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Review

Redox proteomics and the dynamic molecular landscape of the aging brain

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ABSTRACT

It is well established that the risk to develop neurodegenerative disorders increases with chronological aging. Accumulating studies contributed to characterize the age-dependent changes either at gene and protein expression level which, taken together, show that aging of the human brain results from the combination of the normal decline of multiple biological functions with environmental factors that contribute to defining disease risk of late-life brain disorders. Finding the "way out" of the labyrinth of such complex molecular interactions may help to fill the gap between "normal" brain aging and development of age-dependent diseases. To this purpose, proteomics studies are a powerful tool to better understand where to set the boundary line of healthy aging and age-related disease by analyzing the variation of protein expression levels and the major post translational modifications that determine "protein" physio/pathological fate. Increasing attention has been focused on oxidative modifications due to the crucial role of oxidative stress in aging, in addition to the fact that this type of modification is irreversible and may alter protein function. Redox proteomics studies contributed to decipher the complexity of brain aging by identifying the proteins that were increasingly oxidized and eventually dysfunctional as a function of age.

The purpose of this review is to summarize the most important findings obtained by applying proteomics approaches to murine models of aging with also a brief overview of some human studies, in particular those related to dementia.

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1. The dynamic of brain aging

The definition of aging comprises the collection of changes that make human beings progressively more likely to die (Singh and Newman, 2011). Indeed, one hallmark of aging in humans is an age-related increase in mortality. "Normal" brain aging and its association with age-associated brain disorders is an understudied area of research, likely due to the general awareness that aging is an inescapable process which most often overlaps with age-associated diseases, and therefore is not easily regarded on its own. One of the major contributions to aging research came from recent evidences, which permitted the identification of some of the single gene mutations affecting aging and longevity in nematodes, insects, and rodents. These findings demonstrated the presence of a genetic program underlying aging.

"**Aging and death do seem to be what Nature has planned for us. But what if we have other plans?**" *Bernard Strehler*. To address this question, we should take into consideration that post-mitotic neurons are characterized by unique features restricted to the CNS. Indeed, the brain has a higher metabolism than other tissues and utilizes a larger proportion of total oxygen, thus exposing itself to increased risk of free-radical damage. Oxidative damage to neurons include modifications of membrane lipids, proteins and DNA, resulting in inflammation, induction of reactive gliosis and altered Ca^{2+}- and mitochondria-mediated neuronal functions, which together may contribute to the decay of mental capacities with age. Further, with rare exceptions, neurons do not divide but they may exhibit a decreased volume, loss of cell number, and a progressive thinning of cortical thickness 3. The possibility that together with a series of cellular physiological decline, it may also be possible to decipher some "positive" pathways such as the activation of compensatory/protective mechanisms against the deleterious effects of aging (*i.e.*, oxidative stress), and uncharacterized and beneficial late brain-maturation processes also should be considered.

Harman defines aging as the progressive "accumulation of diverse deleterious changes in cells and tissues with advancing age that increase the risk of disease and death." (Harman, 2001). Aging may be considered a physiological decline of multiple cellular functions that progress through life resulting in a decreased resistance to multiple forms of stress, and consequently to increased susceptibility to diseases. Among different types of stressors, one of the prevalent theories centers on free radicals. In the 1950s, the "free radical theory" proposed by Harman stated that aerobic organisms are continuously exposed to free radical attack that damage all cellular macromolecules during their lifespan (Harman, 1956). A revisited version of this hypothesis is the "oxidative stress theory" of aging, which holds that increases in ROS accompany aging may lead to functional alterations, pathological conditions, and ultimately death (Hagen, 2003). Thus, oxidative stress results in an excessive production of ROS as well as reduced capability to counteract them through the activity of both exogenous and endogenous antioxidant defenses. However, despite many reports supporting the notion that ROS are produced in cells and are responsible for the observed damage, a causal link between ROS and aging has still not been clearly established. Other theories have been proposed, and undoubtedly mitochondria play a fundamental role, being crucial components in the control of aging (Kregel and Zhang, 2007). Indeed, it is obvious that incomplete transfer of electrons to oxygen during electron transport chain (ETC) produces ROS that can damage the ETC and other mitochondrial components, including mitochondrial DNA. Together, these events contribute to the exacerbation of a cascade of toxic reactions that are deleterious not only for mitochondria, but for all cellular organelles.

Within this frame, the "cellular senescence theory of aging" emphasizes the importance of cellular signal responses to stress and damage. These signaling responses subsequently stimulate pathways related to cell senescence and death (Toussaint et al., 2002). ROS are able to modulate a plethora of intracellular signals leading to accelerated mitogenesis and premature senescence (Lu and Finkel, 2008; Vigneron and Vousden, 2010). The activation of a subset of redox-sensitive transcriptional factors by age-related oxidative stress is also at the basis of a recent complementary theory, "the molecular inflammatory theory of aging", which culminates in the upregulation of proinflammatory genes resulting in the release of various proinflammatory molecules leading to inflammation processes in different tissues and organs (De la Fuente and Miquel, 2009; Singh and Newman, 2011). By reconciling all these aspects, it is likely that ROS are at the crossroad of multiple signals and are the potential unifying mechanism contributing to many phenomena in aging-related pathologies (Perry et al., 2002; Fortuno et al., 2005; Madamanchi et al., 2005; Moreira et al., 2005). The purpose of this review is to discuss recent evidence that links oxidative stress to biological aging at the molecular level by summarizing proteomics studies performed either on animal models or, with some limitations, in humans.

2. Hallmarks of oxidative stress during aging

2.1. Oxidative stress, mitochondria and brain aging

Aging is a natural process that defines all living organisms. While cell division and oxidative phosphorylation are a requirement that sustains most life on this planet, these processes become deleterious over time. It has been proposed that free radicals generated by oxidative phosphorylation within the mitochondria play a central role in the normal aging process and in conjunction with the decrease in antioxidant defence systems observed (Perrig et al., 1997; Perkins et al., 1999; Rinaldi et al., 2003) may play a major role in the development and progression of many age related neurological diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (ALS) among others (Miquel et al., 1980; Butterfield, 1997; Calabrese et al., 2009, 2010; Texel and Mattson, 2011).

The brain itself is especially vulnerable to the effects of both normal as well as abnormal levels of ROS generation because the brain: (1) consumes high amounts of oxygen; (2) has relatively low amounts of general antioxidant defense systems in comparison to other tissue types; (3) contains many transition metals that may aid in redox cycling; and (4) is high in poly-unsaturated fatty acids (PUFAs). PUFAs in particular are targets of ROS and their oxidation leads to the production of neurotoxic lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA). PUFAs such as arachadonic acid (AA) and docosahexanoic acid (DHA) make up a substantial component of neuronal membranes and participate in many functions such as cell signaling, membrane

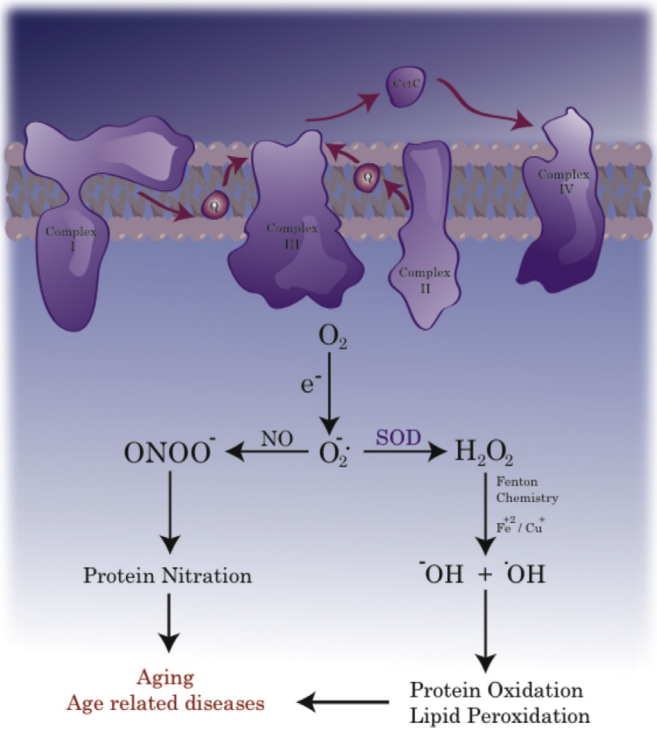

Fig. 1. Oxidative metabolism in mitochondria. Defective mitochondria, as occurs during aging, may lead to overproduction of reactive oxygen species (ROS), highly reactive molecules that can damage lipids, proteins and nuclei acids. Accumulation of oxidized macromolecules has a wide array of physiological (aging) and pathological implications (neurodegeneration).

structure, gene expression and energy production (Perluigi et al., 2010, 2012). AA in particular is an easy target for free-radicals generated within the lipid bilayer due to their abundance of easily extractable allylic hydrogen atoms with the subsequent promotion of radical induced chain reactions that have the potential to injure membrane bound proteins (Butterfield and Stadtman, 1997; Halliwell and Gutteridge, 1984). Moreover, because it is known that the neurons of the brain are mostly post-mitotic, ROS damaged neurons are not able to be replaced, thus, the damage is generally irreversible.

Due to the nature of oxidative phosphorylation requiring the presence of molecular oxygen as the final electron acceptor for ATP production, the potential for the generation of a partially reduced oxygen species along the course of ETC operation is present during normal cellular respiration (Fig. 1). Indeed, the free radical superoxide ($O_2^{\bullet -}$) as well as hydrogen peroxide (H_2O_2) may be produced in small amounts during normal mitochondrial functioning due to the imperfect efficiency of mitochondrial electron transfer; a number that has been estimated to be as high as 2% of consumed oxygen (Chance et al., 1979) but more recent research puts this closer to 0.15% (St-Pierre et al., 2002). Research conducted by different laboratories show that not only is there an inverse relationship between longevity and mitochondrial peroxide production (Sohal and Sohal, 1991), but that the injection of isolated mitochondria from fibroblasts of old animals into young animals may be toxic, promoting senescence (Corbisier and Remacle, 1990), indicating that mitochondrial efficiency may be compromised in aging organisms leading to the increased output of ROS that has been detected in damaged neurons.

Because the mitochondrion is the location of oxidative phosphorylation, the process under investigation for the generation of age related free-radicals, the mitochondria is especially vulnerable to the effects of oxidative stress. For this reason, the mitochondrion employs the use of key antioxidant defense systems to combat the large amount of oxidative stress generated (Sies, 1997). However, due to the imperfect nature of these antioxidant defense systems, both mitochondrial DNA (mtDNA) as well as key metabolic proteins are vulnerable during prolonged or sustained ROS output, resulting in decreased energy production and increased ROS generation as has been demonstrated in rat models (Guerrieri et al., 1996; Babizhayev and Yegorov, 2010; Haynes et al., 2010).

The deteriorating mitochondrion may be a driving force for oxidative stress associated with aging, but evidence shows that the depletion of antioxidant defense systems with age may play an equally important role in the aging process and perhaps in the development of age related neurological conditions. The most abundant thiol antioxidant system within the brain is the glutathione system. Its proper functioning is essential to maintaining a healthy local redox homeostasis (Giordano et al., 2011). Reduced glutathione (GSH) uses its thiol moiety to scavenge both free radicals and xenobiotics within the cell, which leads to the oxidized form of glutathione dimers (GSSG) to increase in concentration. Levels of GSSG are returned to the reduced scavenger form, GSH, through the function of glutathione reductase (GSR); thus a higher GSH:GSSG ratio is evidence of a properly functioning glutathione system (Giordano et al., 2011). Researchers have found evidence of a decreased GSH:GSSG ratio in aging animal models as well as in every brain region tested (Rebrin et al., 2003; Calabrese et al., 2004; Suh et al., 2004, 2005; Zhu et al., 2006; Perluigi et al., 2010). Data such as these have led to the formulation of a hypothesis that either stimulating the production of GSH or the activity of GSR may lead to a higher GSH:GSSG ratio, and as such may serve as a form of therapy for age related neurological diseases such as AD and PD (Pocernich and Butterfield, 2012). *In vivo* and *in vitro* experiments have shown the possibility of this route of therapy as has been demonstrated experimentally (Boyd-Kimball et al., 2005; Chinta et al., 2006; Joshi et al., 2007).

Taken together, these evidence lend support to the hypothesis that aging is an accumulative process in that damage over time builds up to a critical mass promoting system failure that results in mortality as well as age related diseases (Ames et al., 1993; Huang et al., 2010).

2.2. Markers of protein oxidation

Protein oxidation refers to the direct or indirect damage afflicted to a protein during the process of an oxidative insult. There are three major forms of protein oxidation that are detectable by standard methods; these being protein carbonylation, protein nitration, and protein bound-lipid peroxidation products.

Protein carbonylation normally refers to the reactive ketones or aldehydes that are formed as a product of direct free radical induced damage of proteins and can affect both the protein backbone as well as the side chains (Stadtman and Levine, 2000). Direct protein carbonylation may result in the loss of function of the affected protein (Smith et al., 1992; Levine, 2002). This global oxidation process is referred to as “primary protein carbonylation”, a product that would be difficult to detect if not for the use of 2,4-dinitrophenylhydrazine (DNPH). The resulting DNP-hydrazones formed are detectable through immunochemical blotting (Levine et al., 1994; Suzuki et al., 2010). Other methods that may be used to detect protein carbonylation are biotin hydrazide coupled to fluorescein isothiocyanate (FITC)-labeled streptavidin as well as solution based spectrophotometric analysis (Suzuki et al., 2010). While many forms of protein oxidation are irreversible barring clearance from the cell, protein carbonylation may be remedied by carbonyl reductase.

What is sometimes called “secondary protein carbonylation” is an indirect method of protein oxidation in that an intermediate

molecule carries the initial free-radical product to the protein which then initiates the protein carbonylation adduct (Grimsrud et al., 2008; Suzuki et al., 2010). This intermediate vehicle usually takes the form of lipid peroxidation products (MDA, acrolein, HNE, *etc.*) formed from PUFAs that produce reactive α/β unsaturated aldehydes and bind to the protein *via* Michael addition (Butterfield and Stadtman, 1997). This binding of an amphiphilic lipid moiety to a protein may induce a denaturing conformational change that is responsible for loss of function detected in samples with high levels of protein bound lipid peroxidation products (Sayre et al., 1997; Subramaniam et al., 1997; Lovell et al., 2001; Sultana and Butterfield, 2004). The generation of lipid hydroperoxides has been hypothesized to possess the tendency to not only oxidize biomolecules in the lipid membrane in the immediate vicinity, but due to the nature of radical chain reactions, lipid hydroperoxides may also affect lipids and proteins in an adjacent cellular or organelle membrane (Spiteller, 2007). While one result of lipid peroxidation is protein modification, the products of lipid peroxidation themselves are potentially dangerous due to their electrophilic nature but also due to their tendency to act as oxidants (Morrow and Roberts, 2002).

Protein nitration occurs following formation of peroxynitrite ($ONOO^-$) from superoxide ($O_2^{\bullet -}$) and nitric oxide ($NO^\bullet$). The latter is produced by nitric oxide synthase (NOS) with significant vital signaling implications (Shahani and Sawa, 2011). Peroxynitrite, in the presence of CO_2, is known to covalently modify tyrosine residues to produce 3-nitrotyrosine (Butterfield and Kanski, 2001; Butterfield et al., 2007). The formation of 3-nitrotyrosine has been found to inactivate key proteins such as actin, manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/Zn SOD), and tyrosine hydroxylase. In addition, due to the steric constraints placed on the tyrosine residues by the addition of a large nitro group in the 3′ position, difficulty in phosphorylating the tyrosine, *via* tyrosine kinases, may occur (Butterfield, 1997a). Research on aging rats has shown that nitrated tyrosine residues may play an important role in the aging process, as protein nitration levels increase with age in the cerebellum, substantia nigra, hippocampus, semimembranousus and soleus muscle, as well as localized to membrane raft proteins that may play a significant role in cell signaling processes (Dremina et al., 2005; Fugere et al., 2006; Poon et al., 2006a,b; Gokulrangan et al., 2007).

Aside from the general oxidatively induced protein modifications such as those discussed above, protein oxidation/nitrosylation may also result in much more specific modifications such as those of S-nitrosylation and methionine oxidation (sulfoxidation). S-nitrosylation occurs when a reactive cysteine moiety reacts with N_2O_3 to form an S-nitrosothiol (SNO) (Stamler et al., 1992; Broniowska and Hogg, 2012; Raju et al., 2012). SNO, unlike many other forms of radical induced modification, may not only be regulated through the action of nitrosylases and denitrosylases, which either add or remove the NO modification (Seth and Stamler, 2011), but is also maintained at normal levels through a complex homeostatic system of nitrosylated proteins (Lopez-Sanchez et al., 2008). SNO modification has been identified as a method of redox-based cellular signaling (Stadtman, 2006), and an altered SNO-profile in diseases such as Alzheimer disease have been reported (Zahid et al., 2013).

Methionine has been found to be exceptionally prone to oxidation *in vivo* when exposed to high levels of oxidative stress, resulting in the sulfoxidation products Met-sulfoxide [Met(O)] and Met-sulfone [Met(O_2)] (Moskovitz et al., 2011). While the Met-sulfone is generally an irreversible product of sulfoxidation, Met-sulfoxide, may yet be converted back to Met *via* the methionine-sulfoxide reductase (Msr) pathway (Oien and Moskovitz, 2008). Sulfoxidation has been implicated in aging disorders such as Alzheimer disease, in which a large percentage of Aβ in amyloid plaques has been found to contain the sulfoxide modification on Met^{35} (Kuo et al., 2001; Boutte et al., 2006). In fact, the highly reactive nature of Met^{35} in Aβ is of such importance, that it is the central theme within the Aβ induced oxidative stress hypothesis, put forth by our research group (Varadarajan et al., 1999; Butterfield et al., 2010; Butterfield and Sultana, 2011).

3. Redox proteomics strategies

General methods of detecting protein oxidation in homogenates are useful for getting a sense as to the redox state of the cell, however they do not provide an accurate detailed account of specific protein oxidation occurring in a system. Redox proteomics is a set of techniques which allow for the identification of specific target proteins that may be differentially oxidized as a result of oxidative injury. There are two prominent methods that allow for the use of redox proteomics, those being the targeted gel-free enrichment of proteins presenting the oxidative hallmarks discussed above, and the global gel-based approach. For a comprehensive review that includes gel-free enrichment of proteins, the reader is directed to (Butterfield et al., 2012), however for the scope of this review we will be discussing the global gel-based approach.

Gel-based redox proteomics utilizes a two-dimensional gel approach to protein separation. Proteins from a sample are initially separated in the first dimension according to their isoelectric point within an isoelectric potential gradient (IPG) strip. Proteins must be rehydrated and either passively or actively loaded into the IPG strip prior to isoelectric focusing. Once the primary separation is complete, the IPG strip is loaded onto a poly-acrylamide gel and the proteins are forced from the IPG strip to the gel by use of a current, where they are separated according to their respective gel migration rates. This two dimensional separation of proteins according to their migration rates as well as their isoelectric points allow for individual proteins to reach their own *X*- and *Y*-coordinates within the gel, a characteristic that traditional 1-D gel approaches do not allow.

The primary difference between gel-based redox proteomics and gel-based expression proteomics is the use of immunohistochemical methods such as those previously discussed that allow for the detection of common oxidative stress hallmarks. The 2-D gels obtained are transferred to either a nitrocellulose or polyvinylidene membrane where they may be probed with primary antibodies raised against the modification of interest, followed with a secondary antibody raised against the primary antibody that allow for a means of detection. Additional steps must yet be made however to ensure proper protein identification and modification matching. Two gels must be run in parallel on the same sample as the proteins from the first gel that are transferred to the blot and are lost for identification purposes. The second gel is used to match the spots of interest from the blot to those of the gel. This method is performed *via* the spot matching programs such as PDQuest or Dimension Delta 2D which utilize the pixel density of protein spots to compare density changes in samples.

Once spots of interest are located within the gel, the spots must be excised from the gel and digested with trypsin. The tryptic peptides are then subjected to either matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry or electrospray ionization (ESI) tandem (MS/MS) mass spectrometry. Current literature suggests that ESI-MS/MS is more utilized than MALDI-TOF. This is likely due to the fact that not only are the masses of the parental tryptic peptides measured, but the parental peptides are then subjected to an additional dissociation step, collision induced dissociation (CID), that fractures the tryptic peptide into

b- and y-type daughter fragment ions. This additional dissociation allows for the identification of the amino acid sequence and identification of the tryptic peptides as well as potential amino acid sites of modification *via* search algorithms such as MASCOT or SEQUEST. This process is not without its limitations however, as the efficiency of ionization as well as the possibility of a low oxidatively modified peptide abundance is present (Butterfield and Sultana, 2008; Butterfield et al., 2012).

4. Redox proteomics studies in animal models of aging

4.1. SAMP8 mice

The senescence-accelerated mouse (SAM) is the result of a phenotypic selection from a common genetic pool of AKR/J strain of mice, which were noticed to become senile at an early age and had a shorter life span (Takeda et al., 1981). Among certain littermates of AKR/J mice, five of these litters with early senescence were selected as the progenitors of the senescence-accelerated-prone mice (SAMP). Interestingly, SAMP8 mice showed characteristic learning and memory deficits at old age (Flood and Morley, 1993) with low incidence of other phenotypic aging alterations when deficits in learning and memory are developed (Flood and Morley, 1993). The SAMP series includes nine sub-strains, which are characterized by different dysfunctions such as skin abnormalities and alteration of normal behavior. Among these, SAMP8 show a peculiar age-dependent deficit in learning and memory (Yagi et al., 1988; Ohta et al., 1989) and therefore became the main focus of research for studying the molecular mechanisms responsible of age-dependent cognitive impairments. In detail, SAMP8 mice, compared with aged SAMR1 mice, exhibit impairments in learning tasks, altered emotion, abnormality of circadian rhythm (Miyamoto, 1997), elevated deposition of amyloid β-peptide and also increased oxidative stress (Butterfield et al., 1997; Butterfield and Poon, 2005).

In the search of causative factors responsible for cognitive defects, consistent accumulation of the precursor of Amyloid beta peptide (Aβ) in the hippocampus and other brain regions from SAMP8 mice was observed (Kumar et al., 2000). However, SAMP8 mice develop amyloid plaques in the late stage of their life (Morley et al., 2000) after the learning and memory deficits have already become evident (Nomura et al., 1996). Accordingly, Aβ-directed antibody or Aβ-directed antisense (Maekawa et al., 1993; Kumar et al., 2000; Morley et al., 2000) treatment resulted in an improvement of cognitive abilities in 12-month-old SAMP8 mice. Further, treating aged SAMP8 mice with antioxidants, such as lipoic acid, N-acetylcysteine, L-acetylcarnitine, and melatonin, not only decreased oxidative stress in aged SAMP8 brains, but also improved their learning and memory (Okatani et al., 2002; Farr et al., 2003). These results highlight that oxidative damage correlate with impairment of learning and memory as observed in the SAMP8 mouse.

By coupling proteomics and redox proteomics approaches, our laboratory investigated the expression of proteins and their oxidative modifications in the brains from aged SAMP8 mice. We found that the specific protein carbonyl levels of lactate dehydrogenase 2 (LDH-2), α-enolase, creatine kinase (CK), α-spectrin and DRP-2 were significantly increased, and the protein expression level of triosophosphate isomerase (TPI), LDH-2, neurofilament (NF-L), α-spectrin and heat shock protein 86 (HSP86) were significantly changed in 12-month-old SAMP8 mouse brains compared with brains for 4-month-old SAMP8 mice. Overall, these data indicate an alteration of glycolysis and energy-related proteins (α-enolase, LDH-2 and TPI, CK) and structural proteins (α-spectrin, NF-L and DRP-2) (Poon et al., 2004a,b).

Enolase catalyzes the penultimate step of glycolysis by converting 2-phosphoglycerate to phosphoenolpyruvate. Increased oxidation of enolase in AD and animal models of AD was demonstrated (Butterfield et al., 2006a,b, 2012), coupled also with lower enzymatic activity in the brain of subjects with MCI (Butterfield et al., 2006a,b) and subjects with AD (Meier-Ruge et al., 1984; Sultana et al., 2006). Carbonylation of this protein supports the hypothesis of altered energy metabolism as a common theme in aging and neurodegeneration. Since glycolysis is the main source of ATP production in the brain, impairment of glycolysis may lead to a shortage of ATP in brain cells, thus to a multitude of cellular dysfunctions (Butterfield and Lauderback, 2002). ATP is extremely important at nerve terminals for normal neurotransmission and any crucial drop of ATP levels may lead to loss of synapses and synaptic function, both of which can affect propagation of action potentials and contribute to memory loss. Although the main function of enolase is the role it plays in glycolysis, enolase also has roles in plasminogen regulation and activation of the MEK/extracellular regulated kinases (ERK) pro-survival pathways (Butterfield and Lange, 2009).

LDH-2 is a subunit of lactate dehydrogenase (LDH), which is also a glycolytic protein that catalyzes the reversible NAD-dependent interconversion of pyruvate to L-lactate. The five isoenzymes of tetrameric LDH are found in various proportions of different somatic tissues in the combination of the A and B subunit in mammals (Sakai et al., 1987). Previous reports demonstrated that a single mutation in LDH-2 was able to reduce the activity of LDH (Maekawa et al., 1993) suggesting that the LDH-2 subunit is critical for its activity. Lactate appears to be the only oxidizable energy substrate available to support neuronal recovery (Schurr et al., 1997). Although the activity of LDH shows no significant difference in AD compared with age-matched controls (Chandrasekaran et al., 1994), many studies show that LDH activity in rat brains declined with increased age.

TPI isomerizes dihydroxyacetonephosphate to glyceraldehyde-3-phosphate (G3P), a necessary reaction for the continuation of glycolysis and the overall production of ATP. As discussed above, ATP is essential in maintaining neuronal functionalities such as the activity of ATPases, ion-motive pumps, and potential gradients. Our group already showed that TPI is oxidatively modified in late-stage AD (Sultana et al., 2006), but without a significant reduction in its activity. Therefore, if TPI is oxidized, lower activity of TPI is expected to be observed. Data from aged SAMP8 mice led to hypothesize that the increase in TPI levels should compensate for the decreased activity of oxidized TPI. As a result, no net decrease in TPI activity is observed. Accordingly, hypoxia-induced oxidative stress is accompanied by an increase in the TPI protein level (Lushchak et al., 1998). This is consistent with an upregulation of TPI to compensate for its loss of activity by oxidative modification.

CK catalyzes the conversion of creatine to phosphocreatine at the expense of ATP, which is later used in the production of high-energy phosphate that is used for the generation of ATP. Immunochemical approaches in Alzheimer's superior and medial temporal gyri have previously identified CK as a carbonylated protein. Further, using a redox proteomics approach, this protein was also found to be carbonylated in the inferior parietal region of AD brain compared with that of the age-matched control (Castegna et al., 2002). Moreover, CK activity is reduced in AD brain (Aksenova and Burbaeva, 1989). Loss of its activity in AD (David et al., 1998), resulting from its oxidation, suggests decreased energetics in neurons and synaptic terminals.

In line with other studies showing reduced glucose metabolism in aged SAMP8 mice (Shimano, 1998), these findings further confirm that the reduced ATP production is possibly caused by a loss of activity of specific glycolytic enzymes and also of CK oxidation. Since 20% of ATP in brain is produced from glycolysis,

oxidative modification of key glycolytic enzymes would significantly decrease the ATP levels in neurons and synaptic elements; ATP is also critical to the function of the antioxidant systems within the cells.

α-Spectrin, NF-L and DRP-2 are involved in signaling, intracellular trafficking and maintaining the structure of dendrites and axons in neurons. Increased oxidation or reduced expression of these proteins correlate with neuronal atrophy and loss in the posterior cholinergic column, reduction of dendritic spines observed in the hippocampal pyramidal neurons in aged SAMP8 (Kawamata et al., 1998). α-Spectrin plays a critical role in cytoskeletal stability and flexibility, by forming a supporting and organized scaffold for intracellular cohesion with the association of actins (Leto et al., 1988). Spectrins can be proteolytically degraded by calpains and caspases, yielding breakdown products found to be elevated in the brain during aging (Cai et al., 2012). Interestingly, Aβ can also induce spectrin breakdown in cultured rat cortical neurons by activating caspase-dependent proteolysis (Harada and Sugimoto, 1999). An increase of α-spectrin breakdown products was also observed in some regions of the aged Balb/c mice brain (Bahr et al., 1991) supporting the idea that α-spectrin levels may decrease during aging. According to these data, our results show a decreased level of α-spectrin in aged SAMP8 mouse brain, as well as an increased specific carbonyl level. These results are consistent with the notion that degradation of α-spectrin would disrupt the cytoskeleton and affect intercellular and intracellular communications, therefore contributing to the learning and memory deficits observed in SAMP8 mice.

NFs are axonal proteins that provide structural stability to axons and define axonal diameter (Hoffman et al., 1987). NFs are composed of light, medium and heavy subunits and assemble to form long macromolecular filaments. Because of the dynamic nature of NFs within the axon, the individual NF proteins are repeatedly turned over or exchanged within NFs (Okabe et al., 1993). Modification of the NF structure results in the destabilization of the interactions between the NF proteins. Oxidation and nitration of NF proteins lead to alteration of their three-dimensional structure (Gelinas et al., 2000), and these oxidized forms will undergo proteolytic degradation (Grune et al., 1996, 2003). It is likely that oxidative modification could be responsible for the NF abnormalities observed in several oxidative-stress related neurodegenerative diseases such Alzheimer disease, Parkinson's disease, and amyotrophic lateral sclerosis. Consistent with this result, the expression NF-L in SAMP8 brains is significantly decreased in aged SAMP8 brain, suggesting the decreased level of NF-L in brain caused the increased axonal dystrophy in the gracile nucleus observed in aged SAMP8 mice (Kawamata et al., 1998).

DRP-2, also known as collapsin response mediator protein (CRMP-2), plays an important role in maintaining microtubule assembly, cellular migration, and cytoskeletal remodeling. CRMP-2 regulates the formation of microtubules and it is highly concentrated in growing axons. It interacts with the cytoskeleton and mediates signals related to myelin-induced axonal growth inhibition. CRMP-2 has been reported to associate with NFs, which may lead to decreased levels of cytosolic CRMP-2, eventually leading to shortened neuritic and axonal growth, and thus accelerating neuronal degeneration in AD (Yoshida et al., 1998). In addition to AD, decreased expression of CRMP-2 protein also was observed in fetal and adult DS subjects (Weitzdoerfer et al., 2001). Since memory and learning are associated with synaptic remodeling, oxidative modification and subsequent loss of function of this protein could conceivably be responsible for shortened dendritic length and synapse loss (Coleman and Flood, 1987). Shortened dendritic length would likely lead to less neuronal communication with adjacent neurons that could contribute to memory loss and cognitive decline associated with AD (Hensley et al., 2011).

Table 1
Oxidatively damaged proteins in SAMP8 mice and protective effect of treatment with lipoic acid (LA).

Oxidized proteins in SAMP8 mice	Protective effects of LA
Lactate dehydrogenase 2	Reduced oxidation
Alpha-enolase	Reduced oxidation/increased exression
Creatine kinase	–
TPI	–
α-Spectrin	–
CRMP-2	Reduced oxidation
Neurofilament L	Increased exression

4.1.1. *Effect of lipoic acid on SAMP8 mice*

Considering the role of OS in aging and neurodegeneration, ongoing research focuses on development of therapeutic strategies, among which are those that scavenge free radicals by antioxidants (Butterfield et al., 2002). A promising candidate is α-lipoic acid (LA), a coenzyme involved in production of ATP in mitochondria, thought to have antioxidant capabilities (Packer et al., 1997). This remarkable fatty acid is not only a potent antioxidant, but serves as a nexus in the body's antioxidant network by regenerating the other major antioxidants including glutathione (which cannot be taken as a supplement). Neuroprotective properties of LA also rely on its ability to chelate metal ions and to bind to reactive aldehydes, such as 4-hydroxynonenal (HNE) and acrolein (Korotchkina et al., 2001)), which are the major by-products of lipid peroxidation (Esterbauer et al., 1991). It was shown that LA improves memory in aged female NMR1 and 12-month-old SAMP8 mice (Farr et al., 2003). LA can also reverse partial brain mitochondrial decay, RNA/DNA oxidation and memory loss in old rats (Farr et al., 2003).

By using a proteomics approach, it was possible to identify the proteins that are expressed differently and/or less oxidized in the12-month-old SAMP8 mouse brain treated with LA. Our findings have shown that the specific carbonyl levels of LDH-2, DRP-2, and α-enolase were significantly decreased and the expression levels of α-enolase, NF-L, and uMiCK, were increased in brains of aged SAMP8 mice treated with LA in comparison to aged SAMP8 mice, not treated with LA (Table 1). Thus, injecting LA can decrease the specific carbonyl levels of α-enolase, DRP-2, and LDH-2 and can increase the protein levels of uMiCK, α-enolase, and LDH-2 in aged SAMP8 mice brains. These alterations of proteins may contribute to the improvement of learning and memory in LA-injected SAMP8.

4.1.2. *Effect of brain-delivered antisense oligonucleotides in SAMP8*

Similar to the antioxidant studies, previous reports showed that decreasing the production of Aβ by intracerebroventricular (ICV) injection of a 42-mer phosphorothiolated antisense oligonucleotide (AO) directed at the Aβ region of the APP gene can reduce lipid peroxidation and protein oxidation (Poon et al., 2004a,b) and improve cognitive deficits in aged SAMP8 mice. We suggest that *in vivo* levels of Aβ are significantly reduced as a consequence of administration of AO against the Aβ region of APP (Poon et al., 2005a,b), which results in reduced carbonylation of specific proteins such as aldolase, coronin 1a, and peroxiredoxin2.

Very recently, these findings were further extended by testing the effects of ICV injection of AO directed against the PS-1 gene in SAMP8 mice (Fiorini et al., 2013). The results suggest that the treatment with AO directed against PS-1 in old SAMP8 mice restores learning and memory while also reducing Aβ-induced oxidative stress as previously demonstrated to occur in SAMP8 mice (see above). Not only were decreased protein carbonyl and 3-NT levels found, but the majority of the proteins identified as differently expressed and oxidized appear to have been overexpressed and less nitrated in old SAMP8 mice treated by AO directed against PS-1. These proteins are involved in energy metabolism, neuritic

growth, lipid abnormalities, cell cycle regulation, synaptic abnormalities, tau function, and lysosomal function; all these processes already have been reported to be significantly impaired in AD.

A similar AO approach was recently conducted which used AO against the Tau kinase, glycogen synthase kinase-3β (GSK-3β) in brain of SAMP8 mice (Farr et al., 2013). In this case, AO treatment against GSK-3β led to improved learning and memory, and the phase II enzyme transcription factor Nrf-2 was translocated to the nucleus with corresponding elevation of glutathione-S-transferase. Notably, Tau phosphorylation was significantly decreased, consistent with improved learning and memory.

4.2. Animal models of normal aging

In addition to studies of murine models of aging (SAMP8), a parallel proteomics study was performed in different brain regions isolated from senescent rats (28 months) compared with adult rats (12 months) (Perluigi et al., 2010). This animal model of aging is based on a naturally occurring phenotype, which may be particularly useful for the identification of biological markers and to provide new insight into the molecular mechanisms of brain development, aging, and neurodegeneration.

Among the proteins found to be increasingly oxidized, the majority are involved in energy metabolism including glycolysis, the Krebs cycle and ATP production. In addition, protein oxidation also was found to affect components of the cell involved in cell structure, signal transduction, and the cellular stress response, such as Hsp70. The 70-kDa family of stress proteins have been found to play an important role in cellular protection against a variety of stresses by preventing protein aggregation and assisting in the refolding of damaged proteins. Hsp70 is expressed at very low levels in brain under physiological conditions, but is induced by different type of stress including OS (Mancuso et al., 2007). The findings of increased oxidation of chaperones in striatum, cerebellum, and cortex could lead to an increased accumulation of misfolded proteins, one of the characteristic hallmarks in both aging and neurodegenerative disorders. Interestingly, some of the proteins shown to exhibit increased carbonylation in senescent *vs.* adult rats are similar to those already found in AD brain (Butterfield et al., 2012), consistent with the notion that aging is a major risk factor for the development of neurodegenerative diseases. Further, there are currently no proteomics studies on human brain aging. Although the etiologies are different and likely multifactorial, aging and age-related neurodegenerative diseases share some common pathological mechanisms, among which we suggest that energy failure is one of the most crucial for neuronal dysfunction and brain dynamics.

In a similar study, 2D Oxyblot technique was used to investigate the differentially oxidized proteins in the temporal cortex of the old (24 month old) *vs.* young (1 month old) rats (Wang et al., 2010). The temporal cortex is essential for learning and memory and is particularly vulnerable to oxidative damage during brain aging. SOD1, SOD2, peroxiredoxin 1, peptidylprolyl isomerase A, cofilin 1, and adenylate kinase 1 were identified to be excessively oxidized in the old rat temporal cortex. These proteins are associated with antioxidant defense, the cytoskeleton, and energy metabolism. The majority of these proteins have already been demonstrated to be involved in the aging process. Interestingly, this is the first report showing increased oxidation of Cofilin 1. This protein regulates reversibly the polymerization and depolymerization of actin cytoskeleton, the dynamics of which is suggested to be critical in the brains of aging and neurodegenerative diseases (Poon et al., 2006a,b). Recent studies suggested that cofilin-mediated regulation of actin dynamics may correlate with the age-related alterations of ROS and consequently are implicated in cell death.

Toda et al. (2010) applied a proteomic strategy to determine the significance of protein oxidation in the aged mouse hippocampus by focusing on identification of the methionine oxidation product, Metsulfoxide. Most cells posses the specific enzymatic system, methionine sulfoxide reductase (MsrA), to repair damaged protein by reducing MetO (Sharov et al., 1999). However, a sufficient level of activity of MsrA is essential for cells to survive in the presence of ROS (Moskovitz et al., 1998). *msrA* knockout mice have a significantly shorter lifespan than controls (Moskovitz et al., 2001). MsrA activity is significantly reduced in AD brain when compared with the normal control brain, suggesting the involvement of Met sulfoxidation in the process of hippocampal neurodegeneration in AD. Further, studies from aged rat tissues showed that downregulation of *msrA* gene expression was associated with decreased enzyme activity of Msr. Met sulfoxidation might occur on almost all Met-containing proteins under oxidative conditions in cells. However, pathophysiological consequences might vary with site of MetO and degree of conformational alteration in each oxidized protein. Experimental evidence from the hippocamppi of aged mice showed increased MetO of calmodulin, UCH-L1 and nucleoside diphosphate kinase Nm23-M1. The increase in oxidation of CaM might disturb the CaM-dependent calcium signaling in brain function. Oxidation of UCH-L1 and nm23-M1 might also affect ubiquitin recycling in proteasome-dependent protein degradation (Butterfield et al., 2012) and guanosine triphosphate-mediated signal transduction, respectively, in the aged mouse hippocampus.

4.2.1. Effects of L-acetyl carnitine on normal aged rats

Similarly to the approach used for lipoic acid, another study to test the protective effects of antioxidant compounds on age-related dysfunction was performed by supplementation with L-acetylcarnitine (LAC) to rats at young age.

LAC is the acetyl ester of carnitine, the specific carrier of long chain fatty acids for transport across the membranes of mitochondria, where they are oxidized for energy (Thal et al., 2000). LAC is used as a supplement rather than L-carnitine itself because it is better able to pass through cell walls. LAC thus serves as a vehicle for getting L-carnitine into cells, where the L-carnitine (once liberated from the acetyl group) can then deliver fatty acids into the mitochondria. Previous studies showed that LAC levels are reduced in aged brains thus affecting brain levels of ATP, and that chronic administration of LAC ameliorated the cognitive deficit of aged rats (Castorina and Ferraris, 1994). Interestingly, the protective mechanism exerted by LAC does not relate exclusively to its role in energy metabolism, but also due to its structural similarity to acetylcholine. In addition, other mechanisms seem to be involved, including its ability to increase the levels of neurotrophins, which are essential to the maintenance of neural plasticity and are fundamental for memory consolidation. It is likely that LAC could significantly (a) improve metabolic function and at the same time lower free radical production; (b) restore age-related dysfunction of key mitochondria enzymes, essential for energy production; (c) improve memory in old rats (Castorina and Ferraris, 1994).

In order to gain insight into the protective mechanisms of LAC, a parallel proteomics approach to identify the proteins that could be protected from oxidation in aged rats treated with LAC compared with untreated group was used. By measuring markers of protein oxidation, *in vivo* administration of LAC was found to significantly reduce the levels of protein carbonyl, 3-NT, and HNE-binding in all brain regions of rats when compared to control untreated rats (Poon et al., 2006a,b). Among the brain regions analyzed, hippocampus (HP) showed one of the largest reductions of protein carbonyl, HNE, and 3-NT levels in old rats treated with LAC. A similar trend was also evidenced in the cortex (CX), which is another major region affected during aging. Further, both these regions are responsible for maintenance of learning and memory.

Table 2
Altered protein carbonyl levels and altered expression levels in hippocampus of aged SAMP8 mice compared with young rats and protective effect N-acetylcarnitine (LAC).

Altered protein specific carbonyl level in aged rat	Effect of LAC on aged rat
Cofiln 1	Reduced oxidation
Actin	Reduced oxidation
Altered protein expression level in aged rat	Effect of LAC on aged rat
Aconitase 2 ↑	↓
Inositol monophosphate ↑	↑
α-Enolase ↑	↓
Creatine Kinase B ↓	↑
Tubulin α-1 chain ↓	↑

The protective effects of LAC treatment were investigated by applying a parallel proteomic analysis to identify the proteins that were increased in specific carbonyl levels as well as those with altered expression in these brain regions in old rats when compared to those in young rats, following LAC supplementation (Table 2). In detail, LAC treatment was able to modulate the oxidation or the expression of proteins classified in three main categories: (1) antioxidant (Peroxiredoxin, HSC70, gloxylase1, 3-mercaptopyruvte sulfurtransferase); (2) mitochondrial function (aconitase, fumarase, creatine kinase); and (3) plasticity (cofilin, Rad GDP dissociation inhibitor beta, actin) (Poon et al., 2006a,b).

Our findings demonstrated that LAC could reduce oxidative stress by improving antioxidant defense and mitochondrial function in the CNS, thereby improving the neuroplasticity and learning and memory deficits in aged rats.

4.2.2. *Effects of caloric restriction on normal aged rats*

Growing evidences support the view that caloric restriction may be beneficial to prevent age-related complications, mostly those associated with cognitive dysfunction. The mechanisms proposed to explain this protective effect involve the reduction of oxidative stress and sirtuin-based improvement of plasticity in the CNS. A number of studies showed that CR can prevent the age-dependent increase of oxidative stress (Lass et al., 1998; Koubova and Guarente, 2003) and protein oxidation in brains (Dubey et al., 1996; Guo et al., 2000). CR is also believed to contribute to the improvement of age-related learning and memory deficits (Ingram et al., 1987), as well as the improvement of the plasticity and recovery of the CNS (Mattson, 2000a,b). Although the beneficial effects of CR on brains are well established, the mechanisms of its action remains unclear. Two major hypothesis have been proposed: (1) CR may act at the mitochondrial level, reducing mitochondrial metabolism and therefore free radical production; and (2) CR can induce mild metabolic stress response by promoting the release of neurotrophic factors, the repair of oxidative damage, and protecting neurons against apoptosis (Mattson, 2000a,b; Koubova and Guarente, 2003). A number of studies demonstrated a reduction in steady-state oxidative damage to proteins, lipids, and DNA in animals subjected to restricted caloric intake, as a direct consequence of reduced ROS release from mitochondria coupled with enhanced antioxidant defences. To gain insight into the mechanism of such protective effects, the hypothesis was tested that the cognitive improvement of aged rats undergoing CR may result from the protection from oxidative damage of a subset of proteins, which otherwise would be oxidized in normal aged rats fed *ad libitum* (Poon et al., 2006a,b).

To this purpose, different brain regions were evaluated to measure the bulk protein 3-NT, HNE and carbonyl levels of CX, substantia nigra (SN), septum pellucidum (SP), striatum (ST), HP and cerebellum (CB) in CR-rats compared with controls. CR was generally able to reduce protein oxidation in all aged rat brain regions, but statistically significant only in certain regions. Among these, in HP the greatest reduction of protein carbonyl, HNE and 3-NT levels were observed. HP plays an important role in both memory and learning and it is particularly vulnerable to age-related damage. Moreover, synaptic loss and increased protein oxidation in HP is the major pathological hallmark of AD (Scheff and Price, 2003). We also showed that the 3-NT levels of HP were significantly decreased in HP of CR aged rats when compared to age matched control. Furthermore, we identified that CR treatment led to reduced 3-NT levels of specific proteins, namely malate dehydrogenase (MDH), phosphoglycerate kinase (PKG1) and 14-3-3 zeta. In parallel, the expression levels of DLP1 splice variant 1, mitochondrial aconitase (ACO2), dihydrolipoamide dehydrogenase (DLDH), neuroprotective peptide H3 (NPH3), and eukaryotic translation initiation factor 5A (eIF-5A) were increased. Taken as a whole, three major processes seem to be significantly protected: glutamate regulation, mitochondrial function and protein synthesis (Fig. 2).

The ST is best known for its role in movement, but it is also involved in a variety of other cognitive processes involving executive function. ST shows increased protein oxidation and lipid peroxidation as a function of age (Felten et al., 1992). In ST, the specific carbonyl levels of pyruvate kinase M2 (PKM2), alpha-enolase, inositol monophosphatase (INSP1), and F1-ATPase Chain B (ATP-F1B) were significantly decreased in aged CR rats. In contrast, the expression levels of phosphoglycerate kinase 1 (PKG1), inosine monophosphate cyclohydrolase (IMPCH) and F1-ATPase Chain A (ATP-F1A) were significantly increased in the ST of CR rats.

In conclusion, all the results support the hypothesis that CR induces a mild metabolic stress response by increasing the production of neurotrophic proteins, therefore, priming neurons against apoptosis. Further, CR is able to positively modulate glutamate metabolism, mitochondrial functions and protein synthesis machinery. These data provide a valuable insight into the mechanism of CR on oxidative stress reduction and functional improvements in the aged CNS. CR has been suggested as a potential strategy to reduce risk of age-related neurodegenerative disorders which the present study imply maybe due reduction in oxidative damage and changed expression of specific hippocampal and striatal proteins.

4.2.3. *Effect of low vitamin D diet on middle- to old aged rat brain*

In many countries worldwide, intake or production of vitamin D is low. To model what consequences might arise because of low vitamin D, middle to old aged rats were raised on a low-, normal- or high vitamin D diet for 4–5 months. Elevated 3-NT was found in brain of rats on a low vitamin D diet compared to those on normal or high vitamin D diets (Keeney et al., 2013). Further investigation showed that this elevation may involve dysregulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and NF-κB mediated transcription of inducible nitric oxide synthase (iNOS) as indicated by translocation of NF-κB to the nucleus and elevation of iNOS levels. Proteomic techniques were used to provide insights into potential mechanisms underlying these effects. Several brain proteins were found at significantly elevated levels in low VitD group compared to the control and high VitD groups. Three of these proteins, 6-phosphofructokinase, triosephosphate isomerase, and pyruvate kinase, are involved directly in glycolysis. Two others, peroxiredoxin-3 and DJ-1/PARK7, have peroxidase activity and are found in mitochondria. Peptidyl-prolyl cis-trans isomerase A (PPIA or cyclophilin A) has been shown to have multiple roles including protein folding, regulation of protein kinases and phosphatases, immunoregulation, cell signaling, and redox status. Together, these results suggest that dietary VitD deficiency contributes to significant nitrosative stress in brain and may promote cognitive decline in middle-aged and elderly adults.

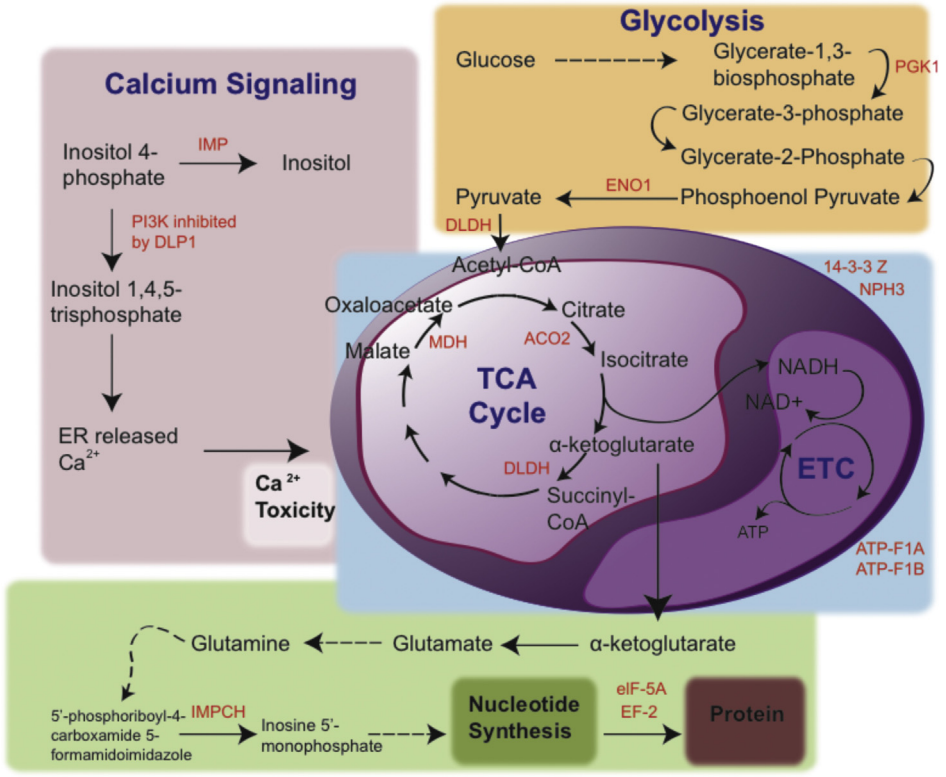

Fig. 2. Caloric restriction effects on cell homeostasis. Proteins modulated (highlighted in red), either at expression level and oxidative modification, by caloric restriction (CR) are involved in different pathways as shown in this simplified diagram. As it can be observed, CR affect crucial cellular functions including mitochondrial metabolism, Ca^{+2} signaling, protein synthesis and glutamate regulation.

4.2.4. Effect of ApoE genotype on aging and neurodegeneration:

One of the major risk factors for the elderly population to develop AD and cardiovascular diseases is the APOE genotype (Bookheimer and Burggren, 2009). The APOE gene has three common alleles, APOE ε2, ε3, and ε4. In comparison with APOE3, which is considered the "normal" genotype, the APOE4 allele increases susceptibility to late-onset AD, while both the APOE4 and APOE2 alleles increase the risk of cardiovascular disease (DeKroon et al., 2011). Among different hypotheses, it appears that ApoE may affect Abeta (Aβ), mainly the aggregation process or efflux from brain. In order to better elucidate temporal changes in protein oxidation resulting from aging and ApoE, a 2D-DIGE method was applied for the simultaneous detection of differently expressed and carbonylated proteins. In detail, hippocampal proteome from young-adult (25–30 weeks) and old (76–97 weeks) mice transgenic for the human ApoE gene (APOE, APOE3, APOE4) isoforms, APOE3 or APOE4, were analyzed to detect changes in the levels of oxidation and total protein expression. Among the proteins identified to be increasingly carbonylated, many have been already associated with AD and aging, including Hsc71, mortalin, γ-enolase, α-enolase, tubulin and actin. By comparing differentially carbonylated proteins, two distinct groups can be discussed: one in which carbonylation increased with age independent of APOE genotype, and the other in which carbonylation was a function of APOE genotype. Taken together, these results suggest that age and APOE genotype influence protein carbonylation in distinct ways, with no significant interaction. In addition, expression levels were in general higher in ApoE3 genotype which corresponded to increased carbonylation, suggesting that an increase in protein carbonylation with the APOE3 genotype may result above all from a general increase in expression level. It is reasonable to propose that little relative change in protein carbonylation between the APOE3 and APOE4 genotypes exists. Intriguingly, the difference in carbonylation between aging and APOE genotype in this study correlates with the differential expression of peroxiredoxin family members. Prdx-2 expression increased in APOE3 mice, Prdx-3 expression increased in APOE4 mice, and Prdx-6 expression increased with age, independent of APOE genotype. It is likely that differential expression of peroxiredoxins may be involved in the independent effect of age and ApoE on protein oxidation.

4.3. Aging in murine olfactory system

Olfactory impairment and anosmia (loss of sense of smell) have been demonstrated to occur with aging in humans (Murphy et al., 2002) as well as with neurodegenerative disorders including AD and PD (Mesholam et al., 1998). Olfactory sensory decline is indexed by a number of neuroanatomical and immunohistochemical changes in the olfactory epithelium (OE) and olfactory bulbs (OB) of aging humans: a reduction in both the levels of xenobiotic metabolizing enzymes as well as HSP70 expression by human olfactory receptor neurons (Getchell et al., 1995). In the OB of elderly humans, high levels of lipofuscin, a pigment that represents the intralysosomal accumulation of undegradable oxidatively modified molecules (Terman and Brunk, 2004), as well as the presence activated microglia, rare amyloid fibrils and neurofibrillary tangles were observed.

In addition to the many neuroanatomical and immunohistochemical changes, several studies have suggested that increased oxidative stress occurs in the olfactory system of elderly humans. In a previous proteomics study, the steady-state levels of proteins in the OBs of 1.5- and 20-month-old mice showed that that 20 proteins were expressed at significantly different levels at the two ages (Poon et al., 2005a,b). To determine whether elevated oxidative stress occurs in the murine OB, redox proteomics analysis was also performed to evaluate protein carbonylation. Interestingly, many

of the oxidized proteins were previously identified as targets of protein carbonylation in another study of young *vs.* old mouse brains (Poon et al., 2006a,b), thus confirming that the specific decline of selected cellular functions are hallmarks of the aging process. The results demonstrate that: (1) both total protein carbonylation and nitration are elevated in the OBs as a function of age; (2) specific proteins that we have identified are targets of carbonylation are localized in neuronal and glial cell types that perform key roles in olfactory sensory processing and cellular homeostasis; and 3) protein nitration targets primarily the vasculature of the OB.

4.4. Canine model of human aging

Aged dogs naturally develop cognitive deficits and display brain pathology quite similar to what is observed in human aging, providing a useful model for studying the molecular mechanisms underlying age-dependent cognitive dysfunction (Head et al., 2000). Nevertheless, aged dogs develop a decline in many different cognitive domains and exhibit individual variability in the aging process. The neurobiological basis for cognitive dysfunction may be related to structural changes that reflect degeneration. Neuron loss and cortical atrophy in vulnerable brain regions of the aged dog may be due to the accumulation of toxic proteins, including Aβ or oxidatively modified lipids, proteins, or nucleic acids. Further, additional pathways also could contribute to neurodegeneration, including mitochondrial dysfunction and cumulative oxidative damage (Head, 2013). However, among putative candidates that trigger neurodegenerative phenomena in the dog brain there is the progressive accumulation of Aβ in diffuse plaques and in the cerebral vasculature.

Growing interest rises from the evidence that canine and human Aβ have identical amino acid sequence (Selkoe et al., 1987). In parallel, Aβ regions show differential accumulation in the dog brain, as it occurs in human. Aβ deposition occurs earliest in the prefrontal cortex of the dog and later in temporal and occipital cortex, similar to previous reports in humans (Thal et al., 2002). Although the brain regions affected by senile plaques are similar in dogs and humans, they are likely to mimic an earlier phase of Aβ deposition (Head, 2013). As a consequence, Aβ plaque deposition becomes severe in the dog brain with evidence of cognitive deficits (Cummings et al., 1996a,b). Thus, aged dogs exhibit key features of human aging, making them particularly useful for studies of molecular mechanisms of both healthy and pathological aging and for development of therapeutics that can be translated into human clinical trials (Head, 2013).

For this purpose, the Head and Butterfield laboratories collaborated (Opii et al., 2008) to test the effect of an antioxidant-fortified diet and a program of behavioral enrichment in the aging canine (beagles) brain as protective treatment to counteract oxidative damage and to restore antioxidant reserve systems. Beagle dogs have the advantage of being easy to handle, capable of learning a broad repertoire of cognitive tasks, and these dogs metabolize dietary nutrients in similar ways as humans. Therefore, aged beagles are a good model for dietary treatments (Cummings et al., 1996a,b).

Four different treatments were compared in 23 age-matched beagle dogs for a period of 2.8 years and markers of oxidative stress in the parietal cortex were analyzed: C/C – control enrichment/control diet, E/C – behavioral enrichment/control diet, C/A – control enrichment/antioxidant diet, E/A – behavioral enrichment/antioxidant diet. All treatments were effective in reducing the levels of brain 3NT and protein carbonyls, but only those in the combined treatment EA showed a significant reduction when compared to control. Further, in order to understand the protective mechanism underlying the combined treatment, EA, redox proteomics analysis showed less oxidation and increased expression of key brain proteins involved in energy metabolism (α-enolase, GAPDH, Fructose bisphosphate aldolase C, CK), antioxidant systems (SOD-1 and GST), and in maintenance and stabilization of cell structure (NF-L, fascin) in the EA treated beagles compared to controls. A significant increase in the activity of antioxidant enzymes, such as GST and SOD, and a significant increase in the expression of HO-1 protein, an important defense system in neurons under oxidative stress (Calabrese et al., 2003), was also demonstrated. The significant decrease in oxidation and expression of some of these key brain proteins was shown to correlate with improved cognitive function in the aged canines undergoing these interventions.

This study supports the notion that oxidative stress may be a key mechanism contributing to decline in memory and cognitive function with age. Further, most of the proteins identified to be modulated by the combined EA treatment were already found to be implicated in the protective effects of antioxidant treatment discussed in the present review (LA, LAC and CR). Taken together, these findings highlight the major cellular functions that once impaired, such as by increased oxidation, contribute to memory and cognitive decline, key features of aging and, if further exacerbated, neurodegeneration.

Reduction in the levels of oxidative stress/damage following this treatment, together with preserving the functionality of energy metabolism, antioxidant systems, and the maintenance and stabilization of cell structure correlate with improved memory and cognitive function observed in the aging canine. These findings suggest possible mechanisms through which the aging canine, provided with the combined intervention of an antioxidant fortified diet and a program of behavioral enrichment, shows improvements in cognitive function (Milgram et al., 2002).

5. Aging and neurodegeneration: focus on Alzheimer's disease

The most comprehensive proteomics study of human aging, or, more correctly, dysfunctional human brain in a disorder associated with aging, has been performed by our group on AD post-mortem human brain. AD is the most common form of dementia in the elderly population, clinically characterized by progressive memory loss, cognitive impairment, loss of language and motor skills, and changes in behavior not due to any other cause. AD brain, CSF, and plasma demonstrate increased levels of OS (Di Domenico et al., 2011; Nunomura et al., 2012; Sultana and Butterfield, 2013). In AD brain, increased OS has been well documented with markers for protein, DNA, and RNA oxidation as well as lipid peroxidation (Butterfield and Lauderback, 2002; Nunomura et al., 2012; Santos et al., 2012).

AD has at least three stages: amnestic mild cognitive impairment (MCI), early-stage Alzheimer disease (EAD), and late-stage Alzheimer disease (LAD) (Selkoe, 2001). Some patients show a presymptomatic phase before MCI, namely preclinical Alzheimer disease (PCAD). Due to the progressive nature of AD, patients are usually diagnosed on the basis of the severity of symptoms during the transition into each progressing stage. Braak staging (scoring) characterizes the severity of this disease based on the distribution of NFTs and neurophil threads and range from I to VI (the higher the stage, the greater the severity of AD). PCAD patients have recently been added to the list of AD stages, and indicate individuals without memory deficits but with remarkable AD neuropathology (Braak scores are III or higher). Due to difficulties in collecting PCAD samples, experimental data on these subjects are limited. However, our laboratory has described proteomics changes in PCAD brain (Aluise et al., 2010) and in the transition from PCAD to amnestic MCI, including identification of proteins with elevated protein carbonylation that conceivably may be involved in memory loss of amnestic

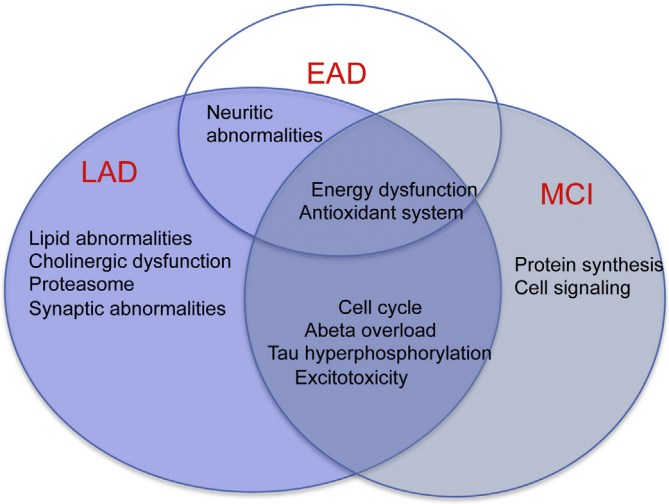

Fig. 3. Venn diagram of oxidatively-modified proteins identified during the progression of AD. Proteins involved in energy metabolism, antioxidant defense system and neuritic abnormalities seem to play a crucial role in the progression of AD. MCI, mild cognitive impairment; EAD, early AD; LAD, late stage AD.

MCI compared with PCAD (Aluise et al., 2011). Amnestic MCI is considered the first clinical stage of AD, but not all AD patients are diagnosed with MCI. Criteria for amnestic MCI include: (a) no dementia; (b) memory deficit corroborated by an informant; and (c) undisturbed activities of daily living. MCI patients can be classified as having amnestic (memory affecting) MCI or non-amnestic MCI (Economou et al., 2007). Braak staging for MCI is typically III or IV, as NFTs begin to form in the hippocampus and neocortex. EAD, an intermediate stage between MCI and LAD, is characterized by increased frontal lobe atrophy, ventricular widening with progressive brain deterioration. Braak scores for this stage typically are between IV and V. Similar to PCAD, EAD samples are rare and therefore experimental evidences still limited. LAD is the final stage of the disease, showing severe memory loss, dementia, behavioral changes, and significant impairment of activities of daily living (Braak staging for these patients is approximately IV–VI). Markers of oxidative stress including DNA oxidation, protein oxidation, and lipid peroxidation are significantly higher in these patients (Butterfield et al., 2001).

The Butterfield laboratory performed a comprehensive redox proteome analysis of different brain regions from AD, MCI subjects and with some limitations also from subjects with EAD and PCAD. By briefly summarizing the major findings of almost ten years of research, it is evident that protein oxidation highly correlates with the clinical features, pathology, and biochemistry of AD (Butterfield et al., 2012). Oxidative dysfunction of proteins involved in energy metabolism, antioxidant response, protein degradation, excitotoxicity, neuronal structure, and mitochondrial abnormalities are likely involved in neurodegeneration at various stages of the disorder (Fig. 3). The identification of these common targets of protein oxidative modification among different phases of disease is consistent with the concept that losses of function of these proteins have an important role both in the molecular basis of this disorder and in the progression of AD. In addition, identification of early molecular events that become dysfunctional in the early stages of AD will drive the development of novel therapeutic strategies aimed at preventing loss of specific functionalities that, even if not the immediate cause of the disease, undoubtedly participate to the chronic accumulation of cellular deficits which ultimately culminate in the loss of cognitive function.

Though it is not possible to make a complete comparison and one of total fidelity between animal and human studies, the redox proteomics findings highlight at least the major cellular functions that seem to be impaired in the aging process and that may be involved in the development of neurodegenerative diseases. We suggest that impairment of energy metabolism is the crucial event found to be altered in both aged animals as well as in AD. Indeed, reduced ATP levels may impact a wide variety of intracellular functions that however at this level are not significantly different compared with young population. Other contributors for the definition of aging as a global reduction of cellular abilities that results in an accumulation of damage are proteins responsible for the maintenance of cellular structure such as tubulin, actin and spectrin. Interestingly, the responsible proteins appear to be much more involved in aging compared with an age-associate disorder like AD. Also involved are proteins that contribute to the stress response system, both antioxidant enzymes and heat shock proteins (HSPs).

Overall, our results demonstrate that aging is fertile ground for development of neurodegenerative disorders, conditions in which deregulation of certain functions occur and that in the presence of additional factors, genetic or otherwise, become exacerbated and ultimately result in neuronal death.

In order to better understand the age-dependent development of AD neuropathology, a comprehensive proteomics analysis of brain from APP/PS1 mice was performed (Sultana et al., 2011). This is consistent with previous reports showing an age-dependent increase in OS markers, including loss of lipid asymmetry, Aβ production and amyloid deposition in the brain of APP/PS1 mice. To further gain insight into the molecular pathways involved in the age-dependent (1, 6, 9, 12 and 15 months of age) decline of cognitive abilities, APP(NLh)/APP(NLh) × PS-1(P246L)/PS-1(P246L) human double mutant knock-in APP/PS-1 mice were analyzed. The selection of age groups was based on earlier studies that demonstrated the most significant age-dependent increase in Aβ levels and levels of OS (Bader Lange et al., 2010). In particular, at 9 months of age these mice show high Aβ levels and increased levels of OS, and we suggest this age to be the beginning of the AD stage. At 6 months of age, Aβ levels and oxidative stress may correspond to MCI status.

It is interesting to note that despite different levels of brain amyloid measured in these mice at different ages, oxidation of specific proteins occurs independently suggesting that some proteins are easily susceptible to oxidation even at low Aβ levels and may be crucial in progression of AD. It well-established that small Aβ oligomers are highly toxic and can induce oxidative stress (Klein, 2006), thus increased oxidation of some proteins, including beta-actin and 14-3-3 zeta/delta/gamma, before Aβ deposition is not surprising and correlate with this notion. Among the proteins with an increased pattern of oxidation as a function of age, our redox proteomics results suggest that oxidation of α-enolase, 14-3-3 protein, and Pin-1 occurs early in the progression of this mouse model of AD and could be important targets to prevent or delay AD. These results match most of the findings obtained by human studies from AD and its early phases (Butterfield et al., 2012).

5.1. *Ubiquitinylation and cysteine nitrosylation during aging and Alzheimer's disease*

Among different types of protein oxidation, nitrosylation has emerged as an important redox-based signaling mechanism (Riederer et al., 2009) (see review (Stadtman, 2006)). S-nitrosylation may occur on single cysteine residues within an acidic/basic or hydrophobic structural motif and may be also subject to oxygen- or glutathione-dependent modifications such as the NMDAr complex or caspases (Choi et al., 2000; Grillari et al., 2006). In response to protein oxidation, degradation of a target protein by the ubiquitin/proteasome pathway occurs by a multistep process to

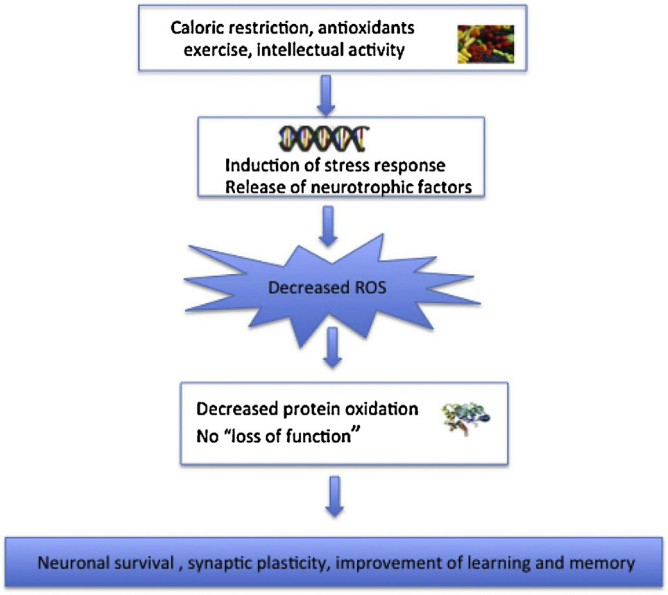

Fig. 4. Promoting healthy aging. Caloric restriction, intellectual activity, exercise and antioxidants are essential to promote neuronal survival and plasticity. Neurons respond to these "positive" stresses by activating signaling pathways that induce the expression of protein chaperones and growth factors which are essential to promote neurogenesis and synaptic plasticity. In parallel, dietary restriction and antioxidant supplementation are able to counteract the age-related increase of oxidative stress and therefore protect proteins from oxidative damage that causes protein dysfunction.

prevent accumulation of oxidized/misfolded protein with altered function.

Riederer et al. (2009) applied one- and two-dimensional gel electrophoresis, involving either general protein stains for protein expression analysis and Western blots for identification of S-nitrosylated, ubiquitinylated or specific proteins such as tau and glial fibrillary acidic proteins (GFAP). S-nitrosylated proteins were also identified by mass spectrometry in human brain tissue, to compare AD samples, at different Braak stages, with age-matched controls; however, no clear-cut change in AD was apparent. Among the relevant modifications, nitrosylation of GFAP correlates with other types of oxidative modifications attributed to GFAP (Boutte et al., 2006; Butterfield, Perluigi et al., 2012; Di Domenico et al., 2013), confirming its susceptibility to undergo irreversible modification. The novel finding is the increased ubiquitinylation of GFAP in AD, consistent with known reactive gliosis in AD. The co-localization of GFAP with β-amyloid may in turn induce an increased degradation of glial proteins. In addition to GFAP, among proteins identified in this study, heat shock proteins, proteins involved in glycolysis, in redox systems, structural proteins including GFAP, and proteins involved in iron transport, channel proteins, ATP and carbohydrate metabolism, a G-protein, synaptic protein, cancer and kinase inhibitors and the proprotein convertase inhibitor were found to be S-nitrosylated. As discussed above, alterations in these functions likely may be involved in the neurodegenerative process.

6. Concluding remarks

Aging is an accumulative process in that damage over time builds up to a critical mass promoting system failure that results in mortality as well as age related diseases. CNS is particularly vulnerable to oxidative injury, and accumulation of oxidative damage in the brain results from either perturbation of redox balance and reduced ability of antioxidant/clearance systems to correctly metabolize oxidized/misfolded proteins. Alteration of intracellular redox homeostasis may in turn stimulate endogenous generation of ROS that will further influence the regulation of a number of physiological functions, including energy metabolism and stress response, and ultimately accelerate the aging process. Understanding the complexity of such molecular interactions may contribute to define the boundary line of "normal" brain aging and development of age-dependent diseases. To reach this goal, redox proteomics studies have been, and still will be, a powerful tool to analyze the impact of protein oxidative modifications on protein function. Interestingly, among the targets of oxidative damage there are members of cellular defense system, including antioxidant enzymes, the proteasome system and stress response proteins. The avoidance of the accumulation of oxidative damage to proteins, as well as lipids and DNA, by the induction of repair processes is an important strategy to maintain healthy brain functions (Fig. 4). The potentiation of such processes, for example by supplementation with natural products (*i.e.*, LA, LAC, antioxidants and low-calorie diet), could be an important means by which to favor healthy aging and decrease risk of development of neurodegenerative disorders.

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