

# The Role of Protein S-Nitrosylation in Alzheimer's Disease and its Treatment

**Keywords:** Alzheimer's disease; Protein S-nitrosylation; Mitochondrial dysfunction; Synapse loss

## Abstract

As a post-transcriptional modification, S-nitrosylation is a covalent binding of nitric oxide to cysteine thiols in proteins to form S-nitrosothiols. Nitric oxide is recognized as an important signaling molecule, and has recently been shown to reversibly nitrosylated proteins. Emerging evidence demonstrates that in Alzheimer's disease, aberrant S-nitrosylation of proteins may exert an effect on mitochondrial fission, A $\beta$  production and synaptic damage, which may directly or indirectly contribute to Alzheimer's disease pathology. In this paper, we review recent findings of an S-nitrosylated protein relationship to the pathogenesis of Alzheimer's disease; discuss the influences of protein S-nitrosylation on Alzheimer's disease progression; and consider indications for protein S-nitrosylation in the treatment of Alzheimer's disease.

Alzheimer's disease (AD) is the most common and leading form of dementia in the aged population, accounting for an estimated 60-80% of all cases [1]. In the United States, approximately 5 million people now suffer from AD, with a 50% increase expected by the year 2025 [2]. AD is recognized as a neurodegenerative disorder although its etiology is not yet fully understood. The majority of AD cases are sporadic, which has a rare relationship with heritance, [3] and only a small proportion of cases are familial, mainly attributed to three genes:  *$\beta$ -amyloid precursor protein (APP)*, *presenilin 1* and *presenilin 2*. A vast amount of cell injury and loss occur in various parts of the brain of patients with AD, particularly in the hippocampus and neocortex [4]. One of the most common characteristics of AD pathogenesis is synaptic deficit, which attributes to intellectual and cognitive decline [5,6]. AD patients experience overwhelming memory loss, cognitive impairment and behavioral changes like aphasia, apraxia, agnosia and executive dysfunction. Currently there is no cure and palliative treatment options are limited. Irrespective of genetics, advancing age is the leading factor associated with AD, with the fact that people over 65 years of age are far more likely to have the disease.

## Neuropathology of Alzheimer's Disease

Although the mechanisms of AD are not fully understood, A $\beta$  plaque, neurofibrillary tangles and neurotransmission deficits have been identified to be its main characteristics [7-9]. A $\beta$  plaque results from aggregation of A $\beta$  peptides that originate from cleavage of APP by  $\beta$ -secretase and  $\gamma$ -secretase [7]. It has been found that A $\beta$  oligomers are toxic, and can cause synapse loss and neuronal cell death [10]. Neurofibrillary tangles are aggregates of hyperphosphorylated tau proteins in the brain [11]. Tau protein is expressed in axons and binds to tubulin, which stabilizes microtubules. In AD patients, hyperphosphorylated tau protein aggregates into insoluble tangles, which have a detrimental effect on the transport system of neurons, resulting in disconnection of neurons and eventually cell death



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[8,9]. Since AD is an age-dependent disease that mainly occurs in the elderly, ageing is a leading factor. The free radicals that can cause oxidative/nitrosative stress contribute significantly to the aging process. Indeed, many studies have reported that the AD brain exhibits increased oxidative/nitrosative stress [12]. Increasing evidence suggests that protein S-nitrosylation plays an important role in the pathophysiological development of AD [13,14].

The glutamatergic and cholinergic systems are highly involved in memory and cognition. Dysregulation of these two systems is implicated in the progression of AD [15,16]. Glutamate is the main excitatory neurotransmitter in the central nervous system. Hyperactivation of N-methyl-D-aspartate (NMDA) glutamate receptor mediates excitotoxicity that contributes to AD pathology [15]. Non-competitive NMDA receptor antagonist memantine is currently used for the treatment of moderate-to-severe AD [17]. Degeneration of cholinergic neurons in basal forebrain has long been reported in AD patients [18], and cholinergic deficits lead to reduced synthesis of acetylcholine [18]. Therefore, a category of currently available drugs for mild to moderate stage of AD are used to enhance acetylcholine levels in the brain, including, for instance, donepezil, rivastigmine and galantamine [19].

## Nitric Oxide and Protein S-nitrosylation

Nitric oxide (NO) is produced from L-arginine catalyzed by nitric oxide synthases (NOS) including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [16,20,21]. This process also requires cofactors/coenzymes such as nicotinamide adenine dinucleotide phosphate, flavine mononucleotide and flavin adenine dinucleotide [22,23]. NO participates in various cellular signaling pathways to regulate a spectrum of brain functions such as neuronal development, synaptic plasticity and apoptosis [24,25]. The actions of NO are multifaceted and mainly classified into two categories: cyclic guanosine monophosphate (cGMP) dependent actions and cGMP-independent actions [26]. Studies have shown that NO at nano molar concentrations is sufficient enough to activate guanylate cyclase and trigger cGMP-dependent signals [26]. NO activated cGMP has been recognized to play critical roles in NO-mediated vasodilation [27]. In addition, NO/cGMP also contributes to the immune system,

neurotransmission processes and regulation of cardiac contractility [28-30]. NO at higher than 50-100  $\mu$ M can modify the thiol group of cysteine residues in proteins via covalently binding and induce protein cysteine S-nitrosylation [26,31-36]. Like other posttranslational modifications, S-nitrosylation is able to trigger conformational changes of various proteins. S-nitrosylated thiols in cysteine residues can be reduced back to free thiols by glutathione or thioredoxin [37]. For instance, S-nitrosothiol group can be denitrosylated by glutathione, forming a reduced protein thiol and S-nitrosoglutathione. S-nitrosoglutathione is rapidly and irreversibly metabolized by S-nitrosoglutathione reductase to glutathione S-hydroxysulfenamide. S-nitrosylated thiols can also be denitrosylated by thioredoxin through its dithiol moiety to form thioredoxin disulfide and nitroxyl or NO, while thioredoxin disulfide can be reduced back to thioredoxin by thioredoxin disulfide reductase [37].

Cysteine residues in proteins are critical in regulating enzymes, transcription factors, protein structures and metal binding. Thiols of cysteine residues are very susceptible to be oxidatively modified by NO radicals. In AD, A $\beta$  toxicity can cause the hyperactivity of NMDA receptors, resulting in increased Ca<sup>2+</sup> influx that activates nNOS and increases NO production [38,39]. In addition, A $\beta$  can increase NO production by activating iNOS in glial cells [25,38,40]. NMDA plays a critical role in the regulation of NO production in AD [41]. Increased NO in AD brains may target a wide variety of proteins. Indeed, many studies have presented compelling evidence implicating NO-induced S-nitrosylation of proteins in the pathogenesis of AD [42-44].

### The Role of S-nitrosylation in AD

Many studies have shown that protein S-nitrosylation is increased in brain of AD patients. Although the exact mechanism of increased S-nitrosylation protein remains largely unknown, the vast majority of

S-nitrosylated proteins are reported to be involved in mitochondrial dysfunction, protein misfolding, synaptic loss and A $\beta$  production (Figure 1).

### S-nitrosylation on mitochondrial dysfunction

Mitochondria are the main production sites for the cellular energy currency adenosine triphosphate. Mitochondrial functioning and energy metabolism are impaired early in the course of AD [45] but this mechanism is not fully understood. It has been reported that excessive S-nitrosylation of mitochondrial proteins may suppress protein functioning, thus compromising, at least partially, mitochondrial functioning, especially in neurodegenerative diseases such as AD where ROS/RNS are overwhelmed at a certain stage of the disease.

Recent studies show that S-nitrosylation of dynamin-related protein 1 (Drp1) is increased in AD. Drp1 is responsible for mitochondrial division and involved in regulation of mitochondrial fission. Mitochondrial functioning requires highly intact structure, in which an intricate balance of fusion and fission is a major determinant. Mitochondrial fusion promotes the mixing of organellar contents and unifies the mitochondrial compartment, both of which guarantee the exchange of mitochondrial DNA as well as various metabolites. Therefore, mitochondrial fusion ensures efficient production of adenosine triphosphate [46]. Fission, on the other hand, breaks down the unified mitochondrion into numerous small morphologically and functionally distinct organelles, leading to mitochondrial fragmentation, a signal of apoptosis [47]. Therefore, the imbalance between mitochondrial fusion and fission contributes to neurodegenerative diseases [48]. Drp1 as a dynamin-related guanosine triphosphatase mediates membrane fission through promoting dynamin dimerization and is a core component of the

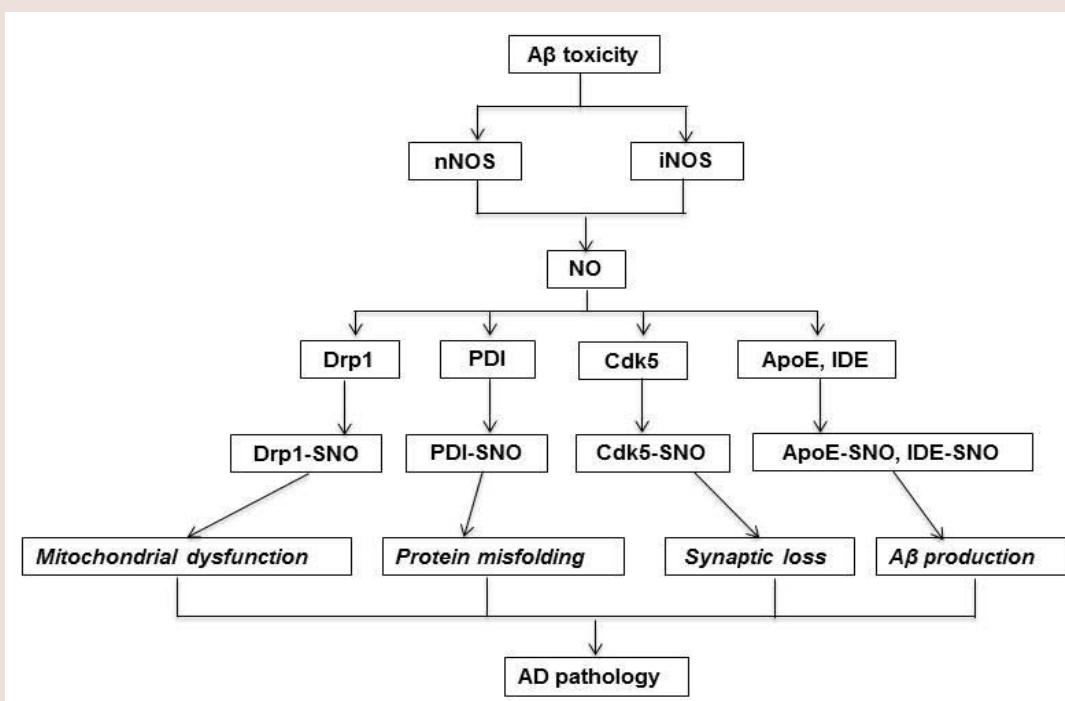


Figure 1:

mitochondrial division machinery and responsible for excessive mitochondrial fission [49,50]. Cho et al. reported that S-nitrosylation of Drp-1 was increased both in mouse cerebrocortical cells treated with oligomeric A $\beta$ <sub>25-35</sub> and in post mortem brains of patients with sporadic AD [42]. Furthermore, Drp1 S-nitrosylation was also increased in human embryonic kidney (HEK) 293 cells transfected with nNOS, and this modification can be blocked by NOS inhibitor N-nitro-L-arginine [42]. This research group also found that Drp1 could not be S-nitrosylated when the Cys644 residue was mutated to Ala (C644A) [42], suggesting that Cys644 is the site for S-nitrosylation. Furthermore, NO donors could increase dynamin dimerization and guanosine triphosphatase activity, but not in Drp1 (C644A) mutated cells. These results suggest that S-nitrosylation of Cys644 residue in Drp1 inhibits Drp1 functioning. A $\beta$  oligomers along with NO donors subsequently accelerated mitochondrial fragmentation and neuronal synaptic damage in HEK 293 cells [42]. These results suggest that S-nitrosylation of Drp1 has a potential detrimental effect on mitochondrial dysfunction in AD.

Zahid et al. also reported that in post mortem hippocampus, substantia nigra and cortex of AD patients superoxide dismutase type 2 can be S-nitrosylated and that superoxide dismutase type 2 S-nitrosylation is increased [13]. Superoxide dismutase type 2 is localized in mitochondria and is an antioxidant metalloenzyme that plays a critical role against oxidative and nitrosative stress in the mitochondrion [51,52]. These findings also support that S-nitrosylation can contribute in part to mitochondrial dysfunction identified in AD.

### S-nitrosylation on protein misfolding

Protein misfolding and aggregation are increased in brain of patients with AD. For instance, neurofibrillary tangles are caused by hyperphosphorylated tau aggregates, while A $\beta$  plaques are caused by A $\beta$  aggregates [7-9]. Recent studies show that S-nitrosylation of protein disulfide isomerase (PDI) in AD contributes to misfolding of proteins. The immature proteins are synthesized on ribosomes, and then translocated to endoplasmic reticulum for proper folding by disulfide bond formation, which makes proteins mature and ready for functioning. PDI catalyzes the correct formation of disulfide bond based on thiol-disulfide exchange reactions [53,54]. PDI also serves as a molecular chaperone that assists in the maturation, transport and folding of secretory proteins [40]. It is evidenced that the absence of proper disulfide bond formation in the endoplasmic reticulum is closely related to down regulation of PDI enzyme activity. Low protein activity of PDI can cause endoplasmic reticulum stress, which is a conventional signal pathway for apoptosis [55]. Therefore PDI can reduce protein misfolding and attenuate endoplasmic reticulum stress, subsequently preventing neuronal cell death.

Takashi et al. reported that PDI could be S-nitrosylated in HEK-293 cells treated with NO donor S-nitrosocysteine (SNOC) or in the HEK-293 cells transfected with nNOS [56]. They further observed that S-nitrosylation of PDI was increased in post mortem brains of subjects with AD when compared to controls [56]. To determine whether S-nitrosylation affects PDI function, they monitored PDI chaperone and isomerase activities, which are two indexes for normal PDI functioning. Chaperone activity was assessed by measuring the aggregation of rhodanese induced by guanidinium. PDI significantly

suppressed the aggregation of rhodanese, while S-nitrosylated PDI also inhibited rhodanese aggregation, but with much lower efficiency [56]. Isomerase activity was then investigated by assessing the renaturation ability of PDI, in which a substrate of an inactivated form of RNase A containing scrambled disulfide bonds was used. The renaturation ability was two-fold in the wild type PDI over the S-nitrosylated PDI, indicating a reduced enzyme activity caused by S-nitrosylation [56]. Therefore, S-nitrosylation can inhibit the PDI enzyme activity and its chaperone activity. Given the major role that PDI plays in endoplasmic reticulum protein folding, it is likely that S-nitrosylation of PDI may impair the protein folding process and exacerbate endoplasmic reticulum stress, ultimately leading to cell death in AD.

### S-nitrosylation on synaptic loss

Neuronal dysfunction and degeneration in AD patients is predominantly in the hippocampus and cerebral cortex [6]. Cyclin-dependent kinase 5 (Cdk5) plays an important role in various neuronal functions including axon guidance, neuronal migration, neuronal survival and the regulation of synaptic spine density [57-60]. Dysregulation of Cdk5 activity contributes significantly to the pathogenesis of neurodegenerative disorders. Hyperactivity of Cdk5 triggered by oxidative stress, A $\beta$  and calcium overload contributes to neuronal death in AD [43].

It has been found that NO donor SNOC increased S-nitrosylation of Cdk5 and Cdk5 enzyme activity in cultured cells [43]. S-nitrosylation sites were further identified at Cys83 and Cys157 residues in Cdk5 [43]. SNOC only increased the protein activity in wild type-Cdk5 cells but not in mutant-Cdk5 cells [43]. This result indicates that S-nitrosylation of Cdk5 increases Cdk5 enzyme activity. Cdk5 is an important modulator of NMDA receptor signaling and neuronal spine loss [43]. It has been found that NMDA can increase Cdk5 S-nitrosylation, which can be abrogated by NOS inhibitor N-nitroarginine, indicating Cdk5 S-nitrosylation is mediated by NMDA receptor. NMDA also more significantly increased spine loss in the wild type-Cdk5/p35 cells than in mutant-Cdk5/p35 cells, suggesting that Cdk5 S-nitrosylation contributes to NMDA-induced dendritic spine loss [43]. Exposure of A $\beta$ <sub>1-42</sub> oligomers induces S-nitrosylation of Cdk5 in cultured rat cortical neurons. This treatment also induces loss of dendritic spines in neurons transfected with Cdk5/p35, but not in mutant-Cdk5/p35. A $\beta$ -induced Cdk S-nitrosylation and spine loss can be completely abolished by adding NOS inhibitor N-nitroarginine, suggesting that Cdk5 S-nitrosylation in AD contributes to the spine loss through NMDA receptors [43]. Recently it was found that S-nitrosylation of Cdk5 can act as an endogenous nitrosylase for Drp1 and induce Drp1 S-nitrosylation by transferring NO group from S-nitrosylated Cdk5 to Drp1, contributing to mitochondrial dysfunction and synapse loss [43]. All of these findings suggest that increased S-nitrosylation of Cdk5 changes its enzyme activity and affects the functioning of other proteins that may contribute to A $\beta$ /NMDA receptor-mediated spine loss and the pathogenesis of AD [43]. In addition both alpha and beta tubulins were identified as targets for S-nitrosylation in human AD postmortem brains [13] that may also contribute to synaptic loss in AD. The functional changes from this S-nitrosylation are not entirely clear. However, since alpha and beta tubulins play an important role

in microtubule structure or cellular architecture, S-nitrosylation of these proteins may be a potential target therapy for the future study.

### S-nitrosylation on A $\beta$ production

Beta-site APP cleaving enzyme 1 (BACE1), insulin-degrading enzyme (IDE) and apolipoprotein E (ApoE) can regulate A $\beta$  metabolism and aggregation. Studies have shown that these three proteins can all be S-nitrosylated and that S-nitrosylation of BACE1, IDE and ApoE are seen to be increased in both cell models and AD post mortem brains [26,61,62], suggesting that protein S-nitrosylation may affect A $\beta$  production and deposition.

BACE1 together with  $\gamma$ -secretase cleaves transmembrane APP to form  $\beta$ -amyloid. Since BACE1 is essential for A $\beta$  production, the expression level and enzymatic activity of BACE1 can change the A $\beta$  deposit. Young et al found that NO donor SNOC increased S-nitrosylation of BACE1 and decreased BACE1 activity in primary cultured cortical neurons [26]. BACE1 S-nitrosylation in AD brains was higher in the mild cognitive impairment stage than in the late stage, which inversely correlates with BACE1 protein levels [26]. These findings together demonstrate a suppressive effect on A $\beta$  load from S-nitrosylation of BACE1.

IDE is a conserved zinc metalloprotease that is responsible for the clearance of various hormones and peptides including insulin and A $\beta$  [63]. It has been evidenced that the NO donor S-nitroso-N-acetylpenicillamine could increase S-nitrosylation of purified rat IDE enzyme and also increase IDE S-nitrosylation in HEK 293T cells transfected with human IDE [61]. Cys110, Cys178, Cys789, Cys819 and Cys966 residues of IDE were identified to be S-nitrosylated. S-nitrosylation of Cys110 and Cys819 leads to complete inactivation of IDE. S-nitrosylation of both Cys789 and Cys966 together also causes conformational change and aggregation of IDE, resulting in the inhibition of IDE activity [63]. It was found that various NO donors could significantly reduce IDE enzyme activity, and insulin and A $\beta$  degradation [61]. This result suggests a potential role of S-nitrosylation on IDE function and A $\beta$  production. Since IDE is a very important modulator for A $\beta$  production, inhibition of IDE activity by NO in the brain may increase the accumulation of A $\beta$ , leading to neuronal death.

ApoE represents a family of proteins including ApoE2, ApoE3 and ApoE4, which are expressed in the brain [64]. ApoE is essential for the normal catabolism of triglyceride-rich lipoprotein metabolites. ApoE isoforms enhance the break-down of A $\beta$ , while ApoE4 is not as effective as the others at promoting these reactions. Therefore gene variation of ApoE4 normally results in increased vulnerability to AD, making it one of the important genetic risk factors for AD pathology [65]. Alexander et al. reported that S-nitrosylation of ApoE2 and ApoE3, but not ApoE4 is increased in the HEK-293 cell line transiently transfected with nNOS, suggesting that ApoE2 and ApoE3 can be S-nitrosylated [62]. Specifically, Cys112 and Cys158 in ApoE2 and Cys112 in ApoE3 were identified as S-nitrosylation sites. S-nitrosylation of ApoE2 and ApoE3 inhibits the binding of ApoE to low density lipoprotein receptors [62]. Since the activities of the ApoE isoforms are essential for the break-down of A $\beta$ , the inhibitory effect caused by S-nitrosylation on ApoE2 and ApoE3 may potentially increase A $\beta$  deposit, contributing to AD pathology.

A $\beta$  plaque, as one of the most important pathological hallmarks in AD, contributes to NMDA receptor hyperactivity, synapse loss and neuronal cell death. NO may regulate A $\beta$  production by S-nitrosylating proteins. In these findings, BACE1 S-nitrosylation potentially suppresses A $\beta$  production, showing a 'beneficial' effect, while IDE S-nitrosylation and ApoE S-nitrosylation potentially increase A $\beta$  production, showing a 'detrimental' effect. These results indicate a potential pharmaceutical way to reduce A $\beta$  production either by S-nitrosylating beneficial proteins or denitrosylating detrimental proteins.

Many S-nitrosylated proteins have recently been identified in AD post mortem brains or AD animal models by high throughput proteomic studies techniques with mainly S-nitrosylated protein enrichment and mass spectrometry [50]. WEB-based Gene Set Analysis Toolkit (WEBGESTALT) and UniProt show that the vast majority of S-nitrosylated proteins are related to signaling pathways, including apoptosis, energy metabolism and redox regulation [13]. In addition, 138 synaptic proteins have been found to be S-nitrosylated in a transgenic animal model, APPV717I [14]. The APPV717I animal model is an AD mouse model over expressing human APP with the London mutation driven by the Thy1 promoter. This finding indicates that S-nitrosylation may have a potential role in mediating neurotransmission.

### Indications for the S-nitrosylation process in the pharmacological treatment of AD

Oxidative stress is one of the main characteristics in Alzheimer's disease. NO can induce protein S-nitrosylation, Protein S-nitrosylation is known to be involved in mitochondrial dysfunction, protein misfolding, synapse loss and A $\beta$  production and therefore may have either a direct or an indirect effect on AD pathology. These findings suggest that the process of S-nitrosylation could be a good target for the pharmacological treatment of AD. These unique characteristics of S-nitrosylation are a significant potentiality for developing new drugs for AD patients.

Antioxidants that can scavenge the excessive NO within the brain may have potential for AD treatment involving S-nitrosylation. Polyphenolic compounds extracted from green tea, magnolia, blueberries and grapes scavenge ROS/RNS including NO and exhibit neuroprotective effects. Studies have shown that these compounds produce beneficial effect on both *in vivo* and *in vitro* in animal models for AD [66-70]. Since inhibition of NOS can reduce NO production, NOS inhibitors may also be beneficial for AD treatment. NOS inhibitors 1400W (N-(3-(aminomethyl)benzyl) acetamide), L-NIL (N-iminoethyl-L-lysine) and L-NAME (N-nitro-L-arginine methylester) were found not only to reduce NO release but also to inhibit A $\beta$  and glutamate-induced neuronal cell death [71,72]. Other compounds have also demonstrated a beneficial effect on AD pathology by inhibiting NOS activity. Rutin is a flavonoid that can dose-dependently decrease A $\beta$ <sub>42</sub>-induced cytotoxicity in human SH-SY5Y neuroblastoma cells. One of its main effects is to decrease NO production by reducing iNOS activity [73]. Ferulic acid ethyl ester was reported to protect neurons against A $\beta$ <sub>1-42</sub>-induced toxicity in primary neuronal cell culture in part by down-regulation of iNOS [74]. Xanthorrhizol reduced ROS generation and glutamate-induced neurotoxicity in the murine hippocampal HT22 cell line, partially by

reducing the expression of iNOS, which consequently resulted in the reduction of NO [71].

NMDA receptors are now considered to be a target for AD treatment as it plays vital role in neuronal excitotoxicity. NMDA receptor activation can increase NO production and that increased NO can then nitrosylate NMDA receptor, resulting in *negative feedback regulation* of NMDA receptor activity. Therefore, alteration of NMDA receptor status may also have a potential for AD treatment. Nitroglycerin, which donates NO, has been shown to limit excessive NMDA receptor activity in rat AD models [73,75]. Nitromemantines are combinatorial drugs of memantine and NO donors. Theoretically memantine works as a homing molecule to allow NO donor to combine with S-nitrosylation sites of the NMDA receptor. Nitromemantines have been proven to be highly neuroprotective in cultured rat primary cortical neurons treated with A $\beta$  [76].

## Conclusions

Protein S-nitrosylation contributes significantly to the pathological development of AD. Protein S-nitrosylation occurs in response to excessive NO production, which may regulate mitochondrial dysfunction, protein misfolding, synaptic loss and A $\beta$  production. The global S-nitrosylation of proteins detected in AD indicates a potential pharmaceutical way forward to development of future AD therapies. However, our knowledge of the molecular mechanisms of S-nitrosylation in AD is by no means complete. In future studies, more work will be needed to elucidate the precise mechanisms of S-nitrosylated proteins in AD pathology. In all, it is an interesting and promising topic for future AD therapies.

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