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Review Article

Oxidative Stress and Proteostasis Network: Culprit and Casualty of Alzheimer's-Like Neurodegeneration

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Free radical-mediated damage to proteins is particularly important in aging and age-related neurodegenerative diseases, because in the majority of cases it is a non-reversible phenomenon that requires clearance systems for removal. Major consequences of protein oxidation are loss of protein function and the formation of large protein aggregates, which are often toxic to cells if allowed to accumulate. Deposition of aggregated, misfolded, and oxidized proteins may also result from the impairment of protein quality control (PQC) system, including protein unfolded response, proteasome, and autophagy. Perturbations of such components of the proteostasis network that provides a critical protective role against stress conditions are emerging as relevant factor in triggering neuronal death. In this outlook paper, we discuss the role of protein oxidation as a major contributing factor for the impairment of the PQC regulating protein folding, surveillance, and degradation. Recent studies from our group and from others aim to better understand the link between Down syndrome and Alzheimer's disease neuropathology. We propose oxidative stress and alteration of proteostasis network as a possible unifying mechanism triggering neurodegeneration.

1. Introduction

Oxidative stress (OS) refers to a condition where reactive oxygen species (ROS) or other oxidants overwhelm the cellular antioxidant defense system, by an increase of ROS production and/or a decrease in the antioxidant response. ROS, such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$), are both radical and nonradical oxygen species formed by the partial reduction of oxygen. The major source of free radicals is the oxidative phosphorylation where electron leakage from the mitochondrial electron transport chain causes the formation of superoxide anion, or they can be released by exogenous sources such as xenobiotic compounds [1]. Oxidative stress damages all macromolecules (carbohydrates, nucleic acids, lipids, and proteins) and is implicated in the pathogenesis and progression of various diseases such as atherosclerosis, cancer, neurodegeneration, and aging as well. Indeed, the rate of generation of ROS in different species roughly correlates with life span and is a major contributor in defining the rate of aging and the development of age-related diseases [2]. The "oxidative stress theory" of aging, proposed by Harman [3], holds that a progressive and irreversible accumulation of oxidative damage impacts on critical aspects of the senescence process, contributing to impaired physiological function, increasing incidence of disease, along with a reduction in life span. Aging cells activate a number of fundamental intracellular processes linked to energy metabolism that function to maintain cellular homeostasis by minimizing oxidative damage that can be detrimental for all cellular components.

Among different targets, free radical-mediated damage to proteins is particularly important in aging and in many age-related neurodegenerative diseases, because in the majority of cases it is a nonreversible phenomenon which therefore requires clearance systems for removal [4]. Further, products of methionine oxidation as well as most other amino acid modifications cannot be directly repaired and must be selectively eliminated to prevent the accumulation of damaged, nonfunctional proteins. Generally, oxidation of proteins could affect protein expression and gene regulation, protein turnover, cell signaling, apoptosis, necrosis, and so forth, eventually leading to loss of cells and function [5].

Another major consequence of protein oxidation is the formation of large protein aggregates, which are often toxic to cells if allowed to accumulate. Insoluble aggregates can be formed as a result of covalent cross-links among peptide chains, as in the case of amyloid β peptide (Aβ) in Alzheimer's disease (AD), α-synuclein in Parkinson's disease (PD), and mSOD1 in amyotrophic lateral sclerosis (ALS). Further, oxidation of proteins increases the susceptibility of a protein to degradation by the 20S proteasomes and, consequently, decreases levels of the proteins in general. However, in certain diseases, oxidation of proteasome components has been reported and defective proteasome consequently leads to accumulation of damaged proteins within the cells [6]. Deposits of aggregated, misfolded, and oxidized proteins accumulate normally over time in cells and tissues and are often present in increased amounts in a range of age-related disorders, such as neurodegenerative diseases.

The protein quality control system (PQC) through degradation of oxidized, mutant, denatured, or misfolded proteins is involved in many biological processes where protein level regulation is necessary. This system allows the cell to modulate its protein expression pattern in response to changing physiological conditions and provides a critical protective role in health and disease. If this process is inefficient damaged/dysfunctional proteins are not efficiently removed and may accumulate. Clearance of oxidatively modified proteins usually occurs through the proteasome system. However, there are also evidences demonstrating that also autophagy pathways degrade oxidized proteins.

In this review, we discuss the role of oxidative stress and alteration of proteostasis network and how they might act synergistically to cause neurodegeneration. We suggest that reduced proteins turnover (as a consequence of inefficient removal by the intracellular quality control system) may be caused by the selective oxidative damage of members of the proteostasis network by focusing our attention on Down syndrome (DS) and AD. By following this view, we highlight the specific pathways, which are in common between DS and AD neuropathology, and propose alteration of proteostasis network as a unifying mechanism of neurodegeneration.

2. Protein Oxidation

Protein oxidation refers to the direct or indirect damage of a protein as a consequence of oxidative insult, often making the protein dysfunctional or nonfunctional [6]. This process inevitably affects protein structure and could lead to the alteration in the secondary and tertiary structure of proteins including dissociation of subunits, unfolding, exposure of hydrophobic residues, aggregation, and backbone fragmentation [7]. Proteins can be oxidized by direct ROS attack, by secondary oxidation products such as the reactive aldehyde (malondialdehyde and 4-hydroxynonenal), formed as final byproducts of lipid peroxidation, or by glycoxidation reactions. Essentially, protein oxidation results from the introduction of aldehyde and ketone groups on side chains—protein carbonylation—the most common type of protein oxidative modification [8]. All amino acid residues are potential targets for oxidation by ROS, with methionine and cysteine residues being particularly sensitive. In the case of methionine, methionine sulfoxide (MeSOX) can be reduced by MeSOX reductases, whereas oxidation of sulfhydryl groups, often resulting in the formation of intra- or intermolecular disulphides, is reduced back by disulfide reductases/isomerases [6]. These are the only known oxidative modifications of proteins that can be enzymatically repaired in mammalian systems.

Oxidation of aromatic amino acids can give rise to various hydroxy derivatives, whereas that of some other amino acids such as lysine, arginine, proline, or threonine residues may yield carbonyl derivatives. As discussed above, protein carbonylation is the most abundant type of protein oxidation that may result in the loss of function of the affected protein [9, 10]. The method for detection of protein carbonylation is through the reaction of protein sample with 2,4-dinitrophenylhydrazine (DNPH) that forms hydrazones, which are detectable through immunochemical blotting, using specific antibodies that recognized hydrazone adducts [11, 12]. Other methods that may be used to detect protein carbonylation are biotin hydrazide coupled to fluorescein isothiocyanate- (FITC-) labelled streptavidin as well as spectrophotometric analysis [11].

Secondly, protein carbonylation can also be caused by an intermediate molecule that initiates the free-radical modification of the protein, which finally results in protein carbonylation adduct [11, 13]. This intermediate oxidant is usually a lipid peroxidation product, such as α/β unsaturated aldehydes (MDA, acrolein, and HNE), that bind to the protein via Michael addition. These types of adducts, similar to what occur with the introduction of carbonyl groups, cause conformational changes of protein tridimensional structure that in turn affect its function. Protein bound lipid peroxidation products are commonly detected in samples by immunochemical methods or by spectrophotometry [14–17]. Though lipid hydroperoxides form at membrane level, they are lipophilic and highly reactive and may initiate radical chain reactions on lipids and proteins in an adjacent cellular or organelle membrane [18].

Protein nitration results when ROS and RNS, such as superoxide ($O_2^{-\bullet}$) and nitric oxide (NO), produce the anion peroxynitrite ($ONOO^-$) which is known to covalently modify tyrosine residues to produce 3-nitrotyrosine (3-NT) [19, 20]. Implications of protein bound 3-NT have been demonstrated to inactivate several key proteins such as actin, manganese and copper/zinc superoxide dismutase (SOD1 and SOD2),

and tyrosine hydroxylase. Another critical consequence of this type of modification is the fact that it may compete with phosphorylation of Tyr residues, via tyrosine kinases [21]. Altered phosphorylation pattern of target proteins may affect significantly several regulatory pathways with pathological implications. Data from aging rats have shown that nitrated tyrosine residues contribute to the aging process, as protein nitration levels increase with age in different brain regions including cerebellum, substantia nigra, and hippocampus, as well as localization to membrane raft proteins that may play a significant role in cell signaling [22–25].

In the last decades, advances of proteomics platforms allowed an accurate and detailed profile of specific protein oxidation occurring in a system. Investigations of oxidative PTMs that occur in vitro or in vivo are currently being performed using focused redox proteomics techniques. Redox proteomics is the branch of proteomics for the identification of specific target proteins that may be differentially oxidized as a result of oxidative injury. Two different types of approaches are mostly applied to a range of biological samples: the targeted gel-free enrichment of proteins presenting the oxidative modification and the global gel-based analysis. The gel-based redox proteomics uses immunochemical methods for the detection of different oxidative stress markers. The two-dimensional gels obtained are transferred to a membrane, probed with primary antibodies against the modification of interest, and followed with a secondary antibody for detection/quantification and analysis. Spot matching programs, like PD-Quest, or Dimension Delta 2D, Image Master, and so forth, compare spot density changes in samples by pixel detection analysis. This sophisticated software allows the multiple comparisons of gels and blots at once. Spots of interest, located within the gel, must be excised from the gel, digested with trypsin, and identified using a peptide mass fingerprinting (PMF) MS approach. The tryptic peptides are subjected to either matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry or electrospray ionization (ESI) tandem (MS/MS) mass spectrometry. In ESI-MS/MS also the parental peptides are subjected to an additional dissociation step, which allows for the identification of amino acid sequence and identification of the initial tryptic peptide as well as potential amino acid sites of modification via searching tools such as MASCOT or SEQUEST. Alternative approaches to gel-based methods, which do not involve gels but rather liquid chromatography separations and MS and tandem MS (MS/MS), have also been developed for redox proteomics studies. A nongel proteomic method requires the digestion of proteins into peptides in solution, the nanoflow LC separation of peptides, and automated MS and MS/MS data acquisition [26].

3. Proteostasis Network

The endoplasmic reticulum (ER) is the site of several homeostatic and biosynthetic pathways that includes Ca^{2+} homeostasis, redox balance, lipid synthesis, and importantly the synthesis of membrane and secreted proteins [27]. Protein synthesis, folding, and trafficking are events coordinated by quality control system to guarantee that only correctly folded proteins leave the ER [28]. When protein misfolding occurs, signaling pathways that promote folding mediated by chaperone proteins are activated. When these pathways fail the misfolded protein is targeted for degradation via ER associated degradation (ERAD) in the cytosol [29]. The two principal ways of intracellular protein degradation belonging to ERAD are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway system [30]. Under conditions where misfolded proteins accumulate within the lumen of the ER, the organelle enters into a state called "ER stress" and the ER responds to this condition by activating a series of complex coordinated signaling pathways, collectively called the unfolded protein response (UPR) [29, 30]. Activation of the UPR affects the expression of different proteins with functions in almost every aspect of the secretory pathway, including folding, quality control, protein entry into the ER, ERAD pathways, and many other effects [31].

3.1. Molecular Chaperones. Molecular chaperones, including heat shock proteins (HSPs), glucose-regulated proteins (GRPs), calnexin (CNX), calreticulin (CRT), peptidyl-prolyl isomerases (PPI), and protein-disulphide isomerase (PDI), are the first line of defence against protein misfolding and aggregation [32]. Chaperones bind to unfolded regions in proteins and keep them in a folding-competent state while preventing aggregation [29]. In addition to their role in folding, some of these chaperones are proposed to act as a quality control system to ensure that only correctly folded proteins proceed to the Golgi for further processing and secretion. The HSP70 family of chaperones recognize, in an ATP-dependent manner, exposed hydrophobic patches of unfolded or misfolded proteins [33]. Crucial members of the HSP70 family are GRP78 and GRP94 [32]. When bound to ATP, GRP78 binds unfolded hydrophobic tracts with low affinity. However, the binding with unfolded proteins stimulates the N-terminal ATPase activity of GRP78 resulting in an ADP-bound form with a much higher affinity for hydrophobic motifs [34]. In the ER, in addition to its role as a folding chaperone, GRP78 also functions as a stress regulator by buffering Ca^{2+} levels [35]. Beyond these activities, GRP78 also regulates the activation of the three transmembrane ER stress transducers: PERK, ATF6, and IRE1. Generally, GRP78 binds these ER receptors, impeding their activation. However, in the presence of exposed hydrophobic residues, GRP78 dissociates from PERK, ATF6, and IRE1 and allows their activation [27]. ER chaperones belonging to the HSP40 family modulate the functions of GRP78 by regulating its ATPase activity as a cochaperone. GRP94 is an ER chaperone belonging to the HSP90 family that facilitates folding through the hydrolysis of ATP [36]. CNX and CRT lectin-like chaperones are involved in the folding of glycoprotein in a process called calnexin cycle [32]. PDI are involved in the formation of disulfide bonds in the ER and include ERp72, ERp61, GRP58/ERp57, ERp44, ERp29, and PDI-P5. These folding enzymes oxidize cysteine residues of nascent proteins and help proteins to form correct disulfide bonds; reduced

folding enzymes are then reoxidized by ER oxidoreductin [37].

3.2. Unfolded Protein Response. The unfolded protein response (UPR) consists of three independent signaling pathways that work in parallel and are activated upon accumulation of unfolded proteins inside the ER [38]. The different ER-resident transmembrane proteins that act as ER-stress sensors define each signaling pathway. These sensors include double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring kinase 1 (IRE1). These three proteins transduce the information about the protein folding status at the ER lumen to the nucleus and cytosol by controlling expression of specific transcription factors and other rapid effects on protein synthesis [27–29]. Under normal conditions, these protein sensors are maintained in an inactive state by binding to the major ER chaperone GRP78 at the side of the ER. When unfolded proteins accumulate in the ER lumen, GRP78 binds to them to keep correct protein folding and is thereby released from PERK, IRE-1, and ATF-6, which are consequently activated. However, it has also been suggested that direct binding of unfolded proteins to the ER-stress sensors leads to activation of the UPR [38, 39]. Sustained ER stress and prolonged UPR activation can lead to the activation of the apoptotic machinery and ultimately to cell death. Thus, despite the beneficial role of the UPR in cellular homeostasis, sustained ER stress leads to the development of pathological conditions [39]. The activation of PERK, upon ER stress, phosphorylates the eukaryotic translation initiation factor 2a (eIF2a) leading to a reduction in global protein and thus decreasing the load of new proteins inside the ER [40]. In addition, activation of PERK promotes the nuclear import of nuclear factor E2 related factor 2 (Nrf2), which activates antioxidant enzyme gene transcription promoting cellular survival [28]. IRE1 presents two genes with homologue sequences: IRE1a and IRE1b. IRE1a is expressed ubiquitously, whereas the expression of IRE1b is limited to gut epithelial cells [41]. IRE1a is a kinase and endoribonuclease, that, upon activation, catalyses the correct splicing of the transcription factor X box-binding protein 1 (XBP1), that regulates a subset of UPR targets genes involved in ER protein synthesis and folding, ERAD, autophagy, and redox metabolism. IRE1 also activates the preapoptotic c-Jun N-terminal kinase (JNK), the apoptosis signal-regulating kinase (ASK1), and the caspase 12. The IRE1-JNK pathway is also required for activation of autophagy after ER stress [31].

Upon the accumulation of unfolded proteins in the ER, ATF6 is released from GRP78 and is trafficked to the Golgi apparatus where it is cleaved by site 1 and site 2 proteases at the transmembrane site. The cytoplasmic part of ATF6, an active transcription factor known as ATF6 p50 (or nATF6), migrates to the nucleus to activate UPR gene expression [42].

3.3. Ubiquitin-Proteasome System. The ubiquitin-proteasome system (UPS) and autophagy-lysosome system, known as autophagy, are the two main paths of protein and organelle clearance in eukaryotic cells [43]. The proteasomal system is located in the cytosol and the nucleus, and it is responsible for the degradation of more than 70–80% of intracellular proteins. Further the UPS not only degrades misfolded, oxidized, or damaged proteins, but also removes proteins involved in many cellular processes, such as signal transduction, cell cycle regulation, and cell death, and, ultimately, regulates gene transcription [44]. Indeed, the UPS employs specialized functions, depending on its localization or on time-dependent regulation.

Most of the proteins are targeted for proteasomal degradation after being covalently modified with ubiquitin, a small protein with 76 amino acids, which is conjugated through the formation of an isopeptide bond between the ε-amino group of a lysine residue of the substrate and the C-terminal carboxylate [45]. This conjugation normally involves three types of enzyme: E1 (ubiquitin-activating enzyme) hydrolyses ATP and forms a thioester-linked conjugate between itself and ubiquitin; E2 (ubiquitin-conjugating enzyme) receives ubiquitin from E1 and forms a similar thioester intermediate with ubiquitin; and E3 (ubiquitin ligase) binds both E2 and the substrate and transfers the ubiquitin to the substrate [46]. In some circumstances, a fourth ubiquitination enzyme, known as the ubiquitin chain elongation factor E4, is necessary, together with the E1, E2, and E3 enzymes to extend a polyubiquitin chain [47]. Polyubiquitin chain then is recognized by the proteasome, a multicatalytic complex indicated as the 26S proteasome. The 26S proteasome is composed of a 20S catalytic core and two 19S regulatory caps on both ends of the 20S core. The 20S proteasome contains four stacked rings that form a barrel-shaped molecule with a central cavity. These stacked rings include two noncatalytic outer rings called α-rings and two catalytic inner rings called β-rings. Three proteolytic activities are confined to the β-rings including chymotrypsin-like, caspase-like, and trypsin-like that may be involved in the neurodegenerative process [48]. 19S contains at least 18 subunits, with a base composed by six ATPases that exert a chaperone-like activity and a lid composed of eight subunits that recognise the polyubiquitin signals. The 19S proteasome binds and unfolds ubiquitinated proteins and opens the entry gate of the 20S proteasome to allow protein in the central cavity [48].

Oxidation of a protein induces several reversible or irreversible modifications in proteins, including amino acid modification, fragmentation, or aggregation, and causes their increased predisposition towards proteolysis [21, 49]. It has been suggested that the oxidation of proteins causes the exposure of hydrophobic moieties to the surface via partial unfolding that are targeted by proteasome [50–52]. While the 26S proteasome degrades polyubiquitinated proteins, the 20S proteasome by itself seems to be sufficient to degrade nonubiquitinated oxidatively modified proteins in an ATP-independent manner; however, the exact mechanism is still unclear [44, 46, 48].

3.4. Autophagy-Lysosome System. Autophagy developed as a self-eating mechanism with a key role in cell survival and in preserving cell metabolic balance [53]. Autophagy

acts as a starvation response to maintain cellular nutrient levels and helps to regulate intracellular organelle homeostasis. Autophagy plays a crucial role in the removal of toxic/aggregate proteins and impaired organelles that could damage cells during stress and its alteration is reported in various human pathologies including neurodegenerative, cancers, and lysosomal storage disorders [54, 55]. Autophagy includes three major types: macroautophagy (indicated simply as autophagy), microautophagy, and chaperone-mediated autophagy (CMA). All three mechanisms share a common destiny of lysosomal degradation but are mechanistically different from one another [56]. Throughout macroautophagy intact organelles and portions of the cytosol are sequestered into a double-membrane vesicle, known as autophagosome. The autophagosome matures by fusing with an endosome and/or lysosome, thereby forming an autolysosome. This final step allows the interaction of the autophagosome cargo with lysosomal hydrolases to allow its degradation. Differently, microautophagy involves the direct engulfment of cytoplasm at the lysosome surface, whereas CMA translocates unfolded proteins directly across the limiting membrane of the lysosome [56, 57]. Pathways leading to organelle-specific autophagy, such as mitophagy, have also been recently described [58, 59].

Macroautophagy (often indicated as autophagy) is an evolutionarily conserved pathway where several autophagy-related (Atg) proteins coordinate vesicle formation that involves three different steps, initiation, elongation, and maturation, followed by fusion with lysosomes to form autolysosome [53]. The initiation involves the formation of a membrane structure termed the phagophore in the cytoplasm at the phagophore-assembly site(s) (PAS) [56]. It has been hypothesized that autophagosomes either can be generated de novo from preexisting intracellular precursor molecules or could arise from other intracellular membrane structures like the ER [60]. Initiation of autophagy is triggered in response to starvation by inhibition of mTOR that leads to the activation of the ULK1 kinase complex. In turn, activation of this complex causes the activation of another complex that comprises (among other proteins) the class III PI3 kinase Vps34 and the protein Beclin-1 [60]. The activity of Vps34, a class III phosphatidylinositol-3-kinase (PI3K), is necessary for the formation of new autophagosomes and is enhanced by its binding to Beclin-1. The other protein complex involved in this stage of autophagosome formation is the ULK1/Atg1-Atg13-FIP200/Atg17-Atg101 complex that plays an important role in Atg proteins recruitment and autophagosome synthesis. The second ubiquitination-like reaction involves the conjugation of microtubule-associated protein 1 light chain (LC3) to the lipid phosphatidylethanolamine (PE). LC3 is cleaved at its C-terminus by Atg4 to form the cytosolic LC3-I, which is conjugated with PE through the action of Atg7 (E1-like) and Atg3 (E2-like) to generate LC3-II [57, 61]. LC3-II is the most widely used marker to analyze autophagy functionality because it specifically associates with autophagosomes only. LC3-II is bound to both sides of the membrane until fusion with lysosomes and after it the LC3-II on the cytosolic face is recycled (to LC3-I) by Atg4, while the LC3-II on the inner face of the membrane is degraded [57]. Autophagosomes are then transferred along microtubules in a dynein-dependent manner to lysosomes, where the fusion forms the autolysosome. Once the autophagosomes fuse with the lysosomes, their cargo is degraded by the lysosomal hydrolases. Acidification of the newly formed autolysosomes is needed for activation of the lysosomal hydrolases and effective proteolysis of substrates, and it is mediated by a vacuolar [H+] ATPase (v-ATPase) [55, 61, 62].

Autophagosome formation is regulated by many signals that fall into two broad categories: mammalian target of rapamycin- (mTOR-) dependent and mTOR-independent [62]. The mammalian target of rapamycin (mTOR) kinase is the main negative regulator of autophagy. Under starvation conditions or rapamycin treatment, mTOR-mediated phosphorylation of Atg13 and ULK1 is inhibited, leading to dephosphorylation-dependent activation of ULK1 and ULK1-mediated phosphorylations of Atg13, FIP200, and ULK1 itself that triggers autophagy initiation [56]. The mTOR-independent autophagy pathways include, among others, JNK1 that induce autophagy by phosphorylating Bcl-2 or Bim and abolishing their inhibitory effects on autophagy [57, 63, 64]. This mechanism might also account for the upregulation of autophagy after proteasome inhibition or ER stress.

Whereas autophagy is a relatively unspecific degradation pathway, chaperone-mediated autophagy (CMA) is highly specific and it is induced by the deletion of the lysosomal receptor LAMP-2a, a key component of CMA [53].

It is recently proposed that the protease Atg4 is the target of ROS and, indeed, upregulation of autophagy tends to reduce the ROS levels and prevents the deleterious effects of elevated ROS levels. Therefore, autophagy induction in response to increased OS levels represents a survival response, which promotes the degradation of oxidized proteins protecting from ROS-induced apoptosis [55, 60, 62]. In contrast, autophagy inhibition exacerbates the toxicity of oxidative stress and the levels of oxidative damage. However, excessive or chronic upregulation of autophagy promotes neuronal death, highlighting the delicate balance between beneficial and deleterious upregulation in autophagy in the cells of the brain [55].

4. Imbalance between Protein Oxidation and Protein Degradation in DS and AD

Down syndrome (DS) is the most frequent genetic cause of intellectual disability characterized by the anomalous presence of three copies of chromosome 21 (Chr21). The neuropathology of DS is complex and likely results from impaired mitochondrial function, defects in neurogenesis, increased oxidative stress, and altered proteostasis [65]. After the age of 40s many DS individuals develop a type of dementia that has the same characteristic of Alzheimer's disease (AD), with deposition of senile plaques, containing amyloid beta (Aβ) peptide, and neurofibrillary tangles (NFTs), composed of hyperphosphorylated Tau. The only difference is that development of AD occurs much earlier in people with DS, with symptoms beginning robust in their late 40s or early 50s. The incidence of AD in people with DS is estimated to be

three to five times greater than that of the general population. The precise mechanisms by which trisomy 21 leads to the early onset of AD remain to be elucidated.

Among putative candidates, growing studies investigated the role of OS as a possible link between DS and AD. Intriguingly, the causes of increased OS conditions are intrinsically related to the map of Chr21, where a number of genes seem to cause enhanced oxidative damage. Among the most powerful ROS-inducer, Cu/Zn superoxide dismutase (SOD1), amyloid precursor protein (APP), the transcription factor Ets-2, S100B, and carbonyl reductase map on Chr21, all genes that at different extent can be directly linked to increased free radical burden [66].

For example, it is not surprising that elevated levels of SOD1 are responsible for increased release of H_2O_2, which, if not efficiently neutralized, may in turn exacerbate the production of other radical species. Elevated levels of OS could also be caused by increased expression of APP and increased release of amyloid-β-peptide (Aβ). Many studies demonstrated that Aβ is associated with the formation of ROS and reactive nitrogen (RNS) species and induces calcium-dependent excitotoxicity, impairment of cellular respiration, and alteration of synaptic functions associated with learning and memory [67]. Numerous cellular and systemic abnormalities in the DS nervous system have been reported, but other unknown factors could contribute to the range of neurological changes in DS. Recently, our group focused on the analysis of the proteostasis network in DS in order to identify molecular pathways that may.

A number of pathologies, such as AD, PD, or HD, presenting the impairment of proteostasis and the increase of unfolded/misfolded proteins have been classified as protein misfolding disorders (PMDs) [68]. Perturbations in the function of mitochondria, ER, and UPS/autophagy degradation pathways are emerging in PMDs as relevant factors in driving the dysfunction of synapses, axonal transport and triggering neuronal loss. In this context the importance of protein folding, surveillance, and degradation systems in neurons is clear since this postmitotic cellular population is highly dependent on the proteostasis network to manage normal and damaged proteins and support vital signaling functions [69].

A close link between proteostasis network and increased OS has been demonstrated. It has been shown that low amount of ROS can activate the dedicated adaptive cellular apparatus that increase the organism's stress resistance. This involves the enhancement of antioxidant and heat shock responses, cell cycle regulation and apoptosis, DNA repair, UPR, and autophagy stimulation [55]. However, disturbances of proteostasis triggers increased ROS formation and depletion of the antioxidant molecule glutathione. In turn a chronic exposure to ROS oxidized proteins as well as specific component of ER, UPS, or autophagy pathways, thus exacerbating the accumulation of unfolded/misfolded proteins. The collective effects of deficient proteostasis network and increased oxidized proteins produce a vicious cycle that trigger the neurodegenerative process [46, 55, 70].

Increased ER stress is observed in postmortem brain samples from AD patients where increased levels of GRP78/BiP and the activation of UPR in the temporal cortex and the hippocampus occur consistently with NFT and Aβ plaque formation [71, 72]. In addition PDI inactivation by oxidative inactivation was shown in AD cases [72].

It is also well documented that ROS directly inhibit the proteasome [73, 74] and that the ubiquitin-proteasome activity declines, being less efficient in the clearance of proteins, with the increase of OS burden. A significant decline of 26S proteasome activity, but not for 20S proteasome, after OS has been demonstrated [75, 76]. Decreased subunit expression, alterations and/or replacement of proteasome subunits, and formation of HNE-cross-linked proteins support the impairment of proteasome activity during AD [46, 73, 74]. Further, it has been demonstrated that increased levels of lipofuscin are able to inhibit the proteasome [48] and the b5i-subunit of proteasome is a target of HNE modification [77]. The inhibition of proteasome in neuronal cell [78] was also demonstrated in p53-mediated cell death and upon caspase activation [79].

The following section summarizes all the experimental data reporting the oxidative modification of proteins belonging to proteostasis network in AD and DS cases.

4.1. Oxidative Damage to Proteostasis Network in AD. When oxidized/misfolded proteins accumulate in sufficient quantity, they are prone to aggregation. These include the amyloid plaques and neurofibrillary tangles in AD. Indeed, modification of the cellular proteostasis may affect significantly the metabolism of Aβ and Tau and in turn neuronal survival [12]. Studies from Professor Butterfield group and from our laboratory suggest that oxidative stress plays a crucial role by affecting the functionality of selected members of the proteostasis network [5, 65, 80, 81]. In detail, redox proteomics studies identified specific targets of OS-induced damage including chaperones, proteasome, protein synthesis machinery, autophagy, and regulatory proteins (Table 1). Further, studies performed on human brain from AD patients and its early phases demonstrated that oxidative damage also targets proteins involved in energy metabolism, antioxidant response, excitotoxicity, neuronal structure, and mitochondrial activity [5]. It is likely that oxidative-mediated dysfunction of these proteins is involved in neurodegeneration at various stages of the disorder [5] by affecting ATP production, axonal growth, and synaptic function among other fundamental neuronal activities. Moreover, this condition is further exacerbated by defective repairing system. Taken together, these data highlight that "stressed" neurons have to challenge increasing protein dysfunction but with reduced ability of PQC (Figure 1).

Chaperones. Central players of the proteostasis system are molecular chaperones that assist misfolded proteins to direct them, if refolds fail, to the protein-degradation system [82]. Environmental stress induces chaperone (heat shock protein, stress protein) expression reflecting the protective role of chaperones as a key factor in cell survival and in repairing cellular damage after stress. Among this wide family, those initially identified as heat inducible were called heat shock

Table 1: List of the proteins members of the proteostasis networks identified oxidatively modified in Alzheimer's-like diseases.

Proteostasis system	Oxidized proteins	Disease
Molecular chaperones	HSP90	AD [5]
	HSP60	AD [5]
	HSP32	AD [87]
	Haptoglobin	AD [94]
	Alpha 2 macroglobulin	AD [94]
	Serum amyloid P	AD [94]
	Clusterin	AD [94]
	HSC71	DS/AD [80]
ER proteins	EF-Tu	AD [101]
	eIF-a	AD [101]
	Pin1	AD [106]
	GRP78	DS, DS/AD [80, 81]
Ubiquitin-proteasome system	UCH-L1	AD [97], DS/AD, and DS [80, 81]
Autophagy-lysosome system	Cathepsin D	DS [81]
	V0-ATPase	DS [81]
	GFAP	DS, DS/AD [80, 81]

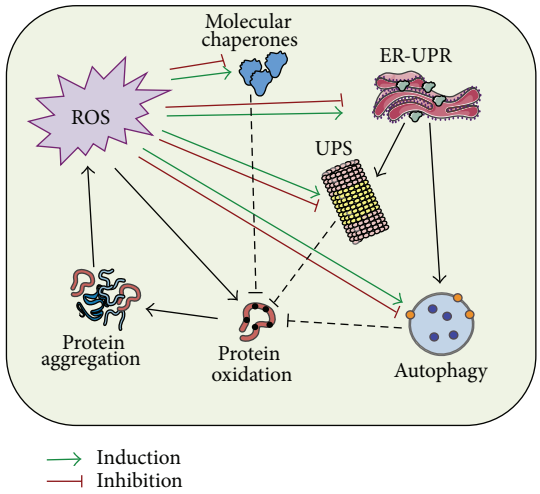

Figure 1: Interplay between oxidative stress and proteostasis network. Low amount of ROS induces the adaptive cellular apparatus such as the heat shock responses, UPR, and autophagy (green arrows). A chronic exposure to ROS leads to accumulation of oxidized proteins as well as damaging (red inhibition arrows) specific component of PQC, thus exacerbating the accumulation of unfolded/misfolded proteins.

proteins (HSPs). There are four large and ubiquitous families of ATP-dependent molecular chaperones, namely, HSP100 proteins, HSP90 proteins, HSP70 proteins, and HSP60 proteins. In addition to the ATP-dependent chaperones, there are ATP-independent chaperones, including small HSPs (sHSPs). There are also dedicated molecular chaperones that remodel a specific substrate protein or complex. The best-known example of folding-related neurodegenerative diseases is Alzheimer's disease. Several studies showed the induction of small heat shock proteins (HSP27, crystallin), HSP70, and ubiquitin (a 6 kDa heat shock protein, which labels damaged proteins and directs them for proteolytic degradation) in neurons affected by AD and in surrounding astrocytes.

Accumulation of chaperones is the response of the affected neuron to eliminate Aβ and Tau. Immunohistochemical studies and expression analyses in AD brain tissue showed that expression levels of a number of HSPs, particularly HSP27 and HSP70, were elevated in affected regions from AD brain tissue, and this elevation appears to be a marker of activated glia and dysregulated/stressed neurons [83, 84]. Protein levels of HSP27, HSP32, HSP60, HSP70, and HSP90 were found to be increased in hippocampus and inferior parietal lobule from amnestic mild cognitive impairment (MCI) patients, suggesting that alteration in the chaperone protein systems might contribute to the pathogenesis and progression of AD [85]. Interestingly, although HSP70 is neuroprotective against intracellular Aβ and is induced in AD brain, this protein is carbonylated in AD, possibly with reduced cellular protection [86].

Several other HSPs have been found to be oxidatively modified in AD, including HSP90 and HSP60 [5], while HSP27 levels are elevated in amnestic MCI [85]. Impairment of these proteins could contribute to proteasomal overload and dysfunction, observed in AD [78]. Aβ-treated synaptosomes show that HSPs are oxidatively modified [87], further accounting for the vulnerability of HSPs to Aβ-induced OS.

Another chaperone member, which has been found to contribute to AD pathology, is the heme-oxygenase 1 (HO-1) [88]. HO-1 is a microsomal enzyme that exists in two main isoforms: the inducible HO-1 and the constitutive HO-2 [89, 90]. HO-1, also known as HSP32, is induced by a variety of stimuli, including OS, heat shock, bacterial lipopolysaccharide, and hemin among others [90]. Conversely HO-2 is sensitive to developmental factors and adrenal glucocorticoids and works as intracellular sensor of oxygen, carbon monoxide, and nitric oxide [89]. The effective contribution of HO-1 induction to cellular antioxidant defense is currently under debate because increasing evidences questioned its protective role in neurodegenerative disorders. Indeed, Hui et al. suggested a potential pathway to explain Tau phosphorylation/aggregation, through excessive iron production mediated by HO-1 overexpression [91]. In addition, Schipper et al. showed that targeted suppression of glial HO-1 hyperactivity may represent an effective neurotherapeutic intervention in AD [92]. Very recent data from Barone et al. [88] showed that HO-1 is increasingly oxidized (PC and HNE modification) in both MCI and AD brain. Along with oxidative modifications of HO-1, the authors observed significant increase of Ser-residue phosphorylation. However, it is difficult to state which posttranslational modification precedes the other. It is likely that OS promotes the increase of Ser-residue phosphorylation in order to activate protein functions, but at the same time HO-1 is a target of oxidative posttranslational modifications, that in turn could impair its function.

Intriguingly, parallel to intracellular chaperones, novel interest is currently devoted to understand the role of extracellular chaperones in AD neuropathology. This is an analogous system existing in the extracellular space that recognizes nonnative proteins and promotes their removal from the extracellular fluid via receptor-mediated endocytosis [93]. Members of the extracellular quality control system are haptoglobin, alpha 2 macroglobulin, serum amyloid P, and also clusterin. Thambisetty and Lovestone discovered that the plasma concentration of clusterin is associated with disease severity, pathology, and clinical progression in AD, as well as with brain fibrillar Aβ burden in nondemented older individuals [94]. Studies form our group demonstrated that haptoglobin, one of the most abundantly secreted glycoproteins with chaperone function, was found to be either increasingly downregulated or increasingly oxidized in plasma from AD and MCI patients compared with controls [95]. We also demonstrated that in vitro oxidation of haptoglobin affects the formation of amyloid-β fibrils, thus suggesting that oxidized haptoglobin is not able to act as an extracellular chaperone to prevent or slow formation of amyloid-β aggregates. These findings suggest that alterations in proteins acting as extracellular chaperones may contribute to exacerbating amyloid-β toxicity in the peripheral system and may be considered a putative marker of disease progression. However, the robustness and the reproducibility of changes of OS stress markers at the peripheral level (CSF, blood, and urine) are still under debate [96].

UPS. Among members of the proteasomal system, a major target of oxidative damage is the ubiquitin-carboxy terminal hydrolase 1 (UCH-L1). UCH-L1 belongs to a family of UCH-L that act in the ubiquitin-proteolytic pathway involved in protein degradation of damaged proteins and has been implicated in many neurodegenerative diseases [97]. UCH-L1 was identified to be carbonylated in AD brain, with a parallel loss of activity, which is consistent with the observed increased protein ubiquitination, decreased proteasome activity, and accumulation of misfiled proteins in AD brains [98]. Thus, it is likely that oxidative inactivation of UCH-L1 possibly contributes to both protein aggregation and exacerbation of OS observed in AD brain. Moreover, UCH-L1 oxidative dysfunction could affect activity of the 26S proteasome, which is known to be reduced in AD [78]. Taken together, the data may lead to hypothesizing that, in degenerating neurons, protein with excess ubiquitination accumulates, the activity of the 26S proteasome decreased, and consequent accumulation of aggregated/damaged proteins is favoured. Additional redox proteomics studies in familial AD confirmed that oxidative damage of UCH-L1 was accompanied by reduced enzyme activity [99].

Protein Synthesis. In addition to protein degradation, protein homeostasis is also regulated by the rate of its synthesis. Several studies have provided indirect evidence that suggests that alterations in protein synthesis may occur in AD [100, 101]. Redox proteomics studies demonstrated that EF-Tu and eukaryotic initiation factor α (eIF-α) are HNE-modified in the IPL of MCI brain [102]. EF-Tu and eIF-α are intimately involved in protein synthesis machinery. Human mitochondrial EF-Tu functions in the translational apparatus of mitochondria and acts as a GTPase by hydrolyzing one molecule of GTP for each A site amino-acylated tRNA of the ribosome. eIF-α, which binds aminoacyl-tRNA to acceptor sites of ribosomes in a GTP-dependent manner, is involved in cytoskeletal organization through the interaction with actin filaments and microtubules [103]. The expression of eIF-α is regulated in aging, transformation, and growth arrest. The dysfunction of the protein synthesis apparatus, mediated in part by the oxidative modifications of both EF-Tu and eIF-α, could significantly compromise neuronal cells homeostasis, thus contributing to the development of neuropathology in AD. Further studies are needed to better understand the impairment of protein synthesis machinery in AD and other neurodegenerative disorders.

Regulatory Pathways. Within this frame, another protein that may play a crucial role in the proteostasis network is Pin1. Pin1 is a regulatory protein that recognizes phosphorylated Ser-Pro or phosphorylated Thr-Pro motifs in target proteins. After binding to this motif on the target protein, the PPIase active site domain of Pin1 catalyses the stereochemical conversion from *cis* to *trans* and vice versa of the Pro residue of the target protein, thereby regulating its activity [104, 105]. Pin1 plays an important role in cell growth and is required for proper progression through the cell cycle in dividing cells [106]. Interestingly, Pin1 is involved in the regulation of phosphorylation/dephosphorylation of Tau protein, APP, and other proteins such as cyclin dependent kinase-5. Pin1 was also identified by redox proteomics as oxidatively modified protein in AD hippocampus [107]. Further, in AD brain, Pin1 colocalizes with pTau and also shows an inverse relationship to the expression of Tau in AD brains [108]. Oxidation and decreased levels and activity of Pin1 could contribute to the formation of both NFTs and plaques (20). According to this proposed role of Pin1 in AD pathology, Zhou and coworkers demonstrated that Pin1 overexpression could restore the function of Tau protein in an AD mouse model [109], supporting the idea that oxidative modification of Pin1 could be one of the initial events that trigger tangle formation and oxidative damage in AD brains. Moreover, given that Pin1 regulates APP processing, it is reasonable to speculate its involvement in the deposition of amyloid plaques.

4.2. Oxidative Damage to Proteostasis Network in DS. Down syndrome individuals are characterized by early accumulation of Aβ and increased OS [110, 111] that are conceivably related to alteration of the proteostasis network as previously discussed. However, only few studies have been conducted to highlight the role of proteostasis impairment in DS pathology. In 2011, Necchi at al. showed that in the cerebellum of Ts65Dn a mouse model of DS proteasome is inhibited as a consequence of increased Aβ levels [112]. Our laboratory employing redox proteomics showed recently that several components of proteostasis network including GRP78, UCH-L1, Cathepsin D, V0-ATPase, and GFAP undergo protein oxidation in the frontal cortex of DS individuals at about 20

years of age [81]. This study proposes the hypothesis that DS brains, prior to significant AD pathology, may show early disturbance of the proteostasis network possibly linked to increased oxidative stress. Recently, we performed a redox proteomics analysis to identify proteins HNE-modified in the brain of DS individuals with and without AD pathology [80]. In detail, we found that UCH-L1, GRP78, GFAP, and HSC71 are targets of HNE modification that contributes to the reduction of their activity. In addition, the accumulation of oxidative damage may lead to the impairment of specific proteins that regulate neuronal integrity, axonal transport, synapse connections, energy production, and antioxidant defense. These oxidative stress-related alterations, as demonstrated also by other groups, are intrinsically, but not exclusively, dependent on triplication of Chr21 genes.

Chaperones/UPR. Members of the Grp family, mainly GRP78, are part of the protective mechanism used by cells to adapt to stress of the ER. Our data, reporting GRP78 increased carbonylation and HNE modification, suggest the alteration of its protein structure that results in the inability to bind to the misfolded proteins that might lead to the dysfunction of UPR system, accumulation of misfolded protein, and risk of cognitive decline [80, 81]. Another member of chaperone family, HSC71, has been found to be oxidatively modified by HNE binding [80]. HSC71 is involved in the degradation of proteins with abnormal conformation by binding to a particular peptide region and labeling it for proteasome-mediated proteolysis [113].

UPS. Upon activation of ER-stress response, UPS mediates ubiquitination and degradation of misfolded proteins, which occur in the cytoplasm [114]. We observed that UCH-L1 is a target of oxidative damage in DS and DS/AD brains, and its oxidative modification likely leads to a decreased function as demonstrated by activity assay [80, 81]. One of the major consequences of aberrant UCH-L1 activity is an impaired proteasome proteolytic system, which will lead to accumulation of damaged proteins and formation of protein aggregates [20, 97, 115–117]. To confirm this hypothesis we measured the trypsin-like, chymotrypsin-like, and caspase-like activities of the proteasome demonstrating decreased levels that suggest a reduced activity of UPS in DS brain [81].

Autophagy. The oxidation of V0-ATPase pump and Cathepsin D (CatD) together with decreased autophagosome formation in DS brain demonstrates the potential involvement of autophagy dysfunction DS-related neurodegeneration [81]. The V0-ATPase pump proton is essential for acidic lysosomal pH and alterations on its activity affect directly lysosome functionality and autophagy, as was previously seen in a PD model [111, 118]. In addition, a recent report showed that V0-ATPase is necessary for amino acids to activate mTORC1, thus suggesting that V0-ATPase is an active component of the mTOR pathway [119]. CatD is normally localized within lysosomes and participates in the degradation of proteins, downstream autophagy, and processing of precursor proteins [120, 121]. A newly recognized member of the autophagy machinery is also GFAP; indeed recent studies demonstrated that GFAP is an important regulator for chaperone-mediated autophagy (CMA). GFAP was proposed to interact at the lysosomal membrane either with the lysosome-associated membrane protein type 2A (LAMP-2A), an important component of the translocation complex, or with the elongation factor 1a (ef1a) [122]. Since CMA is activated as part of the cellular response to oxidative stress required for targeting oxidized proteins to lysosomes, oxidation of GFAP might contribute to disrupt the complex network involved in autophagy processes [123]. This result is consistent with in vitro studies showing the carbonylation of GFAP in synaptosomes treated with Aβ (1–42) [87, 124].

Overall, the above data confirm a close connection between altered proteostasis network and increased OS in DS brain that results in the increase of unfolded/misfolded proteins and the formation of protein aggregates observed in this pathological condition. By comparing results obtained on AD brain, as previously discussed, it is possible to draw a scenario where oxidized proteins overlap between DS and AD brain that strongly support similarities of the neurodegenerative process.

5. Outlook

Growing evidences support the impairment proteostasis network in both DS and AD. Results obtained by pathological analysis of human samples and studies from mouse and cellular models of the diseases support the evidence of a molecular link between protein oxidation/aggregation, the integrity of PQC system (proteasome, UPS, and autophagy), and neurodegenerative mechanisms. Many common pathological hallmarks have been proposed for DS and AD including deposition of amyloid plaques, NFTs, increased oxidative stress, impaired mitochondrial function, and aging effects. Since aging is accompanied by changes in cellular protein homeostasis and an increasing demand for protein degradation, aspects of protein misfolding and protein degradation seem to be relevant in this proposed scenario. One of the major goals in the search for the cause of development of AD-like dementia is to try to outline the cascade of events: what is the initiating factor driving the neurodegeneration, what follows, and what are the end-points. Many pathways have been characterized, but the interplay among the different factors is far from being unravelled. Considering that proteins are the main effectors of all cellular functions, "stressed" neurons have to challenge to maintain correct protein homeostasis. The proteostasis network controls the fine balance between protein synthesis and protein degradation. Degradative machineries, both autophagy pathways and proteasome system, are key mechanisms of vital importance for cell survival under stress conditions. The PQC through degradation of oxidized/misfolded proteins allows the cell to modulate its protein expression pattern in response to pathological conditions and provides a critical protective role. If this process is defective, damaged/dysfunctional proteins are not efficiently removed and may accumulate. Indeed, deposits of aggregated, misfolded, and oxidized proteins are

key hallmarks of neurodegeneration. *What if oxidative damage targets PQC?* A close link between proteostasis network and increased OS has been demonstrated. Low amount of ROS can activate the dedicated adaptive cellular apparatus such as the antioxidant and heat shock responses, cell cycle regulation, DNA repair, UPR, and autophagy. However, disturbances of proteostasis trigger increased ROS formation and depletion of the antioxidants within the cell. In turn, a chronic exposure to ROS leads to accumulation of oxidized proteins as well as damaging specific component of PQC, thus exacerbating the accumulation of unfolded/misfolded proteins. The collective effects of deficient proteostasis network and increased oxidized proteins produce a vicious cycle that may trigger the neurodegenerative process. Consequently, an effect of proteostasis control on neurodegeneration or vice versa may be proposed. Though several evidences on the disturbance of this network are available for AD, further studies are needed to clarify this intricate scenario in DS. Studies from our group may lead to hypothesizing that a close link exists between oxidative stress and proteostasis network that we propose as a unifying mechanism triggering development of Alzheimer's-like dementia.

Experimental approaches employing mouse models clearly demonstrate that stabilization or induction of proteostasis can be neuroprotective. Whether this can be translated into the human condition and, most importantly, whether supporting proteome integrity can be a real target for pharmacological intervention for the prevention and treatment of AD are currently still open. Although there are obviously still many questions to be answered to understand the role of proteostasis in DS and AD, a better understanding of how the proteostasis network is regulated might help to identify targets, which lead to prevention of the deleterious loss of neuronal cells and tissue in AD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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