



## Review Article

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# N-Acetylcysteine: A Natural Antidote for Alzheimer's Disease

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### Abstract

Alzheimer's disease (AD) is the most devastating age-related dementia, which has no effective treatment. Since the pathological hallmarks of AD brains are A $\beta$  plaques and intra-neuronal tau-containing neurofibrillary tangles, most therapeutic approaches, including immunotherapy, are based on the concept that the accumulation of these proteins produces neuronal damage and death. Alternatively, it has been proposed that reactive species can cause oxidative damage including mitochondrial disruption, contributing to AD initiation and progression. Can these cellular dysfunctions be linked by a common pathogenic mechanism susceptible to therapy with the natural compound N-acetylcysteine? A cellular cysteine network (CYSTEINET) has been proposed as a functional and structural matrix of interconnected sensitive cysteine-containing proteins (SCCPs) that in conjunction with reactive species and the cysteine/glutathione cycles can regulate the bioenergetic metabolism, the redox homeostasis, and the cellular survival through different pathways that bear the same regulatory thiol radical. The present paper proposes that cysteinete is impaired in Alzheimer's disease resulting in a functional and structural deregulation of the matrix of interconnected cysteine-containing proteins that result in misfolding, aggregation and accumulation of specific toxic proteins. In this context, the role of N-acetylcysteine to prevent and restore cysteinete deregulation in AD development and progression is discussed.

### Keywords

Cysteine, Thiol, Neurodegeneration, Reactive species, Redox homeostasis, Acetylcysteine, Alzheimer, Cysteinete

## Introduction

Alzheimer's disease (AD) is the most devastating neurodegenerative disorder accounting for 60% to 70% cases of dementia. Most AD cases are sporadic and occur in elderly people over 60 years old. Therefore, like in Parkinson's disease (PD), the principal risk factor for sporadic AD is aging. Cognitive impairments of patients with AD are associated with synaptic deficits and neuronal loss, which have been shown early events in the disease [1-3]. The prevalent hypothesis explaining the physiopathology of AD is the amyloid cascade, which postulates that the aberrant processing of amyloid precursor protein (APP) through amyloidogenic [3-6] pathways yield increased A $\beta$ 1-42 peptide that is the responsible for neurotoxicity, neuronal loss and cognitive impairment in AD patients [7,8]. In addition, intracellular neurofibrillary tangles are formed through the hyperphosphorylation of the microtubule-associated protein tau, which forms part of the cytoskeleton of neurons [9,10].

Since the pathological hallmarks of AD brains are A $\beta$  plaques and intracellular tau-containing neurofibrillary tangles, most therapeutic approaches have been directed towards the inhibition of the aggregation and accumulation of these proteins in the brain. The food and drug administration (FDA) has approved various drugs for AD treatment that can be divided into compounds that directly target amyloid A $\beta$  by active and passive immunization, those targeting the inhibition or modulation of the APP  $\gamma$ -secretase enzyme, and those targeting the APP  $\beta$ -secretase enzyme (BACE1 inhibitors drugs), which have shown controversial and no conclusive results [11,12].

In view of the above, other theories have been elaborated to explain AD etiology and physiopathology including various attempts to integrate the many pieces that form part of the AD puzzle. Our approach is that AD physiopathology is better explained if diverse etiologic factors (genetics, mitochondrial, toxic, metabolic, inflammatory, and age-related) isolated or in conjunction, initiate some biochemical disturbances that converge in a common pathway ending in neurodegeneration. Cysteinete has been proposed as a hierarchical bottom-up biochemical network, composed of

- (a) Reactive species acting as molecular massagers and modulators,
- (b) Cysteine/cystine and reduced/oxidized glutathione (GSH/GSSG) cycles, and
- (c) All peptides and proteins that have "sensitive" cysteine residues in their structure [13,14].

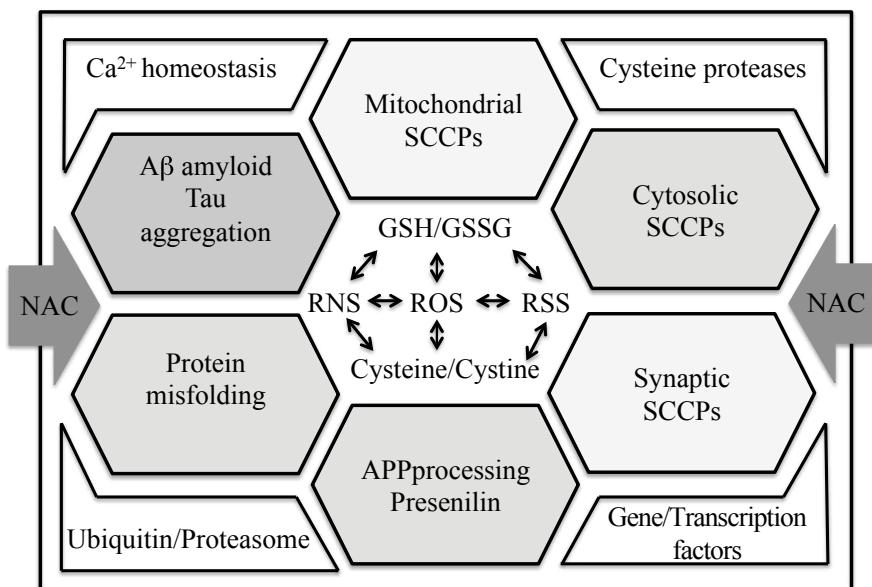
This interconnected matrix of sensitive cysteine-containing

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**Received:** September 02, 2016; **Accepted:** November 03, 2016; **Published online:** November 05, 2016

**Citation:** Martínez-Banaclocha M (2016) N-Acetylcysteine: A Natural Antidote for Alzheimer's Disease. Alzheimers Dis Dement 1(1):4-15

## CYSTEINET



**Figure 1: Cysteinnet deregulation in AD** - Cysteinnet is a hierarchical bottom-up biochemical network composed of reactive species acting as molecular massagers and modulators, cysteine/cystine and reduced/oxidized glutathione (GSH/GSSG) cycles, and all proteins that have “sensitive” cysteine residues in their structure. Sensitive cysteine-containing proteins (SCCPs) have very different metabolic, signaling and structural functions but are regulated by the same thiol radical. These SCCPs can suffer redox modifications (s-glutathionylation, s-nitrosylation, sulfenylation, disulfide bonds formation) resulting in reversible or irreversible changes in the protein function. Cysteinnet deregulation in AD can be prevented and restored by the chronic administration of NAC counteracting the deleterious redox modification of many SCCPs in different pathways. SCCPs (Sensitive Cysteine-Containing Proteins); ROS (Reactive Oxygen Species); RNS (Reactive Nitrogen Species); RSS (Reactive Sulfur Species); GSH (Reduced Glutathione); GSSG (Oxidized Glutathione); NAC (N-Acetylcysteine).

proteins (SCCPs) has very different metabolic, signaling and structural functions in the cell but is regulated by the same thiol radical. These SCCPs use cysteine residues as cellular sensors, which can entangle, at very short timescale, different cellular functions. The central concept is “sensitive cysteines”, which are all cysteine residues in peptides and proteins that can suffer redox modifications (s-glutathionylation, s-nitrosylation, sulfenylation, disulfide bonds formation) under physiological or pathological conditions resulting in a reversible or irreversible change in the protein function [15,16]. Therefore, it is proposed that cysteinnet deregulation is the common mechanism through which different etiologic events or insults overcome age-associated cellular redox homeostasis and evolve toward a vicious circle in age-related neurodegenerative diseases. For example, the ROS overproduction associated with age-related mitochondrial DNA damage and the subsequent impairment in the bioenergetic ability in patients that are homozygote for apolipoprotein E4 may result in a dysfunction of cysteinnet regulation that can entangle diverse pathways facilitating, among others, A $\beta$  amyloid and tau aggregation (Figure 1). In this regard, we propose that cysteinnet deregulation in AD can be prevented and restored by the chronic administration of NAC counteracting the deleterious redox modification of many SCCPs in different pathways.

In the next sections, it has been reviewed the critical role played by redox modification of thiol groups in SCCPs from diverse cellular pathways, and how they can be integrated into the proposed cysteinnet deregulation that may explain AD physiopathology.

### Risk Factors and Cysteinnet Deregulation in AD

#### Aging

Aging is the major risk factor for the development of late-onset AD. Aging is closely associated with oxidative damage to cellular macromolecules produced by reactive species [17-19]. A study in healthy humans aged 19-85 have shown an age-related linear decrease in the cysteine/cystine ratio throughout life, and an

age-related decrease in the total glutathione content and GSH/GSSG ratio after the age of 45 [20]. This show an age-related shift of redox homeostasis toward pro-oxidant conditions, which have critical consequences, not only in many protective antioxidant enzymes that use GSH to counteract the deleterious effects associated with aging [21], but also in many regulatory pathways that depend on the functional integrity of SCCPs [22,23] including mitochondrial ones [24,25]. Therefore, aging increases the rate of irreversible damage to structural and metabolic proteins interfering with normal cellular functions and homeostasis. New quantitative proteomic methods indicate that aging is associated with a shift in redox-regulated proteins resulting in major changes in the glycolytic enzymes as well as in the regulatory enzymes controlling the energy homeostasis in muscular post-mitotic cells [26]. Besides, recent investigations have shown that normal brain aging is characterized by a deregulated pattern of expression of specific aggregation-prone proteins that predispose to A $\beta$  and tau deposition, in contrast to the suboptimal expression of homeostatic proteins [27]. These results suggest that there is an increased susceptibility to protein aggregation in specific brain areas more vulnerable to AD development [27].

In agreement with previous data [28,29], genome-wide gene expression studies have provided evidence of reduced mitochondrial function during aging [30,31] and reduced expression of genes involved in mitochondrial energy metabolism in humans with cognitive decline and AD [32].

Finally, it seems that caloric restriction decreases the body temperature, the rate of metabolism, and the production of reactive species counteracting age-related oxidative damage [33]. Likewise, caloric restriction can stabilize mitochondrial function reducing oxidative damage in neurons in response to different types of genetic and environmental factors [34]. Therefore, age-associated oxidative modification of SCCPs may represent the Achilles’ heels for senescence and age-associated neurodegenerative disorders [35,36].

**Table 1:** Cystein deregulation in AD.

<b>1. Mitochondrial SCCPs dysfunctions</b>
Complex I, IV and V disruption; TCAc disturbance
Reactive species overproduction
Mitochondrial biogenesis
MPTP (mitochondrial permeability transition pore) dysfunction
<b>2. Cytosolic SCCPs dysfunctions</b>
Glyceraldehyde-3-phosphate dehydrogenase
Pyruvate kinase; Phosphofructokinase
Glucose 6-phosphate isomerase
Glycogen phosphorylase
Phosphoglycerate mutase 1
Phosphoglucomutase 2
Protein tyrosine kinases
<b>3. Synaptic proteins</b>
Cysteine string protein α
Synaptophysin
α-synuclein
Soluble NSF attachment protein receptor
Synaptotagmin
<b>4. APP processing and Aβ aggregation</b>
APP mutations and palmitoylation
Presenilin 1 mutations
Presenilin 2 tertiary structure alteration
γ-secretase dysfunction
<b>5. Tau protein aggregation</b>
Tau cross-link
Tau self-acetylation
Microtubule associated protein-2 (MAP2)
<b>6. Calcium homeostasis</b>
Sarco-endoplasmic reticulum Ca <sup>2+</sup> ATPase (SERCA) pump
Inositol 1,4,5-trisphosphate receptor (IP3R)
Ryanodine receptor (RYR)
<b>7. Protein misfolding</b>
Reduced/oxidized disulfide bonds in proteins
Protein disulfide isomerase (PDI)
Mitochondrial import and assembly (MIA) pathway
Other redox-dependent chaperone-like mechanisms (thioredoxin)
<b>8. Ubiquitin-proteasome pathway</b>
E1, E2 and E3 enzymes
Other proteins of the proteasome machinery
Keap1-Cullin-3 ubiquitination system
<b>9. Cysteine proteases</b>
Calpains, cathepsins, and caspases
X-linked inhibitor of apoptosis (XIAP)
<b>10. Transcription factors and genes expression</b>
Heat shock transcription factor 1 (HSF1)
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)
NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)
Ras-ERK pathway

## Apolipoprotein E

The most well known genetic link to late-onset AD is the apolipoprotein (APO) genotype E4 (APOE4) [37]. Individuals with an E4 allele have a reduced lifespan [38] and they have an increased risk to develop AD [39]. There are three alleles of apolipoprotein E that differ in two amino acids. APOE2 contains a cysteine in each position, APOE3 contains a cysteine in one of the positions, but APOE4 does not contain a cysteine in either position. The mechanism by which APOE4 accelerates brain aging may involve decreased antioxidant and neuroprotective properties of this apolipoprotein isoform, since cysteine residues in APOE2 and APOE3, may bind to and detoxify 4-hydroxynonenal, a cytotoxic

product of lipid peroxidation [40]. Therefore, apolipoprotein E isoforms have different potential sites for redox modification and regulation through sensitive cysteine residues. Indeed, apolipoprotein E isoforms can bind to neuronal nitric oxide synthase (NOS) and they can be S-nitrosylated in human hippocampus, possibly regulating the lipid metabolism in AD [41].

## Cystein deregulation in AD

Post-translational oxidative modifications of proteins have diverse functional implications including the control of the redox microenvironment and homeostasis. Since the thiol group of cysteine has a number of oxidation states, it is highly sensitive to environmental, biochemical, and pathological conditions resulting in the reversible or irreversible oxidation of this radical. Therefore, redox modifications of SCCPs can stabilize proteins against further irreversible oxidative damage, or they can be reverted to its physiological state [42]. The reversibility of thiol modifications allows that cysteine residues can function as switches in many regulatory pathways (Figure 1) as well as at many structural sites into the cell (Table 1). However, the irreversibility of thiol oxidation produces cumulative damage to proteins with diverse functional consequences [43,44].

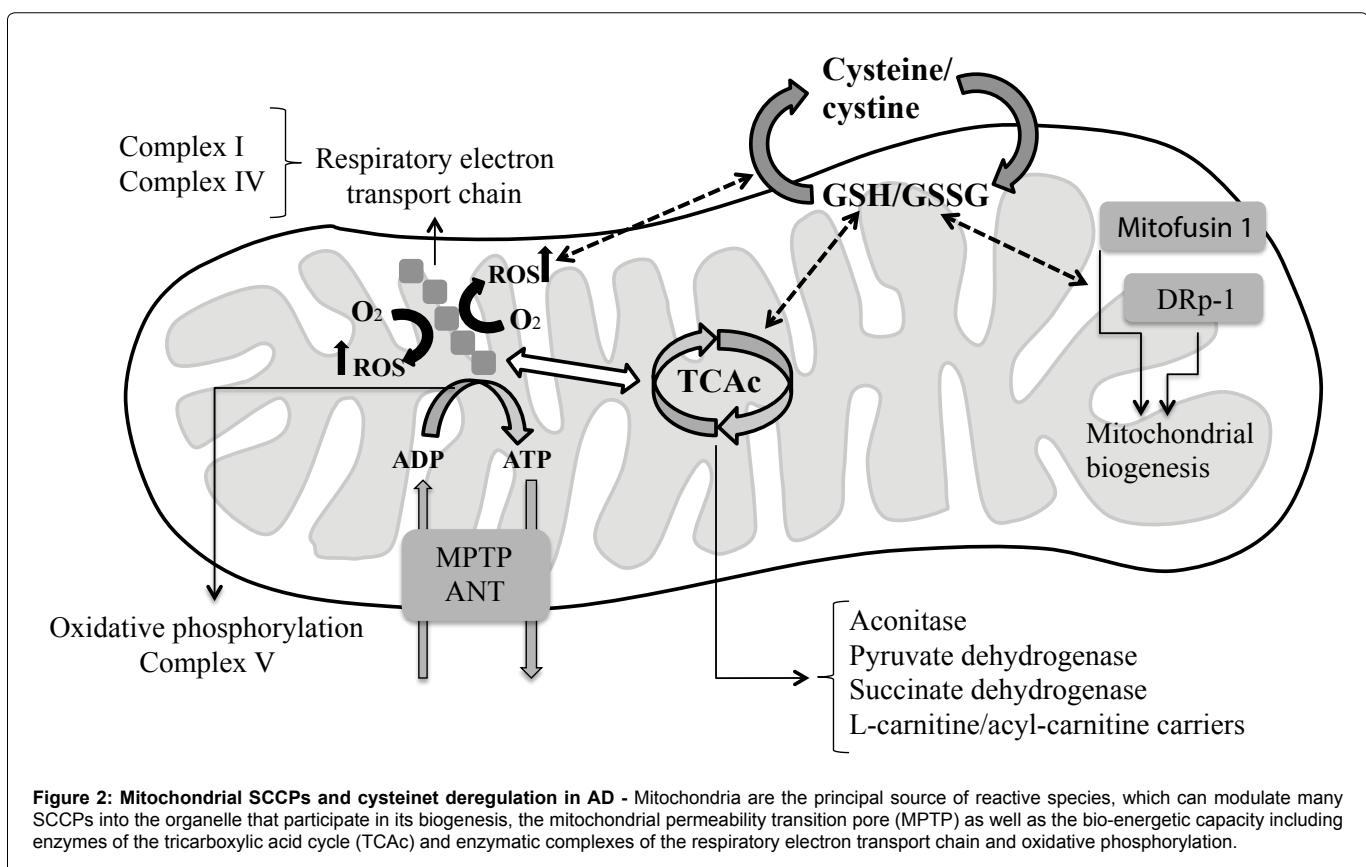
## Reactive species, mitochondrial SCCPs and cystein deregulation in AD

Physiological concentrations of reactive species (reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS)) contribute to regulating cell metabolism and signaling pathways [45,46]. However, AD brains exhibit increased levels of reactive species, which affect proteins that are critical to neuronal survival contributing to the disease physiopathology [47]. In this regard, mitochondrial dysfunction plays a key role in AD physiopathology [48], occurring prior to the onset of clinical symptoms and Aβ plaque formation [48-52]. Indeed, synaptic mitochondria seem particularly vulnerable in aging and AD [52-54]. Mitochondria are the principal source of reactive species, which can modulate many SCCPs into the organelle that participate in the bio-energetic capacity including enzymes of the tricarboxylic acid cycle (TCAc) and enzymatic complexes of the respiratory electron transport chain and oxidative phosphorylation (Figure 2).

Aconitase and pyruvate dehydrogenase can be reversibly inactivated by hydrogen peroxide or S-glutathionylation through the oxidation of sensitive cysteine residues [15,42]. Succinate dehydrogenase can be modified by S-glutathionylation, and L-carnitine/acyl-carnitine carriers have also been found to be S-glutathionylated on Cys136 and Cys155 [15]. The oxidative modification of these enzymes by S-glutathionylation is reversible, and it occurs under physiological conditions. However, during the aging process, when GSH/GSSG ratio into de mitochondria is low, the oxidative modification of these SCCPs may be irreversible (Figure 1 and Figure 2).

Complex I of the mitochondrial respiratory chain can undergo sulfenylation, which decreases its activity. Sulfenylation may suffer further oxidation that irreversibly deactivates Complex I [15]. However, it seems that cysteine residues that are oxidized in Complex I can be protected from further oxidation by S-glutathionylation [42]. This S-glutathionylation of Complex I limits NADH production, and it decreases electron flow through the respiratory chain diminishing ROS generation, which protects this enzymatic complex from irreversible oxidation by reactive species (Figure 2). However, recent data have shown a disruption of Complex I activity at late stages of dysfunction in a mouse model of AD [55].

Complex IV (cytochrome c oxidase) is a key enzyme in the respiratory electron transport chain of the mitochondria, and it



**Figure 2: Mitochondrial SCCPs and cysteinot deregulation in AD** - Mitochondria are the principal source of reactive species, which can modulate many SCCPs into the organelle that participate in its biogenesis, the mitochondrial permeability transition pore (MPTP) as well as the bio-energetic capacity including enzymes of the tricarboxylic acid cycle (TCAC) and enzymatic complexes of the respiratory electron transport chain and oxidative phosphorylation.

has critical cysteine residues that play fundamental roles in metal coordination necessary for the redox-linked proton pumping by the enzyme [56,57]. There is extensive evidence for reduced complex IV activity in AD brain tissue, fibroblasts and blood platelets [58-61].

Oxidative phosphorylation is also modulated by redox modification of the ATP synthase (Complex V) that can be S-glutathionylated on Cys294 of the  $\alpha$ -subunit located in the F1 hydrophilic part of the Complex. Cys294 may also react with the neighboring Cys103 residue to form a disulfide bridge [62]. S-glutathionylation blocks the nucleotide binding to the complex, which results in a decrease in the production of ATP. Therefore, the oxidative modifications of SCCPs can control the mitochondrial ROS generation and the bioenergetic capacity of the cell (Figure 2).

Energy demand in neurons depends on mitochondrial dynamics mediated by fission and fusion mechanisms to generate new mitochondria. In this regard, recent studies have shown that  $\beta$ -amyloid accumulates inside AD brain mitochondria interfering with mitochondrial fusion and fission contributing to the development of AD [63,64]. Among SCCPs that participate in the mitochondrial biogenesis, the integral membrane GTPases Mitofusin 1 [65] and the dynamin family of GTPases dynamin-related protein-1 (Drp-1) are two well-studied examples. S-nitrosylated Drp1 levels are significantly increased in the brain of AD patient compared to controls, and it has been associated with the anomalous fragmentation of mitochondria and the consequent loss of synapses [66,67]. Besides, S-glutathionylation of mitofusin proteins is required to induce mitochondrial hyperfusion, which is regulated by redox micro-environmental conditions [68,69]. Therefore, mitochondrial SCCPs that regulate the structure and biogenesis of the organelle are dependent on the redox modifications of thiol groups in cysteine residues (Figure 2).

Finally, reactive species overproduction can modify the mitochondrial permeability transition pore (MPTP), which is a non-

selective pore that allows the free diffusion of molecules under 1.5 kDa in size [70]. Sulfenylation and S-glutathionylation can induce programmed cell death in the mitochondria through the modulation of MPTP opening [70] that require the disulfide bond formation between specific cysteine residues in the adenine nucleotide translocator (ANT). ANT is a structural component of MPTP [70,71], which exports ATP from the mitochondrial matrix and imports ADP into the matrix [72].

#### Cytosolic SCCPs and cysteinot deregulation in AD

Many extra-mitochondrial proteins are regulated by cysteine redox modifications including many proteins involved in the glucose metabolism such as glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, phosphofructokinase, glucose 6-phosphate isomerase, glycogen phosphorylase, phosphoglycerate mutase 1, and phosphoglucomutase 2, which require cysteine residues for their activity [73] (Figure 1 and Table 1).

A well-studied example of the glycolytic pathway that has been implicated in AD physiopathology is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [74]. GAPDH catalyzes the reversible phosphorylation of G3P, which involves the thiol group of Cys152 [74]. Sensitive cysteine residues of GAPDH are oxidized by hydrogen peroxide, which decreases the stability of the protein, giving rise to monomers, dimers, and other denatured GAPDH products [74]. Furthermore, under pathological conditions glutathione can react with sensitive cysteine residues of GAPDH forming disulfide bonds [74]. GAPDH has been found oxidized in AD brain [74], although some studies have suggested that S-glutathionylation of GAPDH is a mechanism against permanent damage of the protein in the oxidizing environment of the AD brain [74]. Besides, GAPDH can be reversibly inhibited by s-nitrosylation of sensitive cysteine residues by nitric oxide, which can inhibit their dehydrogenase activity [74].

Protein tyrosine kinases (PTK) are a family of enzymes that contain a conserved sensitive cysteine residue. For example, protein

tyrosine phosphatase 1b is redox regulated by cysteine residues placed in the catalytic center of the enzyme, which is reversibly oxidized by hydrogen peroxide to form sulfenic acid as well as by S-glutathionylation [75]. The result of these oxidative changes is an increase in tyrosine phosphorylation [75].

### Synaptic proteins and cysteinot deregulation in AD

Cysteine string protein α (CSPα) is a protein involved in neurodegeneration [76] that regulates vesicle endocytosis, which participates in the regulation of synaptic transmission, and in the maintenance of synapses. CSPα contains a cysteine-rich “string” region for attachment to the synaptic vesicles that depend on its oligomerization [76]. Recently it has been reported that the expression of CSPα is reduced in degenerating areas of the forebrain in post-mortem samples of AD [77].

Synaptophysin is a membrane protein that forms channels in the synaptic vesicle, which depend on sensitive cysteine residues. The molecule contains unstable disulfide bonds that cross-linking with neighboring synaptophysin monomers. Cross-linking of synaptophysin by disulfide bonds are triggered by redox modification, suggesting that native synaptophysin depends on the adequate formation of cysteine associated disulfide bonds to form multimeric complexes in the adequate phospholipid environment [78]. This important protein plays a key role in the synaptic alterations associated with AD and its loss in the hippocampus has been correlated with cognitive decline in AD patients [79]. Besides, synaptophysin concentrations in the frontal cortex of AD patients were significantly lower than in controls [80].

More than 50% of AD patients exhibit α-synuclein accumulation [81]. Although human α-synuclein does not contain any cysteine residues [82], tyrosine to cysteine substitution at critical positions in the α-synuclein protein can increase dimer formation and accelerate protein aggregation and cellular toxicity of α-synuclein [83]. Through a chaperone-like function, α-synuclein may act in synergy with CSP-α in the assembly of the SNARE (soluble NSF attachment protein receptor) complex [84], and this effect involve the binding to synaptotagmin proteins, which have critical cysteine residues in their structure [84,85] (Figure 1).

### APP processing, Aβ aggregation and cysteinot deregulation in AD

In general, there are two clinical forms of AD, the familial or early-onset AD (5% of all AD cases) produced by mutations in APP, presenilin-1 and presenilin-2 genes [86,87], and the highly prevalent sporadic or late-onset AD (95% of AD cases). The discovery that transgenic mice expressing familial human APP and presenilin mutations showed the major features of the human disease [88] contributed to establishing the amyloid cascade hypothesis [89]. Mutations in early-onset cases result in the aberrant processing of APP, leading to the aggregation of Aβ peptides and the subsequent hyperphosphorylation of tau, which seem to be critical factors for AD development [88].

Human presenilin-1 contains five native cysteine residues [90], three cysteine residues are in the transmembrane domains and two are exposed to the cytosol. Cys410 and Cys419 are in the presenilin-1 transmembrane domain 8, and both of these cysteine residues can crosslink with Cys92 in the transmembrane domain 1, contributing to conformational changes [91] that allow the interaction with the active site of γ-secretase, suggesting that these sensitive cysteine residues may support the integrity and hydrophilic environment of this active site [91]. This may have relevance in AD physiopathology since familial cases have shown mutations at three of the five endogenous cysteine residues in presenilin-1 [92,93], which would initiate cysteinot deregulation in early onset cases (Figure 1). On the

other hand, disulfide bridges between residues Cys14-Cys31 and Cys56-Cys65 in presenilin-2, which are absent in the same region of presenilin-1, are critical determinants of the tertiary structure of presenilin-2. Interestingly, these sensitive cysteine residues seem to be important in the regulation of ryanodine receptor (RYR)-mediated Ca<sup>2+</sup> release [94].

Moreover, protein palmitoylation consists of a thioester linkage between a palmitic acid and a cysteine residue to increase the hydrophobicity of the protein facilitating its integration in membranes [95]. APP has two palmitoylation sites at Cys186 and Cys187 that are required for its exit from the endoplasmic reticulum (ER). Cys186 and Cys187 reside within the copper-binding domain of APP, and they can stabilize the domain structure by forming disulfide bonds with Cys158 and Cys133, respectively [96]. Indeed, increased APP palmitoylation may enhance non-amyloidogenic β-cleavage of APP [96].

Therefore, under the view of the present hypothesis, mutations in APP, presenilin 1 or presenilin 2 are the etiologic factors that initiate cysteinot deregulation, which perpetuates and spreads the disequilibrium in the redox homeostasis in cases of early-onset AD (Figure 1).

### Tau protein aggregation and cysteinot deregulation in AD

Twenty years ago it was demonstrated that pathological aggregation of tau protein into paired helical filaments (PHFs) and neurofibrillary tangles are dependent on the intermolecular cross-link of Cys322 [97]. The authors suggested that in addition to the disequilibrium between protein kinases and phosphatases, there is an age-related increase in neuronal oxidative stress that leads to the hyperphosphorylation of the protein (Figure 1). Indeed, it has been shown that peroxynitrite and hydrogen peroxide can induce cysteine oxidations in tau and microtubule associated protein-2 (MAP2) altering the ability of these proteins to assemble with microtubules [98]. Therefore, increased oxidative modification of sensitive cysteine found in brain aging [97] would provide a link for the aggregation of both types of AD deposits, since the amyloid deposits also appear to be related to increased oxidation [97].

Biochemical and kinetic studies indicate that tau catalyzes auto-acetylation mediated by a pair of catalytic sensitive cysteine residues in its microtubule-binding domain [99]. Tau cysteine residues are required for auto-acetylation increasing insoluble tau accumulation by both intra- and intermolecular acetylation mechanisms (Table 1). In fact, it is estimated that tau proteins are about 99% bound to microtubules in mature neurons [99], which inhibit tau acetyl-transferase activity by blocking the cysteine residues that are linked to microtubules. Therefore, it has been suggested that microtubule detachment and activation of tau auto-acetyl transferase activity could represent a pathological event, in which continual self-acetylation gradually shifts the tau-microtubule binding equilibrium toward cytosolic tau accumulation, providing an increased pool of aggregation-prone tau species [99]. Consistent with this model, the levels of acetyl-CoA are reported to be elevated in AD brain [99], which would support increased tau acetyl-transferase activity during the disease progression. Interestingly, methylthioninium, a tau aggregation inhibitor, can prevent the formation of tau filaments and their toxic precursors through oxidation of cysteine residues in the tau repeat domain preventing the formation of disulfide bridges and retaining the tau protein in a monomeric conformation [100]. Additionally, it has been demonstrated that compounds that bind cysteine residues of tau can prevent neurofibrillary tangle-associated brain dysfunction [101].

## Calcium homeostasis and cysteinot deregulation in AD

Calcium ( $\text{Ca}^{2+}$ ) disturbances in AD have been attributed to  $\text{A}\beta$  amyloid up-regulation [102] through the formation of channels in membranes, up-regulation of NMDARs channels, and a mechanism that implicates the prion protein [103]. However, intracellular  $\text{Ca}^{2+}$  levels in neurons depend on the equilibrium between pumping of cytosolic  $\text{Ca}^{2+}$  into the ER lumen by sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) pump, and the release of  $\text{Ca}^{2+}$  from the ER through the activation of two types of  $\text{Ca}^{2+}$  receptors, the inositol 1,4,5-trisphosphate receptor (IP3R) and the RYR (Figure 1). One function of RYR is to amplify the IP3R-mediated release of  $\text{Ca}^{2+}$  from ER enhancing neuronal  $\text{Ca}^{2+}$  signals [104]. IP3R and RYR receptors are the most expressed intracellular  $\text{Ca}^{2+}$  channels regulated by thiol groups of cysteine. RYR1 channel has multiple cysteine residues that can suffer S-nitrosylation and S-glutathionylation, which can destabilize the closed state of the RYR1 complex resulting in an altered release of  $\text{Ca}^{2+}$  to the cytoplasm [105]. Similarly, IP3R contain multiple sensitive cysteine residues that serve as the target of ROS produced in both the ER and the mitochondria [106]. This receptor regulates the cytoplasmic  $\text{Ca}^{2+}$  levels in cells through the modulation of specific cysteine residues within the IP3-binding core and suppressor domain of the protein, changing the active conformation of the receptor [107]. These findings may be relevant having into account that IP3R levels have been found decreased in the hippocampal formation of AD patients, showing significant correlations with senile plaque staging and neurofibrillary tangle pathology [108]. Besides, high glucose levels can oxidize SERCA Cys674 preventing its inhibition by nitric oxide and hydrogen peroxide [109], which can contribute to  $\text{Ca}^{2+}$  disturbance. Interestingly, quantitative mapping of oxidation-sensitive cysteine residues in SERCA from skeletal muscle of Fisher 344 x Brown Norway F1 hybrid rats, were found selectively oxidized during aging [110]. These data suggest an age-related loss of sensitive cysteine residues in SERCA explaining the age-associated decrease in the specific  $\text{Ca}^{2+}$ -ATPase activity [110].

## Protein misfolding and cysteinot deregulation in AD

Under physiological conditions, chaperones help proteins to achieve their three-dimensional (3-D) configuration, but aging and other stressing conditions can disturb the exquisite balance among the synthesis, folding and degradation of proteins resulting in their accumulation. Protein folding depends on the amino acid primary sequence as well as on the cysteine content to form a disulfide bond under different redox conditions [111] (Figure 1). Cysteine thiol groups can switch between oxidized and reduced states contributing to protein folding [112]. In fact, many misfolded proteins contain disulfide bonds [113]. The spontaneous folding process can be particularly slow in the case of cysteine-containing proteins that require the formation of disulfide bonds [114]. Therefore, *in vivo* disulfide bond formation is catalyzed by specialized enzymes such as protein disulfide isomerase (PDI) [115]. This ER enzyme has been found S-nitrosylated in brain samples of sporadic Parkinson and Alzheimer's patients [116]. S-nitrosylation of PDI facilitates further oxidative modifications compromising chaperone/protein folding and accumulation in ER [116].

Mitochondrial biogenesis depends on protein import from the cytosol through mitochondrial translocation pathways (Table 1). Among these pathways, the mitochondrial import and assembly (MIA) pathway targets proteins to the intermembrane space (IMS) and couples import to folding and oxidation resulting in the covalent modification of the incoming protein precursor that incorporates disulfide bonds in the process [117]. For example, Mia40 is a thiol oxidase does not belong to the thioredoxin family, and its catalytic disulfide is arranged in a unique Cys-Pro-Cys motif. The formation of the initially mixed disulfide between Mia40 and a substrate

protein directs the course of its oxidative folding [118], which seem dependent on specific cysteine residues to form the initial enzyme-substrate-mixed disulfide [118]. Therefore, Mia40 and other redox active proteins like thioredoxin, glutaredoxin, and peroxiredoxin into the IMS have been suggested as redox-dependent chaperone-like mechanisms in mitochondria associated with sensitive cysteine residues [118] (Figure 1).

## Ubiquitin-proteasome pathway and cysteinot deregulation in AD

Oxidative damage in proteins and aggregates are recognized and degraded by the ubiquitin-proteasome machinery in a first step to recycling those proteins [119]. The ubiquitin-proteasome machinery is a multistep pathway that involves the activation of the ubiquitin protein through an ATP-dependent process that uses two enzymes (E1 and E2) to form ubiquitin-E2, which binds to target protein by the mediation of the ubiquitin protein ligase (E3) [120]. These steps are dependent on cysteine residues that form consecutive thioester linkages ending in protein degradation in the proteasome [120] (Figure 1). Therefore, the ubiquitin-proteasome multistep pathway is dependent on the physiological redox regulation of thiol groups of sensitive cysteine residues of the proteins implicated in the pathway [121,122]. Accumulation of ubiquitinated protein aggregates occur during normal brain aging and reach pathological levels in neurodegenerative disorders such as AD [123]. A recent study in a mouse model of reduced proteasome activity in the brain showed increased concentrations of those proteins that are elevated in the brain of AD patients. Furthermore, these mice showed deficits in spatial memory suggesting that defective proteasome function participates in the cognitive impairment [124].

## Cysteine proteases and cysteinot deregulation in AD

Emerging evidence shows that cysteine proteases play important roles in AD physiopathology including calpains, cathepsins, and caspases [125].

For example, calpains are cysteine proteases that have been implicated in AD through hyperactivation, which results from several factors, including enhanced intracellular  $\text{Ca}^{2+}$  concentration [126]. Besides,  $\text{A}\beta$  peptides induce calpain activation by elevation of  $\text{Ca}^{2+}$  levels leading to neuronal cell dysfunction and death [125]. Activation of the catalytic center of calpains 1 and 2 is dependent on  $\text{Ca}^{2+}$  allowing the rearrangement among the amino acids cysteine, histidine, and asparagine [127]. Therefore, calcium binding to the protein and the proper oxidation state of sensitive cysteine residues in the catalytic center are necessary to the adequate activities of these proteases [128].

The role of caspases in AD physiopathology has been widely examined, and many proteins of the caspase family are transcriptionally elevated in AD [125]. In this regard, nitric oxide may nitrosylate sensitive cysteine residues on the catalytic center of caspase-3 preventing apoptosis in AD [129]. Besides, inhibitors of apoptosis (IAPs) are a family of proteins that regulate cell survival by binding to caspases to inhibit their catalytic activities [130]. The protein X-linked inhibitor of apoptosis (XIAP) is the most commonly expressed and the most potent endogenous caspase inhibitor among the IAPs, and it can be S-nitrosylated in several neurodegenerative disorders (Table 1). In fact, recent studies have shown a significant increase of S-nitrosylated XIAP in brain samples from PD, AD and Huntington's disease (HD) patient's possibly promoting apoptosis [131].

## Transcription factors and cysteinot deregulation in AD

There are some examples of redox regulation of gene transcription in which cysteine residues form a fundamental part of the regulatory

machinery (Figure 1). It has been shown that disulfide bridges control the formation of the homotrimer of the heat shock transcription factor 1 (HSF1) in mammals, which is translocated into the nucleus where it activates the transcription of both heat shock protein (Hsp) 70 and Hsp90 [132], which have been implicated in AD physiopathology. In oxidizing conditions, the intramolecular disulfide bond prevented the activation of HSF1, but under reducing conditions the intramolecular bond is broken and HSF1 cannot form the required trimer [132].

Nuclear factor (erythroid-derived2)-like 2 (Nrf2) is a transcription factor that regulates the expression of antioxidant proteins protecting against oxidative damage. Nrf2 is kept in the cytoplasm by the Kelch like-ECH-associated protein 1 (Keap1) and Cullin-3, which degrade Nrf2 by ubiquitination [133]. Oxidative modification of critical cysteine residues in Keap1 disrupts the Keap1-Cullin-3 ubiquitination system, and Nrf2 is not ubiquitinated. The result is the translocation of Nrf2 into the nucleus where it combines with a small (musculoaponeurotic fibrosarcoma) Maf protein and binds to the antioxidant response element in the upstream promoter region of many antioxidant genes initiating their transcription [134].

### **N-Acetylcysteine (NAC) as A Natural Antidote for AD**

The mechanisms of NAC actions, at the cellular level, are not completely understood. It has been argued that the principal mechanism explaining NAC beneficial actions is through the restoration of the glutathione pool and its redox capacity [135-137]. NAC does not need the alanine-serine-cysteine system to enter the cells since NAC is a membrane-permeable cysteine precursor that does not require active transport. Once into the cell, NAC is rapidly hydrolysed to yield cysteine [138], which can regenerate total glutathione content and reduce excessively oxidized glutathione levels. Another action of NAC is its direct scavenging ability against reactive species [139,140]. However, little attention has been focused on the effect of NAC on protein associated thiol regulation. NAC can restore the specific activities of mitochondrial complex I, IV and V in synaptic mitochondria obtained from aged mice, which was attributed to the restoration of thiol cysteine in those proteins. Likewise, *in vivo* experiments corroborate that the specific enzymatic activities of some of these complexes were restored by chronic NAC administration increasing ATP and GSH levels and decreasing lipid and protein oxidation in presynaptic terminals [136,141,142]. In the present paper, we have reviewed the functional consequences of redox modifications of different SCCPs that participate in a wide range of pathways implicated in AD physiopathology. In this context, NAC is a safety natural precursor of cysteine that can restore the redox environmental conditions for the adequate function of SCCPs. In fact, glutathione is a sensitive cysteine-containing tripeptide that depends on the cysteine/cystine ratio and the redox microenvironment.

Redox regulation of SCCPs may explain many effects of NAC including the improvement of metabolic function, calcium signaling, protein folding and turnover, which are closely associated with aging and AD development. However, modifications of cysteine residues in proteins by NAC have not been systematically studied yet. For example, NAC can alter the oxidative modifications of plasma proteins like transthyretin (TTR) by interacting with the sensitive cysteine residue of the protein *in vitro* and *in vivo* [142]. The interaction of NAC with TTR seems to be dose-dependent with a biphasic character of NAC-TTR interaction [142,143]. Indeed, TTR can suppress the AD phenotype in transgenic animal models, and it can reduce cerebral A $\beta$  deposition [144]. Moreover, it has been shown that the administration of NAC can ameliorate the onset and severity of premature aging in Bmal1-deficient mice (Bmal1-/-mice). Bmal1 is a circadian clock protein implicated in tissue homeostasis by direct regulation of ROS, and it acts as a transcription factor of key components of the circadian clock. Administration of NAC attenuated the development of the age-associated phenotype of Bmal1-/- mice increasing the average and maximal lifespan of animals [145].

A little-explored mechanism of NAC is the redox regulation of gene transcription and expression pathways dependent on sensitive cysteine residues of transcription factors. Interestingly, NAC can directly modulate the activity of common transcription factors both *in vitro* and *in vivo* [146]. NAC treatment suppressed NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation in oxidative stressed cultured cells, and in clinical sepsis, reducing the subsequent cytokine production. NF- $\kappa$ B is naturally bound to its inhibitor (I- $\kappa$ B) that prevents its nuclear translocation. Dissociation of I- $\kappa$ B following its phosphorylation by the specific kinase of NF- $\kappa$ B (IKK) allows NF- $\kappa$ B transport to the nucleus [146]. In fact, it has been demonstrated that NAC down-regulates the transcription of the APP gene in neuroblastoma cells by decreasing the binding activity of the transcription factor NF- $\kappa$ B [147]. These effects of NAC are mediated through its ability to regulate and modulate the sensitive cysteine residues in those proteins that form part of the proposed cysteinets.

Genome-wide association studies have implicated numerous genes in AD [148] supporting the concept that altered APP misprocessing is only a part of the complex AD physiopathology. Therefore, diverse genetic alterations, risk factors and disturbed pathways may converge on the proposed cysteinets deregulation in AD, which may be prevented and restored by NAC. Taken together, NAC may protect neuronal cells working as an antioxidant but also regulating the oxidation of thiol groups of sensitive cysteine residues in multiple SCCPs, thereby restoring key cellular pathways that maintain neuronal survival. For example, it has been demonstrated that NAC activates the Ras-ERK (extracellular signal-regulated kinase) pathway in PC12 cells preventing neuronal death in the absence of trophic factors by a non-antioxidant mechanism [149]. Since Ras proteins have critical redox-sensitive cysteine residues [150], the authors suggested that NAC directly activates Ras through its reducing capacity [149]. Activation of the Ras-ERK pathway may also lead to the induction of c-fos and c-jun genes [149]. Indeed, NAC can induce c-fos and c-jun transcription in cellular systems [151]. Other studies have shown that NAC can protect human cortical neurons against apoptosis induced by A $\beta$ -amyloid 1-42 [152] as well as inducing p35/Cdk5 pathway activation [153], and reducing phosphorylation/deactivation of MLK3-MKK7-JNK3 signaling cascade [154]. Therefore, in addition to antioxidant properties, and increasing GSH levels, NAC protects against A $\beta$  toxicity through the activation of diverse signaling pathways. Finally, since down-regulation of peptidyl-prolyl isomerasases 1 (Pin1) has been implicated in Alzheimer's disease [155] it was investigated the effect of chronic administration of NAC in human double mutant APP/PS-1 knock-mice. NAC treatment slightly increased the Pin1 levels, possibly decreasing A $\beta$  induced oxidative stress [156].

Other probable beneficial action of NAC administration may be the prevention of age-related protein misfolding and aggregation through the regulation of cysteine oxidative modification associated with aging. The 3-D structures of proteins are related to their function, and conformational changes occur in many proteins when they accumulate in tissues [157]. Conformational changes are most likely to occur in proteins that have repetitive amino acid sequences, such as polyglutamine in HD. In this case, NAC has proven its beneficial effects in animal models [158]. Indeed, accumulation of misfolded proteins can contribute to the pathogenesis of age-related neurodegenerative diseases such as AD, PD and HD, which may be potentially restored by NAC administration. Indeed, NAC have shown beneficial effects in many different diseases including neurodegenerative diseases such as PD, and other brain injuries like cerebral ischemia and hemorrhage, traumatic brain injury, and psychiatric diseases [reviewed in 13,14].

Finally, sulfur compounds, particularly cysteine, inhibit the fibrillation of A $\beta$ 1-40 and A $\beta$ 1-42. Interestingly, aggregates of A $\beta$ 1-40 and A $\beta$ 1-42 induced by cysteine were less cytotoxic than those induced by catechin, which is the most typical inhibitor of amyloid fibril formation [159].

### Preclinical and clinical studies of NAC in AD

AD is an age-associated neurodegenerative disease that has shown oxidative damage in the temporal and cerebral cortex and decreased glutathione concentrations in different cortical areas and the hippocampus [160-162]. Indeed, most clinical trials of antioxidants for the treatment of AD have employed  $\alpha$ -tocopherol or selegiline, an irreversible and selective monoamine oxidase B (MAO-B) inhibitor in an attempt to counteract the pro-oxidant conditions of the disease. Besides, NAC has been tested in some murine models of AD [163,164], but studies in humans with neurodegenerative diseases are scarce.

Some preclinical studies have provided evidence that administration of NAC is beneficial in AD murine models counteracting oxidative damage [163-165] and decreasing A $\beta$ 1-40 and A $\beta$ 1-42 levels [166]. Besides, NAC was able to improve the results in the T-maze foot-shock avoidance paradigm [167]. NAC administration to human double mutant APP/PS-1 knock-mice before the deposition of A $\beta$  in their brains, decreased protein and lipid oxidation, nitration of proteins, and increased glutathione peroxidase and reductase activities compared to age-matched controls [156]. Pretreatment with NAC in cultured neuroblastoma cells can also affect APP processing by influence both  $\beta$ -secretase and  $\gamma$ -secretase activities [168]. In the same study, NAC decreased phosphorylated tau levels in the presence or absence of stressing conditions (hydrogen peroxide and UV light exposition) through a pathway involving the modulation of phosphatases activities [168].

As previously mentioned, AD is associated with mitochondrial disorders including a reduced level of cytochrome c oxidase activity in post-mortem cerebral cortex. Additionally, ROS generation in cybrid cells transferred with mitochondria from AD platelets showed that complex IV defects are associated with the disease [169]. Besides, mitochondrial DNA mutations have also been demonstrated in AD patients [170-172], suggesting a role for mitochondrial DNA damage in the impairment of the oxidative phosphorylation and bioenergetic capacity associated with the disease [173]. Two studies have demonstrated a decrease in the mitochondrial mRNA that encodes complex IV in the temporal cortex and hippocampus of AD patients [174,175]. A study evaluated the effect of lipoic acid and NAC on oxidative and apoptotic markers in fibroblasts from patients with AD and age-matched and young controls. AD fibroblasts showed the highest levels of oxidative stress, and both antioxidants exerted a protective effect as evidenced by the decrease in oxidative stress and apoptotic markers. Moreover, the oxidative damage observed was associated with mitochondrial dysfunction, which was attenuated by both antioxidants. These data suggest that mitochondria must be a major target in AD therapies based on NAC supplementation [176]. Finally, we have demonstrated that chronic oral administration of NAC was able to restore cytochrome c oxidase activity measured in synaptic mitochondria from aged mice, decreasing the age-related oxidative damage in mitochondrial lipids and proteins [136,141,142]. This effect of chronic NAC administration was associated with a delay in age-associated memory impairment in aged animals [177].

In a study on probable AD patients, treatment with NAC for 24 weeks to drive cystine-glutamate antiporter system caused a beneficial trend in all measures tested as well as a significant improvement in some cognitive tests [178]. The release of glutamate from cystine-glutamate antiporter system and the subsequent activation of extra-synaptic NMDA (*N*-methyl-D-aspartate) receptors may contribute

to  $\beta$ -amyloid production and toxicity [179,180]. In contrast, cystine uptake by cystine-glutamate antiporter system seems to exert beneficial effects by lowering  $\beta$ -amyloid stress, preventing apoptosis triggered by oxidative stress, and normalizing the activity of sodium-dependent glutamate transporters [168,181]. Another clinical study in a small group of patients with moderate to late-stage probable AD showed improvements in various neuropsychiatric tests after chronic administration of NAC in combination with folic acid, vitamin B12,  $\alpha$ -tocopherol, S-adenosyl methionine and acetyl-L-carnitine [182]. A case report described a man with probable AD and hyperhomocysteinemia who showed significant clinical improvement after the administration of NAC, vitamin B12, and folinic acid [183].

### Conclusions

Future studies are needed considering the essential role of cysteine residues for the maintenance of protein structure and function and the importance of NAC in various clinical and experimental setting. The present review emphasizes the singularity of NAC as the unique substance that, at present, is widely used in clinical practice, can cross the blood brain barrier (BBB) and can directly interact with key SCCPs into the brain counteracting brain aging and age-related neurodegenerative diseases. Indeed, recent preliminary results have demonstrated a beneficial effect of NAC on the dopamine system associated with the improvement of clinical parameters in PD [184]. In this study, patients received a DaTScan before and after treatment with NAC for 90 days, to measure dopamine transporter (DAT) binding. The study showed a significant increase in DAT binding in the caudate and putamen in the PD group treated with NAC without significant changes in the control group. In addition, the unified Parkinson's disease rating scale score, to measure clinical symptoms, were also significantly improved in the NAC group [184]. Therefore, differential cysteine labeling and global label-free proteomics studies in blood, CSF and brain tissue will be necessary to understand the role of cysteine deregulation in aging and age-associated neurodegenerative diseases. NAC can be potentially used to prevent and restore the deregulation of redox homeostasis through the replenishment of mitochondrial soluble (cysteine/glutathione) and protein-linked thiols, which may restore the mitochondrial bioenergetic capacity and biogenesis. Much remains to be investigated concerning redox signaling and modulation of the mitochondrial function, but it seems that redox regulation by NAC can play a key role in the cysteine deregulation associated with aging, which is the principal risk factor for late-onset AD.

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