Chapter 8
Brain Oxidative Stress in the Pathogenesis and Progression of Alzheimer's Disease

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Abstract Alzheimer's disease is an age-related neurodegenerative disease and is characterized by the presence of senile plaques (SP), neurofibrillary tangles, and synapse loss. Amyloid-beta, one of the main components of SP, has been known to induce oxidative stress and is highly toxic. Using redox proteomics approaches a number of oxidatively modified proteins were identified in AD and mild cognitive impairment (MCI) brain that are consistent with the clinical presentation, pathology, and biochemistry. The identification of key proteins that are highly susceptible to amyloid-beta-mediated oxidation might serve as biomarkers for use in diagnosing and also in the identification of therapeutic targets to prevent or delay this devastating disorder.

Abbreviation

3-NT	3-nitro tyrosine
AD	Alzheimer's disease
ADDL	β-amyloid-derived-diffusible ligands
AGEs	Advance glycation end products
AICD	APPs intracellular c-terminal domain
APC	Anaphase promoting complex
APOE 4	*Apolipoprotein E* allele 4
APP	Amyloid precursor protein
Aβ	Amyloid beta-peptide
AβPP	β-amyloid precursor protein

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BR	Bilirubin-IX-alpha
BRCA 1	Breast cancer type-1 susceptibility protein
BV	Biliverdin-IX-alpha
BVR-A	Biliverdin reductase-A
CBP	Creb response binding protein
CDKs	Cyclin-dependent kinases
CEL	*N*-carboxyethyl-lysine
CML	Nε-(carboxymethyl) lysine
c-MYC	Cellular-myelocytomatosis
CR	Carbonyl reductase
CSF	Cerebral spinal fluid
DMDMAH-1	Dimethylarginine dimethylaminohydrolase 1
DRP-2	Dihydropyrimidinase-related protein 2
EOAD	Early onset-AD
F_2-IsoP	F_2-isoprostane
F_4-NP	F_4-Neuroprostane
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glycer-AGE	Glyceraldehyde-derived AGEs
GPX	Glutathione peroxidase
GRD	Glutathione reductase
GRP	Glucose regulated protein precursor
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3-β
GST M	Glutathione-S-transferase Mu
HNE	4-hydroxy 2-trans-nonenal
HNE-GSH	HNE-glutathione
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2
HSPA8	Heat shock protein A8
iASPP	Inhibitor of apoptosis-stimulating protein of p53 (iASPP)
IDE	Insulin degrading enzyme
IGF-1	Insulin growth factor-1 (IGF-1)
IKKβ	IkB kinase type β
iNOS	Inducible nitric oxide synthase
IPL	Inferior parietal lobule
LDH	Lactate dehydrogenase
LOAD	Late onset-AD
MAPK	Mitogen-activated protein kinase
MCI/aMCI	Non-amnestic/amnestic mild-cognitive impairment
MDA	Malondialdehyde
MDH	Malate dehydrogenase 1 (MDH)
Mdm-2	Murine double minute-2
MMSE	Mini-mental state examination
MnSOD	Manganese superoxide dismutase
MRP3	Multidrug resistant protein-3
mtDNA	Mitochondrial DNA

nDNA	Nuclear DNA
NF-E2	Nuclear factor-erythroid 2
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor kappa-B
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NPrG	1-*N*2-propanodeoxyguanosine
Nrf-2	Nuclear factor related factor 2
PDI	Protein disulfide isomerase
PICALM	Phosphatidylinositol-binding clathrin assembly protein
Pin1	Peptidyl prolyl cis-trans isomerase
PLK	Polo-like kinase
PP2A	Protein phosphatase 2-A
PPIase	Peptidyl-prolyl *cis/trans* isomerase
PRVI	Peroxiredoxin 6
PS-1	*Presenilin-1*
PS-2	*presenilin-2*
Rb	Retinoblastoma protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RSNO	S-nitrosothiols
SBP1	Syntaxin binding protein I
SCF^{skp2}	Skp cullin, F-box containing complex
SOD	Superoxide dismutase
SOD1/SOD2	Superoxide dismutase1/2
Thio-1	Thioredoxin-1
TNF-α	Tumor necrosis factor-α
TPI	Triose phosphate isomerase (TPI)
tRNA	transfer RNA
UCH L-1	Ubiquitin carboxy-terminal hydrolase L-1
UPR	Unfolded protein response
VDAC	Voltage-dependent anion channel

8.1 Introduction

Alzheimer's disease (AD) is the sixth leading cause of death in the USA and is the most common form of dementia. This disease currently is estimated to affect 5.1 million people aged 65 and older in the USA. With approximately 80 million people

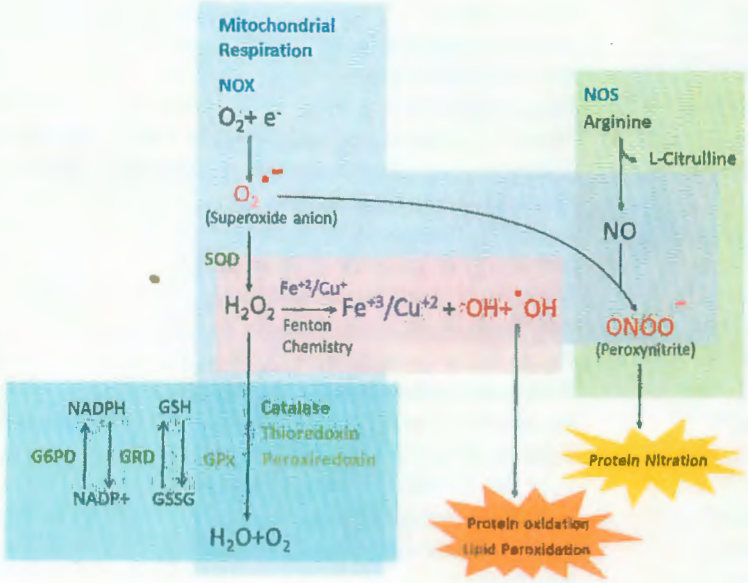

Fig. 8.1 Presented above are important pathways in the production (names represented in *blue*) and breakdown (names of antioxidant enzymes in *green*) of ROS/RNS (compounds represented in *red*) that lead to damaging posttranslational modifications of biomolecules

in the "Baby Boom" population, it is expected that the number of AD patients will increase to as many as 20 million by 2050 unless means to delay the onset or progression of the disease are developed [41] (Fig. 8.1).

8.2 Alzheimer's Disease

AD is histopathologically characterized by the presence of abnormal protein deposits, including senile plaques (rich in amyloid β-peptide [Aβ]) and neurofibrillary tangles (NFT, rich in hyperphosphoryalted tau) [71], and synaptic loss. Structural MRI studies demonstrate early brain atrophy in AD that is predominant in hippocampus, precuneus, temporal and parietal lobes, parts of the frontal cortex and cingulate gyrus. In contrast, the cerebellum typically displays minor, if any, changes in AD brain compared to controls [37]. FDG-PET analysis demonstrates a characteristic pattern of decreased glucose metabolism in parietal-temporal association cortices [62].

Diagnosis of AD is established by criteria outlined by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) in which both possible and probable AD is diagnosed using cognitive examinations with the exclusion of other

possible causes, while definite diagnosis is obtained postmortem or via biopsy. Within the realm of possible and probable AD, there are also subclassifications that have been recognized such as non-amnestic/amnestic mild-cognitive impairment (MCI/aMCI), early onset-AD (EOAD), and late onset-AD (LOAD).

8.3 Mild Cognitive Impairment

MCI is arguably the earliest form of AD. Patients who have developed aMCI have an increased probability of developing clinical AD [58], yet it remains a challenge to identify which patients with MCI will proceed to clinical AD and which patients will not [43]. In this stage of the disease, patients are found to have decreased hippocampal volume based on MRI measurements [42], decreased energy metabolism, increased number of NFTs and subsequent Braak staging (Stage II–III) [11], lowered MMSE scores, increased levels of amyloid deposition correlated with both non-amnestic MCI as well as amnestic MCI, as well as cell cycle protein oxidation and general cycle aberrations [35, 76].

The exact molecular mechanisms that lead to the loss of neurons and development of AD pathology are still unclear. Mutations of *presenilin-1* (PS-1), *presenilin-2* (PS-2), and *APP* genes have been reported to cause familial AD (FAD). In addition, other genes, such as *apolipoprotein E* allele 4 (*APOE 4*), *clusterin* (CLU aka APOJ), *phosphatidylinositol-binding clathrin assembly protein* (PICALM), *endothelial nitric oxide synthase-3*, and *alpha-2-macroglobulin*, have been suggested as risk factors for AD.

A number of hypotheses have been proposed to link the pathologic lesions, neuronal histopathology, biochemistry, and clinical symptoms of AD, including the amyloid cascade, excitotoxicity, oxidative stress, and inflammation hypotheses. All these hypotheses are based, to some extent, on the role of Aβ. The oxidative stress hypothesis for the pathogenesis and progression of AD [14, 18, 55] is based on the observations of increased cellular free radical production in AD. Such production is proposed to exceed the capacity to scavenge or otherwise neutralize these damaging moieties, leading to neuronal dysfunction and death that is mediated by protein oxidation, lipid peroxidation, and nucleic acid oxidation [20].

8.4 Amyloid Beta-Peptide (Aβ), Oxidative Stress, and AD Pathogenesis

The Aβ-induced oxidative stress hypothesis in AD [14, 55] is supported by Aβ-induced elevation of oxidative stress markers in brain, and subsequent neuronal degeneration [38]. Senile plaques are composed of a core of Aβ surrounded by degenerating neurites. Aβ is derived by the proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases. Both Aβ(1-40) and Aβ(1-42) peptides, among other Aβ peptides, are found in human brain and the former two are the most

abundant forms of this peptide. Aβ exists in at least four aggregated states: monomers, oligomers, protofibrils, and fibrils. Recent data suggest that oligomeric Aβ is the toxic species of this peptide rather than Aβ fibrils themselves [39].

Previous studies [13, 18] have identified the single methionine residue at position 35 of Aβ(1-42) as a critical mediator of increased oxidative stress and neurotoxic properties of this peptide. Recent in vivo from our laboratory showed for the first the time methionine is critical to induce oxidative stress and consequently, a key player in amyloid β-induced oxidative stress and AD pathogenesis [81].

8.5 Evidence of Oxidative Stress in Brain of Subjects with AD and MCI

The atrophy, indicative of neuronal loss, observed in AD and MCI brain by MRI studies correlates well with increased levels of oxidative stress markers in AD and MCI brain. Further, the specific targets of oxidative damage reported to date are linked to the biochemical, clinical, and pathological development of MCI and AD, and are discussed further below.

8.5.1 Protein Carbonyls

Brain protein carbonyls are increased in AD [28, 31, 74]. In the frontal cortex of Swedish APP670/671 FAD mutation, increased levels of protein carbonyls, diene conjugates, and lipid peroxides were found [8]. Further, the levels of carbonyl reductase (CR) are increased in brain of AD and Down syndrome subjects [3], suggesting enzyme induction due to increased levels of protein carbonyls. The authors of this report hypothesized a possible role of Aβ in this observed induction; however, the activity of CR was not measured, and the mechanism(s) accounting for increased CR levels were not identified. Since the 20S proteasome is known to degrade oxidized proteins normally, it is conceivable that oxidative inhibition of the 20S proteasome, similar to the case for the 26S proteasome in AD [47], may occur. Reduced degradation of protein carbonyls by the proteasome complex may contribute to the elevated protein carbonyls found in AD brain.

Beta-actin and creatine kinase BB have been identified as specifically oxidized proteins in AD brain using 2D electrophoresis and 2D Western blots [1]. These techniques form the basis of the methodology needed to further examine the role of oxidative modifications of specific brain proteins in AD pathogenesis and have led to the development and use of redox proteomic [32] techniques to identify carbonylated brain proteins in AD [23]. 2D gel electrophoresis coupled to mass spectrometry [32, 77] have allowed the discovery of increased carbonylation of creatine kinase BB, glutamine synthase, ubiquitin carboxy-terminal hydrolase L-1 (UCH L-1), dihydropyrimidinase-related protein 2 (DRP-2, also called CRMP2), alpha-enolase,

and heat shock cognate 71 in AD inferior parietal lobule (IPL) compared to age-matched controls [23]. Subsequent studies of AD hippocampus demonstrated specific carbonylation of peptidyl prolyl *cis–trans* isomerase (Pin1), phosphoglycerate mutase 1, UCH L-1, DRP-2, carbonic anhydrase II, triose phosphate isomerase (TPI), alpha-enolase, and gamma-SNAP compared to age-matched controls [74]. Consistent with the notion that oxidative modification of proteins leads to dysfunction of normal cellular processes in AD, the activities of Pin1, enolase, and carbonic anhydrase II were significantly lower in AD hippocampus compared to matched tissue samples from control subjects [74]. Others [49] using redox proteomics showed significant decreased protein carbonyls in malate dehydrogenase 1 (MDH), glutamate dehydrogenase, 14-3-3 protein zeta/delta, aldolases A and C and increased oxidation of carbonic anhydrase 1. The sample processing in this study did not use detergents, and may have led to identification of fewer oxidized proteins than that seen in other studies as a result of decreased exposure of protein carbonyls. In the IPL of FAD subjects, increased carbonylation of UCH-L1, gamma-enolase, actin, and dimethylarginine dimethylaminohydrolase 1 (DMDMAH-1) have been reported [17]. Others also reported oxidation and accumulation of proteins like UCH L1, ATP synthase, and Cu,Zn-superoxide dismutase in AD brain [28, 82], confirming our prior results.

MCI brain also demonstrates increased levels of protein carbonyls [17, 48]. Redox proteomics studies in MCI hippocampus led to the identification of alpha-enolase, glutamine synthetase, pyruvate kinase M2, and Pin1 as specifically carbonylated proteins recapitulating many of the findings seen in fulminate AD brain tissue [17]. Recent reports have also identified increased specific carbonylation of carbonic anhydrase II, heat shock protein 70, mitogen activated protein kinase I, and syntaxin binding protein I (SBP1) in MCI [78].

8.5.2 Protein Nitration

Increased protein nitration has been reported in AD brain [72], and correlates with increased nitric oxide synthase (NOS) levels, suggesting a role of nitration in AD pathophysiology [36]. Redox proteomics studies identified a large number of proteins that are specifically nitrated in AD hippocampal and IPL compared to control brain, including alpha- and gamma-enolase, lactate dehydrogenase (LDH), neuropolypeptide h3, TPI, and alpha-actin in AD IPL [24], and alpha-enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP synthase alpha-chain, carbonic anhydrase-II, and voltage-dependent anion channel (VDAC) protein in AD hippocampus [79]. These nitrated proteins are involved in various cellular functions such as energy metabolism, structural maintenance, pH regulation, and mitochondrial function. Oxidative modification (i.e., nitration, carbonylation, etc.) may alter protein functionality [79]. Excess nitration of TPI was recently confirmed [40] in hippocampus and frontal cortex of AD subjects, suggesting a link among decreased glucose metabolism via an impaired glycolytic pathway, nitrosylation of TPI, and

the formation of Aβ and paired helical filaments. However, it is not clear why, in spite of oxidative modification, its activity remains unchanged in AD brain. It is possible that this nitrosylation is a nonspecific marker of increased oxidative stress rather than a direct contributor to the development of AD. In contrast, Reyes et al. demonstrated nitration of Tyr 18 followed by Tyr 29 of tau, which is mostly associated with or in close proximity to amyloid plaques [64]. Hence, nitration of proteins may reflect underlying posttranslational modification of proteins in AD.

Consistent with this notion, increased levels of 3-NT in MCI hippocampus and IPL using immunochemistry were reported [80]. There is also evidence for AD-specific nitration of MDH, α enolase, glucose regulated protein precursor (GRP), aldolase, glutathione-S-transferase Mu (GST M), multidrug resistant protein-3 (MRP3), and 14-3-3 protein gamma in MCI IPL [80]. In MCI hippocampus, α enolase, MDH, peroxiredoxin 6 (PR VI), DRP-2, fascin 1, and heat shock protein A8 (HSPA8) were identified as specifically nitrated compared to age-matched controls [80]. A recent study [65] reported S-nitrosyl-cysteine modification of DRP-2, alpha-internexin, glutamate dehydrogenase 1, alpha-enolase, GFAP, MDH, ProSAAS precursor protein, proopiomelanocortin, proenkephalin, and septin in the entorhinal cortex of AD, and suggested that Aβ activation of glial cells surrounding the SP might have led to increased nitrosylation of GFAP contributing to the pathogenesis of AD. Protein disulfide isomerase (PDI), an enzyme that catalyzes thiol–disulphide exchange, has been reported to be *S*-nitrosylated in AD brain [84]. Increased nitrosylation and decreased activity of this protein in AD may lead to alteration in its ability to facilitate disulfide bond formation and rearrangement reactions, increased accumulation of polyubiquitinated proteins, and activation of the ER-resident unfolded protein response (UPR). Recently Cho et al. [27] reported increased levels of S-nitrosylation of dynamin-related protein 1 in brains of subjects with AD and suggested that S-nitrosylation of this protein may trigger mitochondrial fission, consequently adding to known mitochondrial damage in AD, which could contribute to synapse loss and neuronal damage in this disorder.

8.5.3 *Lipid Peroxidation*

Increased lipid peroxidation in AD brain has been identified by measuring elevated levels of free and protein-bound HNE, acrolein, F_2-isoprostane (F_2-IsoP), F_4-neuroprostane (F_4-NP), and isoprostane 8,12-iso-iP$F_{2\alpha}$-VI [56, 86]. Further, the increased levels of another marker of lipid peroxidation, malondialdehyde (MDA), in AD brain have been correlated with the decreased activity of superoxide dismutase (SOD) [22].

Increased levels of adducts of HNE and glutathione (HNE-GSH) were found in AD [85]. In normal cells the HNE-GSH adducts are removed by the combined action of GST, GSH, and MRP-1. However, in AD brain all these detoxification components were targets of HNE modification themselves, leading to decreased

clearance of HNE and subsequent accumulation of HNE protein adducts [50, 75]. Further, the proteasome, which removes damaged proteins from the cells, has elevated HNE- and neuroprostane-conjugation in brain in both MCI and AD [25]. Further, increased levels of lipid peroxidation markers such as thiobarbituric acid reactive substances, MDA, F_2-IsoP, F_4-NP, and protein-bound HNE also were reported in subjects with MCI [19, 48, 56].

Proteomics studies identified regionally specific HNE modification of proteins, i.e., ATP synthase, GS, MnSOD, DRP-2 in AD hippocampus and α-enolase, aconitase, aldolase, peroxiredoxin 6, and α-tubulin in AD cortex [60]. Some of these proteins were previously found to either nitrated or carbonylated in AD [24, 74, 79]. The appearance of different oxidative modifications in common target proteins supports the role of oxidative stress in AD and is consistent with the notion that these specific proteins may be involved in AD.

In MCI hippocampus and cortex, increased levels of protein-bound HNE in neuropolypeptide h3, carbonyl reductase (NADPH), α-enolase, lactate dehydrogenase B, phosphoglycerate kinase, heat shock protein 70, ATP synthase alpha chain, pyruvate kinase, actin, elongation factor Tu, and translation initiation factor alpha were identified by proteomics [63]. Increased lipid peroxidation in AD and MCI brain and a role for Aβ(1-42) in this process were further supported by studies that showed loss of apoptosis-related phospholipid asymmetry in AD and MCI [2]. Noting that the high reactivity of free radicals requires that the initiator of lipid peroxidation must reside in the lipids, the findings above suggest that, in AD and MCI brain, oligomeric and hydrophobic Aβ (1-42) inserts into the membrane of brain cells to cause lipid peroxidation and that such changes are an early event in the pathogenesis and progression of AD.

The proteomics studies suggest that oxidation of proteins is an integral part of the progression and pathophysiology of AD [57]. The appearance of common targets of oxidation of proteins between MCI and AD implies their important roles in loss of cellular energetics, alterations in neurotransmission and cell signaling pathways, as well as SP and NFT formation. In the following section we discussed about some the proteins that critical in the progression and pathogenesis of AD.

8.6 Glucose Metabolism and AD

Enolase, an oxidatively modified protein in AD and MCI brain, is important for regulating glucose metabolism. However, a number of recent studies showed that enolase also plays important roles in cell signaling, Aβ clearance, and activation of cell survival pathways [16]. Oxidative dysfunction of one protein may alter several cellular pathways implicated in the pathogenesis of AD. This point is further illustrated by GAPDH, which is also selectively oxidized in AD [79]. GAPDH is a key enzyme in the glycolytic pathway; oxidation of GAPDH increases the levels of the glycolytic intermediates that are upstream to GAPDH such as

glyceraldehyde-3-phosphate, which can activate the glycation pathway leading to increased formation of methylglyoxal that can react with the biomolecules, causing further damage and altered cellular function. Further, the increased levels of glyceraldehyde-3-phosphate can also lead to activation of protein kinase C pathway [21]. In addition, to up regulation of other pathways the decreased activity of GADPH increases influx of glucose through polyol pathway during which NADPH is consumed, which leads to reduced availability of GSH [29].

GAPDH has recently been shown to play key roles in transcription regulation, cell signaling, and vesicular transportation in addition to binding to other small molecules such as nitric oxide (NO), glutathione (GSH), tumor necrosis factor (TNF)-α. GAPDH also interacts with β-amyloid precursor protein (AβPP) [70]. Hence, oxidative dysfunction of enolase and GAPDH can lead to multiple changes consistent with pathology, biochemistry, and clinical presentations of AD and MCI [15]. Modulation of the cellular pathways altered by the selective oxidation of both GADPH and enolase could prove to be fertile ground for the development of novel therapeutic agents for AD [15, 16].

Recent longitudinal studies have shown a relationship between AD and glucose metabolism disorders [10]. One of the key proteins in regulation of glucose levels is insulin. The regulation of brain levels of insulin is important for proper cognitive function. For example, insulin is known to regulate the expression of *N*-methyl-D-aspartate (NMDA) receptors, one of the calcium (Ca^{2+}) regulating protein [33], that regulates the functions of other proteins that are important in learning and memory process. Further, the levels of insulin also influences the acetylcholine transferase, an enzyme involved in the synthesis of acetylcholine which has been reported to be altered in AD brain and is consequently a key in the etiology of AD [66].

Interestingly, a recent study showed that insulin protects the neuron against β-amyloid-derived-diffusible ligands (ADDL), important for synaptic deterioration, induced oxidative stress [10]. Insulin protects the neuron not by simply binding to ADDL but rather via an insulin dependent signaling mechanism. A pilot study showed that intranasal insulin administration improves memory and attention in patients AD patients without affecting the glucose levels [30]. Interestingly, the enzyme that regulates the insulin levels i.e., insulin degrading enzyme (IDE), is also important in the degradation of Aβ [61]. The reduced levels of insulin may also induce GSK-3beta activity which might lead to increased phosphorylation of tau protein and consequently NFT formations [69]. Hence, the decrease in insulin as reported in diabetes might lead to increased accumulation of Aβ in brain, cognitive impairment, and eventually AD pathogenesis.

In addition to decreasing the IDE levels, decreased activity of the enzymes involved in glucose metabolism by oxidation leads to increased glucose accumulation, which may have toxic effects on neurons through osmotic insults and oxidative stress subsequent to AGE formation. In addition to the effect on neurons, AGEs can also activate the microglia in the CNS, which can promote more free radicals and other inflammatory markers. The role of microglia has been proposed to be one of the underlying mechanism(s) of AD pathogenesis.

8.7 Pin1 and Canonical AD

Proline is an amino acid that may adopt one of two spatial conformations within a peptide. These two conformations are referred to as *cis* and *trans* orientations, of which the isomerization between the two is known to be a slow step in protein folding. Pin1, or Protein Interacting with NIMA (never in mitosis-A), is a PPIase (peptidyl-prolyl *cis/trans* isomerase) of the parvulin subfamily that specifically acts upon protein substrates with the motif of phosphorylated Ser/Thr on the amino-terminal side of an adjacent Pro (pSer/Thr-Pro) [51].

We have recently demonstrated that not only was Pin1 oxidatively modified in age matched control AD hippocampus, but that the activity as well as the protein expression of Pin1 were both found to be significantly reduced [73]. The implications of this finding are vast when considering the large amount of literature that links Pin1 as a crucial regulator of intracellular processes. When considering AD, Pin1 has been shown to regulate both directly and indirectly amyloid precursor protein (APP) and Tau, the proteins of which constitute the primary components of the neuropathological hallmarks β-amyloid plaques and NFTs, respectively. Pin1 has been shown to induce dephosphorylation of Tau at key sites via regulation of protein phosphatase 2-A (PP2A) and the knock down of Pin1 leads to hyperphosphorylation and aggregation of Tau positive NFTs [52].

Interestingly, Pin1 has been shown to act on the intracellular c-terminal domain (AICD) pT668-P of APP to direct processing of this protein toward the non-amyloidogenic pathway [59]. More recently, it has been found that Pin1 interacts with and inhibits the kinase activity of glycogen synthase kinase 3-β (GSK3β) which has been implicated in both Tau hyperphosphorylation as well as directing APP toward the amyloidogenic pathway [52]. The discovery that Pin1 both directly associates with the protein precursors to the classical AD pathological hallmarks as well as the protein kinase systems that contribute to this pathology, illustrates not only the complex and integral control that Pin1 exerts over this system, but also the potential ramifications that an oxidized and dysfunctional Pin1 could have.

8.8 Implications of an Oxidized Pin1 in Context of the Cell Cycle in MCI/AD

In normal non-neuronal cells, the means in which cells divide is through the induction of the cell cycle creating two daughter cells from one parental cell. This process may be broken down into four distinct segments (G_1, S, G_2, M); two growth phases G_1 and G_2 which themselves are built around an S phase during which DNA is replicated, followed by the M phase, or mitosis, which consists of chromosomal as well as cellular separation into two distinct daughter cells. In an effort to control abnormal proliferation of cells through the different phases of the cell cycle, regulatory points exist that the cell must fulfill for division to proceed. These checkpoints exist

between the phases and are maintained by numerous control proteins. Neurons in a typical non-disease brain of an adult remain in what is called a quiescent state (the G_0 phase) wherein the cell cycle has been halted. Neurodegenerative diseases in which the protein Tau is key demonstrate aberrant cell cycle reentry of quiescent neurons and has been shown to proceed through multiple cell cycle check points, yet ultimately failing to complete mitosis and proceeds to apoptosis.

To date, a large number of control proteins important to the cell cycle have been identified as having aberrant expression, localization, or posttranslational modifications in AD [9, 46]. Moreover, cell cycle proteins have been found to have irregular expression in earlier stages of AD. We have reported that levels of CDK2, CDK5, and cyclin G1 were elevated in both the hippocampus IPL in brains of subjects with aMCI, demonstrating that cell cycle changes may appear prior to the classical AD diagnosis [45, 76].

While induction of neurons into the cell cycle by ambiguous means has been shown to be characteristic of AD, the progression and ultimate failure of the neuron to complete the cell cycle remains to be clarified in its own right, possibly due to the vast complexity of cell cycle control. Interestingly, not only does Pin1 interact with and promote cell cycle entry, but Pin1 has also been found to be associated with a number of proteins fundamental to the progression and completion of the cell cycle; associations that could have disastrous effects in vivo due to deficient expression or oxidized Pin1.

Pin1 has been found to regulate the tumor suppressor p53, a protein of significance in controlling cell proliferation, through multiple mechanisms including increasing p53 stability through the interaction of p53 with the E3 ubiquitin ligase murine double minute-2 (Mdm-2), facilitating the DNA binding of p53 to a number of pro-apoptotic promoter sites under conditions of cellular stress, enhancing p53 acetylation by p300 or creb response binding protein (CBP) acetylases, and the dissociation of p53 from the inhibitor of apoptosis-stimulating protein of p53 (iASPP) which is a key inhibitor of p53 [54]. In addition to p53, Pin1 has been implicated as a crucial player in the regulation of phosphorylated retinoblastoma protein (pRb), a key mediator of the G1/S phase transition. Evidence demonstrates that Pin1 plays a crucial role in the association of pRb with CDK/Cyclin complexes and release of E2F transcription factors important for entry into the S-phase [67]. Pin1 has been shown to directly affect the stability of the cell cycle inhibitor $p27^{kip1}$ via interaction with the SCF^{skp2} (skp, cullin, F-box containing complex) E3 ubiquitin ligase complex [87].

The finding of an oxidized and dysfunctional Pin1 makes a significant contribution to the two-hit hypothesis proposed by Zhu et al., providing a direct link in which increased oxidative stress in conjunction with cell cycle abnormalities play vital roles in AD pathogenesis [88].

8.8.1 Heme Oxygenase-1/Biliverdin Reductase-A

HO is an enzyme that oxidizes the heme moiety of heme-associated proteins and exists in two major isoforms arising from different genes: constitutively expressed heme oxygenase-2 (HO-2) and inducible HO-1, also known as heat shock protein-32

(Hsp-32). Using NADPH and oxygen, HO-1 produces ferrous iron, carbon monoxide, and biliverdin-IX-alpha (BV) through the hydrolysis of the tetrapyrrolic ring of heme moieties. Working with BVR-A, HO-1 provides the substrate, BV, for the production of the antioxidant bilirubin-IX-alpha (BR), which has been hypothesized to redox cycle between BR and BV in the presence of BVR-A and ROS, thereby protecting the cell from cytotoxicity [4, 53]. Notably, BVR has also been shown to function as a dual-specificity Ser/Thr and Tyr kinase plugging into the insulin growth factor-1 (IGF-1) and MAPK pathways as well as regulates the expression of various oxidative stress adaptive responsive genes [44, 83]. Research conducted by our laboratory revealed a significant elevation in HO-1, the inducible isoform, in AD hippocampus as well as increased posttranslational modifications including global protein phosphorylation and protein/lipid oxidation in both AD and MCI hippocampus and the classically unaffected AD region of the brain, the cerebellum [5].

We also found that BVR-A expression is significantly increased, has a significant reduction of phosphorylation on Ser/Thr as well as Tyr residues, and displays a significant reduction in both AD and MCI hippocampus [6]. In addition to these findings, it was demonstrated that in AD and MCI hippocampus, BVR-A had significant nitrosative modifications in the form of 3-NT, while simultaneously displaying a marked decrease in both protein bound carbonyls and HNE. Protein bound 3-NT levels correlated with a significant increase in inducible nitric oxide synthase (iNOS), which produces the radical NO, in both AD and MCI hippocampus and which, in the presence of superoxide, produces the peroxynitrite anion shown to be a precursor to protein-bound 3-NT [6]. Because BVR-A has been shown to plug into several important metabolic pathways, including upstream insulin signaling, the effects of impaired or aberrant BVR-A activity could prove disastrous for the neuron in AD.

8.8.2 *Altered p53 and Cellular Redox Status*

p53 is a multifunctional protein that plays a major role in monitoring the state of cell proliferation and stress response through the transcription of key proteins in an effort to prevent damaged DNA from progressing to the next cellular generation [7]. The activity and stability of p53 as a transcription factor is regulated by many independent mechanisms [34, 54]. It is not clear exactly how p53 influences or is influenced by the redox state of the cell, research has shown that p53 plays a dual role, either increasing or decreasing ROS/RNS generation dependent on the cell type involved [7]. It has been hypothesized that p53 activity is dependent upon a threshold level of oxidative stress, having a number of key residues that are redox sensitive, as well as a possible altered or "mutant" 3D conformation upon posttranslational modification that may change the type of genes transactivated by p53. Importantly, we showed that such oxidative/nitrosative posttranslational modifications of p53 do occur in both MCI and AD inferior parietal lobule [26].

Buizza et al. immortalized lymphocytes collected from patients of controls, early onset AD (EOAD), and AD to determine if progression of the disease correlated

with increased oxidative stress, decreased oxidative stress responsive enzyme expression or activity, and altered conformation of p53 [12]. The researchers found that protein bound HNE and 3-NT were significantly elevated ($p<0.05$) in lymphocytes of AD patients, while the difference in oxidative parameters measured in EOAD compared to controls were elevated but not statistically significant. To determine the state of cellular machinery designed to regulate proper cellular redox status, the key proteins superoxide dismutase1/2 (SOD1/SOD2), glutathione peroxidase (GPX), glutathione reductase (GR) had their expression levels and activity measured. It was found that while the expression of these enzymes was not statistically altered, the activity of SOD and GR were found to be significantly decreased while GPX activity was found to be simply decreased. Most interestingly, using conformationally specific anti-p53 antibodies, it was found that an unfolded or altered p53 conformation correlated with an increasingly oxidized/nitrated p53 and diminished SOD activity indicating that the redox status of the cell influences the structural composition of p53 in lymphocytes. This finding gives rise to the potential of a peripheral diagnostic marker for the progression of AD [12].

As discussed, p53 has been found to play a dual role in both increasing and decreasing ROS/RNS dependent on the cell type, as demonstrated in non-neuronal cells by the ability of p53 to induce expression of antioxidant genes beneficial to cellular homeostasis in the absence of exogenous stress; while an increase in oxidative stress may lead to a pro-oxidant and pro-apoptotic p53 [68]. In neurons the threshold level discussed appears to be lower than in non-neuronal cells leading to a pro-oxidant p53 under basal conditions. For this reason, we used p53 knockout [$p53^{(-/-)}$] mice to determine the basal oxidative and nitrosative stress levels as well as antioxidant defense system activation in various neuronal compartments in the absence of p53 [7]. It was found that the oxidative/nitrosative stress was statistically decreased in the p53 null mice, the most drastic changes occurring in the nucleus and mitochondria. The expression levels of the redox protective entities thioredoxin-1 (Thio-1), BVR-A, manganese superoxide dismutase (MnSOD), IkB kinase type β (IKKβ), and nuclear factor kappa-B (NF-κB) were all found to have significantly increased levels, while a significant decrease in nuclear factor-erythroid 2 (NF-E2) related factor 2 (Nrf-2) was observed in the nuclear fraction [7]. The explanation given by the authors was that a loss of p53 appears to perturb the normal cellular homeostasis, resulting in the activation of protective machinery to avoid cellular damage or death. While the overall findings support their conclusions, it is evident that the intricacies of the mechanisms induced upon loss of p53 require further evaluation.

8.9 Conclusion

The oxidative stress elevation at different stages of AD and the appearance of specific protein targets of oxidation such as enolase and Pin1 suggest that specific proteins might be key players in AD pathogenesis (Fig. 8.2). As discussed above, most of the targets of protein oxidation have multiple functions, and suggest a link between metabolic disorders and the initiation and progression of AD (Fig. 8.2).

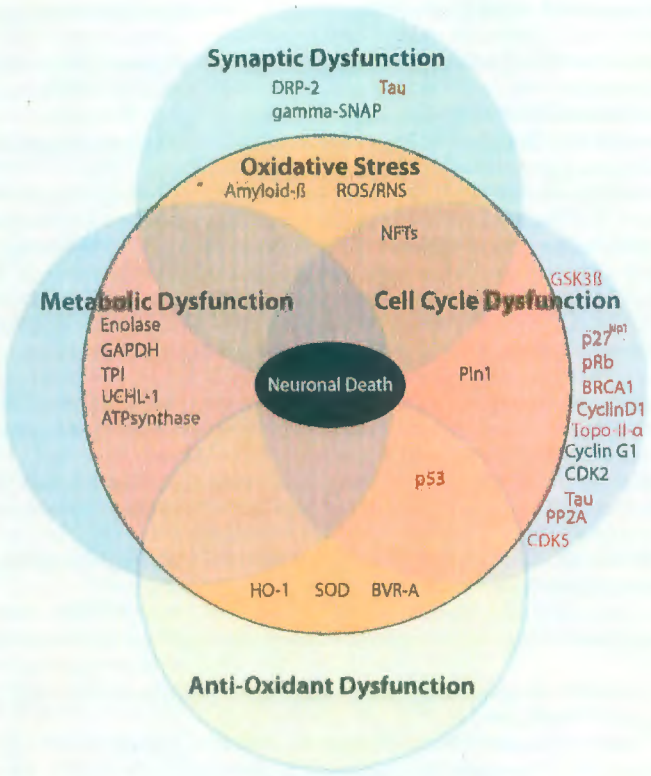

Fig. 8.2 Venn diagram representing selected key proteins implicated in the different dysfunctional systems found within Alzheimer and MCI diseased brains/model systems. Protein names colored *red* represent a direct or indirect regulation by Pin1

There have been many studies tasked with finding ways to use biomarkers in the cerebral spinal fluid (CSF), oxidative stress biomarkers (OSBs) in blood and plasma [89], and protein and lipid oxidation of mitochondria [90] in order to correlate pathology with cognition obtained from examinations such as MMSE. The importance of identifying biomarkers for use in diagnosing the stages of progressing AD is underscored by the fact that known pathological markers such as β-amyloid deposition occur prior to cognitive deficits which could allow for earlier and potentially more effective treatments.

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