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REVIEW ARTICLE

Redox proteomics analysis to decipher the neurobiology of Alzheimer-like neurodegeneration: overlaps in Down's syndrome and Alzheimer's disease brain

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Accumulation of oxidative damage is a common feature of neurodegeneration that, together with mitochondrial dysfunction, point to the fact that reactive oxygen species are major contributors to loss of neuronal homoeostasis and cell death. Among several targets of oxidative stress, free-radical-mediated damage to proteins is particularly important in aging and age-related neurodegenerative diseases. In the majority of cases, oxidative-stress-mediated post-translational modifications cause non-reversible modifications of protein structure that consistently lead to impaired function. Redox proteomics methods are powerful tools to unravel the complexity of neurodegeneration, by identifying brain proteins with oxidative post-translational modifications that are detrimental for protein function. The present review discusses the current literature showing evidence of impaired pathways linked to oxidative stress possibly involved in the neurodegenerative process leading to the development of Alzheimer-like dementia. In particular, we focus attention on dysregulated pathways that underlie neurodegeneration in both aging adults with DS (Down's syndrome) and AD (Alzheimer's disease). Since AD pathology is age-dependent in DS and shows similarities with AD, identification of common oxidized proteins by redox proteomics in both DS and AD can improve our understanding of the overlapping mechanisms that lead from normal aging to development of AD. The most relevant proteomics findings highlight that disturbance of protein homoeostasis and energy production are central mechanisms of neurodegeneration and overlap in aging DS and AD. Protein oxidation affects crucial intracellular functions and may be considered a 'leitmotif' of degenerating neurons. Therapeutic strategies aimed at preventing/reducing multiple components of processes leading to accumulation of oxidative damage will be critical in future studies.

Key words: Alzheimer's disease, Down's syndrome, energy metabolism, protein oxidation, proteostasis network, redox proteomics, trisomy 21.

OXIDATIVE STRESS HYPOTHESIS OF NEURODEGENERATION

Neurodegenerative disorders are a heterogeneous group of diseases of the nervous system that have many different aetiologies. Neuropathologically, such disorders are characterized by selective abnormalities of specific regions of the brain and specific populations of neurons, but OS (oxidative stress) contributes, and eventually exacerbates, the major disease-specific pathogenic process. OS is caused by an imbalance in the redox state of the cell, either by overproduction of ROS (reactive oxygen species)/RNS (reactive nitrogen species), or by decreased antioxidant response. The high lipid content of nervous tissue, together with its high aerobic metabolic activity, leaves the brain particularly susceptible to oxidative damage [1]. In addition, those brain regions that are rich in catecholamines are selectively vulnerable to free radical generation. ROS such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$), are both radical and non-radical oxygen species generated as by-products of aerobic respiration and various other catabolic and anabolic processes [2]. The major sources of free radicals include the mitochondrial oxidative phosphorylation pathway, in which electron leakage from the ETC (electron transport chain) causes the formation of $O_2^{\bullet-}$ [3]. Mitochondrial Complex I and Complex III leak superoxide radical at a steady rate that approximates 1–2 % of oxygen that enters the mitochondrial ETC. This radical, in turn, is converted by mitochondrial-resident MnSOD (manganese superoxide dismutase) into H_2O_2 and O_2. Detection of superoxide by EPR spin trapping and H_2O_2 by

Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; AGE, advanced glycation end-product; APP, amyloid precursor protein; CAT, catalase; Chr21, chromosome 21; CMA, chaperone-mediated autophagy; 2,4-DNPH, 2,4-dinitrophenylhydrazine; DS, Down's syndrome; ER, endoplasmic reticulum; ETC, electron transport chain; FBA, fructose bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GPX, glutathione peroxidase; GRP78, glucose-regulated protein of 78 kDa; HNE, 4-hydroxynonenal; HSC71, heat-shock cognate 71 stress protein; HSP, heat-shock protein; MAPK, mitogen-activated protein kinase; MCI, mild cognitive impairment; MDH, malate dehydrogenase; MeSOX, methionine sulfoxide; NFT, neurofibrillary tangle; 3-NT, 3-nitrotyrosine; OS, oxidative stress; PK, pyruvate kinase; PQC, protein quality control; PTM, post-translational modification; PUFA, polyunsaturated fatty acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, tricarboxylic acid; UCH-L1, ubiquitin C-terminal hydrolase L1; UPR, unfolded protein response; UPS, ubiquitin–proteasome system.

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various fluorescence or electrochemical techniques has been demonstrated (reviewed in [4]). Once released, H_2O_2 is relatively stable and can diffuse through the membrane. In the cytosol, it can be efficiently removed by antioxidant systems such as catalase, glutathione peroxidase and thioredoxin peroxidase. In addition to mitochondrial production, ROS can be released in response to different environmental stimuli such as growth factors, inflammatory cytokines, ionizing radiation, chemical oxidants, chemotherapeutics, toxins and transition metals [5]. Other than mitochondria, a number of cytosolic enzymes are able to generate ROS [6], including the NADPH oxidases, plasma membrane-associated enzymes [7]. The function of NADPH oxidases is to produce superoxide from O_2 using electrons from NADPH.

At physiological conditions, intracellular ROS are kept at low, but measurable, levels that result from the rate of production and the rate of scavenging by various antioxidants [8]. Indeed, ROS, at low concentration, are found to act as signalling molecules in many physiological processes, including cell growth, cell proliferation, redox homoeostasis and cellular signal transduction [9], for example by activating proteins such as tyrosine kinases, MAPKs (mitogen-activated protein kinases) or Ras proteins. It is important to underline that the multifaceted effects of ROS on different cellular processes suggest that oxidant species are not merely detrimental by-products, but also crucial mediators of a variety of signalling pathways [9].

The increase in energy metabolism by aerobic pathways enhances the intracellular concentration of ROS, which in turn trigger the self-sustaining lipid peroxidation cascade, inducing damage to several brain structures, mostly in the presence of compromised physiological defence systems, such as antioxidant enzymes and cell stress response. The rate of generation of ROS in different cellular types correlates with lifespan, and is a major contributor in defining the rate of aging and the development of age-related diseases [6].

Over several decades, researchers demonstrated that OS is a causative or at least collateral factor in the pathogenesis of several neurodegenerative diseases [10]. Considering that aging is the most important risk factor for neurodegenenerative disorders, the free-radical theory of aging may be translated to neurodegeneration as well. Indeed, the progressive and irreversible accumulation of oxidative damage affects the senescence process, contributing to altered physiological functions, increasing incidence of disease, along with a reduction in lifespan [11]. In parallel, changes in redox-responsive signalling cascades and in the expression of corresponding target genes may have a similar or even greater impact on senescence as the direct radical-specific damage of cellular components [9].

Among different targets, free-radical-mediated damage to proteins is particularly crucial in aging and in age-related neurodegenerative diseases, because in the majority of cases oxidative modification is a non-reversible phenomenon that requires clearance systems for removal of oxidized/dysfunctional proteins [11]. Generally, oxidation of proteins could affect different processes including protein expression, protein turnover and cell signalling, eventually leading to cell death [12]. 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 Aβ (amyolid β peptide) and hyperphosphorylated tau in AD (Alzheimer's disease). 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.

Once neuronal homoeostasis is disturbed by the increasing burden of oxidized proteins, as a result of both physiological and pathological aging, protective mechanisms, including protein degradation systems, sustain cellular homoeostasis by repairing or removing the oxidized products [13]. However, reduced activity of these defence mechanisms may render the cell incapable of efficiently removing oxidized biomolecules, resulting in their accumulation. Two major pathways are responsible for the proteolysis of intracellular proteins, either for damage and self-renewal: the UPS (ubiquitin–proteasome system) [14] and the autophagy–lysosome pathway [15]. The UPS is located in the cytosol and the nucleus, and it is responsible for the degradation of more than 70–80 % of intracellular proteins. Most of the proteins are targeted for proteasomal degradation after being tagged with a polyubiquitin chain that, in turn, is recognized by the proteasome. Experimental evidence suggests that failure of the UPS may contribute to neurodegeneration. However, additional factors, including other protein degradation pathways and mitochondrial dysfunction associated with a decline in ATP levels, may contribute to cellular viability.

In addition to the UPS, the other primary pathway for protein breakdown in the cell is through autophagy. Autophagy includes three major types: macroautophagy (indicated as autophagy), microautophagy and CMA (chaperone-mediated autophagy). All three mechanisms share a common destiny of lysosomal degradation, but are mechanistically different from one another [16–18].

Protective up-regulation of the UPS, macroautophagy and CMA occurs in response to OS to remove oxidized proteins. However, in the presence of higher free radical levels, degradation of oxidized proteins may be inefficient [19]. Indeed, oxidative modifications induce cross-linking/misfolding that may block the entrance of protein substrates in the proteolytic cavity of the proteasome, as well as potentially inhibiting the overall activity of the proteasome [20]. In addition, oxidatively modified proteins may directly damage the autophagic degradation system and ROS can damage the lysosomal membrane [13]. Some oxidatively modified aggregated species are resistant to degradation by proteases and accumulate within lysosomes. There, the proteins resistant to proteolysis become a potential new source of free radicals, further damaging the lysosomal structure.

Intriguingly, all of these processes more or less rely on ATP consumption to occur efficiently. Mitochondrial dysfunction is an early event in the pathogenesis of neurodegenerative diseases. Reduced ATP levels, increased ROS, impaired calcium buffering and altered mitochondrial permeability are characteristic mitochondrial defects of degenerating neurons [21]. For example, in the case of the proteasome, ATP binding to the 19S proteasome component promotes the association with the 20S particle and opens a gated channel into the 20S particle that allows substrate entry and access to the peptidase sites [22]. Furthermore, the polyubiquitination sequence is ATP-dependent since ubiquitin is activated by E1 in an ATP-dependent fashion. Also in the case of autophagy, the proton-pumping activity of V-ATPase is responsible for acidification of the lysosome/vacuole [23].

Below we discuss evidence that supports the interplay between genetic and endogenous factors related to protein oxidation, protein aggregation and protein dysfunction, coupled with reduced energy metabolism, in development of AD. The chemistry of protein oxidation, redox proteomics approaches and results obtained on human post-mortem brain from DS (Down's syndrome) and AD cases are discussed.

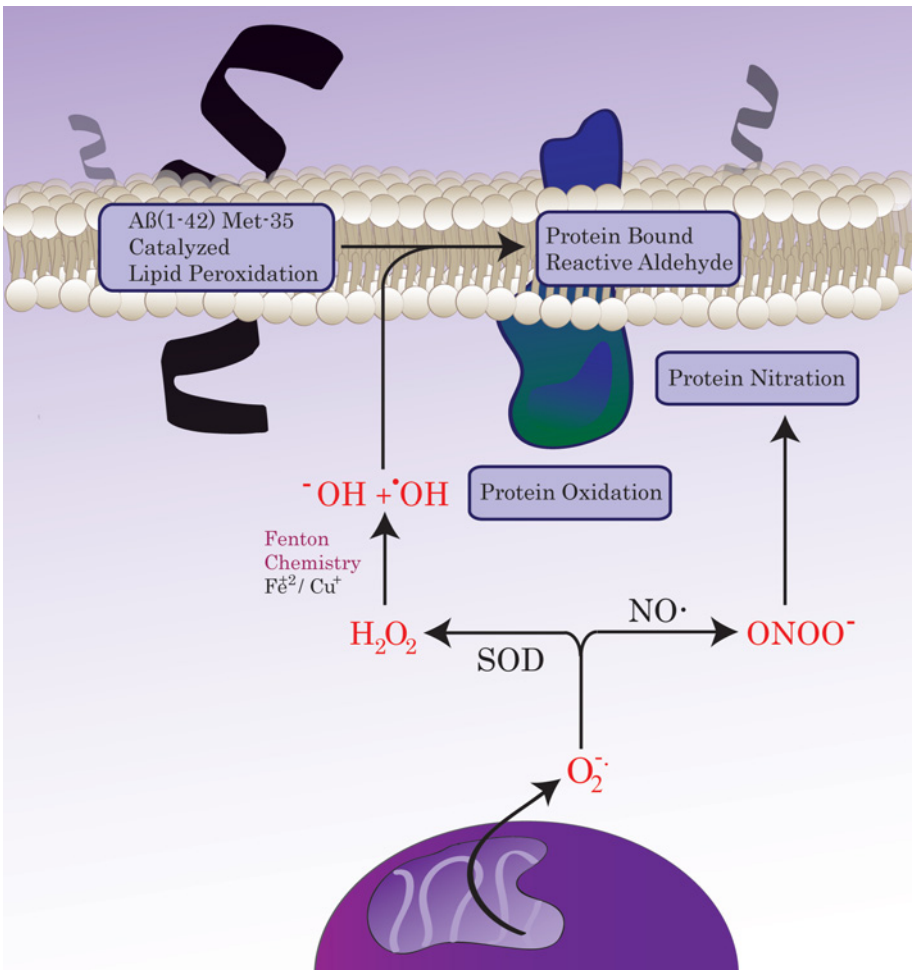

Figure 1 ROS-induced protein modification

ROS/RNS are highly reactive species that can irreversibly modify proteins. Among different types of protein oxidation, protein nitration, protein carbonylation and protein bound to alkenals are the most commonly assayed. These type of modifications are crucially involved in both aging and neurodegeneration.

PROTEIN OXIDATION: CHEMISTRY AND BIOLOGY

The oxidation of proteins by ROS within a cell has long been linked with not only the normal aging process [24–26], but also with a multitude of diseases that range from cancer [27,28] to neurodegenerative diseases such as AD [29–31]. As with other PTMs (post-translational modifications) of proteins, the oxidation or nitration of a peptide chain may alter the 3D conformation of the peptide resulting in a gain or loss of function. This process inevitably affects protein structure and could lead to the alteration in the secondary and tertiary structure resulting, for example, in dissociation of subunits, unfolding, aggregation and backbone fragmentation [32]. Proteins can be oxidized by direct ROS attack, or by secondary oxidation products such as the reactive aldehyde, formed as final by-products of lipid peroxidation, or by glycoxidation reactions. All amino acid residues can be attacked by ROS, but methionine and cysteine residues are particularly sensitive. In the case of methionine, low levels of ROS lead to formation of MeSOX (methionine sulfoxide) that in turn can be reduced by MeSOX reductases. In addition, oxidation of thiol groups, often resulting in the formation of intra- or inter-molecular disulfides, are reduced back by disulfide reductases/isomerases [33]. Since these two oxidative modifications can be enzymatically repaired in mammalian cells, it is likely that they play a key regulatory role and sense the change to cells in the redox environment. Indeed, a number of signalling pathways, such as JNK (c-Jun N-terminal kinase), p38 and MAPK kinases, are strongly responsive to redox regulation [9]. However, the interplay between individual redox-sensitive signalling proteins to redox-regulated processes *in vivo* is quite complex.

While ROS plays a much larger cellular role in both signalling and anti-microbial defence when tightly regulated by functioning ROS-scavenging mechanisms, a loss of such scavenging mechanisms or an unhealthy increase in the cellular oxidants result in damage that takes the form of protein, lipid and nucleic acid oxidation (Figure 1) [30,34–38].

When ROS act directly upon a protein, protein carbonylation is a result, often forming reactive aldehydes or ketones by way of primary or a secondary mechanisms [39–41]. Primary protein carbonylation may occur via α-carbon hydrogen extraction, oxidation of the peptide backbone resulting in fragmentation, and oxidation of amino acid side chains. Secondary protein carbonylation on the other hand takes place with the addition of reactive aldehydes such as acrolein and HNE (4-hydroxynonenal) that are produced from mechanisms of lipid peroxidation [42]. The α/β-unsaturated reactive aldehydes serve as electrophiles that react with protein side chains such as lysine, histidine and

cysteine via Michael addition. Protein carbonyls however may be more than just cell stressors, as new information points to protein carbonyls as a potential form of redox signalling through a reversible enzyme-mediated system [43,44]. In the laboratory, a common method to determine the level of protein carbonylation in a biological sample is through the use of 2,4-DNPH (2,4-dinitrophenylhydrazine) to form hydrazones which are detectable with anti-2,4-DNPH-sensitive antibodies [39]. This method may be used in conjunction with 1D or 2D gel Western blots as well as the dot/slot blot.

Unlike the aqueous environment of the cytoplasm, the lipid bilayer and its hydrophobic nature exclude many polar oxidants that would otherwise damage bilayer integrity. However, the bilayer is not impervious to OS as not only can non-polar oxidants such as H_2O_2 or O_2 pass into the bilayer, but oxidants may also be created within the bilayer as is hypothesized in the Aβ-induced OS hypothesis in AD [45,46]. Aβ-(1–42) inserts into the bilayer and serves as a catalytic producer of ROS, initiating a process called lipid peroxidation [47,48]. One method for the initiation of lipid peroxidation, free-radical-mediated lipid peroxidation, occurs when a carbon-centred radical is produced on a PUFA (polyunsaturated fatty acid) by the abstraction of an allylic hydrogen by some form of radical present within the bilayer, for example the sulfuranyl free radical on Met^{35} of Aβ-(1–42). Oxygen, which lacks a dipole moment, diffuses into the lipid bilayer where it may react with the carbon-centred radical to form a lipid peroxyl-radical [49,50]. The lipid peroxyl-radical may then abstract an allylic hydrogen from an adjacent polyunsaturated lipid which propagates the chain reaction and forms a lipid hydroperoxide that may then undergo cleavage forming an array of possible reactive aldehydes such as F_2-isoprostane, HNE and 2-propenal (acrolein). HNE for example is primarily produced from arachidonic acid, an omega-6 PUFA with inflammatory and signalling properties of its own [51]. In neuronal cells under Aβ toxicity an increase in HNE to concentrations of 5–10 μM was demonstrated within the lipid bilayer [52]. Other PUFAs that are of importance in the generation of lipid peroxidation products are LA (linoleic acid), DHA (docosohexanoic acid) and cholesterol, among others [50]. Although reactive aldehydes are a main by-product of lipid peroxidation, other deleterious effects such as loss of lipid asymmetry and apoptosis may follow [53]. Reactive aldehydes deplete the cell of nucleophilic compounds that serve as antioxidants (GSH, lipoic acid and thioredoxin) by covalently modifying proteins via Michael addition in a secondary protein carbonylation reaction [54]. Protein-bound and free reactive aldehydes are being investigated as potential markers of OS and disease progression in conditions ranging from neurodegeneration to brain infarction [55,56].

NO (nitric oxide), a molecular radical and important second messenger produced from L-arginine using one of three NOS (NO synthase) variants, is vitally important in some biological pathways such as vasodilation, inflammation and the immune response [57–59]. High concentrations of NO, through secondary reactions with ROS, may go on to produce other RNS such as NO_2 (nitrogen dioxide), N_2O_3 (dinitrogen trioxide) and $ONOO^-$ (peroxynitrite). These compounds react with certain metal cofactors of enzymes, the peptide backbone and specific amino acids such as tyrosine, cysteine and tryptophan [60]. 3-NT (3-nitrotyrosine), a PTM of tyrosine, is a well-recognized marker of nitrosative stress. The formation of 3-NT is the product of the reaction between $ONOO^-$ and CO_2 which form both NO_2, a free radical, and the carbonate radical ($CO_3^{\bullet -}$) through the intermediates nitrosoperoxycarbonate and nitrocarbonate [61]. 3-NT formation has recently been linked to systemic autoimmune disorders such as lupus through the generation of endogenous antibodies against native proteins that may be nitrated [61]. Nitration of tyrosine may also result in stearic hindrance that blocks a potential phosphorylation at the 4′ para-position, affecting the potential of tyrosine to be phosphorylated, resulting in a potential for decreased tyrosine signalling [41]. A decrease in tyrosine signalling may result in the progression of neurodegenerative diseases [57,62,63].

Cysteine is another common target of PTM by NO, forming a reversible S-nitrosylation (R-SNO) product through a proposed interaction of a cysteine anion with NO_x, $ONOO^-$ or NO covalently bound to a metal [60]. S-Nitrosylation has been found to be an important signalling modification mediated by NO, whereas aberrant S-nitrosylation has been implicated in neurodegenerative diseases, among others [64,65].

Taken together, it is clear that ROS can damage proteins, and eventually accelerate their proteolytic degradation, with two divergent purposes: (i) a regulatory mechanism, for example in the degradation of the transcription factor subunit [i.e. HIF-1α (hypoxia-inducible factor-1α) and the NF-κB (nuclear factor κB) inhibitor IκB], the inhibition of protein tyrosine phosphatases and the regulation of vascular tone; and (ii) to irreversibly damage proteins leading to loss or gain of function.

REDOX PROTEOMICS APPROACH

In order to gather insights into the state of the proteome affected by diseases or therapies, the use of proteomics is an invaluable tool. The study of proteomics in a given system is a complex undertaking as the proteome is fluid and dynamic and is affected by both intrinsic and extrinsic factors that combine to shape the composition of the proteome at any given period in time. Therefore any insights gained from applications of proteomics must have all factors taken into account that could influence the expression and/or PTMs on the proteins in the system. Such factors could take the form of stressors such as age, oxidative insult, gene activation, drug administration or disease. In order to control for such factors, the proteome of a treated/disease state should always be compared against an age-matched control. A common method in which to conduct an expression proteomics experiment is by way of the gel-based method. This method utilizes a 2D approach, in that proteins are separated in the first dimension according to their isoelectric points before separation in the second dimension according to their migration rates through a polyacrylamide gel. By separating the proteins in two dimensions, each spot in the subsequent gel will likely result in one protein, unlike a separation in one dimension that may result in a band of many proteins. Protein spot density programmes, such as PDQuest, allow for the comparison of the relative concentration of protein within a specific spot which provides insight into the levels/expression of a specific protein within the samples when compared with a control.

Redox proteomics uses similar methodology to that of expression proteomics; however, two identical gels are run instead of one. After 2D separation, the first gel is set aside for spot-density analysis while the second gel is transferred to a nitrocellulose membrane via Western blot techniques to undergo immunohistochemical analysis. Once the transfer is complete, the membrane is blocked using BSA and probed with a primary antibody to the redox modification of interest, followed by a secondary antibody to recognize the primary antibody. Once the blot is developed, spots may be analysed for relative changes in spot density which correlate with increased or decreased oxidative modification [12,66]. The spots on the blot may then be compared with the spots on the gel.

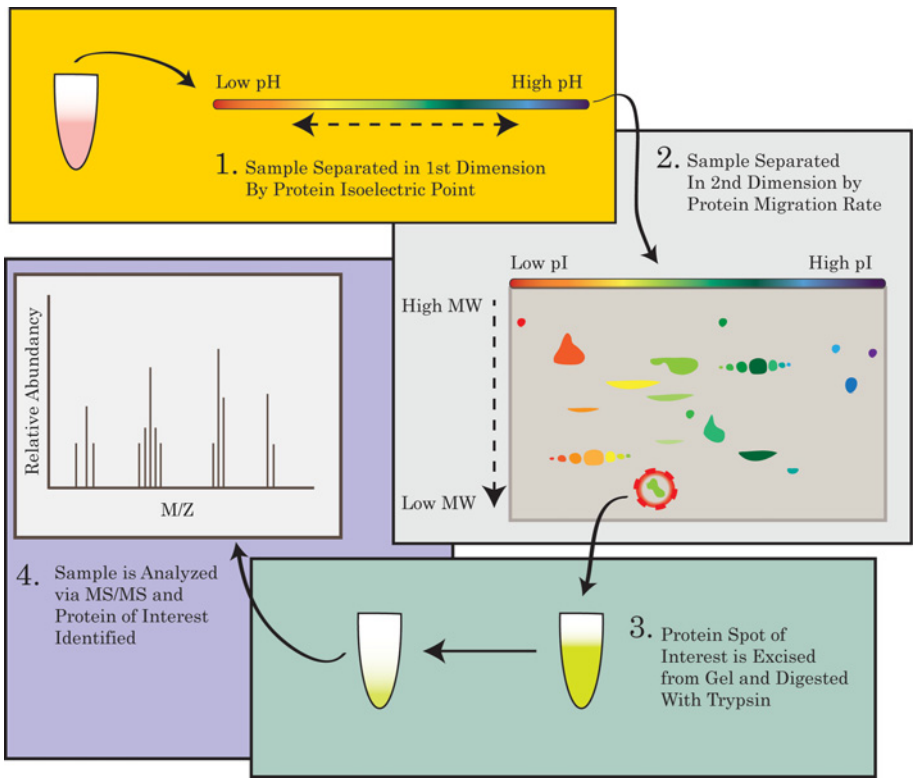

Figure 2 Redox proteomics

The workflow of a redox proteomics analysis involves four principal steps. 1. Isoelectrofocusing. 2. Second-dimension electrophoresis. 3 Proteins of interest, selected by 2D image analysis software, are excised from the gel and digested with trypsin. 4. MS analyses coupled to database searching leads to protein identity. MW, molecular mass.

The identification of the spot of interest is achieved through spot excision followed by tryptic digestion and MS/MS identification. Finally, a selection of proteins is designated for confirmation via Western blot to validate the MS/MS results. An example of the redox proteomics experimental workflow is shown in Figure 2.

OXIDATIVE STRESS: A COMMON PATHOLOGICAL FEATURE OF DOWN'S SYNDROME AND ALZHEIMER'S DISEASE NEURODEGENERATION

In recent years, much interest has been devoted to understanding the common neuropathological features of DS and AD. The relationship between DS and AD is complex. Studies have shown that, by the age of 40, almost all people with DS have evidence of brain changes characteristic of AD, with deposition of senile plaques, containing Aβ and NFTs (neurofibrillary tangles), composed of hyperphoshorylated tau, and also cholinergic and serotonergic reduction [67–70]. DS is the most frequent chromosomal abnormality that causes intellectual disability, resulting from the presence of an extra complete or segment of chromosome 21 and is termed trisomy 21 (Chr21). The high incidence of AD symptoms and pathology in DS individuals is thought to be caused by the extra copy of chromosome 21, which encodes some of the genes already known to be associated with AD brain abnormalities, including APP (amyloid precursor protein), SOD1 (Cu/Zn superoxide dismutase), CBS (cystathionine β-synthase) and S100β, among others.

Accordingly, the 'gene-dosage effect' of DS suggests that phenotypic alterations result from the overexpression of a subset of genes and their encoded proteins [71,72]. Research has also demonstrated that many DS features, including the development of AD, are due to the complex effects of multiple Chr21 genes and their interactions with genes on other chromosomes. By combining these two different hypotheses, it is likely that, in this dysregulated scenario, the effects caused by some dosage-sensitive genes are amplified and result in a plethora of different phenotypic traits according to the 'number and dose' of genes involved. Results obtained by the analysis of DS cases and the development of a number of mouse models of the disease support both hypotheses. Interestingly, the development of AD occurs much earlier in people with DS, with symptoms beginning to be robust by the fifth decade of life, and an incidence three to five times greater than that of the general population [73].

This complex scenario is an attractive field of research, and the precise molecular pathways by which trisomy 21 leads to the early-onset of AD remain to be elucidated. Several studies supported the view that increased OS conditions may contribute to accelerated senescence and neuropathology in DS individuals [67]. Accordingly, different markers of oxidative damage are elevated in brain tissue from DS (reviewed in [74]). Indeed, DS may be regarded as a chronic OS condition, due to the fact that higher levels of free radicals are caused by a number of trisomic genes, that directly or indirectly, participate to create an imbalance between ROS production and clearance. Among ROS inducers, SOD1, APP, the transcription factor Ets-2, S100β and carbonyl reductase, map on Chr21 [74]. SOD1 catalyses the dismutation of O_2^- to O_2 and H_2O_2, the latter in turn neutralized by CAT (catalase) and by GPX (glutathione peroxidase) to water [21]. CAT and GPX are generally expressed at lower levels in brain compared with other tissues [22] and this reduced 'buffer' activity may contribute to the inefficient removal of increasing levels of

H_2O_2 in DS. In turn, accumulation of H_2O_2, in the presence of Fe(II) or Cu(I), leads to hydroxyl radical formation that damage membrane lipids, proteins and nucleic acids; in addition, the direct interaction of Fe(II) or Cu(I) with lipid hydroperoxides can also generate hydroxyl radicals [75].

However, as also shown by a proteomics study from Gulesserian et al. [76] increased OS in fetal DS brain is not only a consequence of SOD1 overexpression, which alone cannot explain the generalized increase in oxidative damage. To search for other major OS-inducers, another gene triplicated in DS, APP, was examined. As expected by a gene-dosage effect, DS individuals with a triplication of the APP gene have increased production of Aβ. Both the levels of Aβ-(1–42) and Aβ-(1–40) are higher in DS plasma than controls [77], and also the ratio of Aβ-(1–42)/Aβ-(1–40) is lower in DS than in controls. Aβ and tau lesions affect several brain regions in DS, including prefrontal cortex, hippocampus, basal ganglia, thalamus, hypothalamus and midbrain, and are believed to underlie the development of cognitive decline and dementia. In DS fetal tissue increased APP and elevated Aβ levels are found that lead to mitochondrial dysfunction [78]. Interestingly, Aβ itself can be oxidized, and plaques in the aged DS brain contain a significant amount of oxidized Aβ [79]. Oxidized Aβ in DS brain is observed within microglia as small aggregates, suggesting reduced clearance from the brain.

Elevated OS markers have been measured in peripheral nervous system and central nervous system samples from DS patients and relevant animal models. Levels of TBARS (thiobarbituric acid-reacting substance), total protein carbonyls, and AGEs (advanced glycation end-products) are increased in the cortex from DS fetal brain compared with controls. In addition, accumulation of 8OHdG (8-hydroxy-2-deoxyguanosine), oxidized proteins and nitrotyrosine, was observed in the cytoplasm of DS [80]. At the systemic level, the amount of $iPF_{2\alpha}$ (isoprostane 8,12-iso-$iPF_{2\alpha}$), a specific marker of lipid peroxidation, is elevated in urine samples from adults with DS [81]. In addition, levels of AGEs, dityrosine, H_2O_2 and nitrite/nitrate are significantly higher in urine samples of DS compared with age-matched controls [82]. Previously, we also found in the amniotic fluid from DS pregnancies that the levels of protein carbonyl and lipid peroxidation were increased, coupled with reduction of GSH and Trx (thioredoxin) levels, and induction of the HSP (heat-shock protein) response [83].

As an essential link to OS, mitochondrial dysfunction is observed as redox imbalances occur, due to the major role of mitochondria in oxygen metabolism, as mentioned above, and this is the case in DS. It is well recognized that mitochondrial dysfunction is a crucial event for neurodegeneration. Mitochondria are known to play a central role in many cell functions including ATP generation, intracellular Ca^{2+} homoeostasis, ROS formation, as by-products of oxidative phosphorylation, and apoptosis. Neurons are particularly dependent on mitochondria because of their high energy demand, and it is likely that neurons are sensitive to mitochondrial dysfunction. The role of mitochondrial dysfunction in AD is well-established [21,78,84,85]. Moreover, altered mitochondrial activity has been reported in DS fibroblasts [86] and mitochondrial DNA mutations were found in DS brain tissue [87]. In addition, both DS neurons and astrocytes display an abnormal pattern of protein processing consistent with chronic energy deficits [88]. These deficits are further complicated by the fact that a number of proteins that cause neurodegeneration, including Aβ, interact with mitochondria or affect mitochondrial function. Taken together, both DS and AD neurons are vulnerable to: increased ROS levels, decreased mitochondrial function, decreased ATP production and increased Aβ load. All these events may participate in a self-sustaining vicious cycle thus ultimately leaving neurons highly susceptible to death.

Redox proteomics studies performed on brain from DS and AD subjects identified a common framework of oxidized proteins that suggest a number of similarities in the neurodegenerative process. Below, the impact of oxidative damage to members of the proteostasis networks and energy metabolism are discussed in light of their putative role in development of AD and commonalities of oxidatively modified brain proteins between AD and DS with AD.

OXIDATIVE DAMAGE TO THE PROTEOSTASIS NETWORK IN DS AND AD

When oxidized/misfolded proteins accumulate in sufficient quantity, they are prone to form aggregates [33]. Aggregates of this type include amyloid plaques and neurofibrillary tangles in both DS and AD. The formation of aggregates affects several intracellular pathways that negatively impact metabolism and protein turnover. It is not completely clear why or how toxic protein aggregates occur, but irreversible damage to proteins from OS seems to be involved in physiological aging and neurological degeneration [12]. One interesting aspect of this complex process is that ROS can be generated during early phases of protein self-aggregation [89]. Indeed, the production of H_2O_2 from Aβ has been demonstrated by a number of studies [89]. Furthermore, early oligomeric forms of protein aggregates, with their associated redox-active metals, may directly interact with cell membranes, cell-surface receptors or various intracellular target molecules possibly through the generation of ROS [89]. Considering that ROS damage induces protein aggregation, which in a vicious cycle further leads to ROS release, the ability of the intracellular PQC (protein quality control) system to efficiently remove toxic aggregates is vital to fight against neurodegeneration.

Preserving protein homoeostasis, or 'proteostasis' requires several parallel strategies that involve refolding, degradation or clearance of misfolded polypeptides [90]. Any condition leading to increased protein misfolding load may result in the disturbance of the proteostatis network, a crucial aspect of cell physiology. Thus it is essential for the cell to mount a rapid and robust response to restore protein homoeostasis by both the up-regulation of quality control components and the removal of misfolded/aggregated proteins.

Central players of the proteostasis system are molecular chaperones that assist misfolded proteins to direct them, if refolding fails, to the protein-degradation system [91]. Environmental stress induces chaperone (HSP and stress protein) expression, a key protective factor for cell survival to repair cellular damage after stresses. Among this broad family, those initially identified as heat-inducible were called HSPs. There are also dedicated molecular chaperones that remodel a specific substrate protein or complex.

AD is one of the best-known examples of misfolding-related neurodegenerative diseases and has also been named a 'protein misfolding disease', along with other brain disorders. Accumulation of chaperones may be one response of the affected neuron to eliminate Aβ and tau [92,93].

Intriguingly, redox proteomics studies performed on human brain from DS and AD subjects demonstrated that a number of oxidized proteins are in common between the two pathologies, namely: GRP78 (glucose-regulated protein of 78 kDa), UCH-L1 (ubiquitin C-terminal hydrolase L1), HSC71 (heat-shock cognate 71 stress protein) and GFAP (glial fibrillary acidic

protein). Among the components of the proteostasis network, GRP78, UCH-L1, cathepsin D, V_0-ATPase and GFAP were increasingly carbonylated in the frontal cortex of DS individuals at approximately 20 years of age compared with their age-matched controls [94]. These initial findings suggest the hypothesis that younger cases with DS may already show disturbance of the proteostasis network, possibly linked to increased OS, many years before the appearance of AD neuropathology.

We next extended our redox proteomics investigation to include older DS cases with AD pathology, and we identified HNE-modified proteins. We analysed post-mortem frontal cortex from four different groups: DS individuals with and without AD pathology compared with their age-matched controls [95]. The comparison entails both age- and genotype-dependent variables, discriminating age-dependent effects with those intrinsic to DS. Specifically, we found that GRP78, GFAP, UCH-L1 and HSC71 are targets of HNE-modification in DS brain.

Chaperones/UPS

Members of the Grp family, mainly GRP78, participate in protective mechanisms activated by cells to adapt to stress of the ER (endoplasmic reticulum). Under conditions in which misfolded proteins accumulate within the lumen of the ER, the organelle enters into a state called 'ER stress' and activates a series of complex co-ordinated signalling pathways, collectively called the UPR (unfolded protein response) [96,97]. The increased carbonylation and HNE modification of GRP78 in DS with AD brain suggests that alteration of protein structure may possibly reduce the ability of GRP78 to bind to misfolded proteins with consequent accumulation of misfolded protein and risk of cognitive decline [94,95]. Another member of the HSP chaperone family, HSC71, is oxidatively modified by HNE binding [95]. HSC71 is involved in the degradation of proteins with abnormal conformation by binding to a particular peptide region and labelling it for proteasome-mediated proteolysis [98].

Upon activation of the ER-stress response, UPS mediates ubiquitination and degradation of misfolded proteins, which occur in the cytoplasm [99]. We showed that UCH-L1 is a target of oxidative damage in DS, DS/AD, MCI (mild cognitive impairment) and AD brains, and that its oxidative modification likely leads to a decreased function as measured by an activity assay [100,101]. One of the major consequences of aberrant UCH-L1 activity is altered proteasome activity, which leads to accumulation of damaged proteins and consequently formation of protein aggregates [100,102–105]. To confirm this hypothesis, the trypsin-like, chymotrypsin-like and caspase-like proteasome activities were measured and each activity was reduced in DS brain compared with controls [94].

Taken together, the redox proteomics results from our laboratory [12] predict that in degenerating neurons: (i) proteins with excess ubiquitinylation accumulate; (ii) the activity of the 26S proteasome is decreased; and (iii) consequent accumulation of aggregated/damaged proteins is favoured. All of these characteristics are observed in brains of subjects with both AD and DS with AD.

Autophagy

Emerging evidence highlights the role of autophagy in aging and neurodegeneration, and several therapeutic strategies aimed at restoring autophagy have been tested in different models [106]. Among mediators of the autophagic cascade, we identified the V_0-ATPase pump and cathepsin D as oxidatively modified, together with decreased autophagosome formation, in younger DS brain before the neuropathology of AD is evident. These modifications appear to be restricted to DS brain. However, GFAP is oxidatively modified in DS, DS/AD and AD brain [12,101]. GFAP, in addition to being recognized as a marker of astrocytic activation, is a newly recognized regulator of the autophagy machinery and important regulator for CMA [107]. GFAP is proposed to interact at the lysosomal membrane either with LAMP-2A (lysosome-associated membrane protein type 2A), an important component of the translocation complex, or with EF1a (elongation factor 1a) [107]. Since CMA is one of the cellular mechanisms activated to resist OS required for targeting oxidized proteins to lysosomes, oxidation of GFAP might contribute to disruption of autophagic flux [108]. This result is also consistent with *in vitro* studies showing the carbonylation of GFAP in synaptosomes treated with Aβ-(1–42) [109,110].

Overall, the above results confirm a close connection between imbalance between increased protein oxidation and reduced ability to remove oxidized/misfolded proteins (Figure 4). A key player that seems to disrupt this fine-tuned equilibrium is OS that is not only a challenge to neuronal cells with increasing amounts of ROS and ROS-damaged by-products, but also contributes to a general failure of the defence system through oxidative modifications, i.e. reduced activity, of selected members of the proteostasis network. This proposed scenario requires further elucidation in that some of the above-mentioned activities require ATP to occur efficiently.

ENERGY METABOLIC DYSFUNCTION IN DS AND AD BRAIN

Glucose is the principal source of energy for the brain, which utilizes 20 % of glucose metabolism and consumes more than 30 % of the inspired oxygen, although the brain accounts for only 2 % of the total body weight. Glucose metabolism is essential for healthy brain function and even a small interruption of glucose metabolism causes brain dysfunction and memory loss [12]. Emerging evidence supports the notion that AD is tightly linked to metabolic disorders in which brain glucose utilization and energy production are impaired. Both obesity and Type 2 diabetes significantly increase the risks of cognitive decline and development of AD, consistent with the notion that impaired brain glucose metabolism plays a significant role in disease pathogenesis [111–113]. APP and Aβ cause decreased activity in mitochondrial respiratory chain complexes, decreased activity of several mitochondrial enzymes and also induce ROS production [80,114–117]. In addition, a number of studies on AD human specimens and/or animal and cell culture models suggested that increased levels of OS are able to impair key players of the glucose metabolic pathway [45,118–120]. This metabolic and oxidative compromise may render neurons susceptible to excitotoxicity and apoptosis, and also induce hypothermia, causing abnormal tau phosphorylation through differential inhibition of kinases and phosphatases [121]. Reduced glucose metabolism might also affect autophagy and protein degradation pathways, as already discussed above in both AD and DS, which respond to alterations of cell energy metabolism [122]. Moreover, dysfunction of mitochondria has been reported to alter APP metabolism, increasing the intraneuronal accumulation of Aβ peptide and enhancing the neuronal vulnerability [78,85,123].

Redox proteomics studies on AD brain demonstrated the oxidation of α-enolase, MDH (malate dehydrogenase), FBA A/C (fructose bisphosphate aldolase A/C), ATP synthase and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Similarly, PK

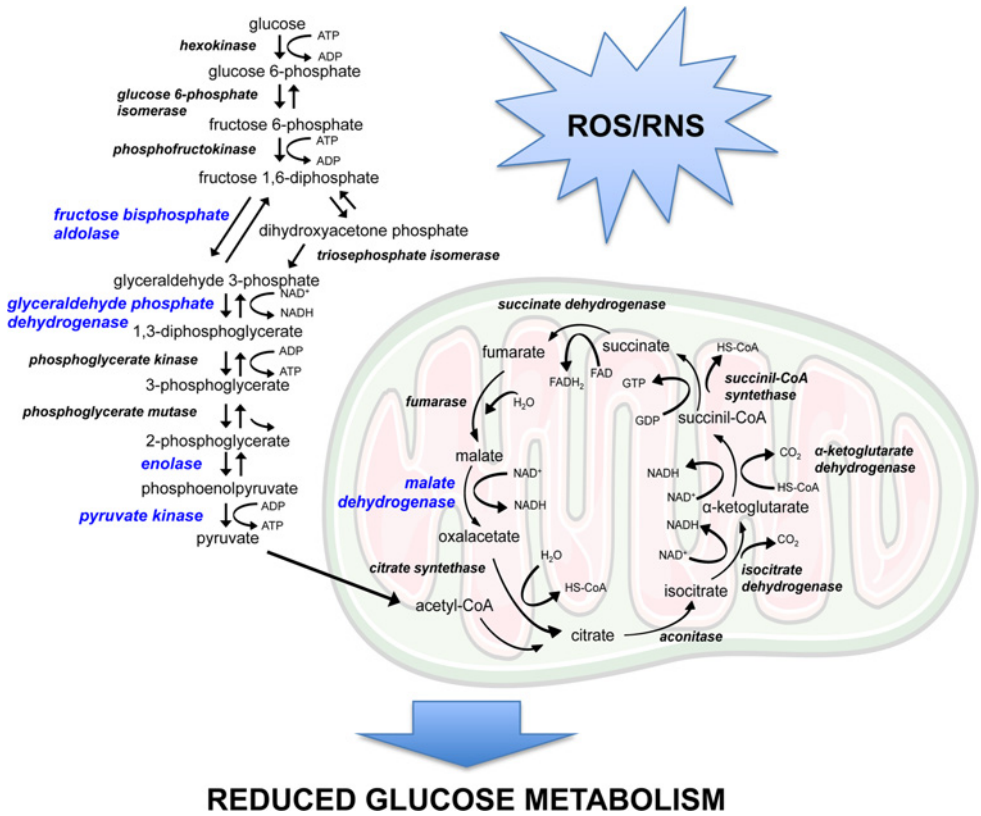

Figure 3 Energy metabolism failure

Increased protein oxidation of energy metabolic enzymes. Specifically, the oxidation of glycolytic enzymes, highlighted in blue, and TCA cycle enzymes lead to reduced activity which culminates in reduced glucose metabolism and decreased synthesis of ATP.

(pyruvate kinase), MDH, α-enolase, FBA C and TPI (triose phosphate isomerase) were found increasingly oxidized in amnestic MCI brain [103,124–128], indicating that impaired glucose metabolism is an early event in the progression of AD. The oxidative modification of energy-related proteins correlates with reduced cerebral metabolic rate of glucose in brain of MCI and AD patients in FDG (2-fluoro-2-deoxy-D-glucose)-PET (positron-emission tomography) studies, suggesting that oxidative damage of protein involved in glycolysis, the TCA (tricarboxylic acid) cycle and ATP synthesis might be a crucial event in the reduction of glucose metabolism [122,129]. Among the proteins found oxidatively modified in AD and MCI subjects, PK, α-enolase, MDH, GAPDH and FBA A/C also were identified by redox proteomics to be carbonylated or HNE-bound also in DS, and DS with AD brain, suggesting the common occurrence of reduced glucose utilization, mitochondrial deficit and increased OS in the development of AD-like dementia [95,101].

Interestingly, the reported oxidation of MDH, GAPDH and α-enolase is consistent with chronic dysfunction of energy metabolism that starts early in the DS population and increases in the presence of AD pathology in DS [95]. In agreement, redox proteomics performed in DS transgenic mice [130] identified lipid-peroxidation-derived modification of a number of proteins belonging to energy metabolism pathways, such as ATP synthase mitochondrial F1 complex b subunit, α-enolase and TPI, thus confirming the involvement of impaired energy consumption in DS. All the above-mentioned proteins rely, to varying extents, on ATP utilization and are closely related to energy metabolic pathways, and their oxidation eventually culminate in reduced ATP production, probably contributing to the loss of synapses and synaptic function at nerve terminals. Consistent with this notion, a recent study showed a significantly decreased level of synaptophysin in DS with AD brain [131]. Thus we propose that these changes contribute to neurodegenerative processes and cognitive decline [12,67,74]. Specifically, the common proteins found oxidized in AD and DS brain are: FBA, GAPDH, α-enolase, PK and MDH.

FBA cleaves fructose 1,6-bisphosphate and produces the two glycolytic intermediates, G3P (glyceraldehyde 3-phospate) and DHAP (dihydroxyacetone phosphate). Fructose 1,6-bisphosphate is neuroprotective and preserves GSH in cortical neurons during OS conditions [132]. FBA catalyses a critical step, as it generates two substrates that are used to eventually produce two molecules of ATP and more in the TCA cycle and ETC.

GAPDH is known in glycolysis for the conversion of glyceraldehyde 3-phosphate into 1,3 bisphosphoglycerate and, consequently, in energy production. In addition, inhibition of GADPH can lead to accumulation of trioses, with subsequent non-enzymatic conversion into MG (methyl glyoxal), a highly reactive α-ketoaldehyde that readily oxidizes proteins, lipids and other cellular components, leading to further cytotoxicity [133]. Furthermore, GAPDH is known to facilitate APP and tau function normally [134], so oxidized GAPDH probably loses this ability and conceivably contributes to the hallmark neuropathology of AD and of DS with AD.

α-Enolase catalyses the penultimate step of glycolysis by converting 2-phosphoglycerate into phosphoenolpyruvate. Although the main function of enolase is its role in glycolysis,

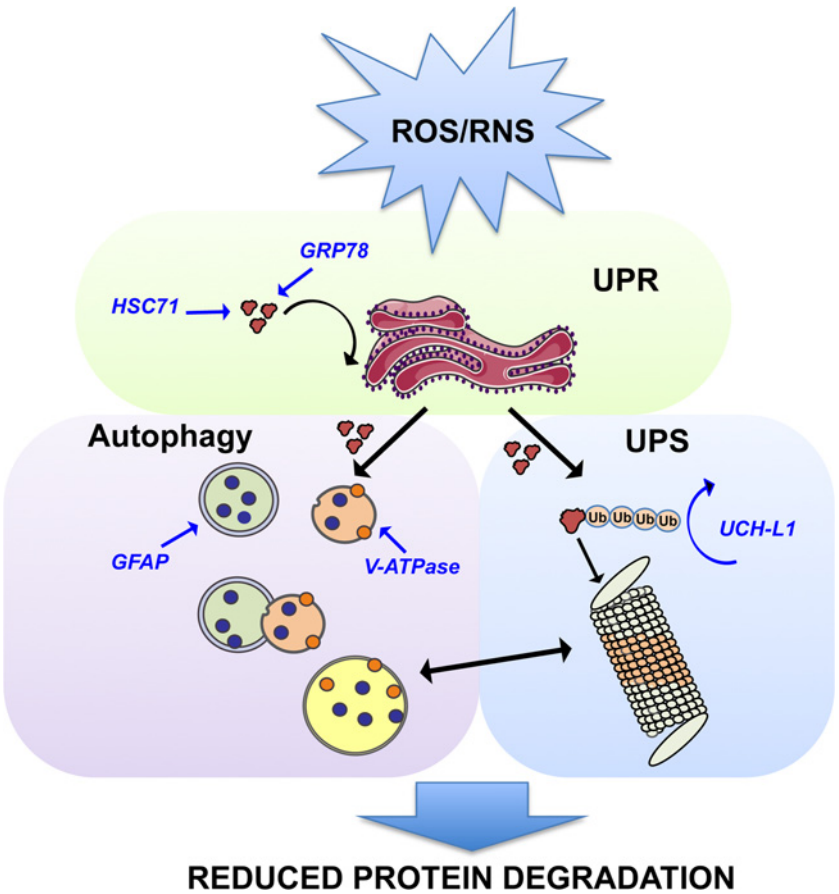

Figure 4 Reduced protein degradation

Impairment of protein degradation machinery. Redox proteomics studies identified oxidatively modified proteins in DS and AD brain that are members of the protein degradative system, called the proteostasis network. Specifically, UCH-L1 is involved in the proteasome pathway, GRP78 and HSC71 in the UPR, and GFAP in autophagy. Dysfunction of all these three processes contributes significantly to accumulation of oxidized/misfolded proteins. Ub, ubiquitin.

this enzyme also plays a role in plasminogen regulation (which leads to plasmin, a protease that cleaves Aβ) and activation of the MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase]/ERK pro-survival pathways [135]. Therefore loss of activity of enolase activity secondary to oxidative modification can contribute to Aβ-mediated neurotoxicity and decreased pro-survival mechanisms.

PK catalyses the final step in glycolysis, the conversion of phosphoenolpyruvate into pyruvate with the concomitant transfer of the high-energy phosphate group from phosphoenolpyruate to ADP, thereby generating ATP. Under aerobic conditions, pyruvate can be transported to the mitochondria, where it enters the TCA cycle and is further metabolized to produce considerably more ATP through oxidative phosphorylation [136].

MDH catalyses the reversible oxidation of malate to oxaloacetate by NAD^+ in the TCA cycle. MDH links glycolysis to the ETC by transferring NADH to NADH dehydrogenase (Complex I) through the malate–aspartate shuttle, resulting in the production of ATP [12].

Protein modification of these glycolytic and TCA cycle enzymes may disrupt neuronal energy metabolism and ion homoeostasis, thereby impairing ion-motive ATPases, signal transduction, membrane lipid asymmetry [53], and glucose and glutamate transporters [137], supporting the hypothesis of altered energy metabolism as a common theme in AD neurodegeneration.

Data from DS and early AD brain suggest that OS and related oxidative damage of metabolic enzymes occurs before the onset of symptoms in AD and robust Aβ plaque formation [83,114,120]. Thus it is reasonable to suggest that reduced glucose utilization (Figure 3) and the consequent mitochondrial deficits are early OS-induced events strongly contributing to the neurodegenerative process that culminate in AD pathology and might represent one of the primary OS-related links between DS and AD pathology.

CONCLUSIONS AND FUTURE DIRECTIONS

The role of OS in neurodegeneration is well recognized, but in the case of DS and AD neuropathology, genetic similarities, due to the fact that some of the genes responsible for the familial form of AD are encoded by Chr21, provide the basis to better understand specific dysregulated pathways. Results obtained by the analysis of human specimens and studies from mouse and cellular models of the disease reveal a molecular link between protein oxidation/aggregation, the integrity of the PQC system (proteasome, UPS and autophagy), dysfunction of energy metabolism and neurodegeneration. Many common pathological hallmarks exist between DS and AD, including deposition of amyloid plaques, NFTs, increased oxidative damage and impaired mitochondrial function, among others. Intriguingly,

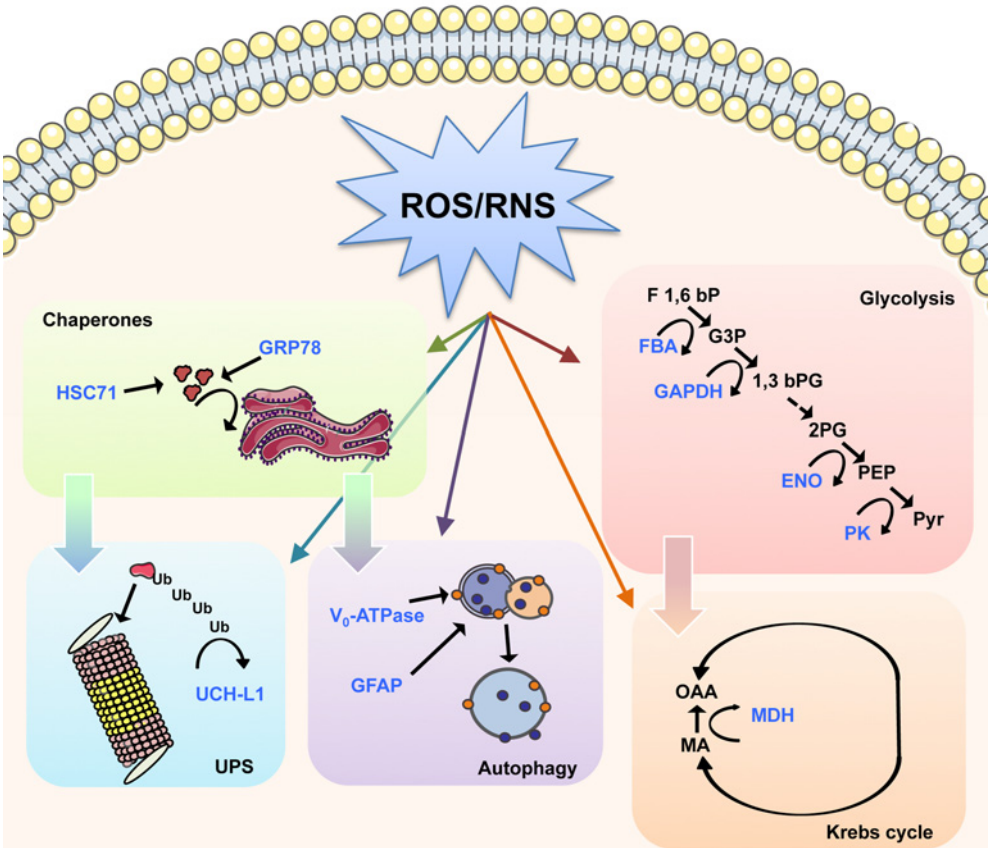

Figure 5 Protein oxidation overlap in DS and AD neuropathology

Identification of oxidized proteins in AD and DS brain suggest common dysregulated processes with oxidative stress being a 'leitmotif'. These findings support a molecular link among protein oxidation/aggregation, the integrity of protein quality control (UPS and autophagy), dysfunction of energy metabolism and neurodegeneration.

we propose that all of these processes seem to be joined by a 'leitmotif' – OS – since they are all the cause and/or the consequence of increased free-radical burden. If a low amount of ROS can activate the protective cellular apparatus such as the antioxidant and heat-shock responses, cell cycle regulation, DNA repair, UPR and autophagy, then chronic exposure to ROS causes irreversible damage to all intracellular macromolecules. Among these, protein oxidation impairs multiple cellular functions by a largely irreversible process that results in altered, mostly reduced, protein activity. It is likely that stressed neurons have the challenge of increasing loads of oxidatively damaged proteins, which overwhelm the ability of the proteostasis network. This, in turn, promotes further accumulation of damaged proteins, increasingly prone to aggregation, ultimately resulting in neuronal death. Alteration of protein homoeostasis, coupled with increasing demand for protein degradation, and reduced ATP production may produce a vicious cycle that may accelerate the neurodegenerative process (Figure 5). Dementia associated with AD in DS occurs at an earlier age than that of sporadic AD, yet there are common oxidative alterations in the proteostasis network and glucose metabolism between these two conditions, implying that these processes are intimately associated with dementia and potentially targets for therapeutic intervention. In addition, other than proteostasis and glucose metabolism, redox proteomics studies allowed the identification of oxidized proteins belonging to several dysfunctional pathways among which, detoxification systems, excitotoxicity or synapse function, that highly correlates with DS and AD pathological features supporting the role of protein oxidative damage in neuron degeneration and cognitive decline. This scenario suggests that any pharmacological intervention may be more effective if it engages more than one molecular pathway, and drugs that target not only specific mechanisms but also the interplay among them might be beneficial. Current therapies are moving towards the use of formulations containing compounds able to modify several oxidative aspects of the disease. Therefore a complete knowledge on the pathways affected by oxidative damage occurring during neurodegeneration is needed.

Redox proteomics [138] undoubtedly played a crucial role in the detection of proteins oxidatively modified by PCO, 3-NT and/or HNE-modified during neurodegeneration allowing deciphering of the disease-specific oxidative footprint. The continuous technical advances in the field will allow better understanding of the role of oxidative stress and redox signalling in biological processes related to both physiological or pathological conditions.

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