older and with over 5 million cases in the US [1]. Characterized by extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles, the disease involves the progressive loss of neurons and neural synapses amid complex pathophysiological alterations in the metabolism of neurons and glial cells [2, 3]. Implicated in such metabolic changes is the increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) including the gasotransmitter nitric oxide (NO·). Elevated levels of NO· in particular have been demonstrated to result in post-translational modifications (PTMs) of proteins associated with mitochondrial dysfunction and bioenergetic compromise [4].

Cysteine is an amino acid particularly vulnerable to modification in oxidative stress conditions, capable of undergoing a plethora of reversible modifications including S-nitrosylation (SNO) and S-glutathionylation (PSSG) [5, 6]. Although it has a rare amino acid frequency of 2.6% in humans [7], cysteine is known to be important to signaling and cellular redox status and is often found near the center of enzyme active sites [8]. SNO is believed to be specifically regulated and dysfunction of physiological SNO regulation can be critical to the pathogenesis of neurological disease [9, 10]. Due to its low endogenous abundance and reversible nature, however, SNO presents challenges for *in vitro* analysis [11].

Proteomics, the study of the entire complement of proteins in cells, tissues, or organisms, offers techniques for the investigation of labile protein modifications such as SNO. Carefully designed high-throughput mass spectrometric methods allow cysteine modifications to be probed across hundreds and potentially thousands of proteins. Identified proteins may then be thoroughly investigated to elucidate the function of the cysteinyl modification, allowing a methodological approach in unravelling the roles of cysteine modifications in disease states. This review discusses the physiological function of NO· and SNO

in the brain, common proteomic methodology for the analysis of SNO proteins, and findings of aberrant SNO in AD. Additionally PSSG, another cysteine PTM that is less studied in AD, will be presented.

Physiological and Pathological NO·

NO· is synthesized from L-arginine and oxygen via neuronal nitric oxide synthase (nNOS, NOS-1) with NADPH and calcium-calmodulin as cofactors within the brain. NO· also may be synthesized from inducible NOS (iNOS, NOS-2) or endothelial NOS (eNOS, NOS-3) under certain conditions [12]. Physiologically, NO· increases the activity of soluble guanylyl cyclase (sGC) to increase guanosine 3',5'-cyclic monophosphate (cGMP) signaling and nitrosylating thiols of target proteins. Pathologically, NO· nitrosylates aberrant thiols and reacts with superoxide to form peroxynitrite leading to tyrosine nitration [13]. The overall effect of NO· depends on the concentration of NO·: at low nanomolar concentrations, NO· provides pro-survival effects while at micromolar concentrations NO· exhibits pro-death effects due to unique radical reactivity of NO· and its capability to cross nonpolar membranes and access buried protein residues [14]. Thus, NO· exhibits a dual role which may be neuroprotective under physiological conditions or neurodestructive under pathological conditions of increased RNS as highlighted in **Figure 1**. A detailed description of NO· physiological function and its role in synaptic transmission and various diseases are reviewed elsewhere [14, 15].

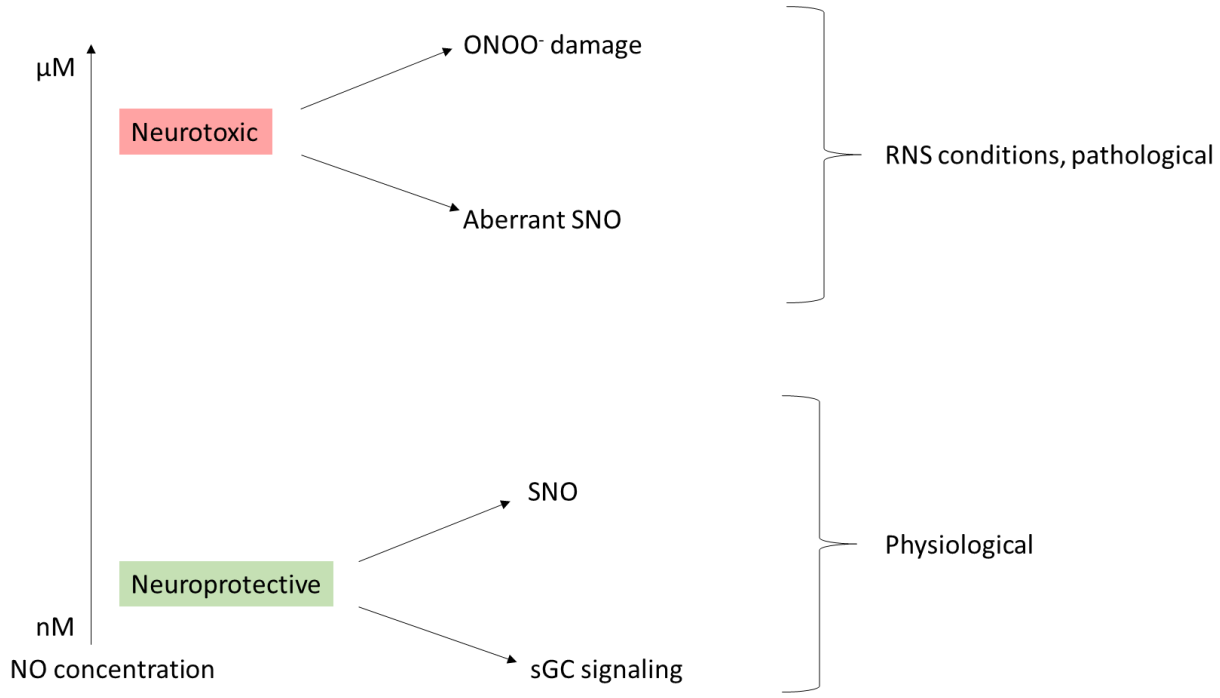


Figure 1. The dual effects of NO· concentration on cell viability. At low concentrations NO· exhibits neuroprotective effects via soluble guanylyl cyclase (sGC) signaling and physiological SNO while at RNS concentrations NO· exhibits neurotoxic effects via aberrant SNO and the generation of damaging peroxynitrite.

As neural NO· is produced largely by nNOS, Ca^{2+} levels are indirectly responsible for NO· levels. The nNOS isoform (as well as eNOS) requires greater than homeostatic Ca^{2+} levels in order to bind calmodulin for NO· synthesis. The iNOS isoform lacks the Ca^{2+} requirement and produces NO· at homeostatic conditions but is not known to be expressed in neurons at physiological conditions [12, 16]. A primary source of short-term neural Ca^{2+} increase is the N-methyl-D-aspartate receptor (NMDAR), a nonselective cation channel which opens in response to glutamate binding. Stimulation of neurons by glutamate thus indirectly activates nNOS and increases NO· levels [17, 18]. Under pathological AD conditions, $A\beta_{1\text{-}42}$ oligomers stimulate α7 nicotinic acetylcholine receptors of neighboring astrocytes, resulting in a release of glutamate which in turn stimulates neuronal NMDAR's. This flood of glutamate known as excitotoxicity disrupts neural Ca^{2+} homeostasis and generates RNS through the overproduction of NO· [19].

S-Nitrosylation

The putative formation of SNO results from the non-enzymatic reaction of a protein thiolate anion (RS^-) with a nitrosonium cation (NO^+), the latter formed from the metal-catalyzed oxidation of NO· [20-22]. Radical recombination of protein thiyl and NO· radicals has additionally been proposed as a source of SNO [23]. Due to the non-enzymatic formation of SNO and contrasting endogenous SNO formation, several regulatory processes have been proposed: the subcellular environment, site specificity, and rate of denistrosylation. Random diffusion has been viewed as the primary transport mechanism and limits NO· distribution from generation sites. Thus, the formation of SNO may be regulated by the compartmentalization of SNO proteins in close proximity to NOS [24]. The protein microenvironment of SNO residues influences the favorability of SNO formation, although variations in SNO proteins across variables such as pK_a, hydrophobicity, solvent accessibility, charge of nearby residues, and presence or lack of metal coordination suggest an array of potential mechanisms for selective SNO formation [25]. Although labile, many SNO modifications do not spontaneously reverse *in vivo* and must be reduced enzymatically, such as by the thioredoxin or S-nitrosoglutathione (GSNO) reductase systems. The selective enzymatic reduction of physiologically stable SNO-sites may thus provide an additional layer of SNO specificity [26].

Recently, the paradigm of non-enzymatic SNO formation has been challenged by a model of S-nitrosylases in which NO· enzymes generate proximity-based SNO proteins which then propagate the SNO modification throughout the cell via transnitrosylation [27]. Knocked out genes in *E. coli*, that were induced during aerobic respiration on nitrate (ARN), resulted in variations in global SNO levels but not in NO· generation [27]. The greatest reduction in SNO occurred during knockout of the hybrid cluster protein Hcp [27]. Further analysis reinforced the S-nitrosylase role of Hcp, suggesting an ubiquitination-like model in which an NO· synthase (e.g. the nitrate reductase NarGHI) synthesizes NO·, NO· S-nitrosylates Hcp, then Hcp transnitrosylates proteins with transnitrosylase ability such as glyceraldehyde

3-phosphate dehydrogenase (GADPH). Due to the existence of mammalian transnitrosylases and S-nitrosylase metalloproteins, this S-nitrosylase framework may have applicability beyond bacterial systems [27].

Indeed, the propensity of certain SNO-proteins to transfer the NO moiety to other proteins is well-established and has been proposed as a mechanism for the selectivity of S-nitrosylation [28-30]. Chemically, this may be achieved due to the potential variability in bond character and differential stabilization of resonance structures based on the SNO microenvironment [31, 32]. Environments promoting nitrogen electrophilicity would favor a transnitrosylation reaction to another thiol while environments promoting sulfur electrophilicity would favor disulfide formation with another thiol such as glutathione (GSH) [31, 32]. Despite the establishment of transnitrosylases, human S-nitrosylases remain less investigated. Hemoglobin serves as an example of an established S-nitrosylase, capable of binding NO· to heme, S-nitrosylating Cysβ93 of hemoglobin, and transferring SNO to other protein or peptide thiols [33]. Another example raises the formation of a S-nitrosylation complex consisting of iNOS, S100A8, S100A9, and target proteins, whereby iNOS generated NO·, S100A8 and S100A9 directed target site-specificity, and S100A9 became S-nitrosylated and transnitrosylated 95 target proteins identified by proteomic analysis [34]. The framework of enzymatic S-nitrosylation however, requires broader evidence in human systems. Should the enzymatic SNO formation model hold, it may provide new scope into the investigation of S-nitrosylation in neurodegenerative diseases such as Alzheimer's disease (AD).

Attempts to assign SNO motifs based on primary or secondary sequence have largely failed to account for all SNO sites identified in the corresponding studies [25, 29, 35, 36]. Rather, the local microenvironment of target thiols accounting for tertiary and quaternary structure appears to best account for SNO site selectivity [37]. Indeed, computational tertiary structure analysis of 1250 known human SNO sites demonstrated ~86% of SNO sites were physically near charged residues without a

primary sequence motif consensus [29, 38]. However, the specific local conditions of SNO formation remain to be fully elucidated and warrant further investigation.

In the context of AD, SNO proteins have been studied largely on a single to several protein basis with thorough investigations into the role each SNO protein plays in disease pathology. High-throughput proteome-wide studies of specific brain regions or structures have additionally been performed although to a much smaller extent than targeted investigations [39-45]. Taken as a whole, investigations into SNO proteins in AD present pervasive SNO dysregulation in AD brain tissue, suggesting the modification plays a critical role in disease pathogenesis. The specific effects of SNO in AD vary from protein to protein which is discussed in-depth in later sections. Also discussed are the findings of high-throughput studies, noting the full role of SNO in AD remains incomplete.

S-Glutathionylation

Glutathione is a tripeptide consisting of glutamate, cysteine, and glycine; S-glutathionylation refers to the covalent attachment of glutathione to a protein at a cysteine residue via a disulfide bridge between the protein cysteine and glutathione cysteine. Glutathione exists in a cycle with glutathione disulfide (GSSG); GSH enzymatically reacts with PSSG to remove the S-glutathionylation modification and form GSSG which is then enzymatically reduced into 2 molecules of GSH. This allows glutathionylation to act as a regenerating cellular redox buffer against ROS [46]. Intracellular GSH can vary from 0.2-10 mM and is found at 1-2 mM for most cell types [47]. The ratio of GSH to GSSG meanwhile varies with cellular localization. Mitochondria for example typically exhibit a higher GSH:GSSG ratio than the cytosol, resulting in a more reducing environment [48].

Historically, the glutathionylation/deglutathionylation cycle has been viewed as a process which acts mostly as a buffer against ROS/RNS via reducing aberrant cysteine modifications and thereby preventing the formation of damaging irreversible cysteine modifications. Recent perspectives suggest

glutathionylation has a deeper role in cellular physiology with potentially pervasive functions in cell signaling and alteration of protein activity [49]. Glutathionylation may play a deep role in the pathology of AD [50-57] and thus warrants more thorough investigation.

Proteomic Methods for SNO and PSSG Protein Identification

Shotgun proteomics

Shotgun proteomics is the analysis of complex peptide mixtures [58]. A typical shotgun analysis entails experimental workup of the proteins of interest, separation by liquid chromatography (LC), analysis by tandem mass spectrometry (MS/MS), and data analysis with bioinformatic software. Typical MS and MS/MS analysis identifies full peptide *mass to charge* (*m/z*) ratios and *m/z* ratios of peptide fragments which allow determination of the peptide sequence [59]. A variety of shotgun proteomics strategies have been developed for the relative quantification of cysteine PTMs. Isotopic labelling involves chemically modifying target peptides with reagents synthesized to contain light or heavy isotopes. As these labels are chemically identical but possess different masses, the mass difference can be detected within MS and thus distinguish experimental groups in a pooled sample [60]. Isobaric tags such as tandem mass tag (TMT) involve the attachment of multiple equal mass chemical groups which produce unique mass ions during MS/MS analysis [61]. As displayed in **Figure 2A**, TMT consists of three groups: a protein reactive group for attachment to primary amines (or protein thiols, shown in **Figure 2B**), a mass reporter to produce a unique mass ion during MS analysis, and a mass normalizer to ensure each tag possesses the same exact mass [61]. TMT reagents are based on the location of heavy isotopes within its molecular structure. Only the mass reporter group is cleaved and detected during MS analysis, so each tag achieves a unique reporter ion mass by increasing the number of heavy isotopes in the mass reporter group while decreasing the number of heavy isotopes in the mass normalizer group [62]. Isotopic labeling and isobaric tagging thus allow relative quantification across multiple experimental

groups in a single sample. Shotgun proteomic methods targeted to cysteine PTMs have incorporated these techniques.

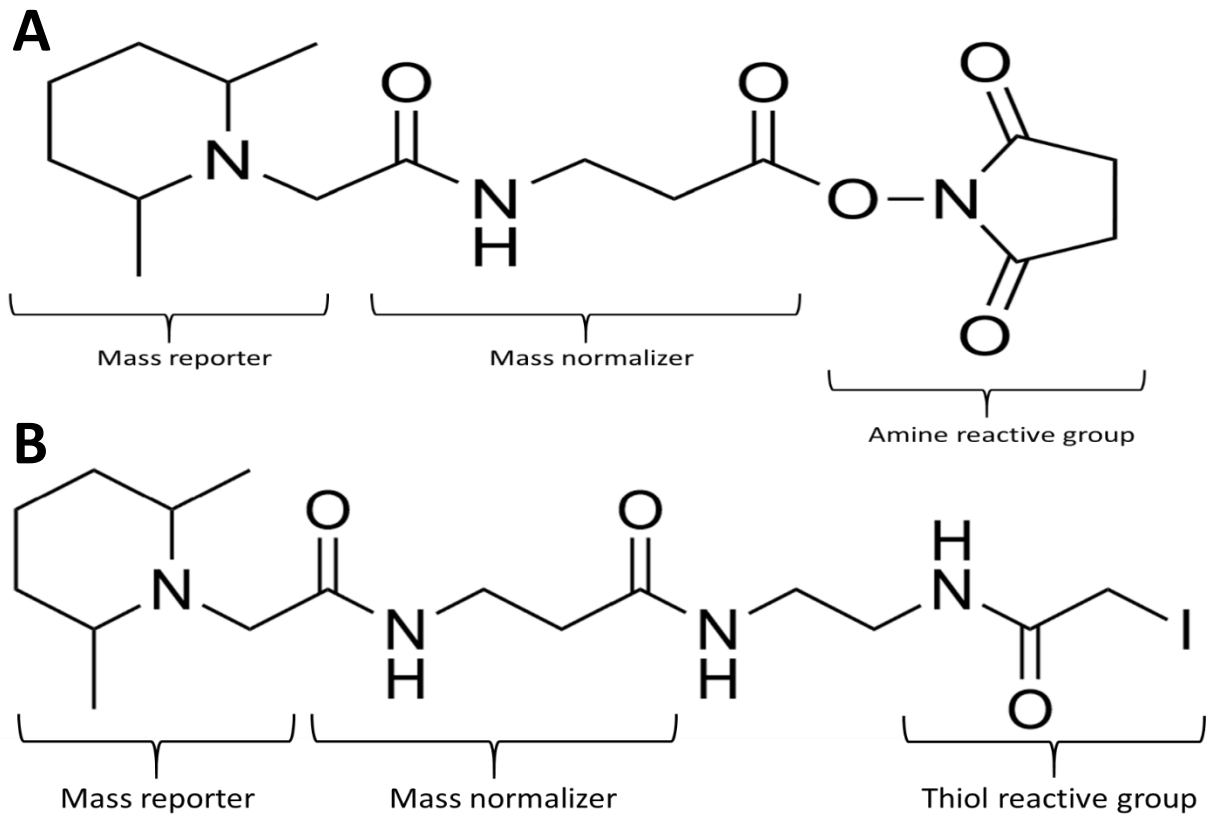


Figure 2. Structure of tandem mass tags (TMT). TMT consists of a mass reporter, mass normalizer, and amine reactive group (**A**). Cys-reactive TMT reagents such as iodoTMT (**B**) replace the amine reactive group with a thiol reactive group.

SNO

Various methods are aimed at the *in vitro* and *in vivo* study of SNO. In the context of AD, chief among these methods are variations of the biotin switch technique (BST), summarized in **Figure 3**. The BST involves irreversibly alkylating all free cysteines, selectively reducing SNO, and biotinylating reduced SNO cysteines with a thiol-specific biotin reagent [63, 64]. For samples with artificial induction of SNO,

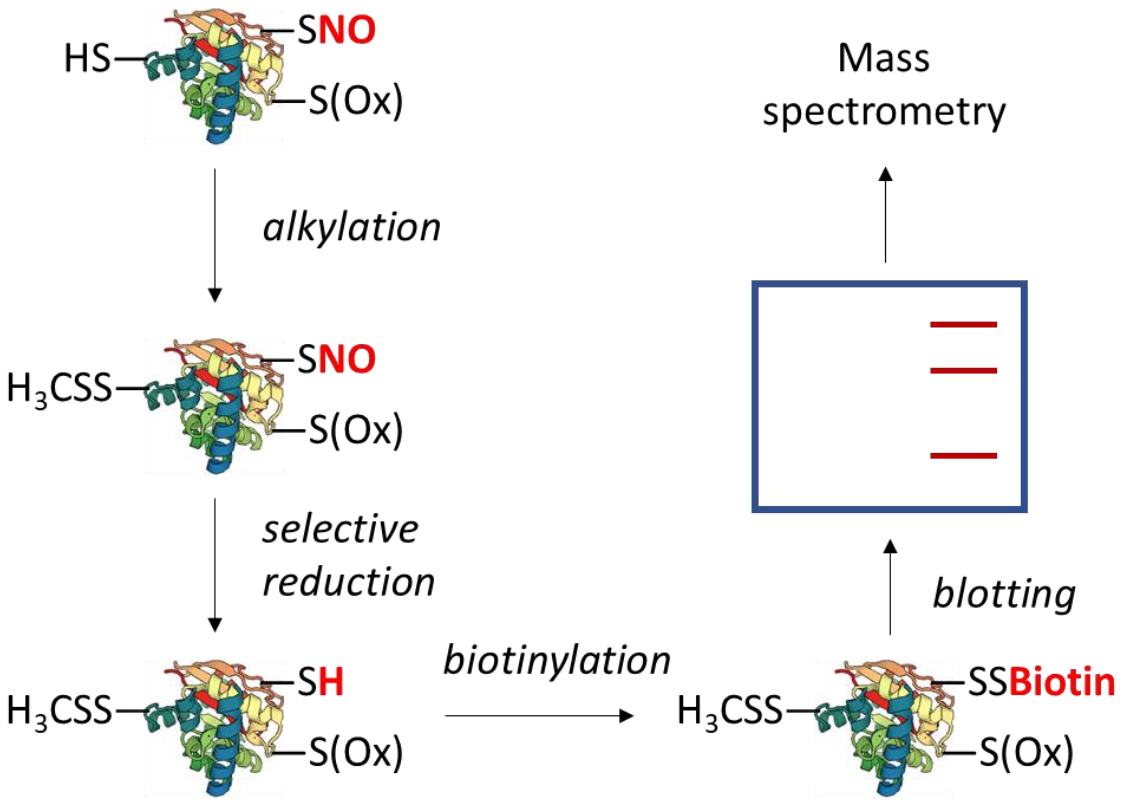


Figure 3. A generalized schematic of the biotin switch technique. The technique entails irreversible alkylation of free thiols, selective reduction of SNO, and biotinylation of SNO sites. Biotinylated proteins may be subject to blotting with anti-biotin or enriched with streptavidin followed by specific protein blotting. Bands can be excised, trypsinized, and subjected to mass spectrometry. Common variations include choice of thiol alkylating agent or replacement of biotin with an agent such as iodoTMT. Methods based on the biotin switch technique typically utilize modifications to the labelling and enrichment strategies, such as replacing biotin/streptavidin with a thiol-active resin (SNO-RAC) or direct tagging of SNO without prior selective reduction (SNOTRAP).

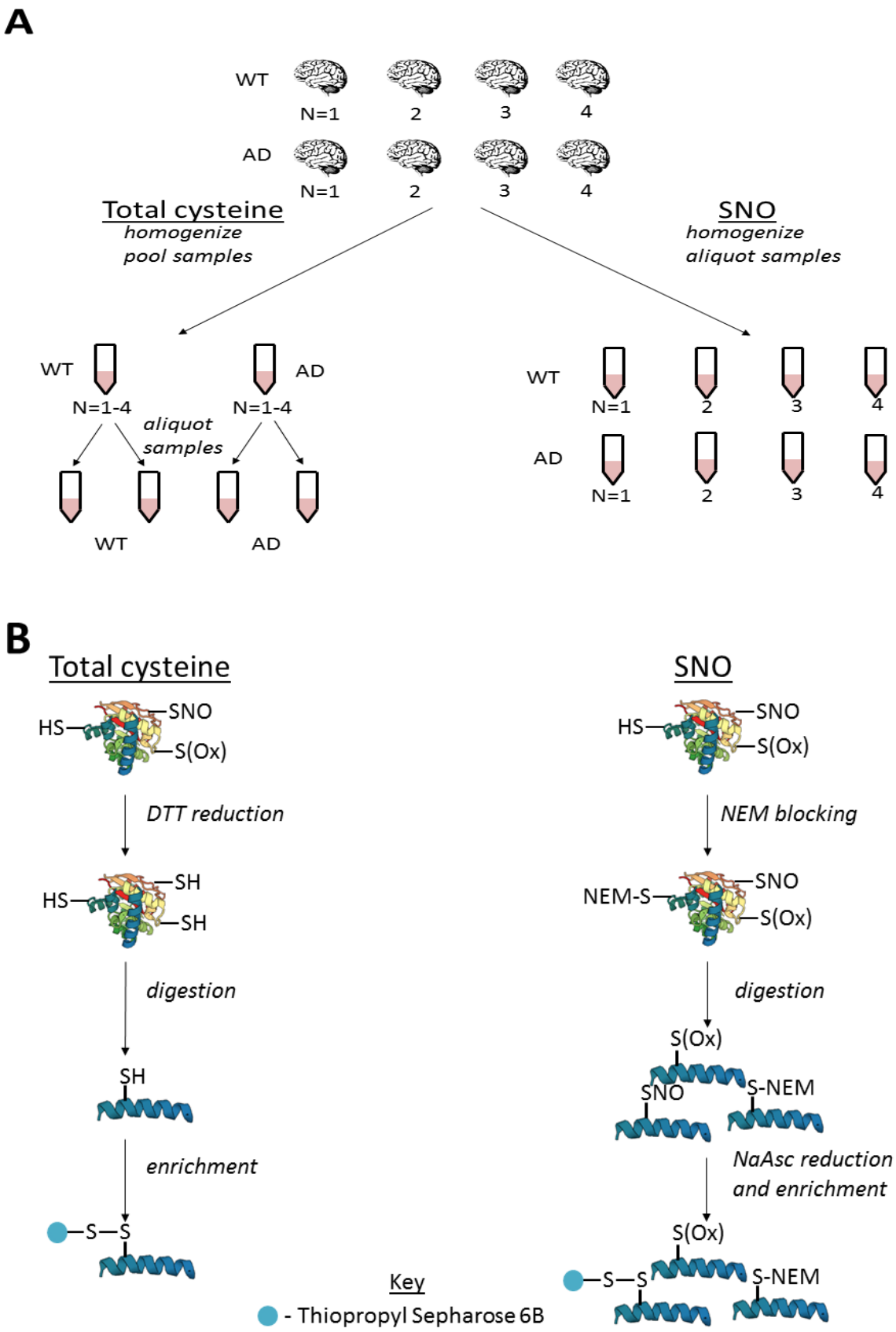
SNO proteins can be detected with anti-biotin immunoblotting but may not reflect *in vivo* SNO. For endogenous SNO, the low abundance of the modification typically requires streptavidin enrichment followed by Western blotting for specific proteins of interest [63, 64]. Variations to the BST include different choices of cysteine alkylation agents (e.g. N-ethylmaleimide) [45, 65, 66], replacement of biotin with iodoTMT and anti-TMT Western blotting [67], and predominate use of MS analysis in place of blotting techniques. SNO-Site Identification (SNOSID), a high-throughput proteomic modification to BST, added tryptic digestion after the biotin labelling step to allow selective pulldown of SNO peptides followed by LC-MS/MS analysis [68]. Another noteworthy modification to BST is SNO resin-assisted

capture (SNO-RAC) [69], an SNO-specific method more sensitive to high mass (>100 kDa) proteins. SNO-RAC replaces biotin labelling and streptavidin enrichment with a thiol-specific resin, allowing the condensation of labelling and pulldown into a single step with protein analysis performed via Western blot or MS [69]. Drawbacks to the BST include many potential sources of error, generation of artifacts if not performed in darkness due to the nonspecific reaction of ascorbate with disulfides in light, and sensitivity to transition metals in solution due to nonspecific side reactions with absorbate [70]. SNO-RAC suffers from similar drawbacks, remaining technically challenging despite a smaller number of sample preparation steps [69]. SNOSID has similar drawbacks but offers the ability to identify unknown endogenous SNO proteins, indirect identification of SNO sites, and is amenable to complex mixtures [10]. Utilizing an anti-SNO antibody allows the direct confirmation of the presence of SNO proteins but the antibodies available are suitable for immunohistochemistry, not immunoprecipitation or Western blot [71].

Several laboratories have developed other methods for studying SNO in AD [40, 41, 43] which we have recently reviewed [72]. Capillary gel electrophoresis with laser induced fluorescent detection (CGE-LIF) is a method for the detection of SNO, replacing the biotin labelling step of BST with Dylight 488 maleimide to allow fluorescent detection with sensitivity in the picomolar range [43]. CGE-LIF however lacks the ability to determine protein identities without use of a separate method such as BST [43]. SNOTRAP is a technique similar to SNOSID, which follows free thiol alkylation with reaction of the SNO moiety with a triphenylphosphine thioester probe linked to biotin, allowing streptavidin enrichment followed by LC-MS/MS analysis. SNOTRAP offers more direct identification of SNO sites than SNOSID but remains technically challenging [40].

Our laboratory developed oxidized cysteine-selective combined precursor isotopic labelling and isobaric tagging (OxcyscPILOT), summarized in **Figure 4**. As displayed in **Figure 4A**, four samples of wild type (WT) and four samples of AD are selected for SNO analysis while pooled samples for WT and AD

mice are generated for total cysteinyl analysis. As summarized in **Figure 4B**, pooled samples are reduced, digested, then enriched while SNO samples are NEM blocked, digested, then simultaneously selectively reduced and enriched. As displayed in **Figure 4C**, all samples are subjected to on-resin isotopic labeling and on-resin isobaric tagging to allow multiplexing during MS analysis. The use of light or heavy N-terminal dimethylation produces an ~8 Da per charge mass shift between light and heavy samples in the initial MS scan. The second MS scan analyzes peptide fragmentation while the third MS scan analyzes TMT reporter ion signal, producing a spectrum with TMT signals from the WT and AD pools and eight randomized SNO samples. OxcyscPILOT overcomes several limitations inherent in other proteomic SNO methods. First, the inclusion of a pooled WT and AD standard allows SNO levels to be normalized to the total cysteine proteome within individual spectra without the need to adjust for variability between scans. Second, multiplexing allows the statistical power of 4 biological replicates per WT and AD without increasing analysis time by a factor of 8. Third, the site occupancy of SNO can be calculated to determine the level at which a protein is SNO-modified versus unmodified. Although developed with TMT^{6}-plex reagents, the use of TMT^{11}-plex would allow the procedure's multiplexing capability to increase to 18 samples or 9 biological replicates per WT and AD [41, 73].


A
WT
N=1
2
3
4
AD
N=1
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SNO
homogenize
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WT
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aliquot
samples
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AD
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AD
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Total cysteine
HS
SNO
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DTT reduction
HS
SH
SH
digestion
SH
enrichment
S—S
SNO
HS
SNO
S(Ox)
NEM blocking
NEM-S
SNO
S(Ox)
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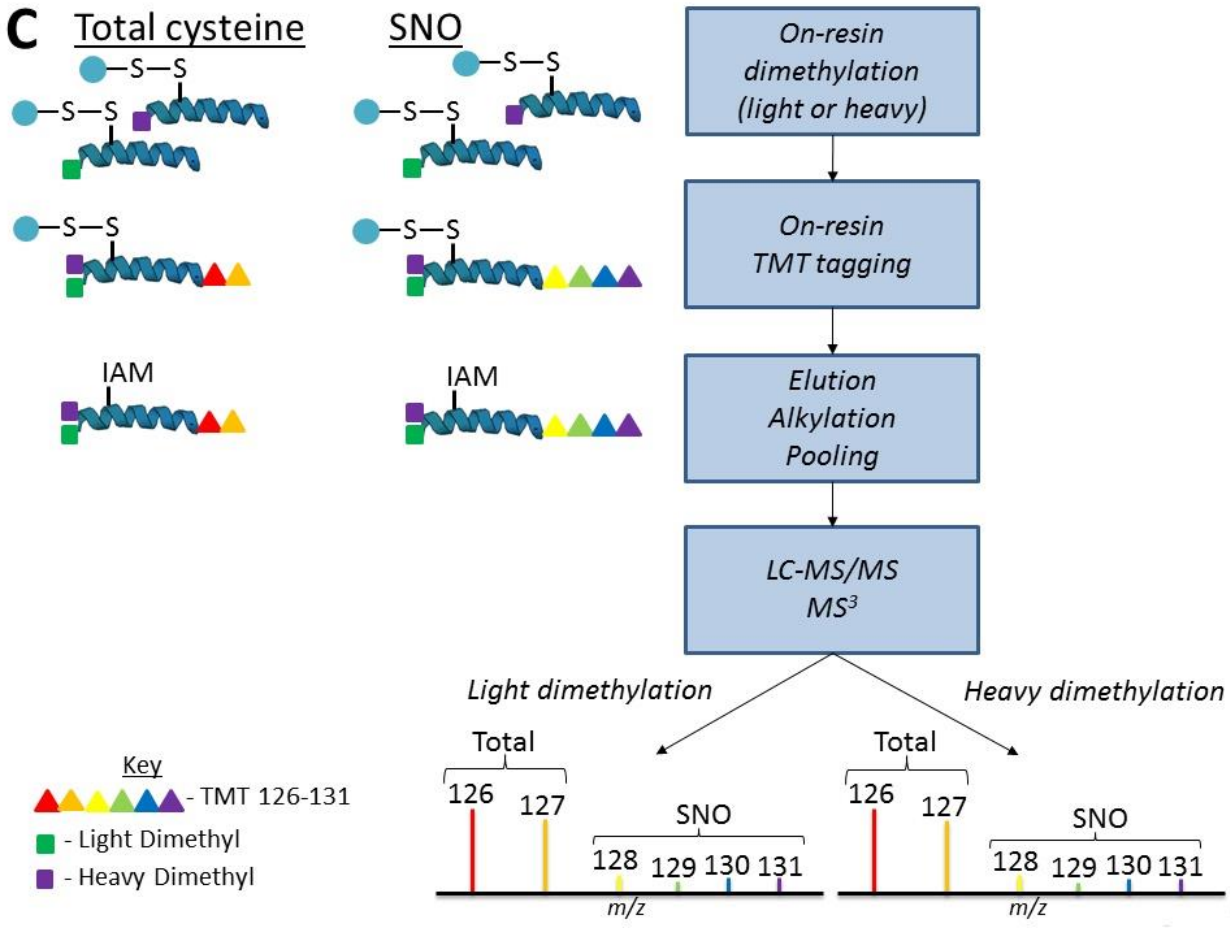

Figure 4. A schematic workflow for OxcyscPILOT. Basic steps include NEM-blocking (SNO)/reduction (total), tryptic digestion, selective reduction and enrichment (SNO)/enrichment (total), dimethylation, TMT tagging, elution, alkylation, and LC-MS/MS and MS3 analysis. Originally published in [73].

PSSG

Several techniques have been utilized for the analysis of S-glutathionylation in the context of AD. Direct methods include Western blotting techniques utilizing an anti-GSH antibody and direct detection via MS by searching for a peptide mass shift corresponding to the attachment of GSH. The former suffers from low sensitivity while the latter is not easily amenable to complex mixtures [49]. A notable indirect method summarized in **Figure 5** alkylates all free thiols followed by selective reduction of S-glutathionylation with a mixture of glutaredoxin-3, glutathione reductase, NADPH, and glutathione [74]. The free thiols are then alkylated with a biotin-linked reagent, allowing streptavidin pull-down

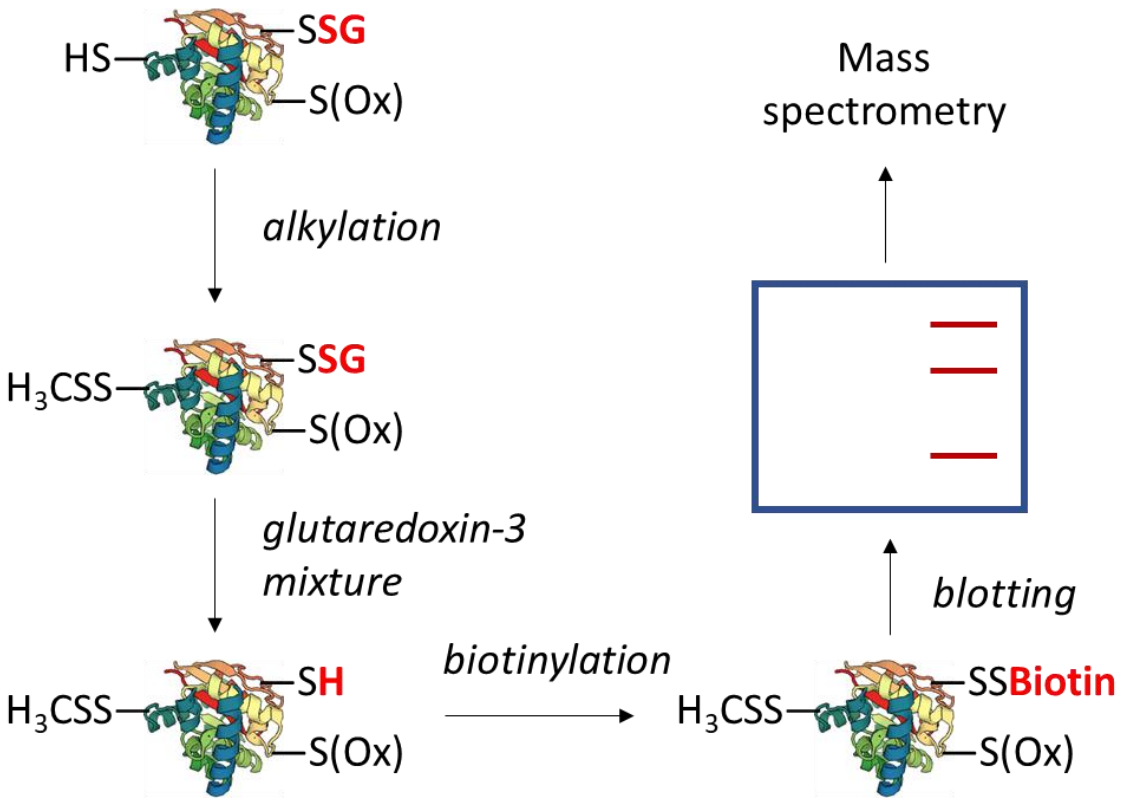

Figure 5. A generalized schematic of indirect PSSG detection. The technique entails irreversible alkylation of free thiols, enzymatic reduction of PSSG, and biotinylation of PSSG sites. Biotinylated proteins are enriched with streptavidin followed by staining. Bands are then excised, trypsinized, and subjected to mass spectrometry.

followed by gel separation and MS [74]. Another CGE-LIF method replaces the biotinylation step with Dylight 488 maleimide to allow fluorescent detection of PSSG in the attomole range [53, 75]. Both indirect methods however lack complete specificity due to glutaredoxin-3's nonspecific reduction of non-PSSG disulfides, thus necessitating inclusion of positive and negative controls for high confidence in the identification of PSSG proteins [49].

SNO proteins in AD

A wide array of SNO proteins have been investigated in the context of AD, ranging from thorough studies of single proteins to high-throughput identification of multiple SNO proteins using proteomics. The majority of investigations have targeted single to several proteins, typically utilizing BST

Table 1. List of selected SNO proteins in AD.

Protein	Function	SNO	SNO in AD	References
Beta-secretase 1	Amyloidogenic processing	Loss of function	↓	[76, 77]
Caspase 3	Apoptosis	Transnitrosylation	N/A	[78]
Cyclin-dependent kinase 5	Cell survival, proper synaptic spine density	Loss of synaptic spine density, transnitrosylation of Drp1	↑	[67, 79, 80]
Dexras 1	Forms a complex with nNOS	Contributes to Aβ toxicity	N/A	[81]
Dynamin-related protein 1	Initiator of mitochondrial fission	Increased mitochondrial fission	↑	[41, 67, 80, 82-85]
Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis, apoptotic signaling	Increased apoptotic activity	↑	[39, 67, 86]
Insulin degrading enzyme	Degradation of insulin and Aβ	Reduction of function	↑	[52, 85]
O-linked N-acetylglucosaminyltransferase	Catalysis of Ser/Thr-linked O-GlcNAcylation	Reduction of function	N/A	[87]
Phosphate and tensin homologue	Negative regulation of P13k/Akt pathway	Increased PTEN degradation	↑	[88]
Protein disulfide isomerase	Protein folding	Loss of function	↑	[89-91]
Ryanodine receptor	Intracellular calcium release	Increased calcium leak	↑	[92, 93]
X-linked inhibitor of apoptosis	Inhibition of caspases 3, 4, 7	Loss of function	↑	[78]

assay coupled with in-depth exploration of the function and effects of identified SNO-proteins in cell models and/or animal models. These studies often combine *in situ* cell studies, animal model probing, and proteomic analysis of postmortem AD brain tissue [52, 67, 76-94]. On the other hand, high-throughput proteomics studies are typically performed in AD tissues, generating hundreds of SNO sites

that will require deeper experimental investigation. Below, the findings of targeted studies for major proteins in AD are discussed. Additionally, the overall findings of high-throughput investigations are summarized in **Table 1** and **Figure 6** presents possible crosstalk between the results of targeted studies.

Cyclin dependent kinase 5 and dynamin related protein 1

Cyclin dependent kinase 5 (Cdk5) is a neural protein involved in cell survival, synaptic plasticity, pain signaling, and drug addiction [95]. For proper function, Cdk5 requires binding of the activator p35 [95]. During neurotoxic conditions such as Aβ exposure, p35 may be cleaved into p25 and p10 which results in the hyperactivation of Cdk5 and neurotoxicity in AD [95]. SNO of Cys83 or Cys157 of active Cdk5 enhanced activity in HEK293 cells and is likely a contributor to NMDAR-mediated dendritic spine loss [79]. During Aβ exposure, neuronal cultures exhibited NMDAR-mediated SNO of Cdk5. Levels of SNO-Cdk5 were elevated in Tg2576 AD mouse brains. A further study demonstrated transfection of neural cultures with SNO-immune Cdk5 resulted in reduced neural spine loss when treated with Aβ [79]. Additionally, SNO-Cdk5 was increased in postmortem human AD brain [79]. NO· production and thus SNO-Cdk5 likely resulted primarily from extrasynaptic NMDARs activation rather than synaptic NMDARs activation [80]. Further, inhibition of miRNA-132 (miR-132, known to be significantly downregulated in AD) in neural culture increased SNO-Cdk5 and nNOS with confirmation of decreased miR-132 expression in postmortem human AD temporal cortex [67]. Interestingly, SNO-Cdk5 can favorably transnitrosylate dynamin related protein 1 (Drp1) [79].

Drp1 is a protein which participates in mitochondrial dynamics by initiating mitochondrial fission via the formation of complexes on the mitochondrial membrane, known to be induced by NO· [96-98]. Cerebrocortical neurons treated with $A\beta_{25-35}$ induces pathological mitochondria fragmentation with SNO-Drp1 formation. Endogenous SNO-Drp1 in Tg2576 AD mice and postmortem AD human brains was elevated. SNO-Drp1 exhibits increased GTPase activity via a direct GTPase assay and mutant C644A (the

endogenous SNO site) Drp1 suppressed NO·-induced mitochondrial fragmentation [82]. A later study found no increase in GTPase activity of Drp1 due to SNO and detected no significant difference in SNO-Drp1 levels in postmortem human brain between AD and controls [94]. The methodology however may have introduced artifactual oxidation to endogenous Drp1 from ambient air [83]. Direct GTPase assays found increased GTPase activity from SNO in close Drp1 homologues [83].

Further studies lend additional support for SNO-Drp1 in AD [67, 80]. SNO-Drp1 was increased and Drp1 was decreased in peripheral blood lymphocytes of patients with AD or mild cognitive impairment (MCI) [84]. While more focused on insulin degrading enzyme (IDE, refer to following section), incubation of rat cortical cultures or rat cortico-hippocampal slices with $A\beta_{1-42}$, high glucose, or both, resulted in SNO-Drp1 formation at levels comparable to AD brains [85].

Protein disulfide isomerase

Protein disulfide isomerases (PDIs) are a family of endoplasmic reticulum (ER) chaperones which catalyze thiol-disulfide exchanges and rearrangement reactions for the proper folding of proteins via two catalytic -Cys-Gly-His-Cys- domains [99-101]. SNO-PDI inhibits PDI (specifically PDIA1) activity and may be generated by NMDAR-mediated excitotoxicity, contributing to the unfolded protein response with sustained nitrosative stress. SNO-PDI was found in postmortem AD and Parkinson's disease brains, suggesting SNO-PDI may contribute to neuronal cell death in neurodegenerative diseases [89]. Incubation of dopaminergic SH-SY5Y cells or primary cultured hippocampus neurons with $A\beta_{25-35}$ resulted in elevated RNS and induced formation of SNO-PDI [90, 91] and reduced PDI activity which could be prevented with neohesperidin, an ROS-scavenger [91].

Glyceraldehyde 3-phosphate dehydrogenase and sirtuin 1

GADPH is a glycolytic enzyme with roles in activating apoptosis when S-nitrosylated [102-104]. Sirtuin 1 (SIRT1) is a nicotinamide adenosine dinucleotide-dependent class-III deacetylase that deacetylates Lys174 of tau protein and thus ameliorates tau aggregation [105, 106]. Treatment of WT mice with $A\beta_{1-42}$ induced the formation of SNO-GADPH which could transnitrosylate and deactivate SIRT1 which led to increased tau aggregation. Increased SNO-GADPH was present in postmortem AD cortical samples [86] consistent with other studies [67].

Insulin degrading enzyme

IDE is a zinc metalloendopeptidase responsible for the degradation of both insulin and $A\beta_{1-42}$ [107, 108]. NO· donors inhibit insulin degradation by IDE up to 70% and Aβ degradation by 25% with micromolar concentrations of NO· donors. Excess insulin almost fully inhibited IDE-mediated Aβ degradation and IDE was SNO-modified *in situ* [52]. Treatment of rat cortical cultures, rat hippocampal slices, or human neural cultures with glucose, $A\beta_{1-42}$, or both elevated SNO-IDE due to NMDAR-mediated nitrosative stress. SNO inhibited the Aβ-degrading function of IDE and significant levels of SNO-IDE in postmortem AD brain were observed [85].

O-linked N-acetylglucosaminyltransferase

O-linked N-acetylglucosaminyltransferase (OGT) is a catalyst of Ser/Thr-linked O-GlcNAcylation [109]. Aβ treatment of human neuroblastoma SK-N-MC cells elevated SNO-OGT and reduced global O-GlcNAcylation. OGT associated with nNOS after Aβ treatment while hyper O-GlcNAcylation appeared to prevent NMDAR-mediated calcium ion influx. SNO-OGT may contribute to tau hyperphosphorylation [87].

Beta-secretase 1

Beta-secretase 1 (BACE1) is a membrane aspartyl protease responsible for the N-terminal cleavage of amyloid precursor protein (APP) in the amyloidogenic processing of APP [110, 111]. Treatment of rat primary cortical neurons with <100 nM of NO· donors suppressed BACE1 transcription while 0.1-100 μM treatment did not influence transcription but deactivated BACE1 via SNO [76, 77]. SNO-BACE1 was reduced in the entorhinal cortices of postmortem human AD brains [76]. Additionally, GSNO treatment of bEND3 cells induced the formation of SNO-dynamin 2, increasing Aβ uptake and clearance [77].

X-linked inhibitor of apoptosis and caspase-3

X-linked inhibitor of apoptosis (XIAP) is an inhibitor of apoptosis via direct inhibition of caspases 3, 7, and 9 while caspase 9 is an inducer of apoptosis and caspases 3 and 7 are executors of apoptosis [112]. NO· donors can induce SNO of Cys450 of XIAP, inhibiting its E3-ligase and antiapoptotic activity. SNO-XIAP in AD postmortem brains was increased and SNO-caspase-3 can transnitrosylate XIAP [78].

Ryanodine receptor

Ryanodine receptors (RyRs) are endoplasmic reticulum (ER) channel proteins responsible for creating Ca^{2+} efflux from ER calcium stores into the cytosol [113]. Blocking NO· synthesis increased synaptic depression in presymptomatic 3xTg-AD mice. NO· synthesis may result in SNO-RyR and increased RyR-mediated calcium release which may contribute to stress conditions in late AD [92]. Further investigation into RyR-mediated calcium leak found SNO as part of RyR remodeling in postmortem AD brain and the APP/presenilin 1 (PS1) AD mouse model [93].

Dexamethasone-induced Ras-related protein 1

Dexamethasone-induced Ras-related protein 1 (Dexras1) is a small GTPase known to modulate signaling cascades important to neurogenesis in the hippocampus [114]. SNO-Dexras1 formed on Cys11 in primary hippocampal neuron cultures after incubation with $A\beta_{1-42}$ and in the hippocampus of 4-month APP/PS1 mice. Dexras1 mutated to lack the SNO site, resulted in reduced impairment in $A\beta_{1-42}$ mice, suggesting SNO-Dexras1 contributes to the neurotoxicity of $A\beta_{1-42}$ [81].

Phosphatase and tensin homolog

Phosphatase and tensin homolog (PTEN) is a tumor suppressor phosphatase negative regulator of the P13K/Akt pathway [115, 116]. SNO-PTEN was found in the entorhinal cortexes of MCI and AD [88]. Both exogenous and endogenous NO· donors could induce stable SNO-PTEN in rat cortical cultures which resulted in ubiquination of PTEN and subsequent degradation by the ubiquitin-proteasome pathway. Due to PTEN's neuroprotective effect, SNO-induced degradation of PTEN may contribute to neurodegenerative processes in AD [88].

Table 2. SNO pathways identified in AD.

Pathways of SNO proteins	Brain region	References
Metabolism	Hippocampus, substantia nigra, cortex, cerebellum, whole brain	[40, 41, 65]
Signaling pathways	Hippocampus, substantia nigra, cortex, whole brain	[41, 65]
Apoptosis	Hippocampus, substantia nigra, cortex	[65]
Redox regulation	Hippocampus, substantia nigra, cortex	[65]
Glycolysis/gluconeogenesis	Synaptosomes	[39]
Calcium homeostasis	Synaptosomes	[39]
Ion and vesicle transport	Synaptosomes	[39]
Synapse function	Hippocampus, cortex, cerebellum	[40]
AD pathology	Hippocampus, cortex, cerebellum	[40]

High-throughput SNO and modified cysteine studies

Numerous studies aimed at the proteome-wide identification of SNO proteins and other oxidative cysteine modifications have been performed. Forty-five endogenous SNO proteins were identified in AD hippocampus, substantia nigra, and cortex and are mostly involved in metabolism, signaling pathways, apoptosis, and redox regulation [65]. Endogenous SNO proteins of synaptosomes of WT and hAPP AD mice were profiled, identifying 138 SNO proteins of which 38 were detected only in the hAPP mice [39]. These proteins were generally at higher levels of SNO and included pathways such as glycolysis/gluconeogenesis, calcium homeostasis, and ion and vesicle transport [39]. SNO proteins were analyzed in the cortex, hippocampus, and cerebellum of CK-p25 AD mice, finding 313 endogenous SNO sites located on 251 proteins with 135 SNO-proteins unique to neurodegeneration [40]. The affected pathways included synapse function, metabolism, and AD pathology [40].

Our laboratory identified and quantified the SNO-proteome of the whole brain of the APP/PS1 mouse model, finding 135 SNO proteins of which 11 proteins were differentially modified between WT and AD [41]. Significantly modified proteins previously identified in AD were ADP/ATP translocase 1 [39],

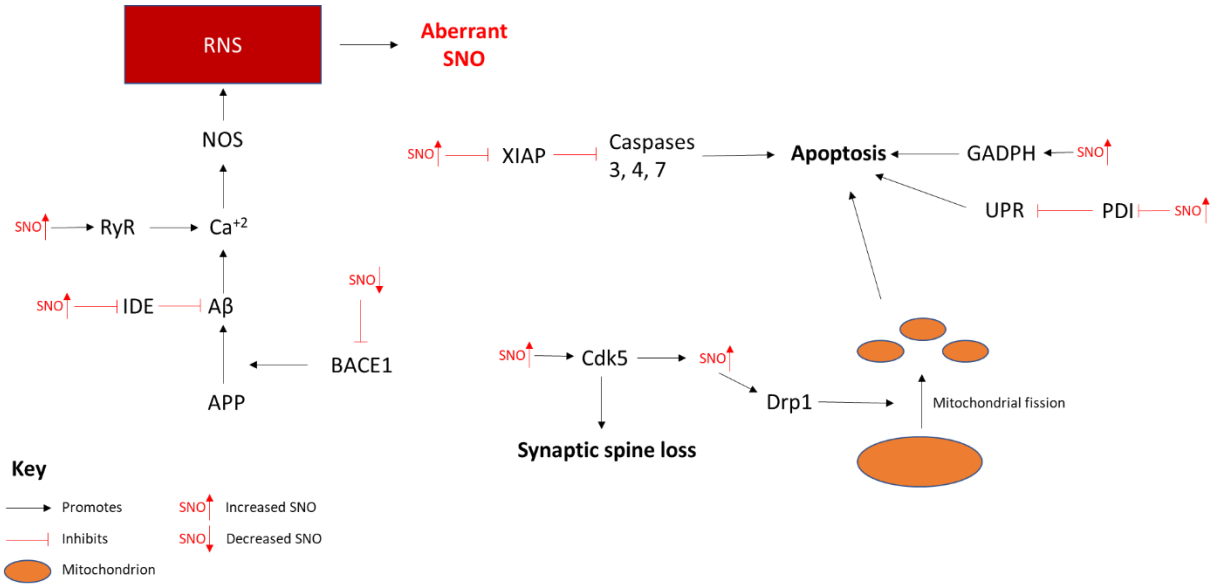

Figure 6. The effects of aberrant SNO in the AD brain. This is based on endogenous SNO protein quantification in mouse models or postmortem AD neural tissue. SNO may be inhibitory or enhance protein activity depending on the target protein. Abbreviations: APP- amyloid precursor protein; BACE1 – beta-secretase 1; Cdk5 – cyclin-dependent kinase 5; Drp1 – dynamin-related protein 1; GADPH – glyceraldehyde 3-phosphate dehydrogenase; IDE – insulin degrading enzyme; NOS – nitric oxide synthase; PDI – protein disulfide isomerase; RyR – ryanodine receptor; UPR – unfolded protein response; XIAP – X-linked inhibitor of apoptosis.

ras-related C3 botulinum toxin substrate [39], 14-3-3 protein zeta/delta [65], glutamine synthetase [39, 65], myelin proteolipid protein [39], citrate synthase [39], and 2',3'-cyclic-nucleotide 3'-phosphodiesterase [39] while newly discovered SNO proteins were septin 5, myc box-dependent-interacting protein 1, isocitrate dehydrogenase, and 14-3-3 protein gamma. Most site occupancies of significant SNO proteins were below 1% with the exception of the 14-3-3 proteins which were 5-7% for 14-3-3 gamma and 28-54% for 14-3-3 zeta/delta [41]. Modified pathways consisted primarily of metabolism and signal transduction [41]. We additionally identified and quantified the cysteine proteome of 14-month APP/PS1 mouse livers, finding 2259 unique proteins with 65 proteins differentially expressed between WT and AD [118]. We identified and quantified the reversibly oxidized cysteine proteome of 14-month APP/PS1 mouse livers by alkylating free thiols, reducing oxidized cysteines, and enriching previously oxidized cysteines followed by LC-MS/MS analysis [119]. We found 828 reversibly oxidized cysteine sites with 19 sites differentially expressed between WT and AD without

identification of specific PTMs [119]. Modified pathways consisted of amino acid metabolism, carbohydrate metabolism, lipid metabolism, citric acid cycle and respiratory electron transport, and biological oxidation [119].

The SNO proteome of the entorhinal brain tissue of postmortem AD patients was probed but there was no significant difference between AD and control, perhaps due to the sensitivity of the employed method [42]. Several studies targeted the SNO proteome without providing identifications for individual proteins. A highly sensitive SNO detection method was developed and demonstrated utility via quantifying the SNO protein amounts in the cerebrum of 5-month B6Cg-Tg AD mice compared to WT [43]. A 2D micro-capillary gel electrophoresis fingerprint of the SNO proteome of 11-month B6Cg-Tg AD mouse brains was also later generated [120]. Proteins identified as significantly S-nitrosylated between AD and control across multiple high-throughput studies include GADPH [39, 40], glutamine synthase [40, 41], ADP/ATP translocase 1 [39, 41], and 2',3' cyclic nucleotide 3' phosphodiesterase [39, 41]. BV2 microglia cells treated with $A\beta_{25-35}$ for 24 hours changed cysteine redox status for 60 proteins in pathways such as cell death and survival, inflammatory response, or cellular growth and proliferation [45].

As displayed by **Figure 7**, high-throughput SNO studies exhibit a small degree of overlap of SNO proteins and largely identify novel SNO proteins in AD. Given the heterogeneity of the brain, some difference between SNO proteomes is expected and illustrates the need to spatially investigate SNO in brain. The validity of shotgun proteomics methodology in probing SNO is shown in **Figure 7** because more than 350 SNO sites in AD have been determined. However, the largescale identification of unknown SNO proteins demonstrates the need for future studies to further understanding of SNO in AD.

Table 3. PSSG proteins and pathways identified in AD.

Protein	Function	PSSG	PSSG in AD
α-enolase	Glycolysis, apoptotic signaling	Reduction of function	↑
α-crystallin B	Heat-inducible chaperone	Reduction of function	↑
Deoxyhemoglobin	Oxygen transport	Increased oxygen affinity	↑
Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis, apoptotic signaling	Reduction of function	↑
Na,K-ATPase	Sodium-potassium pump	Reduction of function	N/A
Insulin degrading enzyme	Degradation of insulin and Aβ	Reduction of function	N/A
p53	Tumor suppressor	Potential decrease in formation of active tetramer	↑
Transthyretin	Thyroxin/retinol transport	N/A	↑

PSSG Proteins in AD

S-glutathionylation is less studied in the context of AD. Postmortem AD inferior parietal lobule (IPL) was probed, revealing significant increase of PSSG in deoxyhemoglobin, α-enolase, α-crystallin B, and GADPH [50]. GADPH and α-enolase activity was reduced in AD IPL [50]. Selective PSSG of the monomeric and dimeric forms of p53 were observed, suggesting that PSSG may prevent the formation of the more active p53 tetramer [51]. Like SNO-IDE; PSSG-IDE likely inhibits IDE degradation of Aβ [52]. Global PSSG levels in AD-Tg mice were compared to WT. PSSG levels were increased in AD cerebrum but decreased in AD hippocampus and whole blood [53]. Measurement of PSSG levels of 1, 5, and 11-month old AD-Tg mice were increased in the brains of all but 5-month old mice and global PSSG was increased in the blood of all age groups [54]. Principle component analysis could distinguish AD from WT with both brain PSSG and blood PSSG [54]. Blood collected from AD patients included a significant decrease in

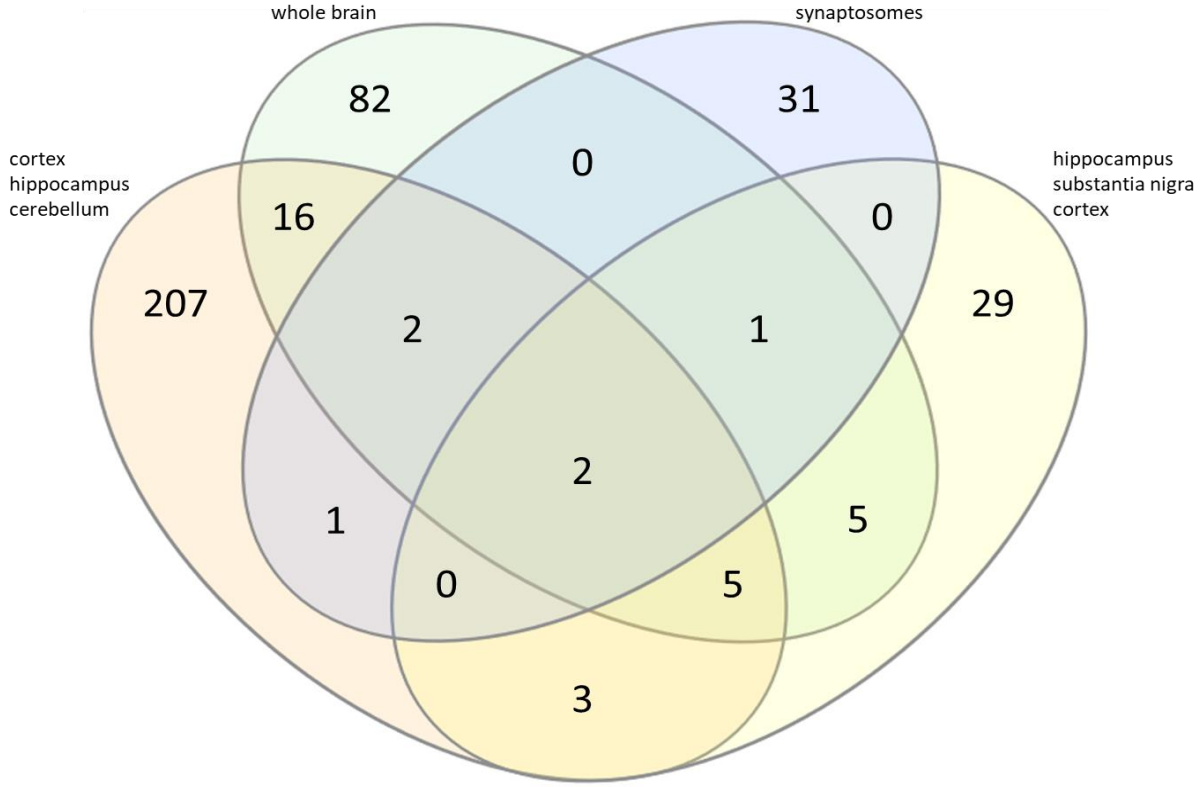

Figure 7. Overlap between identified SNO proteins in AD in high-throughput proteomic studies. These studies targeted the cortex, hippocampus, and cerebellum [40]; whole brain [41]; synaptosomes [39]; and hippocampus, substantia nigra, and cortex [65]. Diagram created with InteractiVenn [117].

GSH/GSSG ratio which may affect glutathionylation [55]. Transthyretin in cerebral spinal fluid from patients with AD, MCI, normal pressure hydrocephalus, or healthy controls possessed significant oxidation occurring on Cys10 in AD and MCI [56]. Oxidation types included PSSG, S-cysteinylation, and S-cysteinylglycinylation [56]. Incubation of SH-SY-5Y cells with $A\beta_{42}$ increased PSSG of the α-subunit of Na,K-ATPase with enzyme inhibition, suggesting PSSG had an inhibitory effect [57]. These findings are summarized in **Table 3**.

As PSSG remains relatively understudied in AD, further studies are required to better assess how PSSG may be involved in AD. With the advent of high-throughput proteomic studies and the development of indirect PSSG methods amenable to MS, a logical next step would be a high throughput study aimed at a proteome-wide analysis of PSSG in AD.

Conclusions

Overall, shotgun proteomic studies offer a useful starting point for characterizing large numbers of SNO or PSSG proteins. Altered SNO levels of BACE1, IDE, and RyR likely contribute to impaired calcium ion homeostasis, indirectly increasing NO· production and maintaining RNS conditions whereas other studied SNO modifications likely contribute to the observed pathophysiology in AD. The full role of SNO in AD may thus be more fully elucidated with proper future study into different brain regions and neural cell types, across different disease stages to track alterations in SNO with disease progression, and into peripheral organs such as liver which may play a role in altered AD metabolism. Once SNO proteome-wide studies identify potential SNO dysregulation, targeted studies can determine the roles individual dysregulated SNO proteins play in disease pathology. Follow-up studies are required to expand upon the current results of high-throughput SNO experiments as proteins such as glutamine synthase and 2',3'-cyclic-nucleotide 3'-phosphodiesterase have been repeatedly identified as significantly modified. While cell studies offer clues into the function of SNO proteins *in vivo*, such studies require endogenous confirmation of SNO proteins in AD tissue. Due to clear detrimental effects of SNO of proteins such as Cdk5 and amelioration of pathological features when prevented, SNO provides a potential avenue for novel drug development.

As a less studied modification in AD, PSSG may benefit greatly from high-throughput proteomic studies. Considering PSSG is viewed as a buffer against ROS/RNS stress and a mechanism by which aberrant cysteine modifications can be avoided, it would be interesting to discover if aberrant SNO might influence PSSG. Potential crosstalk between both modifications has not been investigated in AD and may reveal another layer of complexity to dysregulated cysteine modifications. Considering cysteine PTMs such as SNO and PSSG as potential drug targets in AD is an exciting direction for the field.

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