Investigation into the Molecular Complexity of Alzheimer's Disease: An Amyloid- β Centered Approach

Federica Cioffi

INVESTIGATION INTO THE MOLECULAR
COMPLEXITY OF ALZHEIMER'S DISEASE:
AN AMYLOID- β CENTERED APPROACH

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Table of contents

Chapter 1 Introduction to Amyloid- β peptide and Alzheimer's disease	1
1.1. Protein structure and folding.....	2
1.2. Protein misfolding and related disease	5
1.3. Amyloid-beta peptide.....	6
1.4. Alzheimer's disease	11
1.5. Scope of the thesis	19
1.6. References.....	20
Chapter 2 Searching for improved peptide inhibitors preventing conformational transition of amyloid- β_{42} monomer to a β -sheet conformation	31
2.1. Introduction.....	32
2.2. Materials and Methods.....	34
2.3. Results and Discussion	37
2.4. Conclusions.....	44
2.5. Acknowledgments.....	46
2.6. Supplementary Information	46
2.7. References.....	47
Chapter 3 Benzodifurans for biomedical applications: BZ4, a selective anti-proliferative and anti-amyloid lead compound.....	51
3.1. Introduction.....	52
3.2. Materials and methods	55
3.3. Results.....	58
3.4. Conclusions.....	65
3.5. Future Perspective.....	66
3.6. Acknowledgements.....	66
3.7. Supplementary Information	67
3.8. References.....	70

Chapter 4 Characterization of insulin-degrading enzyme-mediated cleavage of A β in distinct aggregation states.....	77
4.1. Introduction.....	78
4.2. Material and methods.....	79
4.3. Results and discussion	82
4.4. Conclusion	93
4.5. Acknowledgements.....	93
4.6. Supplementary	95
4.7. References.....	97
Chapter 5 Molecular mechanisms of oxidative stress and antioxidative agents in Alzheimer's disease	103
5.1. Introduction.....	104
5.2. The definition of oxidative stress.....	105
5.3. Generation of reactive oxygen and nitrogen species and their neutralization.....	106
5.4. Physiological roles of ROS and RNS	116
5.5. Effect of oxidative stress on biomacromolecules	117
5.6. Consequences of oxidative stress on a mitochondrial level..	120
5.7. How oxidative stress derails in Alzheimer's disease.	121
5.8. AD related oxidative stress	123
5.9. Genetic and other factors that correlate oxidative stress to Alzheimer's disease	129
5.10. Putative molecular mechanisms of Alzheimer's disease-related oxidative stress	142
5.11. Oxidation of tau and A β	155
5.12. Oxidative stress as biomarker	166
5.13. New biomarkers	173
5.14. Oxidative stress therapeutics.....	179
5.15. Conclusion	194
5.16. References.....	195

Chapter 6 IFN γ and A β act in a concerted manner to induce microglial IL-1 β secretion and loss of synchronous neuronal firing activity.....	251
6.1. Introduction.....	252
6.2. Materials and Methods.....	253
6.3. Results.....	257
6.4. Discussion and conclusions	263
6.5. Acknowledgments.....	265
6.6. Supplementary Information	266
6.7. References.....	267
Chapter 7 Characterization of the interaction between Amyloid- β and Interferon- γ	271
7.1. Introduction.....	272
7.2. Materials and Methods.....	273
7.3. Results.....	276
7.4. Discussion	282
7.5. Acknowledgments.....	284
7.6. Supplementary Information	285
7.7. References.....	286
Chapter 8 General discussion.....	289
8.1. Overall insights on AD	290
8.2. The multifactorial character of AD.....	290
8.3. Towards a multi-target treatment of AD.....	294
8.4. Conclusive remarks and future perspectives.....	295
8.5. References.....	297
Appendix.....	301
List of abbreviations	303
Summary	305
Nederlandse samenvatting	309

Acknowledgements	313
About the author	317
List of publications	319

Chapter 1

Introduction to Amyloid- β peptide and Alzheimer's disease

1.1. Protein structure and folding

The central dogma of molecular biology describes the flow of genetic information from DNA to RNA, to make a functional product, a protein. This dogma suggests that the DNA is transcribed into messenger RNA (mRNA) inside the nucleus of the cell. Subsequently, the mRNA is transported to the cytoplasm where it is translated to proteins by the ribosomes.

There are 20 different standard L- α -amino acids (aa) used by cells for protein construction. These aa are joined together in long chains by peptide bonds between the amino group of one aa and the carboxylic group of another. Sequences with fewer than 50 aa are generally referred to as peptides. There are four levels of protein structure (Fig. 1.1). The linear chain of aa constitutes the primary structure of a protein. Within the protein chains there are regions that fold into regular structures called secondary structures. One type of motif is called α -helix and in this fold the backbone forms a spiral shape with about 3.6 aa per turn. The helix is stabilized by hydrogen bonds that occur between each hydrogen of the amide group and the oxygen of the carboxylic group. Another common type of secondary structure is the β -pleated sheet. β -pleated sheets are formed from largely extended polypeptide strands that make intra-molecular hydrogen bonds between the backbone groups of the neighboring strand. The β -strands can interact in two ways to form a β -sheet. Either the aa in the aligned β -strands run in the same direction, in that case the sheet is described as parallel, or the aa in successive β -strands can have alternating directions, in which case the β -sheet is called antiparallel. The tertiary structure of a protein describes the way the whole chain folds itself into a three-dimensional shape. Several types of interactions are involved in the formation of the tertiary structure of a protein, e.g. physical interactions including ionic interaction, hydrogen bonds, van der Waals forces and chemical interactions in the form of disulfide bridges. Finally, the quaternary

structure of a protein consists of multiple subunits. A major force stabilizing the quaternary structure is the hydrophobic interaction. Additional stabilizing forces include interactions between side chains of the subunits, including electrostatic interactions between ionic groups of opposite charge [1]. The final shape adopted by a newly synthesized protein is typically the most energetically favorable one. In fact, right after synthesis, the polypeptide remains in a denatured state, which is characterized by high conformational entropy. As proteins fold, more and more energetically favorable physical contacts are formed and the protein loses its conformational entropy resulting in a net gain in free energy. Finally, they reach their ultimate form, which is unique and compact [2].

Figure 1.1. Levels of protein structure. Primary structure, the sequence of amino acids, determines secondary and tertiary structure. Quaternary structure is created by assembling smaller proteins into a large structure. (Figure modified from Particle Sciences Technical Brief: 2009: volume 8. Particle – Drug Development Services).

Despite the long-standing assumption that the unique three-dimensional structure of a protein determines its function, it has been recognized that there is a whole class of proteins which do not conform to this assumption as they lack a defined three-dimensional structure. These are classified as intrinsically disordered proteins (IDPs) [3]. There is a huge body of research showing that IDPs are often functionally important in the cell [4–6]. IDPs have a large interaction surface area and are characterized by high flexibility facilitating their interaction with other proteins. They play a pivotal role in cell signaling, transcription, translation and cell cycle regulation [7,8].

1.1.1. Regulation of protein folding

The cell has evolved exquisite machineries to aid the synthesis, folding, trafficking and clearance of proteins [9]. To cope with misfolding and unfolding of proteins, cells have developed a protein quality control system, which comprises both chaperones and proteases and is responsible for limiting this burden [10,11]. Chaperones are molecular helpers that assist other proteins with acquiring their correct and active structure. These specialized molecules are also able to rescue misfolded proteins back to their normal conformation [12] or guide them through a degradation process [13].

1.1.2. Regulation of intrinsically disordered proteins

Due to their unusual structural features and important functional properties, the presence and abundance of IDPs in a cell needs to be carefully monitored. Several recent studies have investigated how IDPs are regulated in a cell. Levels of these disordered proteins are regulated by controlled expression. The transcription rate of mRNAs encoding IDPs does not significantly differ from the rate with which mRNAs encoding ordered proteins are produced. However, the IDP-encoding transcripts were generally less abundant than the transcripts encoding ordered proteins due to the increased decay rates of the former. Cellular levels of IDPs are tightly regulated also at a protein level.

IDPs were shown to be less abundant than ordered proteins due to the lower rate of protein synthesis [14]. In principle, the amount of intrinsically disordered proteins is regulated by post-translational modifications, e.g. phosphorylation that leads to structural changes such as folding [15] and ubiquitination [16].

1.2. Protein misfolding and related disease

Proteins are essential in life as they carry out several functions important in all biological processes. They act as catalysts, they transport and store other molecules such as oxygen, they provide mechanical support and immune protection, they generate movement, they transmit nerve impulses, and they control growth and differentiation. Folding in proteins happens spontaneously [17]. However, the folding process is not failproof and proteins may not fold correctly. In fact, around 20% of newly synthesized proteins are unable to attain the correct fold [18]. Misfolded proteins are not only a product of inefficient folding but can also be formed as a consequence of stress conditions. Changes in the cellular environment due to aging, pH variations, as well as genetic mutations, are known to cause protein misfolding or unfolding [19]. This misfolding can lead to aggregate formation and protein configurations that gain toxic function. Protein misfolding and aggregation is a particular problem in the brain, being the underlying cause of several severe and incurable age-related disorders [20]. Aggregation-prone IDPs, such as amyloid-beta ($A\beta$) and tau can lead to the etiology of Alzheimer's disease (AD), α -synuclein (α -syn) aggregation is involved in Parkinson's disease [21] and misfolded protein huntingtin to Huntington's disease [22].

1.3. Amyloid-beta peptide

A β is a 4 KDa peptide which has been widely studied as it plays a crucial role in the development of AD. Aggregated forms of A β can assemble into for the disease characteristic plaques.

1.3.1. A β generation

The generation of A β is a physiological process that occurs in healthy subjects as well as in AD patients. A β monomers have been suggested to act as a modulator of synaptic activity and have neuroprotective functions. The A β peptide is generated from a larger precursor protein, named amyloid precursor protein (APP), which is embedded in the cell membrane. APP can undergo proteolytic processing via two separate pathways. The first one is the non-amyloidogenic pathway (Fig. 1.2A), in which APP is cleaved by α -secretase. Enzymes which have shown α -secretase activity are: ADAM9, ADAM10 and ADAM17. Their characteristic is the ability of cutting within the A β domain, preventing the generation of A β . This cleavage leads to the production of an N-terminal ectodomain (APPs α) in the extracellular space and a C-terminal fragment of 83 amino acids (aa) which remains within the membrane (C83). The latter is further cleaved by the γ -secretase complex (presenilin-1, nicastrin, anterior pharynx-defective 1 and presenilin enhancer 2) to generate a small fragment called P3 peptide and a C-terminal fragment (CTF). In the amyloidogenic pathway (Fig. 1.2B), APP is first cleaved by β -secretase (BACE1) releasing the soluble APP portion (APPs β) and retaining the last 99 amino acids of APP in the membrane (C99). Subsequently, the γ -secretase cuts off the remaining protein in the membrane to produce A β peptide outside of the cell and a CTF in the intracellular space [23,24] .

Figure 1.2. Proteolytic processing of APP. A) Non- amyloidogenic pathway. B) Amyloidogenic pathway.

The proteolytic activity of the γ -secretase complex can result in A β peptide of different lengths, from 38 to 48 amino acids. However, the most predominant A β forms are A β_{40} and A β_{42} [25,26].

1.3.2. Aggregation of A β

A β has been identified as a primary component of extracellular brain plaques (large accumulations of A β) and has been postulated to cause synaptic loss and subsequent neuronal degeneration in AD. Therefore, the characterization of the aggregation behavior of this peptide has been the subject of scientific research. While the 40-residues peptide is the most abundant isoform in a healthy brain, studies have shown that A β_{42} is the most amyloidogenic. The presence of the last two amino acids (isoleucine and alanine) makes A β_{42} more hydrophobic, aggregate at a faster rate and the resulting aggregate species have high cell cytotoxicity [27–29]. Once produced, A β can aggregate to form polymorphic fibrils and a variety of intermediate assemblies,

including oligomers and protofibrils, both *in vitro* and in human brain tissue (Fig. 1.3).

According to the amyloid cascade hypothesis, A β fibril deposits are the cause of nerve cell toxicity in Alzheimer's [24,30,31]. However, more recent research supports the idea that small, soluble aggregates of A β , called 'oligomers' or 'protofibrils' negatively affect neuronal functioning. While some oligomer species can undergo a fibrillization process evolving into fibrils (on-pathway) that seem relatively harmless, alternatively, cytotoxic hold in assemblies that do not transform into fibrils and are referred to as off-pathway oligomers are formed [32,33].

Figure 1.3. Schematic representation of the aggregation mechanism. Monomers associate into oligomers and form a critical nucleus. The critical nucleus is the starting point for further polymerization. Figure modified from [34].

1.3.3. Conversion of A β monomers into fibrils

Studies aimed at the investigation of A β aggregate structures are hampered by the supramolecular polymerization into oligomeric and protofibrillar intermediates and the insolubility of the fibrils. The non-crystalline state and insolubility of the A β aggregates make them not amendable to direct studies using traditional methods. However, soluble A β has been characterized using nuclear magnetic resonance (NMR), circular dichroism (CD) while fourier transform infrared (FTIR), atomic force microscopy (AFM) and transmission electron microscopy (TEM) have been used to determine the structure of fibrillar A β . In fibrils, A β possess a β -turn- β motif. A β consists of an

unstructured and flexible part between aa 1 and aa 17 and a β -turn conformation which involves aa 18 to 42, stabilized by a salt bridge between aa 23 and 28 (Fig. 1.4). Additionally, hydrophobic interactions and intermolecular bonds are responsible for contributing to the aggregated structure of A β [35].

Figure 1.4. Conversion of A β monomers into fibrils. Residues 1-17 constitute a highly disordered and flexible part (grey). Residues 18-42 form the β -turn region stabilized by a salt bridge from aa 23 to aa 28 (red dashed line) and hydrophobic bonds (green residues). Additional intermolecular contacts occur between aa 13 and 40, 42 and 35 (blue dashed line). Figure modified from [35].

Fibrils, formed by A β , assume β -sheet structure with β -strands oriented perpendicular to the fibril axis [36]. A β fibrils are polymorphic at a molecular level [37], meaning that fibrils with different morphology, e.g. symmetry, thickness, twist period and protofilament orientation have also different intermolecular structure [38]. This structural diversity may correlate with variations in disease development [39]. Fibril polymorphisms could be the reason why some report significant toxicity for A β fibrils and other do not. Therefore, different fibril polymorphisms can associate with different degree of toxicity [40]. Moreover, fibrils have been shown to disassemble in smaller

assemblies, such as protofibrils and oligomers, which can in turn be responsible for neurotoxicity.

1.3.4. A β degradation

An imbalance between A β production and elimination plays a key role in AD pathogenesis. In order to degrade dysfunctional or excessively produced peptide and restore its balance, the human body has several control mechanisms to prevent A β accumulation including: proteasomal degradation [41], phagocytosis [42,43], receptor-mediated clearance through the low-density lipoprotein receptor-related protein 1 (LRP1) [44], proteolytic degradation [45] and the glymphatic pathway [46–48]. Enzymes with A β degrading activity have been reviewed by Saido and Leissring and the most studied are illustrated in table 1.

Table 1: Some proteases able to cleave A β [49].

Protease	Localization	Known A β substrate	References
Insulin-degrading enzyme (IDE)	Extracellular space, endoplasmic reticulum, endosomes, lysosomes, mitochondria	Monomers	[50,51]
Neprilysin (NEP)	Extracellular space, endoplasmic reticulum, Golgi	Monomers oligomers	[52,53]
endothelin-converting enzyme (ECE)	Extracellular space, endoplasmic reticulum, endosomes	Monomers	[54]
Matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9)	Extracellular space, endoplasmic reticulum, Golgi	Fibrils	[55,56]
Cathepsin B	Extracellular space, endosomes, lysosomes	Fibrils	[57]

Proteolysis of A β in AD patients has been shown to be decreased particularly by neprilysin (NEP) and Insulin-degrading enzyme (IDE) [58] and therefore these enzymes have received the most attention to date. Several insights have emerged from the study of NEP in APP transgenic mice. Genetic deletion of

NEP resulted in increased A β levels, while its upregulation in a J20 line of APP transgenic mice 14-months old, showed prevention of A β plaque formation [51]. Similarly, IDE knockout mice developed increased endogenous levels of A β in the brain [51]. Other enzymes under investigation for their capability of cleaving A β include: Matrix-metalloproteinases (MMPs) [55,56], endothelin-converting enzymes (ECE) [54], and Cysteine protease Cathepsin B [57]. Enhancing the removal of formed A β is as important as balance its production. Thus, studies and therapies towards A β clearance using proteolytic enzymes have gained considerable recognition in the last years.

1.4. Alzheimer's disease

In 1901 the German psychiatrist Alois Alzheimer described the first case of the disease which was named after him. He described the case of Auguste Deter with profound memory loss, unfounded suspicions in relation to her family, and other worsening psychological changes. The study of her brain at autopsy revealed a dramatic brain shrinkage and the presence of deposits in and around the cells [59].

Alzheimer's disease (AD) is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills, and eventually affects the ability to carry out the simplest tasks. In the final stage, AD can lead to death [60]. Nowadays, 47 million people worldwide are living with dementia. Estimates are that the number of patients will be 76 million by 2030 and 131.5 million by 2050 [61]. By far, AD is the most prevalent neurodegenerative disease and represents a great challenge for society and health-care systems.

In the following sections, several aspects of AD will be discussed.

1.4.1. Diagnosis

The diagnosis of AD is highly complex and involves cognitive assessment throughout questionnaire, knowledge of the family medical history, neurological examination and brain imaging [62]. The latter includes magnetic resonance imaging (MRI). MRI uses radio waves and a strong magnetic field to produce detailed images of the brain. MRI scans are used to rule out other conditions that may account for or be adding to cognitive symptoms. In addition, they may be used to assess whether shrinkage in brain regions implicated in Alzheimer's disease has occurred [63].

Another common imaging technique is positron emission tomography (PET). During a PET scan, a low-level radioactive tracer is injected in a vein. The tracer may be a special form of glucose that shows overall activity in various brain regions. Glucose is the main source of energy of the brain and therefore it is used as biomarker for impaired brain metabolism and synaptic activity in AD patients. This can show which parts of the brain are not functioning well [64]. Moreover, new PET techniques are able to detect level of amyloid plaques and tau tangles in the brain through the radiotracer Pittsburgh compound-B [65].

Currently, the presence of early stage dementia is also confirmed by analysis of established biomarkers in the cerebrospinal fluid (CSF) and plasma [66]. A biomarker is defined as a biologic feature that can be used to measure the presence or progress of disease [67]. Validated CSF biomarkers to diagnose AD include: A β , total tau and phospho-tau-181. It is generally accepted that low A β levels, in combination with increased total tau and phospho-tau-181 in CSF, represents a significant indication of sporadic AD [68]. Other candidate biomarkers under investigation are also related to A β metabolism, neuronal and synaptic degeneration, inflammation, and oxidative stress [69].

1.4.2. Pathology

An Alzheimer's brain is mainly characterized by the presence of abnormal plaques and tangles [70]. The latter, occurs in the entorhinal cortex, while A β plaques initially develop in the hippocampus, area of the brain that is essential for forming memories. That is why short-term memory loss is usually one of the first symptoms of Alzheimer's disease. Plaques and tangles tend to spread through the cortex as the disease progresses. At the final stage brain mass is significantly reduced as a result of large-scale neuronal loss and nearly all brain functions are affected. AD is characterized by three stages: mild, moderate and severe (Fig. 1.5). In the early stage (Fig. 1.5A), before symptoms can be detected with current tests, plaques and tangles form in brain areas involved in: learning, memory, thinking and planning. In the moderate stage (Fig. 1.5B), the hippocampus develops more plaques and tangles than were present in early stages. As a result, individuals develop problems with memory or thinking serious enough to interfere with work or social life. Many people with Alzheimer's disease are first diagnosed in this stage. In advanced Alzheimer's disease (Fig. 1.5C), most of the cortex is seriously damaged. The brain shrinks dramatically due to widespread cell death. Individuals lose their ability to communicate, to recognize family and to care for themselves [71].

Figure 1.5. Progressive illustrations of brain degeneration caused by Alzheimer's disease. The damage develops first in one or more sites in the basal temporal and orbitofrontal neocortex (A). Later, it spreads throughout the hippocampal region (B). In severe cases of AD, A β plaques affects almost all areas of the brain (C). (Figure taken from: Alzheimer's Association@.)

1.4.3. Familial and sporadic AD

AD is classified, based on etiology, onset of symptoms, pathophysiological, biochemical and genetic alterations, into familial (FAD) and sporadic (SAD) cases [72]. Genetic mutations leading to early-onset (<65 years old) AD involve genes encoding for APP, presenilin-1 (PSEN1) and presenilin-2 (PSEN2). However, individual with these genetic mutations represent less than 5% of AD cases [23]. The majority of AD cases is sporadic and is due to a combination of environmental, genetic factors and ageing [73].

1.4.4. Hallmarks of AD

One of the pathological hallmarks of AD includes elevated levels of toxic A β oligomers which represent the precursor of the A β plaques detected upon anatomy of the AD brain [74]. Other than A β peptide containing plaques, characteristic pathologic features of AD are: the accumulation of neurofibrillary tangles [1], mitochondrial dysfunction [75], oxidative stress [76] and inflammation [77].

1.4.5. A β plaques deposition and aberrant tau

Extracellular amyloid plaques (Fig. 1.6, blue arrow) predominantly consist of self-assembled A β forms generated *in vivo* by the amyloidogenic proteolytic

cleavage of APP [78]. The classical amyloid cascade hypothesis assumes that pathological assemblies of A β are the primary cause of both AD forms and all other neuropathological changes (cell loss, inflammatory response, oxidative stress, neurotransmitter deficits and at the end loss of cognitive function) are downstream consequences of A β accumulation. Neurofibrillary tangles (NFTs) consist of intracellular protein deposits made of hyperphosphorylated tau protein (Fig. 1.6, red arrow). Tau protein is a microtubule-associated protein which is involved in stabilization and promotion of axonal microtubules but when hyperphosphorylated it loses its physiological role and aggregates into tangles which exert a toxic function and impact on healthy neurons [79,80].

Figure 1.6. Tissue sample from an AD patient brain reveals AD pathology. Extracellular A β plaques (blue arrow) in concomitance with NFTs, which are mainly composed of hyperphosphorylated tau (red arrow). Brain sections represents the hippocampus area. Figure modified from [81].

1.4.6. Mitochondrial dysfunction

Mitochondria are subcellular organelles that play a fundamental role in neuronal cell survival or death because they are regulators of both energy metabolism and cell death pathways. Extensive literature exists supporting a role for mitochondrial dysfunction and subsequent oxidative damage in brain neurodegeneration. In particular, oxidative damage has been postulated to be one of the earliest events in AD [82]. Mitochondria are the primary site for ATP production a multi-step process that can lead to the formation of damaging byproducts, termed reactive oxygen species (ROS), which accumulate with aging. Hippocampal neurons, important for memory and learning processes, are especially vulnerable to mitochondrial dysfunction. This is because neuronal cells are rich in mitochondria as a result of their high energy consumption. Moreover, Baloyannis reported that, in AD cases, mitochondrial pathology correlates substantially with the dystrophic dendrites, the loss of dendritic branches, and the pathological alteration of the dendritic spines [83].

1.4.7. Neuroinflammation

Inflammation of the nervous system is an early and important phenomenon in AD pathogenesis. Even though this process is meant to have a protective role against damaging insults, prolonged inflammation, as observed in AD, can be the cause of neurodegeneration [84]. The main cell types involved in the neuroinflammatory response are astrocytes and microglia. Microglia are generally in a resting state also called “M0”. Upon exposure to stimuli, they undergo a morphological change and they acquire specific functions. Depending on the nature of the trigger, the microglia change towards a “M1” phenotype which is pro-inflammatory and is characterized by the upregulation of specific receptors that recognize harmful substances, followed by the release of inflammatory mediators e.g. interferons (IFNs), interleukins (ILs), colony stimulating factors (CSFs), transforming growth

factors (TGFs) and chemokines (CKs) [85]. This phenotype is counterbalanced by the “M2” anti-inflammatory state expressing tissue repair factors with a neuroprotective function [86]. A diagram of polarization states of microglia is illustrated in fig. 1.7.

Figure 1.7. Phenotypes and activation of microglia. Under stress conditions, microglia undergo structural changes and become activated. Primed microglia are characterized as either an M1 classically activated phenotype or an M2 alternatively activated phenotype. Figure taken from [87].

It is known that macroglia play a pivotal role in maintaining brain tissue homeostasis by removal of dead neurons, bacteria, lipoproteins and A β itself [88–92]. One of the main genes that caught researcher’s attention and that is involved in microglial phagocytic activity, is Trem2 which is a major sensor for apoptotic cells and aberrant lipids [91–94]. In fact, TREM2-deficient microglia showed reduced uptake of A β -lipoprotein complexes *in vitro* [92] and less evidence of A β internalization *in vivo* [95], suggesting a protective role of TREM2 in AD. On the other hand, initial studies from Wisniewski and co-workers showed that activation of microglia is harmful to AD brain as these cells colocalize in proximity to A β plaques while numerous

inflammatory factors are upregulated in the damaged area of the brain, inducing additional neuronal injury [96].

1.4.8. Current treatments

Unfortunately to date, all the medications approved by the Food and Drug Administration (FDA) only provide temporary cognitive improvement with minor effects on the disease pathology [97]. These include three cholinesterase inhibitors namely Donepezil, Galantamine and Rivastigmine which are used for the treatment of mild-to-moderate AD. These drugs induce increased levels of acetylcholine which favors synaptic neurotransmission. Another drug available on the market is Memantine which helps improving memory, attention and the ability to perform simple tasks. It functions by blocking NMDA receptors (NMDAR) which become inaccessible to glutamate. Following the glutamate-NMDAR stimulation, the neurons depolarize and calcium ions enter the neurons. Abnormal levels of Ca^{2+} starts a signal cascade leading to loss of synaptic function and cell death. These phenomena clinically correlate with cognitive impairment in AD brain [98,99].

An alternative strategy to address AD that was under clinical investigation was based on reducing the amount of plaque using immunotherapy [100]. However, this study was interrupted due to occurrence of meningoencephalitis [101] and the absence of improvement in cognition or delay in disease progression.

1.4.9. Challenges in AD treatment

Due to the estimated increasing number of people suffering from AD [61] and the lack of a proper treatment, there is urgent need for developing an efficient therapeutic approach. The development of therapeutics is hampered by the multifactorial, complex character of this disease. In fact, most current drugs,

aiming at improving patients' quality life, focus on isolated targets which present only part of the disease spectrum.

1.5. Scope of the thesis

This PhD thesis lays the foundations for a novel therapeutic approach by investigating four early disease markers, including: A β deposition, A β degradation, oxidative stress and neuroinflammation, opening new avenues for a multi-target treatment of AD.

The thesis starts with an overview on A β and its role in Alzheimer's disease (Chapter 1). Four main sections follow the introduction. The first section consists of a description of chemical compounds designed to inhibit A β aggregation and toxicity (Chapters 2 and 3). The second section consists of Chapter 4, which investigates the ability of IDE to cleave different A β aggregation species.

Next, the third section reviews the role of oxidative stress in AD and the drugs, currently under clinical trials, aiming at relieving this condition in patients (Chapter 5).

In the last section, the potential link between neuroinflammation and neurodegeneration in AD is explored. In this context, Chapter 6 investigates the microglial IL1- β secretion and loss of neuronal synchronous activity upon exposure to A β and interferon gamma (IFN γ) while Chapter 7 describes the biophysical interaction between A β and IFN γ .

Last, the closing section (chapter 8) consists of concluding remarks and gives some perspectives on promising AD research avenues for the future.

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PART 1:

Anti-aggregation compounds

Chapter 2

Searching for improved peptide inhibitors preventing conformational transition of amyloid- β_{42} monomer to a β -sheet conformation

A series of novel mimetic peptides were designed, synthesised and biologically evaluated as inhibitors of $A\beta_{42}$ aggregation. One of the synthesised compounds, termed compound 7 most potently inhibited cytotoxic $A\beta_{42}$ aggregation as demonstrated by thioflavin T fluorescence and cytotoxicity assays. To better understand the effect of compound 7 on $A\beta_{42}$ aggregation, the early stage interaction between compound 7 and the $A\beta_{42}$ monomer was investigated by replica exchange molecular dynamics (REMD) simulations. Our theoretical results revealed that compound 7 can elongate the helical conformation state of an early stage $A\beta_{42}$ monomer and thus helps preventing the formation of β -sheet structures. It does this by interacting with key residues in the central hydrophobic cluster of amino acids (CHC) in the $A\beta_{42}$ peptide.

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Cioffi F contributed to the experimental results presented by testing the anti-aggregation and antitoxic properties of the compounds (figures 2.2, 2.3 and 2.4). Compounds were designed and synthesized by the group of Daniel Enriz and shipped for testing by Cioffi.

2.1. Introduction

Alzheimer's disease (AD), a complex, progressive, and irreversible neurological disorder, is the most prevalent neurodegenerative disease in humans [1]. By 2030, the number of people with the disease is expected to rise to more than 70 million worldwide [2] [3]. Unless there is a breakthrough in treatment, nearly one in every 2–3 people over 85 years of age will attain AD. Although there is some controversy [4], the most widely accepted theory regarding the etiology of AD is the “amyloid hypothesis” which features the accumulation of fibrillar A β in the brain as the primary driving force of AD pathogenesis. Additionally, this hypothesis poses that AD pathology initiates because of an imbalance in A β production and clearance. This imbalance may result from altered expression or processing of amyloid precursor protein (APP) or changes in A β metabolism [5]. Various A β peptide isoforms arise upon processing of APP, with lengths varying from 37 (A β ₃₇) to 46 (A β ₄₆) amino acids. It is widely accepted that A β ₄₀ and A β ₄₂ are the main produced species with A β ₄₂ being more amyloidogenic and toxic form [6].

In the last fifteen years, growing evidences suggests that small oligomeric aggregates of A β , rather than fibrillar products are the most cytotoxic species in neurodegenerative disease [7,8].

Thus, strategies to prevent or destabilize the formation of A β aggregates, both fibrils and oligomers, are promising approaches in the field of drug discovery against AD. Such approaches may include the blockage of protein–protein interactions responsible for the generation of toxic A β aggregates [9] or the inhibition of conformational transitions between the native disordered conformation of A β and the aggregation-related β -sheet structures [10,11]. Insight into conformational transformations that trigger misfolding and amyloid formation is useful in the design of compounds that target these transitions [12,13]. A broad range of anti-A β aggregation agents and peptidic

inhibitors [14–16], nanoparticles [17], small molecules [18–20] have been designed recently to target protein misfolding.

In order to design new molecules that prevent A β ₄₂ aggregation more effectively, the detailed underlying molecular mechanism of inhibition of A β ₄₂ aggregation should be elucidated. Despite the significant progress in experimental methods that give molecular insights into the interaction between A β ₄₂ and anti-aggregation compounds, experiments only give a limited picture of the effect of small molecules on the early stage of the aggregation. Computational simulations, especially molecular dynamics (MD) can provide atomic level description of conformational transitions involved in peptide–ligand interactions. Recently, a large number of computational studies have been carried out to elucidate the fundamental molecular mechanism of unfolding of the native conformation of A β ₄₂ and to characterize the inhibition mechanisms of current A β aggregation inhibitors at atomic level [20].

In the search for new anti-aggregation agents, Daniel Enriz and co-workers have reported a series of peptides with potent inhibitory activity against the formation of A β ₄₂ aggregates [21,22].

These compounds were based on a molecular modelling study using as molecular target a pentameric A β aggregate [23]. Among these compounds, **DZK** (*N* α ,*N* ϵ -Di-Z-L-lysine hydroxysuccinimide ester, Fig. 2.1) presented the most potent activity in inhibiting fibril formation, therefore in the present work this compound was taken as the starting structure in the search for improved inhibitors addressing aggregation at the level of A β monomers.

Figure 2.1. **DZK** structure (core: in magenta, R1: in blue, R2: in red, R3: in green). Structure features of compounds **1-8**

We aim at developing new compounds that have an inhibitory effect on the formation of A β ₄₂ fibrils using a combination of theoretical-experimental studies. In a first step, taking compound **DZK** as a starting point, Enriz's team designed and synthesised several structurally related molecules. In the second step, the anti-aggregation properties of these new compounds were evaluated by spectroscopy, while their capacity to inhibit A β ₄₂-induced toxicity was monitored in an *in vitro* viability assay. To obtain insights into the molecular level interaction between the active compound and the monomeric A β ₄₂ peptide a molecular modelling study was performed.

2.2. Materials and Methods

2.2.1. Thioflavin T fluorescence assay

Amyloid aggregation was measured by a Thioflavin-T (ThT) fluorescence assay, a common technique which allows monitoring fibril formation [24]. A β ₄₂ was dissolved using a previously published solubilisation procedure using HFIP, DMSO and separation on a desalting column [25]. A β ₄₂ concentration was adjusted to 25 μ M using PBS buffer, pH 7.4 and a final

concentration of 12 μM ThT in a 96-well plate. Fluorescence intensity was measured at 37 °C using an automated well-plate reader (TECAN Infinite 200 PRO) at an excitation wavelength of 450 nm and emission detection from 480 to 600 nm. Measurements were performed as independent triplicates. Fluorescence readings were recorded in triplicates. Recorded values were averaged and background measurements (buffer containing 12 μM ThT and compound) were subtracted. Statistical significance of the results was established by P-values using two-tailed t-tests (GraphPad Software).

2.2.2. Cytotoxicity assay

SH-SY5Y cells were grown in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin and 1% nonessential amino acids (Gibco). Cells were seeded in a 96-well plate at 25,000 cells/well and maintained in phenol-red free DMEM/F12 (L-Glutamine, 15mM HEPES) supplemented with 1% penicillin/streptomycin and incubated at 5% CO_2 . Samples containing 25 μM $\text{A}\beta_{42}$, in presence or absence of 50 or 0.75 μM of compound **7**, were pre-incubated at r.t. for 2 h and added to the cells. As a death control an 8% SDS solution was included. The plates were incubated at 37°C for 48 h, followed by addition of CellTiter-Blue® Reagent (20 μl /well) and incubation for 4 h. The fluorescence intensity (excitation wavelength 560 nm and emission wavelength 590nm) was measured using a TECAN Infinite 200 PRO fluorescence plate reader. The medium background values were subtracted from the values obtained in experimental wells.

2.2.3. Molecular modelling

PDB entry 1IYT was selected as starting monomeric conformation of $\text{A}\beta_{42}$. This conformation was determined by NMR measurements in apolar environment [26], being characterized by two helices between the 8-25 and the 28-39 residues. The starting structure was a highly helical conformation of the monomer in order to start the simulation from a close state of the

cleavage, which according to the literature [27] can occur in an α -helical state of the A β section in the Amyloid Precursor Protein. Compound **7** was optimized first at molecular mechanical level, with PRCG (Polak-Ribiere Conjugate Gradient) method [28] using MacroModel from the Schrodinger suit and it was further optimized with Gaussian09 [29] at HF/6-31g level of theory. The atomic charges were taken from the quantum calculation and attached to the structure parameters with the Antechamber [30] program. Other force constant parameters of compound **7** were based on the GAFF parameter set [31].

2.2.4. Simulation details

Sampling conformational space of monomeric A β_{42} in the presence and absence of compound **7**, Replica Exchange Molecular Dynamics (REMD) simulations were carried out in explicit solvent with the GROMACS 5.1 package using the AMBER99SB-ILDN force field for the A β_{42} peptide. A dodecahedron box with 1.2 nm distance between the box and the solute was taken and solvated with explicit TIP3P water molecules. The system was neutralized with Na⁺ ions and further Na⁺Cl⁻ ion pairs were added to mimic the physiological (0.15 M) salt concentration. Both systems (A β_{42} and A β_{42} + ligand) were energy minimized with 50000 steepest-descent steps. After minimization the system was heated up and 200 ps long NPT and NVT equilibrated at 315 K. For REMD simulations, 48 replicas were taken in the range of 315K and 400K using the temperature distribution provided by the web server of D. Van der Spoel [32]. Each replica was 250 ns long and temperature coupling was applied using velocity rescaling with a stochastic term [33] (0.1 ps time constant), as well as the isotropic Parrinello-Rahman barostat [34] with 0.5 ps time constant. P-LINCS algorithm [35] was selected for hydrogen atom connection constrains and in the electrostatic interaction the Particle Mesh Ewald (PME) method [36] was applied.

2.2.5. Ensemble analysis

Two time periods were selected for analysis from the lowest temperature (315 K) replicas: the first 50 ns and the last 150 ns period. The recently published Dihedral-based Segment Identification and Classification method (DISICL) [37] was applied to calculate the secondary structure distribution for each residue. The contact maps are based on the probability of contacts over the selected periods. Two residues were considered to be in contact if their centre of mass distance is equal or lower than 0.4 nm.

2.3. Results and Discussion

2.3.1. Searching new inhibitors preventing conformational transition of A β ₄₂ monomer

In search for new inhibitors with improved potency to inhibit A β ₄₂ aggregation, Daniel Enriz and his team previously reported compound **DZK** (Fig. 2.1). This compound was used as initial structure from which further structures were explored. The general structure of this compound consists of four parts: the core (amino acid Lys) and three substituents located at its α -carboxylic (R1), α -amine (R2) and ϵ -amine group (R3). The strategy chosen for the design of the eight new structures was based on the modification of natural amino acids with aromatic or hydrophobic substituents hypothesised to interact with the central hydrophobic cluster (CHC) of the A β ₄₂ peptide. Compounds **1**, **2** and **3** presented minor variations compared to **DZK** by maintaining the Lys core and introducing new substituents in R1=Z (compound **1**); R2=Fmoc (compound **2**); and R3=Boc (compound **3**). Compounds **4** and **5** not only presented modified substitution patterns with R2=R3=Fmoc but also extra residues at R1 like Phe (compound **4**) and Ala-Gly (compound **5**). Finally, compounds **6**, **7** and **8** presented major variations losing the characteristic Lys core. Compound **6** core was formed by the dipeptide Phe-Gly; compound **7** by Asp-Tyr; and compound **8** by Glu-Tyr.

These last three structures were modified in their N-terminal moieties with the Fmoc group as substituent.

To evaluate the inhibition of A β ₄₂ aggregation into amyloid fibrils, a ThT study was performed in which A β ₄₂ peptide was incubated for seven days at 37°C in the absence and presence of this new series of compounds. Of the eight studied mimetic peptides only compound **7** was considerably more potent at inhibiting ThT-positive A β ₄₂ aggregation compared to the previously reported **DZK** (Fig. 2.2) [21]. Namely, 100 μ M DZK was needed to reduce the amount of ThT positive aggregates by 50% while compound **7** reached similar inhibitory activity at a 3 μ M concentration.

Figure 2.2. A β ₄₂ amyloid aggregation is affected by compound **7**. Dose-response amyloid fibril formation of 25 μ M A β ₄₂ incubated in the presence of compound at 37° C for 7 days was monitored using ThT fluorescence intensity at 485 nm. Values represent results of three independent replicates. Statistical significance of the results was established by P-values using paired two-tailed t-tests. ***P < 0.0005.

To obtain further insight into the binding of compound **7** to early A β ₄₂ aggregate species a ThT assay was performed monitoring fluorescence

intensity at different times of incubation. Similar to the 7-day incubation shown in Fig. 2.2, after 1.5 and 5 hrs ThT-positive A β ₄₂ aggregate formation was diminished suggesting its potential interaction with A β ₄₂ monomers or early A β aggregated species (Fig. 2.3). Although, after 24 hrs, there is a trend towards a reduction in the amount of ThT-positive fibrils, these changes are not significant.

Figure 2.3. Dose-response amyloid aggregation of 25 μM A β ₄₂ incubated in the presence of compound **7** at 37° C for 1.5, 5 and 24 hours was monitored using ThT fluorescence intensity at 485 nm. Values represent results of three independent replicates. Statistical significance of the results was established by P-values using paired two-tailed t-tests. Statistical significance levels were * P < 0.05, **P < 0.005 and ***P < 0.0005.

The inhibitory effect of compound **7** on A β ₄₂ aggregation was further established using a cytotoxicity assay. Based on the result of the ThT measurements, showing that compound **7** affects A β at a monomeric levels or early aggregated species, we wanted to test whether it is able to inhibit the

well-established toxicity of oligomeric A β [7,8]. A cell viability assay, Cell Titer-Blue, demonstrated that 25 μ M (based on monomeric concentration) of oligomeric A β_{42} induced significant loss of viability in an SHSY-5Y neuroblastoma cell line. Co-incubation of A β_{42} oligomers with compound **7** at a concentration as low as 0.75 μ M resulted in complete prevention of A β_{42} -mediated cell toxicity. Moreover, the compound itself was found to be non-toxic at concentrations up to 200 μ M compared to SDS 8% which was used as a control for cell death (Fig. 2.4).

Figure 2.4. Cell viability assay using the CellTiter-Blue reagent with SHSY-5Y cells exposed to A β_{42} oligomers in presence or absence of compound **7**. Controls include 8% μ M solution of sodium dodecyl sulfate, full-length A β_{42} , compound and cells in buffer. The results are represented as mean values $\pm$ standard error of the mean. Values represent results of three independent replicates. Significant p-values are indicated by: *P < 0.05, **P < 0.01 and ***P < 0.001.

2.3.2. Molecular Modelling

In order to have an atomistic insight into the experimentally hard to realize early stage effect of the most promising ligand, computational investigations were carried out regarding the interaction of compound **7** and the monomeric A β ₄₂. MD analysis was applied to the computationally derived A β ₄₂ peptide ensemble, which represent the conformational space immediately after the enzymatic cleavage as well as for a more general state of the A β ₄₂ monomer. Two parts of the trajectories were examined using replicas at the lowest temperature (315 K). The simulations were started from a highly helical experimental conformation of the peptide which mimicked an initial stage after cleavage and the first 50 ns were selected to see how the presence or the absence of the ligand affected the helical content. Secondly, the last 150 ns parts were considered from the same replicas to examine the effect of the ligand on the conformation space of the A β ₄₂ monomer characterized by a higher disordered content.

2.3.3. Maintenance of the original helical content

As was outlined in Barrera Guisasola's previous publications [21,38], a possible strategy to decrease the toxicity of the A β ₄₂ peptide is to avoid its transition to a β -sheet conformation, which is related to the formation of toxic oligomers. This goal can be achieved by stabilizing the early stage conformation of the A β ₄₂ monomer characterized by its high α -helical or random coiled content. Therefore, we employed the model from the PBD entry 1IYT as our starting configuration, which has been determined in an apolar environment and is characterized by two α -helical regions (residues 8-25 and 28-39). Considering the first 50 ns from the REMD simulations we found that compound **7** inhibited the loss of helical structure especially on the C-terminal region compared to the pure A β ₄₂ monomer simulation (see Fig. 2.5).

Figure 2.5. The α -helical content of the $A\beta_{42}$ peptide in the presence (red) and the absence (blue) of compound **7** taking the first 50ns of the trajectories at the lowest temperature.

To elucidate the contact between the protein and the ligand we calculated their centre of mass distances (the resulting contact distribution is shown in Fig. S1, Supplementary material). It was found that the largest number of contacts between the ligand and the monomer peptide was found within the helical regions in particular the one comprised between the residues 16 to 20. As Fig. 2.5 presents, the helical content also increased partly in this region between $K^{16}LV^{18}$ residues. It is worth to note that the hydrophobic interaction between the β -strand $K^{16}LVFFA^{21}$ and $N^{27}KGAIIG^{33}$ regions has important role in the formation of the β -hairpin conformation.

2.3.4. Secondary structure elements progression

To obtain insight into the effect of compound **7** on the monomeric state of $A\beta_{42}$ peptide, the secondary structure elements between the ligand+monomer and the free monomer were compared using the last 150 ns of the replicas with the lowest temperature. The difference between the probability of α -helix (red), 3_{10} -helix (blue) and β -strand (green) for each residue is presented in Fig. 2.6.

Figure 2.6. Difference between the probability of secondary structure elements adopted by the A β ₄₂ peptide in the presence and absence of compound **7** taking the last 150ns of the trajectories at the lowest temperature. Colour code: α -helix (red), 3_{10} -helix (blue) and β -strand (green).

This analysis showed that, in the presence of compound **7**, the β -strand content was significantly diminished at K¹⁶LVFFA²¹ (CHC), S²⁶N²⁷ and A³⁰I³² segments. These residues are mainly involved in the formation of hairpin structures in the A β ₄₂ toxic aggregates [39,40]. This difference observed with the free ligand system had as counterpart increased 3_{10} -helix propensities between S⁸GYEYH¹³ and CHC region. We also observed higher α -helical distribution between N²⁷KGAIIG³³ residues. These results are consistent with theoretical [41] and experimental [42] studies which posit that the preservation of these helical regions can stop oligomerization at the dimer stage by impeding unfolding and β -sheet formation.

2.3.5. Contacts between A β ₄₂ monomer and compound **7** during REMD simulations

Compound **7** was divided into three fragments namely Fmoc, Asp and Tyr-OH. These segments presented different interactions with the A β ₄₂ monomer *i.e.* hydrophobic (Fmoc, Tyr-OH), ionic (Asp) or hydrogen-bond (Tyr-OH,

peptide-bonds). Then, the centre of mass distances between each residues of the protein and the three fragments was calculated, where the critical distance for a contact was defined as less than or equal to 0.4 nm. Fig. 2.7 shows the contact distribution per residue between the A β ₄₂ monomer and the three major parts of the ligand. The highly aromatic Fmoc group often forms contacts with residues 12-19 and 28-38 providing a putative explanation for the inhibitory capacity of compound **7** on the conformational transition of the A β ₄₂ monomer.

Figure 2.7. Distribution of the contacts between compound **7** and the A β ₄₂ monomer in the last 150ns of the trajectory taking the lowest temperature.

2.4. Conclusions

In previous work Enriz's group reported that a small molecule, **DZK**, possesses a potent inhibitory effect on A β ₄₂ aggregation [21]. Here, we investigated experimentally a new series of mimetic peptides based on the **DZK** molecule complemented with a detailed theoretical analysis for a selected case. Among the tested molecules, compound **7** was more potent in inhibiting the formation of ThT-positive A β ₄₂ aggregates than the original

DZK molecule. A more extensive evaluation including a ThT assay monitoring fluorescence intensity at different times and a cell viability assay allowed us to obtain further insight into the inhibitory effect of compound **7** on the A β ₄₂ aggregation process.

Moreover, to obtain molecular insights into the aggregation inhibitory interaction between A β monomers and compound **7**, different computational modelling techniques were employed to identify the interactions of compound **7** with A β ₄₂ monomer and look for possible explanations of how this compound affects the conformational transition of the peptide into to a β -sheet structure. These simulations enabled us to obtain a picture for the experimentally hard to attain, very early stage interaction with the ligand at atomic level. REMD simulations showed that compound **7** diminished the formation of aggregation-prone structures by maintaining helical content which were reproduced by our ligand-free control simulations. It was also found that the β -strand structure was significantly diminished at residues K¹⁶LVFFA²¹. Regarding helical structures, the α -helical distribution was higher at N²⁷KGAIIG³³ residues and 3₁₀-helix propensities were increased at N-terminal in the presence of compound **7**. These secondary structure results are in good agreement with experimental [50] and theoretical [51] studies where it had been indicated that the loss of helical content can be observed during the early stage of toxic species formation.

In conclusion, our experimental and theoretical work demonstrated that compound **7** interferes at early steps of the A β aggregation process and has a nearly two orders of magnitude higher inhibitory effect on A β aggregation as its predecessor, **DZK**.

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2.6. Supplementary Information

Figure S1

Figure S1. Distribution of the contacts between compound **7** and the Aβ₄₂ monomer in the first 50ns of the trajectory taking the lowest temperature.

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Chapter 3

Benzodifurans for biomedical applications: BZ4, a selective anti-proliferative and anti-amyloid lead compound

In order to evaluate benzodifuran-based molecules for their anti-cancer and anti-amyloid activities, we explored a novel compound (BZ4) in comparison with other known benzodifuran analogs, previously studied in Roviello's group. Our results show that BZ4 exerts an antiproliferative activity on different cancer cells without affecting non-cancerous control cells and alters the aggregation properties of β -amyloid ($A\beta$), as ascertained by fluorescence spectroscopy. An overall, qualitative picture of the mechanism of the biological activities of BZ4 is discussed in light of circular dichroism, fluorescence data, and the metal ion-binding properties of BZ4.

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Cioffi F contributed by testing the anti-aggregation and antitoxic properties of the compounds (figures 3.5, 3.6 and S2-S4). Compounds were designed and synthesized by Roviello's group and shipped for testing by Cioffi.

3.1. Introduction

Benzofuran derivatives are heterocyclic compounds found in natural sources, as well as synthetic materials. They have been extensively studied as therapeutic agents for several cancer forms. Benzofurans show a marked therapeutic activity which is associated with their binding to receptors including the estrogen receptor alpha (ER α) [1] and with their potent aromatase inhibitory activity [2,3]. Due to the increasing need to develop new effective anticancer drugs and taking into account the great potential of benzofuran compounds, we explored the anticancer potential of their tricyclic analogs, benzodifurans. These compounds are mostly investigated in the field of new materials, especially as organic dyes in the development of solar cells [4], and only few studies have been addressed thus far at their biological activity [5–7]. Recently, the synthesis and structural characterization of various substituted benzodifuran derivatives, as well as their biological activity has been reported in the literature. These compounds showed interesting antiproliferative effects on different cancer cell lines, also with cell-selective activity [8]. Among the three novel benzodifurans previously described, only **BZ1** (Figure 3.1) was able to bind Cu²⁺ ions, suggesting that copper ion deprivation is not a mechanism of action common to all these compounds and that probably each benzodifuran derivative exerts cytotoxic effects by virtue of different mechanisms or different combinations of them [8]. Overall, these benzodifuran derivatives proved to be valuable lead compounds in anticancer therapies, but their poor solubility (in some cases limited also in dimethyl sulfoxide) impaired further investigations into their biological activity.

Thus, with the aim of improving the solubility of **BZ1**, the most promising compound among the previously investigated benzodifuran derivatives [8], we here designed a new analog with longer oligoethylene glycol chains,

named **BZ4** (Figure 3.1). **BZ4** may have better solubility and in general a good hydrophilic-lipophilic balance for the studied benzodifuran compounds, as previously demonstrated for other small molecules [9–11].

Figure 3.1. Chemical structure of **BZ1**, **BZ2**, **BZ3** and **BZ4** derivatives.

The synthesis and characterization, by spectroscopic and spectrometric techniques, of **BZ4** is reported by Roviello's group in the original paper (doi: 10.4155/fmc-2018-0473). In search for effective antineoplastic small molecules [12,13], we explored the antiproliferative activity of **BZ4** on different human cancer and normal control cells. We compared the observed effects to the previously investigated **BZ1**, **BZ2** and **BZ3** derivatives (Figure 3.1) [8]. Furthermore, to shed light on the potential biological targets and mechanisms at the origin of the biological activity of **BZ4**, we evaluated the ability of **BZ4** to bind various DNA model systems (in single strand, duplex and G-quadruplex forms) and/or Cu^{2+} ions.

Moreover, benzofuran compounds are also screened as potential drugs for the treatment of AD. Thus, we evaluated the ability of **BZ4** to inhibit A β toxic aggregates formation. Aggregation and deposition of A β peptide leads to pathogenic plaques observed in the brain of Alzheimer's disease (AD) patients, the most common form of neurodegenerative diseases [14]. A β_{40} and A β_{42} are the two prevailing isoforms of A β peptide in the human brain, of which the longer A β_{42} isoform more readily self-assembles into aggregates [14,15]. Aggregation of A β_{42} peptides is an early event in the disease progression of AD. Thus, any strategy interfering with the A β self-assembly is potentially of high therapeutic interest in order to retard or prevent AD onset [15]. Several compounds of different nature were identified in the past as effective inhibitors of amyloid formation [16,17], and among these, a pivotal role is played by aromatic or heteroaromatic compounds such as curcumin or quinone derivatives [18,19]. Interestingly, compounds containing benzofuran moieties have been described to bind amyloid plaques, as well as neurofibrillary tangles [20]. They can play an important role in decreasing A β -induced toxicity by interfering with A β binding to plasma membrane [21], as well as inhibiting A β aggregation [22,23]. A specific recognition site for benzofurans on beta-amyloid, different from the recognition site of non-benzofuran A β de-aggregators, is held responsible for the dissolution of amyloid fibrils by benzofurans [24].

Benzofuran-based derivatives were previously shown to interact with *in vitro* generated A β_{40} aggregates with subnanomolar binding affinity [25]. However, these compounds also showed slow wash out and long retention in the brains of control mice suggesting non-specific interactions with brain constituents other than A β aggregates. Redesign of benzofuran-based derivatives aimed at retaining their high binding affinity but improving their selectivity is thus required.

In this context, we study the effect of the benzofuran compound BZ4 on the aggregation of A β into amyloid fibrils and on A β aggregate toxicity on primary mouse cortical neurons.

The A β ₄₂ anti-aggregation potential of BZ4 as well as of the previously described benzodifurans [8] were evaluated by means of fluorescence assays (thioflavin T, ThT).

3.2. Materials and methods

3.2.1. MTT assays on HeLa, Hep-G2, HDF, WM266 cells.

HeLa, Hep-G2, and HDF cells were maintained in DMEM and WM266 in RPMI supplemented with 10% FBS, 2mM L-glutamine, 100 μ g/mL streptomycin in a humidified 5% CO₂ atmosphere at 37 °C as previously reported [26].

BZ4 (10 mg) was further purified on a prepacked C18 mini-column and 2 mg of the collected compound were dissolved in DMSO, so to have a ca. 2 mM stock solution. Cisplatin was dissolved in 0.9% NaCl aq. solution.

A total of 2500 HDF cells and 3000 for all the other cancer cell lines were plated in 96-well tissue culture microplates and incubated at 37 °C for 24 h. The benzodifuran or cisplatin solution was added to the cells at the proper concentration. As control, an equal amount of DMSO or 0.9% NaCl, respectively, was used. MTT assay was performed after 24 h of treatment.[27] In short, for each well, 100 μ L of a MTT solution at a 0.5 mg/mL final concentration, diluted in medium without phenol red were added and incubated for 4 h. Then, the MTT solution was discarded and formazan crystals were dissolved in isopropanol containing TRITON-X100 (10%) and HCl (0.04-0.1 N). Finally, the absorbance was determined at 570 nm by an automated spectrophotometric plate reader. The experiments were repeated at least in triplicate.

3.2.2. Primary Cortical Neuron Culture preparation.

These cultures were prepared from E14-16 mouse embryonic brains. Cells were seeded in a 96-well plate at 60.000 cells/well on polyethylenimine (PEI) precoated plates. To prepare the culture medium, neurobasal medium (Gibco) was supplemented with 2% (v/v) of B27-supplement, 0.5 mM glutamine, and 0.2% P/S. Cells were first cultured in this medium for 48 h after which cytosine arabinoside (Sigma) was added at a final concentration of 10 μ M for 24 hours in order to inhibit the growth of non-neuronal cells. Afterwards, the medium was substituted with fresh neurobasal medium and after a week the neuronal cells (<1% contaminating glial cells) were treated for the MTT assay performed as described before.

3.2.3. CD experiments

For the experiments with DNA, a 2 μ M solution of the oligonucleotide - dissolved in a phosphate buffer solution containing NaH_2PO_4 (10 mM) and KCl (100 mM), pH 7.2 - and a benzodifuran 1.5 mM stock in DMSO were used. The CD titration curves were recorded after the addition of increasing aliquots of the ligand (0.5 equiv. at a time, 0.65 μ L) up to 5 equiv. (10 μ M concentration) to the various DNA solutions. Each sample addition was followed by a suitable equilibration time after which the spectra were recorded. In all cases, no DMSO contribution to the oligonucleotide CD spectra was detected. We obtained the melting curves recording the CD at 290 nm (in case of G4) or 276 nm (for duplex DNA) upon temperature increase with a scan rate of 1 $^{\circ}\text{C}/\text{min}$. T_m values were obtained by the first derivative method.

3.2.4. Copper(II) binding experiments

The binding experiments were monitored by UV/Vis spectroscopy as follows: **BZ4** was used at 25 μ M in PBS at 25 $^{\circ}\text{C}$ and pH 7.2, in order to have a stable UV signal over the experiment period. Then, 2 equivalents of a Cu^{2+} solution (withdrawn from a 10 mM stock solution of copper(II) chloride) were added.

Thereafter, UV/Vis spectra were registered for an overall period of 3 h subsequent to the addition of the metal salt. Each experiment was performed in duplicate.

3.2.5. A β peptide solubilization

Solutions of recombinant A β_{42} peptide (rPeptide) were prepared according to a previously published procedure [28]. In short, A β was sequentially dissolved in hexafluoroisopropanol (HFIP) and DMSO. The DMSO was removed from the A β solution by using a HiTrapTM desalting column (GE Healthcare) and elution with PBS at pH 7.4. We measured the A β_{42} concentration by the Coomassie (Bradford) Protein Assay kit and, afterwards, the final concentration required for the subsequent experiments was achieved by dilution in PBS buffer. A β peptide aggregation, in the presence or absence of **BZ4**, was evaluated at 37 °C under quiescent conditions.

3.2.6. Thioflavin-T assay

Amyloid fibril formation was measured by a ThT fluorescence assay. We adjusted the A β_{42} concentration to 25 μ M diluting the peptide solution with PBS buffer (pH 7.4), while a final ThT concentration of 12 μ M was realized in a 96-well plate—(Greiner flat bottom transparent black, Sigma – cat. M9685). The emitted fluorescence intensity of ThT was measured upon excitation at a wavelength of 450 nm by detection of emission in the wavelength range 480 - 600 nm. The maximum fluorescence intensity at 485 nm was reported as a function of different concentrations of **BZ4**. We performed measurements in triplicate, averaged the values recorded and subtracted background measurements that corresponded to buffer containing 12 μ M ThT and the tested benzodifuran compound.

3.3. Results

3.3.1. Antiproliferative activity of BZ4

The novel benzodifuran derivative **BZ4** was tested for its antiproliferative activity using the MTT assay (Figure 3.2). Three human cancer cell lines (HeLa, Hep-G2, WM266) and non-cancerous human dermal fibroblasts (HDF) were incubated with 10 μ M **BZ4** for 24 h, using cisplatin at the same concentration as a positive control. While **BZ4** induced moderate cytotoxic activity on the three cancer cell lines, no effect was observed on HDF cells. In control experiments, cisplatin reduced the viability of non-cancerous HDF by 33%. This selective negative effect of BZ4 on the viability of cancer cells was similar to what was previously found for other benzodifuran derivatives [8]. The observed selectivity makes **BZ4** an interesting starting compound to develop new potential anticancer drugs.

Figure 3.2. BZ4 is selectively cytotoxic effect to cancerous cell lines. Cells were incubated with the tested compounds at 10 μ M concentration at 37° C for 24 h. Proliferating cells are reported as percentage respect to vehicle treated cells ('control') and are expressed as means $\pm$ SE. All cell experiments were carried out at

least in triplicate. Statistical significance was analyzed using Student's *t* test, unpaired, two-sided with (* $p < 0.05$). HDF: human dermal fibroblast; HeLa: Human cervix adenocarcinoma cells; HepG2: Liver hepatocellular cells; WM266: Malignant melanoma. In order to elucidate the possible bioactivity mechanism of **BZ4**, its ability to bind various DNA model systems and biologically relevant metal ions, specifically involved in cancer, as well its aggregation properties, were also evaluated.

3.3.2. Studies on the interaction with DNA

Oligonucleotide binders play a key role in the development of new anticancer drugs as they interfere with tumor cells growth. Small molecules that can bind to duplex DNA, forming DNA/small molecule complexes, exhibit increased stability compared to free DNA, and can induce cell death by interfering with the cells ability to interact with the DNA [29]. In particular, quadruplex DNA formed in telomers, induced or stabilized by synthetic molecules, blocks the telomerase that would elongate telomers in cancer cells. This is a well-known mechanism for anticancer strategies [30–32]. For this reason, great interest has been recently devoted to the synthesis of molecules able to selectively bind with nucleic acids strands [33–36]. The interaction of **BZ4** with DNA in solution was here analyzed using the following model systems: i) three random coil, single stranded oligodeoxyribonucleotides (ODNs **A**, **B** and **C**), ii) a bimolecular 12-mer duplex (from the association of complementary ODNs **A** and **B**), and iii) a unimolecular G-quadruplex (G4) obtained by folding of a tract of the human telomere sequence (the 26-mer **tel₂₆**), an important target in anticancer strategies. ODNs **A** and **B** contain consecutive purines in their sequence, while ODN **C** does not contain consecutive purines. All the interaction studies were carried out in a 100 mM KCl-phosphate buffer solution (pH 7.2), analogous to recent works [8,37,38]. As the benzodifuran did not show a CD signal at the chosen concentrations (data not shown), we

followed the CD signal relative to DNA as a consequence of the interaction with our compound in order to evaluate the ability of the latter to induce secondary structure perturbation in the DNA model. CD experiments were carried out by adding increasing equivalents of **BZ4** to each selected DNA system (2 μM concentration). Thus, we monitored the difference in the CD spectrum of the ODNs after each addition. In Figure 3.3 and S4, the overlapped CD spectra of the ODN systems alone and in the presence of 5 times higher concentration of **BZ4** (final point of the titration experiments) are reported.

Figure 3.3. DNA binding spectroscopic studies. **a)** Overlay of CD spectra of the single strand ODN and **(b)** the G4 tel₂₆ (2 μM each) in the absence (black lines) and presence (red lines) of 5 equiv. of **BZ4**, in a 100 mM KCl-phosphate buffer solution (pH 7.2). Each measurement was repeated three times (the error on the calculated ΔCD value was ± 0.07 mdeg). In the figure a representative example for each system was reported. CD: Circular dichroism; ODN: Oligodeoxyribonucleotide.

Minor changes in the CD spectra of the single strands containing consecutive purines in the sequence (**A** and **B**, Figure 3.3a and S1a) were observed in the CD spectra, whereas no effect in the case of **C** (the ODN without consecutive purines; Figure S1b) was found.

Regarding the structured DNA systems, the spectra with **BZ4** produced minute variations in the CD spectrum of the G-quadruplex structure (Figure 3.3b), and no effect on the duplex **A/B** conformation was observed (Figure S1c). CD is routinely used to investigate interaction of synthetic drugs with nucleic acids as well as proteins. CD signal will not change if no interaction between the ligand and the DNA occurs. On the contrary, when a ligand affects significantly the secondary structure of a DNA, the CD spectrum of the unbound DNA will be different from the one in which the ligand is added. It thus seems unlikely that the DNA-binding is the main mechanism of action for the observed antiproliferative activity of **BZ4**.

3.3.3. Ability of **BZ4** to bind copper II ions

Considering the crucial role that copper plays in tumor progression [39] and the observed effect of copper chelators as anticancer drugs by blocking tumor angiogenesis and growth in animal models [40], we also investigated the capability of **BZ4** to bind copper(II) ions. UV can allow one to understand if there is an interaction between the compound and copper ion by comparing the UV spectra before and after the ligand addition. Differences in these spectra, and particularly the appearances of new UV bands, are typical of copper complexation. The UV/Vis-monitored binding experiments were performed by incubating **BZ4** with 2 equivalents of a Cu^{2+} solution and spectra were recorded over a 3 h period.

As showed in Figure 3.4a, the incubation of **BZ4** with CuCl_2 for 20 min resulted in the appearance of a new band at 550 nm in the UV-Vis spectrum, indicative of copper (II) binding in agreement with literature reports [41,42].

It is interesting to note that the benzodifuran **BZ1** spectra showed similar changes in the presence of copper II ions, whereas these spectral changes were absent in experiments with **BZ2** and **BZ3** indicating these two compounds were not able to bind Cu^{2+} , as we previously reported [8]. Analyzing the structures of all the benzodifurans (Figure 3.1) and their copper-binding ability, we conclude that the Cu^{2+} coordination involves the amino groups on furan rings, as well as the ether oxygen atoms of the ester chains. In analogy to **BZ1** [8], we can hypothesize a hexacoordinated copper(II) dimeric complex (2 **BZ4** molecules *per* Cu^{2+} , Fig. 3.4b), as repeating unit of a multimeric supramolecular network which could arise due to the symmetric nature of **BZ4**.

Figure 3.4. Copper (II) binding studies. **A)** Overlay of UV-Vis spectra of a **BZ4** solution (25 μM) in PBS (black line), and a solution containing both **BZ4** (25 μM) and CuCl_2 (50 μM) in PBS at different time points (5 min after Cu^{2+} addition = red line, 20 min = blue, 1 h = magenta, 3 h = green); **B)** hypothesized coordination of the copper(II)-**BZ4** complex.

3.3.4. Ability of **BZ4** to interfere with $\text{A}\beta$ aggregation propensity

To assess the anti-aggregation property of **BZ4**, $\text{A}\beta_{42}$ aggregation was monitored at different time points (1.5, 5 and 24 h) upon incubation at 37 $^{\circ}\text{C}$, in the presence or absence of **BZ4** using ThT fluorescence. ThT is a dye

commonly used to follow the aggregation of A β ₄₂, as it generates a characteristic fluorescence emission at 485 nm upon binding to amyloid β -sheet structures [43,44]. The incubation of the peptide alone generated an increase in ThT fluorescence over time, indicating that the peptide self-assembled into ThT-binding fibrils. On the other hand, when A β ₄₂ was incubated with **BZ4** at various concentrations, fibril formation was significantly inhibited, as shown by the lower ThT fluorescence intensities (Figure 3.5). The effect of **BZ4** on A β ₄₂ aggregation is very well visible at concentrations from 25 μ M.

Figure 3.5. BZ4 exerts an inhibiting effect on the amyloid fibril formation of A β ₄₂. Solutions containing A β ₄₂ at a concentration of 25 μ M were incubated in the presence and absence of **BZ4** at 37 °C for 1.5, 5 and 24 h. Amyloid fibril formation was detected using ThT fluorescence intensity measurements at a detection wavelength of 485 nm. The reported values represent the results obtained for three independent experiments. The statistical significance of the replicates was assessed by p-values using paired two-tailed t-tests (GraphPad Software) with *p < 0.05 and **p < 0.005.

Anti-aggregation activity of **BZ4** was compared with those of the previously synthesized benzodifuran derivatives of Figure 3.1 [8]. Interestingly, compounds **BZ1**, **BZ2** and **BZ3** showed no significant inhibitory activity on A β ₄₂ aggregation, indicating that the specific structural features of **BZ4** may be essential for this effect (Figures S2-S4).

Having found this activity only for **BZ4**, in view of further exploring its potential as a therapeutic agent in neurodegenerative diseases, we tested the cytotoxicity of BZ4 on primary mouse cortical neurons using the MTT assay. We found that **BZ4** was not affecting the viability of the cells up to the maximum tested concentration of 20 μ M, compared to cytotoxic staurosporine (Figure 3.6).

Figure 3.6. Cell viability assay using the MTT protocol on primary mouse cortical neurons exposed to 1, 2 and 20 μ M concentrations of BZ4. A 2 μ M solution of staurosporine and non-treated cells were used respectively as negative and positive controls. Statistical significance was analyzed using Student's t test, unpaired, two-sided. All the experiments were performed in triplicate (* $p < 0.01$).

Interestingly, in analogy to **BZ4**, another polycyclic aromatic compound, imidazoquinoline dactolisib (NVP-BEZ235), also showed antiproliferative activity [45]. Additionally it also showed significant neuroprotective effects as evidenced by the reduced hippocampal neuronal death induced by A β as well as this compound could improve the memory deficits induced in mice submitted to hippocampal A β injection [46]. The dual activity of dactolisib relies on the PI3K/mTOR pathway involved in protection against neurodegeneration in hippocampal neuronal cultures and mice whilst inducing an antiproliferative effect [46].

3.4. Conclusions

In this paper, we report the evaluation of the properties of a new benzodifuran derivative, named **BZ4**, including anticancer and anti-amyloid activities, as well as its ability to interact with several DNA model systems. **BZ4** was designed starting from a previously reported benzodifuran derivative (**BZ1**), showing biological activities, by elongating the two oligoethylene glycol chains, with the aim of improving its solubility in aqueous solutions and, in general, the hydrophilic-lipophilic balance of the molecule. The newly synthesized compound showed significant antiproliferative activity on a panel of human cancer cells, but not on non-cancerous HDF or primary neuronal cells. The antiproliferative activity of **BZ4** could be due to its ability to act as a copper (II) ion chelator, as the formation of a Cu²⁺-**BZ4** complex was observed by UV spectroscopy. Moreover, thioflavin T fluorescence assays showed that **BZ4** inhibits the aggregation of A β peptide, one of the most relevant targets in current therapeutic strategies to treat AD. In contrast, fibril formation was not significantly affected by **BZ1** and **BZ2**, and was potentiated by incubation of A β peptide with the most apolar derivative of the benzodifuran series (**BZ3**). These aspects, along with the absence of toxicity

on neuronal primary cells, provide an interesting picture of the properties of this compound. This novel species could be a valuable core compound to generate libraries of optimized analogs in the search for new anticancer and anti-Alzheimer drugs investigating the final benzodifuran effective extra- and/or intracellular targets and its potential mechanism of action.

3.5. Future Perspective

Overall, these findings indicate **BZ4** as a valuable compound to evolve potential candidate drugs for anticancer and β -amyloid-associated neuropathies and strongly encourage further efforts in the search for benzodifuran derivatives with pharmacological activity.

Besides the therapeutic applications that could derive from the present work, in future also the diagnostic potential of benzodifurans in disease imaging could be investigated, in analogy to previous reports on aromatic molecule applications in this area [47–51].

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3.7. Supplementary Information

Figure S1

Figure S1: Overlapped CD spectra of the single strands ODN B and C, and the duplex A/B (2 μ M each structure) in the absence (black lines) and presence (red lines) of 5 equiv. **BZ4**, in phosphate buffer at pH 7.2 containing 100 mM KCl. Each titration was repeated three times (the error on the calculated ΔCD value was about ± 0.07 mdeg): in the figure a representative example for each system was reported.

Figure S2

Figure S2: Dose-response amyloid fibril formation monitoring of 25 μM Aβ₄₂ solutions incubated with **BZ1** at 37 °C for 1.5, 5 and 24 h using ThT fluorescence detection at an emission wavelength of 485 nm.

Figure S3

Figure S3: Dose-response amyloid fibril formation monitoring of 25 μM $\text{A}\beta_{42}$ solutions incubated with **BZ2** at 37 °C for 1.5, 5 and 24 h using ThT fluorescence detection at an emission wavelength of 485 nm.

Figure S4

Figure S4: Dose-response amyloid fibril formation monitoring of 25 μM $\text{A}\beta_{42}$ solutions incubated with **BZ3** at 37 °C for 1.5, 5 and 24 h using ThT fluorescence detection at an emission wavelength of 485 nm.

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PART 2:

Proteolytic Cleavage of A β

Chapter 4

Characterization of insulin-degrading enzyme-mediated cleavage of A β in distinct aggregation states

To enhance our understanding of the potential therapeutic utility of insulin-degrading enzyme (IDE) in Alzheimer's disease (AD), we studied *in vitro* IDE-mediated degradation of different amyloid-beta (A β) peptide aggregation states. Our findings show that IDE activity is driven by the equilibrium between A β monomers and higher order aggregates. Moreover, we identify Met₃₅-Val₃₆ as a novel IDE cleavage site in the A β sequence and show that A β fragments resulting from IDE cleavage form non-toxic amorphous aggregates.

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Cioffi F contributed to the mass spectrometry measurements (figures 4.2, 4.3, 4.4 and 4.5), cell viability assay (figure 4.6E) and SDS-PAGE (figure S1).

4.1. Introduction

Alzheimer's disease (AD) patients generally show a decreased clearance of the amyloid-beta (A β) peptide compared to healthy control subjects [1]. The impairment of A β clearance, in some cases combined with an increased A β production, results in accumulation and assembly of A β into aggregated forms in the brain [2]. Therefore, lowering the A β burden by increasing A β clearance, *e.g.* by upregulating the activity of A β -degrading enzymes, provides a promising avenue for AD treatment [3,4].

Many proteases have been reported to cleave A β *in vivo* or *in vitro*, with insulin-degrading enzyme (IDE) and neprilysin (NEP) being the primary A β -cleaving enzymes [5–8]. Deficiencies of NEP and IDE resulted in the elevation of endogenous A β levels in the brain [9]. Also, a genetic association has been reported between single nucleotide polymorphisms in IDE and late-onset AD [10] and the deposition of A β was inversely correlated with NEP expression in TgCRND8 APP-transgenic mice [11]. IDE, a Zn²⁺-metalloendopeptidase, has the ability to cleave A β intra- and extracellularly and may prove valuable as a target to upregulate A β clearance in impaired AD patients [12–14]. Administration of NEP or IDE reduces A β accumulation in the brain [8,15–17]. Intravitreal delivery of NEP reduced ocular levels of A β _{1–40} and A β _{1–42} in a dose-dependent manner [18]. Moreover, the membrane-bound form of NEP was observed to be active, apart from intracellular, towards extracellular residing A β as a result of facing of the active site towards the extracellular space [11] while it was demonstrated that IDE actively regulates extracellular levels of A β [19]. Therefore, drug compounds are under development that can modulate IDE activity to selectively enhance A β degradation, without affecting degradation of other IDE substrates such as insulin and amylin [20–22]. In view of the development of IDE-based AD therapies, more insight into the A β -degrading

capacity of IDE is required. It has previously been reported that IDE is capable of degrading monomeric A β [23–26], but the effect of IDE on A β aggregates such as oligomers, suggested to be the primary toxic species in AD [27], and fibrils, is less well-documented in the literature. Moreover, the properties of IDE-induced fragments from A β are currently unknown. To extend our understanding of the potential therapeutic utility of IDE, we therefore monitored and characterized IDE-mediated cleavage of A β *in vitro* using biophysical techniques, at different time points during the A β aggregation process.

4.2. Material and methods

4.2.1. A β peptide solubilisation

A β _{1–40}, A β _{1–42}, ¹⁵N-labeled A β peptides (rPeptide), A β _{1–35}, A β _{36–40}, and A β _{36–42} (JPT Peptide Technologies GmbH) were dissolved according to the standard procedure developed and validated in our laboratory [28]. In short, A β peptides were dissolved in hexafluoroisopropanol (HFIP). HFIP was evaporated using nitrogen gas and the peptide film was redissolved using dimethyl sulfoxide (DMSO). The peptide was separated from DMSO by elution from a HiTrapTM desalting column (GE Healthcare) into the appropriate filtered buffer (Thioflavin T (ThT) fluorescence measurements: PBS pH 7.4, electrospray ionization mass spectrometry (ESI-MS): 100 mM NH₄HCO₃ pH 7.4. The resulting samples were kept on ice until experiments started with a maximum lag time of 30 min. Peptide concentration was determined using the Coomassie (Bradford) Protein Assay kit and diluted to the required final concentration. Aggregation of A β peptides occurred at 37 °C under quiescent conditions.

4.2.2. Proteolytic degradation of A β by IDE

Recombinant human IDE (cat. no. 2496-ZN-010) was commercially obtained from R&D Systems and tested for purity using SDS-PAGE, both under

reducing and under non-reducing conditions and visualized by silver stain (Figure S1). IDE was added to A β samples at a final concentration of 36 nM at the beginning of the aggregation process, after 3 h, and after 21 h and later. Under these conditions but in the absence of IDE, samples comprise mainly A β monomers (0 h), oligomers (3 h), and (proto)fibrils (21 h and later), as demonstrated by Broersen et al. [28]. IDE-mediated cleavage of A β was monitored using ThT fluorescence and ESI-MS.

4.2.3. ThT fluorescence to monitor peptide aggregation/cleavage

The fibril formation kinetics of 10 μ M peptide sample preparations were monitored *in situ* by measuring fluorescence of ThT (12 μ M) at 37 °C in a Greiner 96-well plate using a FLUOstar OPTIMA fluorescence plate reader (BMG LABTECH GmbH, Germany) at an excitation wavelength of 440 nm (9 nm bandwidth) and an emission wavelength of 480 nm (20 nm bandwidth). Fluorescence readings were recorded for three independent samples. Recorded values were averaged and background measurements (buffer containing ThT, with or without IDE) were subtracted.

4.2.4. Identification of generated A β fragments upon IDE cleavage using ESI-MS

ESI-MS was used to identify A β fragments generated by IDE [29]. Positive-ion mass spectra were recorded on an orthogonal acceleration quadrupole time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA) equipped with a standard electrospray probe (Z-spray) and controlled by a datasystem running MassLynx 4.1 (Micromass, Manchester, UK), which was also used for spectra analysis and peptide fragment identification. Samples were diluted 1:10 in acetonitrile:water (1:1) prior to immediate infusion using a syringe pump with a flow rate of 5 μ l/min. Cone voltage was set to 30 V, capillary voltage was 3 kV. Scan time was set to 4.9 s with an inter-scan time of 0.1 s. At least ten spectra were acquired for two independent experiments and averaged for each condition.

4.2.5. In silico prediction of peptide aggregation

The statistical mechanics-based algorithm TANGO predicts protein aggregation [30]. For each residue in a protein, TANGO computes the percent occupancy of the β -aggregation conformation. Proteins possessing segments of at least five consecutive residues populating the β -aggregated conformation (each with a score higher than 5 %) are considered to have the tendency to aggregate [30]. TANGO calculations were performed via <http://tango.switchlab.org/> for the peptides under study at a pH 7.4, 298.15 K, 0.02 M ionic strength, and without N- or C- terminal protection.

4.2.6. Transmission electron microscopy of peptide aggregates

After 24 h of incubation at a concentration of 10 μ M, peptides (5 μ l) were adsorbed to carbon-coated Formvar 400-mesh copper grids (Agar Scientific) for 1 min. The grids were washed, blotted, and stained with 1 % (w/v) uranyl acetate. Samples were studied using transmission electron microscopy (TEM) with a JEM-1400 microscope (JEOL Ltd., Tokyo, Japan) at 80 kV. Reported TEM images are representative of three independent experiments.

4.2.7. Dotblot with toxic oligomer-specific A11 antibody

At specific aggregation time points, 5 μ l peptide was spotted onto a nitrocellulose membrane. The membranes were blocked in phosphate buffered saline containing 0.2 % Tween-20 (1 h, 25 °C), and incubated (overnight, 4 °C) with primary A11 antibody (Invitrogen), diluted 1:4000 in 100 mM Hepes, pH 7.0 [31]. After incubation (0.5 h, 25 °C) with a secondary anti-rabbit-HRP-tagged antibody (Promega), diluted 1:5000 in phosphate buffered saline containing 0.05% Tween-20, the membranes were visualized using the ImmobilonTM Western chemiluminescent HRP substrate system. Experiments were carried out in duplicates.

4.2.8. Cytotoxicity of A β fragments

SH-SY5Y cells were grown in DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin and 1% nonessential amino acids (Gibco). Cells were

seeded in a 96-well plate at 25,000 cells/well and maintained in phenol-red free DMEM/F12 (L-Glutamine, 15mM HEPES) supplemented with and 1% penicillin/streptomycin and placed incubated at 5% CO₂. Samples containing A β ₁₋₄₀, A β ₁₋₄₂, A β ₁₋₃₅, A β ₃₆₋₄₀, and A β ₃₆₋₄₂ were pre-incubated at room temperature for 2 h, diluted in DMEM/F12 and added to the cells at a final concentration of 25 μ M. An amount of 2 μ M staurosporine (Sigma-Aldrich) was used as positive control. The plates were incubated at 37°C for 48 h, followed by addition of CellTiter-Blue® Reagent (20 μ l/well) and incubation for 4 h. The fluorescence (excitation: 560nm and emission 590nm) was measured using a TECAN Infinite 200 PRO fluorescence platereader. The medium background values were subtracted from the values obtained in experimental wells. Statistical significance of the results was established by P-values using two-tailed t-tests (GraphPad Software). Values represent results of three independent replicates. Statistical significance levels were *P < 0.05, **P < 0.01 and ***P < 0.001 compared with the control group cells.

4.3. Results and discussion

4.3.1. A β aggregation state determines susceptibility to degradation by IDE

The effect of IDE on various A β aggregated species was initially assessed by monitoring changes in ThT fluorescence, as this is a commonly used reporter dye for fibril formation [32]. In the absence of IDE, A β ₁₋₄₀ aggregation at 37 °C was marked by a strong increase in ThT fluorescence, indicative of fibril formation. In contrast, addition of IDE to monomeric A β ₁₋₄₀ prevented ThT-positive fibril formation within the time frame of the measurements, as the ThT background signal did not increase. This shows that IDE is capable of degrading A β ₁₋₄₀ monomers into fragments that do not form ThT-positive aggregates upon prolonged incubation (Fig. 4.1A), and is in agreement with previous studies [23–26]. In contrast, IDE addition at later A β aggregation

time points, enriched in oligomers (3 h) or (proto)fibrils (21 h) [20], induced a partial decrease in ThT fluorescence suggesting that ThT-positive aggregates were retained in the solution in the presence of the enzyme. The cleavage of A β_{1-42} by IDE showed many similarities with the observations for A β_{1-40} . IDE-mediated A β_{1-42} cleavage was characterized by a strong inhibition of A β_{1-42} fibril formation when IDE was added at the beginning of the aggregation process. However, a small increase in ThT fluorescence was apparent, reflecting the higher aggregation propensity of A β_{1-42} compared to A β_{1-40} . The decrease in ThT fluorescence when IDE was added at later aggregation time points was also detected for A β_{1-42} , but less pronounced compared to A β_{1-40} (Fig. 4.1B). This partial decrease suggests (i) degradation of ThT-positive A β aggregates by IDE, or (ii) degradation of monomers in solution that are in equilibrium with ThT-positive aggregates, causing dissociation of ThT-positive aggregates to restore this equilibrium. Recent structural reports of IDE point in the direction of the latter hypothesis, as the crystal structure of IDE resembles a clamshell with a large internal chamber formed from two bowl-shaped halves connected by a flexible linker. IDE engulfs and degrades its substrates within this catalytic chamber whose size is limited to accommodate only relatively small peptides, i.e. consisting of maximally 70 amino acids [33–35]. The ThT fluorescence data reported here would therefore be in agreement with the hypothesis that IDE exclusively degrades monomeric A β , because oligomeric and fibrillar forms of A β are too large to completely fit into this previously identified catalytic chamber [33–36]. Biological significance can be addressed to this observation as the presence of IDE appears to induce a displacement in monomer-aggregate equilibrium, potentially steering the A β assembly pathway away from toxic oligomer formation.

Figure 4.1. A β aggregation state determines susceptibility for IDE-mediated degradation. Aggregation of 10 μ M (A) A β ₁₋₄₀ and (B) A β ₁₋₄₂ was monitored by ThT fluorescence. IDE (36 nM) was added at the beginning of the A β aggregation process (sample comprising mainly A β monomers), after 3 h (enriched in oligomers), and after 21 h (enriched in (proto)fibrils).

4.3.2. A β degradation by IDE *in vitro* results in multiple fragments reported *in vivo*

To investigate which fragments are formed upon IDE-mediated cleavage of A β , the cleavage fingerprints of A β ₁₋₄₀ and A β ₁₋₄₂ by IDE were determined using ESI-MS. Our results show that monomeric A β ₁₋₄₀ and A β ₁₋₄₂ are cleaved at multiple and similar sites (results for A β ₁₋₄₀ in Fig. 4.2 and for A β ₁₋₄₂ in Fig. S2), which is in agreement with previous *in vitro* studies [23–26,37]. Several A β fragments reported here have also been detected in the cerebrospinal fluid and plasma of AD patients [38–41]. The data reported here confirm the recently discovered cleavage sites Gly₃₃-Leu₃₄ and Leu₃₄-Met₃₅ [37] and identify Met₃₅-Val₃₆ as a novel cleavage site, resulting in the release of

fragments A β ₁₋₃₅ and A β ₃₆₋₄₀/A β ₃₆₋₄₂. For several cleavage sites however, only the corresponding N- or C-terminal fragment was detected in the mass spectrum, as not all fragments are ionized efficiently, were aggregated or degraded prior to entry in the mass spectrometer. As the N-terminal fragment of novel cleavage site Met₃₅-Val₃₆ could not be observed, the observation of this cleavage site was further confirmed using additional MS/MS analysis by comparing mass spectra of the IDE-induced fragments A β ₃₆₋₄₀/A β ₃₆₋₄₂ with that of synthetically derived peptide fragments (Fig. 4.3). Moreover, fragments A β ₁₄₋₂₈, A β ₁₅₋₂₈, and A β ₂₀₋₂₈ were detected, indicating that one A β peptide can be cleaved by IDE at least twice, e.g. at His₁₃-His₁₄ and consecutively at Lys₂₈-Gly₂₉ (Fig. 4.2 - Fig. S2). As one of the roles of IDE is to completely inactivate hormones such as insulin [42], this could explain why it cleaves its substrates at multiple sites, to ensure elimination of any residual activity.

Figure 4.2. IDE-mediated cleavage of monomeric A β results in multiple fragments. Assignment of ion peaks detected by ESI-MS associated with monomeric A β 1-40 (10 μ M) in (A) absence and (B) presence of 36 nM IDE, after 15 min of incubation at 37 °C. (C) Overview of the detected A β 1-40 and A β 1-42 sites susceptible to IDE proteolysis.

IDE addition to aggregated A β resulted in similar fragments compared to addition of IDE to monomeric A β , but there was a considerable amount of non-degraded A β peptide left in the sample (denoted by asterisks in Fig. 4.4). This can be seen by the higher intensities of the ion peaks corresponding to full-length A β (A β^{3+} at m/z 1443.0, A β^{4+} at m/z 1083.3) compared to the most intense generated fragment A β_{20-40} (A β^{2+}_{20-40} at m/z 1017.5) in Fig. 4.4B. This implies that increasing A β fibril maturity correlates with a decreased propensity to IDE-mediated cleavage.

Figure 4.3. Confirmation of A β fragment 36-40/36-42. MS/MS of (A) synthetically derived A β_{36-40} fragment, (B) A β_{36-40} fragment obtained upon IDE-mediated cleavage of A β_{1-40} , (C) synthetically derived A β_{36-42} fragment, and (D) A β_{36-42} fragment obtained upon IDE-mediated cleavage of A β_{1-42} .

Figure 4.4. Longer incubation times of A β prior to IDE addition result in a decrease in susceptibility of A β for IDE-mediated cleavage. Assignment of A β_{1-40} fragments detected by ESI-MS. IDE (36 nM) was added to an A β_{1-40} (10 μ M) buffered solution at (A) $t = 0$ h and (B) $t = 24$ h of the aggregation process, and incubated for 1 h at 37 $^{\circ}$ C. Samples were centrifuged for 15 min at 13200 rpm prior to ESI-MS analysis and the data depicted here correspond to the soluble fractions. Non-degraded A β peptide remaining in the sample is indicated by an asterisk.

4.3.3. Fragments generated by IDE-mediated A β degradation are aggregation-prone

The properties of the obtained fragments upon IDE cleavage of A β have not yet been fully explored. This is however crucial in light of the development of IDE-based therapeutic AD strategies, as one must assure that fragments generated upon increased proteolytic activity are not aggregation-prone themselves.

ThT fluorescence data indicate that no ThT-positive aggregates were formed from the A β fragments obtained upon IDE cleavage of monomeric A β (Fig. 4.1). However, ThT fluorescence may not provide the most sensitive read-out to detect small quantities of aggregation, possibly formed only by a specific subset of fragments. Alternatively, resulting aggregated fragments might have less or no affinity for ThT. Previous studies indicate that the ThT dye binds to surface side-chain grooves running parallel to the axis of the β -sheets, that are characteristic of amyloid fibrils [43]. A model has been proposed in which five aligned aromatic and/or hydrophobic residues are critical for ThT binding [32]. It is thus possible that the A β fragments studied here lack the minimal ThT binding site.

Accordingly, ESI-MS revealed the presence of several fragments in the pellet fractions, obtained after prolonged incubation of A β with IDE, demonstrating their aggregation propensity (Fig. 4.5).

Next, the properties of the newly identified A β fragments (A β ₁₋₃₅ and A β ₃₆₋₄₀/A β ₃₆₋₄₂) were investigated in more detail. TANGO, a statistical mechanics-based algorithm, predicts protein aggregation for A β ₁₋₃₅ and to a lesser extent for A β ₃₆₋₄₂ (Fig. S3). Both peptides contain one or both of the aggregation nucleating regions previously reported for A β ₁₋₄₂, i.e. the regions encompassing residues 17-21 and 30-42. In contrast, no aggregation propensity was predicted for A β ₃₆₋₄₀. To assess the validity of the *in silico* predictions, the aggregation behaviour of the peptides was monitored using

ThT fluorescence at 37 °C. A β_{1-35} displayed a ThT sigmoidal curve, characteristic of A β aggregation, but with an extended lag phase of 10 h and a lower final ThT fluorescence intensity compared to A β_{1-42} (Fig. 4.6A-B). In contrast, A β_{36-40} and A β_{36-42} did not stain ThT-positive, even after 72 h of incubation (Fig. 4.6B). However, TEM imaging revealed amorphous aggregates for all fragments, in contrast to the dense network of amyloid fibrils formed by A β_{1-42} (Fig. 4.6C). As these amorphous aggregates show less or no sensitivity to ThT staining, they might lack the channels/sites necessary for ThT binding [32]. As a final assessment of the properties of these peptide fragments, a dot blot analysis was carried out with the oligomer-specific A11 antibody. A β oligomers have previously been reported to be responsible for the neurotoxicity and cognitive defects in AD patients [27]. A high A11 response was detected for A β_{1-42} (included as a reference) after 1.5 h of incubation (Fig. 4.6D), which has been demonstrated to correspond to a toxic A β oligomer-enriched fraction [28]. Aggregation into higher aggregates coincided with a decrease in A11 intensity. In contrast, A11 reactivity was not detected in the case of any of the A β fragments. Cell viability assays further showed that these aggregated forms of A β fragments were not toxic to neuroblastoma cells (Fig. 4.6E). In summary, although the fragments under study do not form A11-positive oligomers, they do result in non-toxic amorphous aggregates with no or less ThT staining (compared to A β_{1-42}). The aggregation-prone character of these A β fragments should be considered when developing modulators to enhance the proteolytic activity of IDE.

Figure 4.5. A β fragments aggregate in time and are detected in the pellet of the A β 1-40-IDE sample. IDE (36 nM) was added to aggregated A β 1-40 (10 μ M), that was pre-incubated for 24 h at 37 $^{\circ}$ C, and ESI-MS spectra of the pellet fractions (obtained after 15 min of centrifugation at 13200 rpm) were obtained after (A) 1 h and (B) 24 h of incubation with IDE. Whereas the pellet fraction only displayed ion peaks corresponding to full-length A β 1-40 after 1 h of IDE incubation, cleavage fragments were observed in the pellet after 48 h of incubation, indicating that generated A β fragments aggregate in time.

Figure 4.6. IDE-induced A β peptide fragments form amorphous aggregates with less/no staining for ThT. (A) Aggregation propensity of peptides monitored by ThT fluorescence, at 37 °C. The aggregation of A β_{1-35} is shown by a sigmoidal ThT fluorescence curve and a lag phase of approximately 10 h. In contrast, shorter peptides A β_{36-40} and A β_{36-42} do not form ThT-positive aggregates. (B) ThT intensities after 72 h of incubation. (C) After 24 h incubation time at a concentration of 10 μ M, TEM images depict a dense fibril network for A β_{1-42} , whereas the peptide fragments form smaller aggregates with an amorphous-like appearance. Scale bars represent 500 nm. (D) Dotblot analysis shows A11 intensity for A β_{1-42} , in particular for the early aggregation species ($t = 0-5$ h). No A11 intensity was detected for any of the peptide fragments. (E) Aggregated IDE-generated A β fragments A β_{1-35} , A β_{36-40} and A β_{36-42} are not cytotoxic upon incubation at a concentration of 25 μ M with neuroblastoma SH-SY5Y cells. Controls include a 2 μ M solution of staurosporin, full-length A β_{1-40} and A β_{1-42} and cells incubated with buffer.

4.4. Conclusion

In conclusion, we show that A β monomers, either alone in solution or in equilibrium with higher aggregates, are cleaved at multiple sites by IDE. We report a new A β cleavage site, i.e. Met₃₅-Val₃₆, and show that the resulting A β fragments form non-toxic amorphous aggregates that are not or less sensitive for ThT staining. The generation of A β fragments that are aggregation-prone must be considered when developing IDE-based strategies targeting AD.

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4.6. Supplementary

Figure S1

Figure S1. Purity and quality of commercially obtained IDE and NEP evaluated using reducing and non-reducing SDS-PAGE (12% running gel, silver staining). Under non-reducing conditions a mixed population of monomer and dimer can be observed for IDE while NEP is primarily observed as a dimer. Under reducing conditions IDE is observed as a band at 105 kDa and NEP at 102 kDa.

Figure S2

Figure S2. IDE-mediated cleavage of monomeric A β_{1-42} results in multiple fragments. Assignment of ion peaks detected by ESI-MS associated with monomeric A β_{1-42} (10 μ M) in the presence of 36 nM IDE, after 1 h of incubation at 37 $^{\circ}$ C.

Figure S3

Figure S3. Prediction of aggregation propensity of full-length A β_{1-42} and IDE-induced A β fragments. The algorithm TANGO predicts aggregation for A β_{1-42} and A β_{1-35} , with two aggregation nucleating regions encompassing residues 17-21 and the C-terminal residues. A β_{36-42} is predicted to aggregate to a lesser extent, whereas no aggregation propensity is detected for A β_{36-40} .

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PART 3:

Oxidative Stress

Chapter 5

Molecular mechanisms of oxidative stress and antioxidative agents in Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder that can cause dementia in elderly over 60 years of age. One of the disease hallmarks is oxidative stress which interconnects with other processes such as amyloid beta deposition, tau hyperphosphorylation and tangle formation. Oxidative stress levels rise in early stages of the disease and detection assays to establish oxidative stress marker levels have been developed for plasma, serum or cerebral spinal fluid as well as functional imaging. This review discusses current thoughts on molecular mechanisms that may relate oxidative stress to AD and identifies factors that may be causative of oxidative stress *in vitro*, *in vivo* and in clinical studies. Translation of this knowledge into agents serving to relieve oxidative stress levels as ancillary treatment of AD is highlighted. Based on extensive literature review and transcriptome analysis we conclude that the molecular mechanistic association between AD and oxidative stress is partially known. With multiple observations demonstrating oxidative stress as a downstream effect of AD, it is debatable whether agents targeting oxidative stress primarily will alleviate pathogenesis. Further, transport of antioxidants to the targeted site in the brain across the blood-brain barrier is challenging and possibly largely determines the currently observed limited activity of trialed antioxidant formulae in clinical trials.

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This chapter will be submitted as review.

5.1. Introduction

AD is a neurodegenerative disorder with memory deficits, and executive dysfunction as characteristic clinical features [1]. AD primarily affects patients older than 60 years of age. Clinical investigations show that hallmarks of oxidative stress are observed early in the progress of AD [2–7]. Related to this, pre-symptomatic AD has been associated with components of mitochondrial deficiency resulting in disturbed bioenergetics [8]. Apart from causing a reduction in the generation of ATP, mitochondrial deficiency lays at the basis of oxidative stress by resulting in excessive production of reactive oxygen species (ROS). These ROS, in turn, have been related to membrane damage, cytoskeletal alterations, and cell death [9]. The precise nature of the association between oxidative stress and AD pathology is unknown although some molecular mechanisms have been suggested. Apart from features of oxidative stress, progress of AD coincides with signature extracellular accumulation of aggregated amyloid beta ($A\beta$). $A\beta$ is generated by cleavage of amyloid precursor protein (APP). This cleavage can result in an accumulation and subsequent self-assembly of the $A\beta$ peptide generating soluble oligomers. Next to $A\beta$ assemblies, intracellular neurofibrillary tangles containing hyperphosphorylated tau are considered a hallmark for AD. The accumulation of tau tangles is suggested to be a downstream effect, brought about by glycogen synthase kinase 3 ($GSK3\alpha$) activity mediated by $A\beta$ oligomers [10]. This review will focus on oxidative stress in AD and its relation to AD-specific hallmark events such as $A\beta$ oligomer formation, plaque deposition and neurofibrillary tau tangle formation. ROS generation under physiological conditions and how production and/or neutralization of these species is compromised in a neurodegenerative condition such as AD will be covered. Recent evidence on the molecular link between increased levels of ROS and $A\beta$ deposition will be discussed in the context of the

cellular environment. Additionally, the biomarkers that are used to report on oxidative stress in lab experiments as well as clinical trials will be discussed. Based on clinical trials which aim at relieving oxidative stress with compounds, this review will shed light on the state-of-the-art developments in the field of antioxidant therapeutics.

5.2. The definition of oxidative stress

Oxidative stress is the result of an increased level of cellular ROS and is a physiological age-related feature [11,12]. An increased generation of ROS and reactive nitrogen species (RNS) alone may not provide sufficient leverage to cause pathology. As long as the antioxidant defense system is sufficiently capable of scavenging the generated reactive species increased ROS production generally does not pose a problem. However, in some cases aging-related increased production of free radical species coincides with a decreased capacity of the endogenous antioxidant defense system [13,14]. ROS and RNS are reactive because they are free radicals with an unpaired valence electron and, as such, they are capable of oxidizing biomacromolecules in the cellular environment including proteins, lipids, RNA, and DNA. Examples of ROS include superoxide ion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and peroxy radical ($OH^{\cdot}$), while examples of RNS are nitric oxide (NO) and peroxynitrite ($ONOO^-$). Even though normal aging coincides with increased levels of oxidative stress, many diseases demonstrate a more extensive increased level of oxidative stress including cancer, Parkinson's disease, atherosclerosis, myocardial infarction, sickle cell disease, Down's syndrome, ischemic processes, depression, and diabetes mellitus [15–18]. A common trope in the dark on all these diseases is the causal relationship of oxidative stress with the various pathologies.

5.3. Generation of reactive oxygen and nitrogen species and their neutralization

Mitochondrial and non-mitochondrial sources are responsible for generating ROS and RNS in any living cell. Generally, the formation of ROS and RNS are counteracted by a wide range of measures to neutralize the potential damaging effects of these species. The main mechanisms of ROS generation are illustrated in figure 5.1.

Figure 5.1. Main cellular mechanisms of ROS generation. **1) Oxidative phosphorylation:** ATP is produced by the electron transport chain and the enzyme ATP Synthase. The high energy electrons, generated from the NADH carrier, are transferred through the inner mitochondrial membrane enzyme complexes while pumping out hydrogen ions into the inner mitochondrial space. Complex I can also contribute to $O_2^{\cdot-}$ production in cases of high protonmotive force and a reduced Quinone (Q) level. The final product is the reduction of oxygen to form H_2O . However, if the oxygen does not gain all the electrons available, the formation of ROS can occur. **2) Fenton reaction:** Hydrogen peroxide that is free in the cytoplasm can interact with Fe^{2+} to generate hydroxyl radical ($OH^{\cdot}$). **3) Membrane-bound oxidase:** NADPH oxidase is generally present in the cell membrane where it mainly

generates superoxide (O_2^-). **4) Free metal ions:** Free copper and zinc ions are present in limited amounts in the cytoplasm and can oxidize H_2O_2 to $O_2^{\cdot-}$. **3) ROS generation in peroxisomes:** a certain amount of radical species is also produced by the peroxisome. This organelle consists of xanthine dehydrogenase (XDH) that converts xanthine to uric acid. However, XDH can undergo changes towards an oxidase form (XOD) that promotes the reduction of O_2 to $O_2^{\cdot-}$. Other oxidative events that occur in the peroxisome include the β -oxidation of fatty acids by acyl-CoA enzymes and the enzymatic reaction of flavin oxidases. Superoxide anion ($O_2^{\cdot-}$) can cause damage to the DNA. ROS can also translocate into the extracellular space and activate the microglia leading to neuroinflammation and additional ROS production.

5.3.1. Mitochondrial source of reactive oxygen

Mitochondrial adenosine triphosphate (ATP) generation provides the cell with the energy required for a multitude of energy-costly essential processes [12]. Mitochondria are also the main sites for ROS production. ROS is a byproduct of the generation of ATP by means of a process termed oxidative phosphorylation. During this process, 1–2% of the oxygen that is consumed is released as ROS [19,20]. The electron-transport chain (ETC), the apparatus which is responsible for the process of oxidative phosphorylation, is located within the mitochondrial inner membrane [13]. The high energy electrons derived from the process of glycolysis and the citric acid cycle are transferred to three respiratory enzyme complexes that sequentially transfer the electrons to build up a steep proton gradient within the intermembrane space. Subsequently, these protons flow back into the mitochondrial matrix down their electrochemical gradient driving ATP generation by the enzyme ATP synthase complex [21]. The transport of electrons through the various electron accepting and donating complexes is not a one hundred percent efficient process and involves low level leakage of electrons affecting mitochondrial coupling efficiency. Electron leakage occurs when electrons transferred through the ETC exit prior to their reduction of oxygen to water

at cytochrome c oxidase, reacting instead with oxygen resulting in the formation of low to moderate levels of superoxide ($O_2^{\bullet-}$) although very low levels of hydrogen peroxide and hydroxyl radicals are also formed. Theories explaining superoxide production from the various ETC-involved enzyme complexes have been reviewed [21]. Ultimately, the produced ROS will be reduced to water through a series of reduction events. Conditions of mitochondrial damage and hypoxia have been associated with increased electron leakage [22]. The proton leaks into the mitochondrial matrix by direct diffusion through the membrane, or by being transported by the anion carrier protein adenine nucleotide translocase (ANT) [23,24], or by an inducible mechanism being catalyzed by ANT or uncoupling protein (UCP) [21,25,26]. The processes of proton and electron leakage are associated, *i.e.* the generation of $O_2^{\bullet-}$ is sensitive to a decrease in proton motive force which is the result of proton leakage. This association is also referred to as partial uncoupling. The dissipation of protons by UCPs and ANT lowers the mitochondrial proton gradient across the inner membrane, as illustrated in figure 5.2. This, in turn, will lead to a reduced transport of electrons resulting in a lower production of ROS. An increase in proton leakage is a thus physiological feedback response to ROS overproduction [27].

Figure 5.2. Oxidative phosphorylation via electron transport chain (ETC) and proton leak via uncoupling proteins in mitochondria. The ETC pumps protons across the inner mitochondrial membrane into the mitochondrial intermembrane space. Subsequently, protons flow back into the mitochondrial matrix down their electrochemical gradient through the enzyme ATP synthase complex, which drives the synthesis of ATP by coupling of inorganic phosphate (Pi) to ADP. Alternatively, uncoupling proteins (UCPs) can cause dissipation of the strong proton gradient via an independent pathway, bypassing ATP synthase complex, that does not result in ATP generation.

5.3.2. Non-mitochondrial sources of reactive oxygen species

Besides mitochondria other sources have been identified to generate ROS. For example, different from neurons, astrocytes do not depend on the process of oxidative phosphorylation to generate energy; their energy supply relies on glycolysis [28] which takes place in the cytoplasm. Yet, the level of ROS formation in astrocytic cells significantly exceeds that of neurons which is a direct result of a low abundance of the enzyme NADH: Ubiquinone Oxidoreductase Core Subunit S1 (NDUFS1) in astrocytes, compared to neurons [29]. NDUFS1 is responsible for the assembly of complex I into a supercomplex in neurons whereas the free complex I in astrocytes is related to a higher generation of ROS [29]. This high level of astrocytic ROS generation is compensated for by an elaborate machinery consisting of antioxidant compounds and enzymes as well as the stabilization of an antioxidant transcriptional activator, nuclear factor erythroid 2-related factor (Nrf2) [30]. In this way, next to providing them with highly specialized tools to neutralize their own generated ROS, astrocytes also provide antioxidative activity to neurons in their close vicinity, which are poorly equipped with antioxidant machinery. Astrocytic synthesized glutathione (GSH, 1-glutamyl-1-cysteinyl-glycine) induced by Nrf2-mediated gene expression is exported from the astrocyte, degraded, and the products of this degradation

are taken up by a recipient neuron which assimilates the resulting fragments into neuronal GSH. Other non-mitochondrial sources of ROS are free metal cations such as copper (Cu^+) and zinc (Zn^{2+}) that can interact with oxygen as required intermediate in various metal catalyzed oxidation reactions. These metals participate in regulating synaptic functions and have catalytic roles in enzymes. Excess levels of copper and zinc may lead to their direct interaction with oxygen resulting in $\text{O}_2^{\bullet-}$ formation and oxidative stress [20].

The peroxisome has been identified as an important cellular site regulating the metabolism of both ROS and RNS, and abnormalities in these organelles have been related to a number of oxidative stress related disorders [31,32]. Mitochondria and peroxisomes interact intensively as biogenesis of the peroxisome requires mitochondria-derived vesicles and the components controlling their fission machineries are shared [33]. The mitochondria and peroxisomes also interact functionally as they cooperate to maintain homeostasis of ROS. Peroxisomes have been identified to generate H_2O_2 as a by-product of the process of fatty acid β -oxidation.

5.3.3. Generation of reactive nitrogen species

RNS is highly reactive and is mostly generated downstream of ROS formation. When generated at abundant levels, NO was shown to interact with ROS increasing the probability to form RNS. NO-induced oxidation of proteins and other biomolecules results in the formation of peroxynitrite (ONOO^-) [12]. ONOO^- subsequently forms other RNS like nitrogen dioxide (NO_2^*) and nitrosoperoxy carbonate (ONOOCO^{-2}) [13]. Those RNS readily react with the amino acid tyrosine to form nitrotyrosine, a component which has been detected at elevated levels in the AD brain [34,35]. The main production site of RNS, similar to ROS, are the mitochondria. Alternatively, RNS can be generated in the peroxisome upon interaction of nicotinamide adenine dinucleotide phosphate (NADPH) with the amino acid arginine

leading to nitric oxide (NO) as by-product. Peroxisomes play an essential role in lipid metabolism in mammalian cells, for example in α - and β -oxidation, and the generation of bile acids, docosahexaenoic acid and ether-phospholipids, the details of which have been reviewed before [31,36]. By-products of these enzyme-catalyzed metabolic processes include ROS and RNS [37], and, for this reason, peroxisomes are abundant in the anti-oxidant enzymes.

Next to lipid metabolism as a source of RNS, pro-inflammatory cytokine interleukin- 1β can induce RNS formation by triggering the generation of inducible nitric oxide synthase (iNOS) in the microglia which mediates NO synthesis increasing NO levels [35]. In return, it was reported that a superoxide generating factor in cells, pyocyanin, inhibits the secretion of interleukin- 1β into the extracellular space upon inflammasome activation. Normally, upon assembly, the inflammasome induces interleukin- 1β secretion as downstream effect [38]. This observation is of interest as it may provide a molecular mechanism by which oxidative stress and neuroinflammation, another early AD hallmark, are interlinked.

5.3.4. The endogenous antioxidant system

The cellular antioxidant defense system acts by donating an electron to reactive species in this way reducing their harmful effects [39]. The endogenous antioxidant defense system can be divided into enzymatic and small molecule antioxidants. Cellular antioxidants are under homeostatic control; *i.e.* a decrease in one antioxidant may be compensated for by an increase of a different antioxidant. An extensive review covering the topic of ROS detoxification in the brain has been published before [40], and, as such, this paragraph solely serves to provide an introduction to this topic directly related to AD.

5.3.5. Endogenous enzymatic antioxidants: superoxide dismutase, catalase and glutathione peroxidase

One of the most powerful and widely studied enzymatic antioxidants is superoxide dismutase (SOD) which eliminates ROS in the cytosol and mitochondria. All SOD variants require a catalytic metal for their activation. Intracellular antioxidant activity is performed by Cu/Zn SOD1 and manganese Mn SOD2 isoforms, while extracellular ROS removal is performed by Cu/Zn SOD3 [41] (Fig. 5.3). Generally, SOD acts by reducing two reactive superoxide anions into less reactive H_2O_2 . Subsequently, H_2O_2 gets reduced to radical $\text{OH}^\bullet$, by catalase. Finally, glutathione peroxidases (GPx), which is a group of selenium containing enzymes, catalyzes the reduction of $\text{OH}^\bullet$ to water [13].

Figure 5.3. Endogenous enzymatic antioxidants. SOD is responsible for reducing O_2^- to the less reactive H_2O_2 . SOD1 isoform localizes in the cytoplasm, SOD2 in the mitochondria and SOD3 extracellularly. Antioxidant activity of SOD1 and 3 depends on Cu/Zn while SOD2 activity is enhanced by Mn. GPx further convert H_2O_2 into water.

5.3.6. Endogenous small molecule antioxidants

Glutathione. Levels of one of the prime brain thiol-containing antioxidants, GSH, were shown to be decreased upon aging with more profound reduction in individuals suffering from AD compared to age-matched controls. GSH is

a tripeptide consisting of L-glutamate, L-cysteine and glycine synthesized by the enzymes γ -glutamylcysteine synthetase and glutathione synthetase [42]. The reduced form of GSH effectively acts as -OH scavenger. Upon oxidation GSH converts into GSSG. Oxidative stress induces the expression of the NADPH-dependent enzyme glutathione reductase, which reverts oxidized GSSG to its reduced form GSH [43]. mRNA expression levels of gamma-glutamyl cysteine synthetase vary per region in the brain with high expression levels in cortex, cerebellum and hippocampus and low expression in the neostriatum of mice [44,45], and it was suggested that these regional differences in GSH generation can explain regional differences in susceptibility to oxidative stress [45]. In 1980 it was shown that reduced GSH contents in kidney, heart and liver decreased with aging in a mouse model [46]. Later it was shown that these reduced GSH levels could be explained by decreased gene expression in aging rat lung, kidney, liver [47] and brain [47] of the rate-limiting enzyme in GSH synthesis, gamma-glutamylcysteine synthetase. In a study investigating GSH and GSSG levels in human AD patient lymphocytes, decreased GSH levels went hand in hand with increased GSSG levels indicative of elevated peripheral oxidative stress levels [48]. Further reduction in GSH levels was found in erythrocytes from elderly patients with MCI and patients diagnosed with AD compared to a control group [49]. GSSG/GSH ratios are often employed as biomarker for oxidative stress and a study in comparing basal blood levels of GSSG/GSH ratios in control, mild, moderate or severe dementia patients showed a significant correlation of GSSG/GSH ratio with progression of disease [50]. A 2016 study in 74 human subjects demonstrated that GSH levels of autopsied brains did not significantly decrease with aging [51] suggesting that brain and peripheral antioxidant levels may not necessary be associated. Another study investigating GSH and GSH transferase levels in AD brains found no

difference in total brain levels of these two factors compared with age-matched controls [52]. Even though total brain levels of GSH may not be affected by AD, another study showed region-specific differences by identifying increased GSH levels in the hippocampus and midbrain of AD patients without significant differences in GSSG levels [53].

Melatonin. Melatonin, or N-acetyl-5-methoxytryptamine, is involved in various homeostatic functions to aid cellular protection. It is an electroactive neurohormone with antioxidant activity that is synthesized and secreted in the brain from mitochondria of pinealocytes, cells of the pineal gland [54–56]. Melatonin is synthesized under circadian control directly regulated by arylalkylamine N-acetyltransferase which is a serotonin N-acetyltransferase [56–59]. Also the metabolites of melatonin, including N¹-acetyl-N²-formyl-5-methoxykynuramine (AFMK) and N¹-acetyl-5-methoxykynuramine (AMK) demonstrate antioxidant activity, either directly by scavenging a variety of free radicals including hydroxyl, peroxy, superoxide, peroxide and ONOO- [60,61], or indirectly by inducing antioxidant enzymes including SOD, GPx and GSH reductase [62], increasing GSH synthesis [63], and inhibiting prooxidant enzymes RNS, xanthine oxidase and myeloperoxidase [64]. Even though aging is related to a decrease in CSF melatonin levels, presenile and senile AD patients demonstrated an even stronger reduction in melatonin levels that was shown to be dependent on apolipoprotein genotype [65], one of the strongest identified genetic correlates with AD.

Thioredoxin. Thioredoxin (trx) is a 12 kDa protein which is expressed in many organisms and supports the regulation of oxidative stress levels by maintaining cellular redox homeostasis [66]. Mammalian cells contain three isoforms of Trx, all of which contain the redox active site Cys-Gly-Pro-Cys, although their cellular localization varies. The Trx1 isoform has been identified in the cytosol [67], Trx2 in the mitochondria [68], and SpTrx is

mainly present in spermatozoa [69]. Trx acts by reducing disulfide bonds in oxidized proteins, such as peroxiredoxin (Prx). Trx can subsequently be reduced by the enzyme thioredoxin reductase (TrxR) and NADPH. These three proteins together form a system termed the “Thioredoxin-peroxiredoxin system” which generally aims at protecting cells from the injurious effects of ROS [70].

5.3.7. Transcriptional control of the endogenous antioxidant system by Nrf2

The neuron-glia unit, the main interaction site between neurons and cells of glial origin such as astrocytes, regulates oxidative stress levels through an intimately linked intercellular mechanism for redox homeostasis [71,72]. Brain oxidative stress levels are maintained within strict limits as a result of the powerful astrocytic nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) homeostatic pathway [73]. Upon translocation to the nucleus, Nrf2 binds to antioxidant response element (ARE), a promotor element present on antioxidant genes [74]. Nrf2 degradation is controlled by ubiquitin-mediated degradation, which, in turn, is regulated by cytoskeleton associated Kelch-like protein, Keap1 [75–78]. In the absence of oxidative stress, Nrf2 is transcriptionally inactive as its activity is repressed by Keap1 [78]. Under conditions of oxidative stress Keap1 is oxidized inhibiting the degradation of Nrf2. Transcriptional activity of Nrf2 was shown to decline upon aging [79,80]. One study showed that AD progression was linked with haplotype allele variation in NFE2L2 gene promotor which codes NRF2 [81] while therapeutic administration of a lentiviral vector encoding for human Nrf2 was shown to improve cognitive dysfunction in APP/PS1 [82] and APP/PS1DeltaE9 mice [83]. Furthermore, a recent transcriptomics study demonstrated that NRF2 knockout leads to early onset cognitive dysfunction, plaque deposition and tau tangle formation [84]. Other recent experimental evidence linking AD to the Nrf2 pathway showed that methysticin, a

kavalactone activating the Nrf2 signaling pathway, reduced neuroinflammation, loss of memory and damage as a result of oxidative stress in the hippocampus of APP/Psen1 mice [85]. Even though the Nrf2 signaling pathway is highly active in astrocytic cells, this pathway is virtually absent in cells of neuronal origin [30,86] while the capacity of neurons to degrade Nrf2 is high as a result of abundant neuronal expression of the protein cullin 3 which leads to destabilization of neuronal Nrf2 [30].

5.4. Physiological roles of ROS and RNS

Even though ROS has traditionally been linked to pathological conditions it has been recognized that ROS is not only a toxic by-product of the ATP generating pathway. The redox potential, which describes the affinity for electrons, of ROS and RNS is also key to their cellular function and the chemical reactivity of different ROS and RNS varies. The level of ROS is determined by an interplay between their production and scavenging, allowing ROS levels to be maintained within a certain range. Keeping ROS within this range is critical for the cell to control several processes such as cell proliferation, signal transduction reactions, cell differentiation and physiological cell death. A physiological role of hydrogen peroxide was first demonstrated in plant cells where hydrogen peroxide is involved in lignin biosynthesis [87–89]. Subsequently, superoxide and hydrogen peroxide were shown to differentially regulate gene transcription in bacteria [90]. Also in the human body it was demonstrated that production of ROS is a normal physiological process that is essential for some pro-survival signaling pathways. In these pathways ROS act as intracellular second messengers at low and moderate levels. For example, the mitogen-activated protein kinase (MAPK) pathways are influenced by ROS. Here they regulate redox signaling in the cardiovascular system with serine or threonine – specific protein

kinases. Nuclear factor – kappa B (NF- κ B) is also activated by ROS as phosphorylation and degradation of NF- κ B inhibitors leads to inhibition of caspase-dependent cell death pathways [41]. Moderate ROS levels were also observed to play a crucial role in immune system response. For example, phagocytes contain NADPH oxidase which transfers electrons to oxygen molecules, producing superoxide ions and hydrogen peroxide that kills the pathogens in the phagosome [91]. Moreover, the thyroid gland uses H_2O_2 to oxidize iodine (I-) to iodine (I₂) for thyroid hormone production. The thyrocyte cells contain the NADPH complex which produces a certain amount of H_2O_2 that is meant to accumulate in an extracellular compartment, called follicle lumen, where they can not damage the cells of the thyroid gland [92]. Low ROS levels further play a role in the insulin signaling cascade [93], regulation of cerebral vascular tone [94], cellular adhesion processes [95], amplification of the immune response [96], circadian regulation [97], and apoptosis [98].

5.5. Effect of oxidative stress on biomacromolecules

Even though a multitude of experimental evidence shows that low levels of ROS are crucial for normal physiological functioning, a divergence from physiological ROS levels to an increased abnormal level can be associated with a number of related pathologies which all imply oxidative damage of various cellular compartments and molecules.

5.5.1. Membrane associated damage

AD-related excessive ROS formation may damage structure and function of membrane-associated macromolecules such as lipids and proteins in several regions of the brain. A recent publication reported the use of small angle x-ray scattering to experimentally assess the impact of oxidation on a POPC biomimetic membrane, which showed that the artificial membrane was

thinning as a result of oxidation [99]. These findings were consistent with an earlier molecular dynamics study showing that various lipid oxidation species in model lipid membranes led to an increase in membrane fluidity with a decreased thickness of the lipid bilayer without inducing pore formation [100]. Another study showed that, upon exposure of giant unilamellar vesicles, cholesterol and unsaturated lipids became hydroperoxidized leading to formation of phase separated domains [101]. These biomimetic models may translate well to the observed response of cellular membranes to oxidative stress as analysis of lipid rafts in AD brain tissue samples showed that increased levels of membrane-associated oxidative stress were associated with accumulation of cholesterol and ceramides into clustered microdomains, an effect that could be prevented by the antioxidants vitamin E and ceramide inhibitors [102]. Further, extracellular A β aggregation in close proximity of the cell membrane was demonstrated to induce membrane-associated oxidative stress. This process involves lipid peroxidation and generation of aldehyde 4-hydroxynonenal (HNE), a neurotoxic aldehyde that can be detected at early stages of disease progress in the AD brain [103]. HNE levels were observed to be proportional to the extent of neuronal lesions [27,104].

5.5.2. Oxidative damage to proteins

HNE can covalently modify Cys, Lys, and His residues in proteins by two different processes: Schiff's base formation and Michael addition. Schiff's base formation entails the reaction of the aldehydic group of HNE with an amino group of the targeted protein. Michael addition involves the addition of HNE double bond to a protein side chain. As a direct result of these covalent protein modifications the function of ion-motive ATPases, glucose and glutamate transporters, and GTP-binding proteins was found to be impaired [105]. In this way, oxidative damage to proteins may lead to disturbances in cellular homeostasis and impaired energy metabolism [105].

Low levels of membrane-associated oxidative stress were further found to render neurons vulnerable to excitotoxicity and apoptosis which possibly provides a mechanistic association between the A β -mediated oxidative damage to membranes and proteins and the loss of neuronal viability.

5.5.3. Tau (hyper)phosphorylation

One member of the mitogen-activated protein kinases (MAPKs) family, namely p38, is activated during A β -mediated oxidative stress. Among the different roles of p38, it was observed to induce tau phosphorylation in a primary neuronal model, which could be prevented by pretreatment with an inhibitor of p38 or vitamin E, [106]. These findings were confirmed *in vivo* using a transgenic APP/PS1 mouse model for AD in which the presence of amyloid plaques and phosphorylated tau were analyzed [107].

5.5.4. Oxidative damage to DNA and RNA

AD-related increased levels in oxidative stress are reflected by extensive oxidative damage to nucleic acids. The nucleotide bases of which the double-stranded DNA is made up can be oxidized by ROS/RNS leading to alterations in DNA structure. Oxidative stress can affect DNA in various ways resulting in base lesions, intrastrand cross-links or by the attack of the highly reactive OH $\cdot$ on the DNA [108]. The most frequently oxidized base is guanine as it is characterized by a high oxidation potential, followed by cytosine [109]. The major oxidation product of guanine is 8-hydroxydeoxyguanosine (8-OH-dg) which is also used as a biomarker to detect levels of oxidative stress (see paragraph 5.12.2). ROS/RNS can also damage mitochondrial DNA (mtDNA) which is highly susceptible to oxidative stress as a result of reduced complement of DNA repair pathways available in mitochondria compared to the nucleus. Oxidation of mitochondrial DNA is observed in a number of pathologies, including AD [110]. In contrast to oxidative DNA damage, which has been widely investigated, RNA damage has been less well studied. RNA presents a target of ROS-induced oxidative damage causing reduced

translation efficiency and damage to ribosomes. The oxidation of the base guanosine produces 8-hydroxyguanosine (8-oxoG) [111]. High levels of 8-oxoG were observed in neurons within the hippocampus, subiculum, entorhinal cortex, and frontal, temporal, and occipital neocortex in autaptic brain tissues of patients affected by AD [112]. Moreover, RNA oxidation was found to be significantly increased in the hippocampus, cortical neurons, white matter and in the frontoparietal cortex of aged rats [113]. These findings imply a role of oxidative-stress induced damage of DNA and RNA in neurodegenerative disease and aging.

5.6. Consequences of oxidative stress on a mitochondrial level

Neurons in the brain are rich in mitochondria to meet their high demand for energy thus making the brain susceptible to oxidative stress associated with mitochondrial dysfunction [41]. Various routes by which energy production at a cellular level may be derailed resulting in excessive production of ROS have been suggested in literature. Neurons originating from AD brains and containing defective mitochondria show loss of dendritic spines, and abbreviation of dendritic arborization [114]. Various publications consistently report that A β plays a central role in inducing these pathological hallmarks by inhibiting key mitochondrial enzymes in the brain [115].

Differences in CA1 hippocampal mitochondria structure have been detected using 3-dimensional electron microscopy. Instead of the uniformly elongated mitochondrial morphology observed in wild type mice, in mice carrying mutations for presenilin-1, APP and mutant tau protein, but also selectively in the hippocampus of human AD patients [123], mitochondria appear to have an ovoid or teardrop profile. Further, and AD mouse model and AD patients show the presence of multiple small mitochondria and exaggerated

mitochondrial division [116] suggesting that the mitochondrial fission process is altered in AD. The mitochondrial fission process relies on dynamin related protein 1 (Drp1) and mitochondrial fission protein 1 (Fis1) [117,118]. In line with this thought, recent research observed the presence of elongated interconnected organelles where multiple teardrop shaped mitochondria were connected by thin double membranes. This structure, referred to as “Mitochondria-on-a-string (MOAS)”, has been identified in an AD mouse model together with increased Drp1 phosphorylation, causing incomplete fission. Even though altered mitochondrial fission processes in terms of neurodegenerative diseases have been viewed primarily as a pathological feature, using cardiomyocytes Drp1 induced mitochondrial fission was shown exert a protective effect against cellular apoptosis by enabling the cells to meet altered energetic demands[23]. An alternative role of Drp1 was suggested with the observation that reduced association of Drp1 with the mitochondrial membrane induced a lack of mitochondrial fusion, which, in turn, induces high levels of mitochondrial oxidative stress [119]. The fusion process should be in balance with mitochondrial fission to maintain mitochondrial homeostasis. Mitochondrial fusion is mediated by inner membrane fusion factor optic atrophy-1 (OPA1). Addition of H₂O₂ to an osteosarcoma and a cardiomyoblast cell line lead to inhibited mitochondrial fusion as a result of loss of OPA1 activity through cleavage mediated by metalloendopeptidase OMA1 [120,121].

5.7. How oxidative stress derails in Alzheimer’s disease.

5.7.1. Oxidative stress in normal aging and repair mechanisms

Oxidative stress and metabolic impairment generally increase with advancing age resulting in functional impairment of tissue, an observation that served as the basis for the free radical theory of aging that was first published in 1956

[122]. This theory proposes that damaging effects of free radicals are responsible for aging-related loss of cellular function as a result of a decreased ability of aged cells to neutralize the impact of oxidants. ROS-induced damage is subject to a multitude of repair mechanisms [123]. Oncogenic activation of oxidative stress in a normal human fibroblast was shown to activate repair mechanisms for damaged DNA [124]. Also at a protein level the proteasome has been demonstrated to play an active role in degradation of oxidized proteins in cultured liver cells [125], hematopoietic cells [126], K562 chronic myelogenous leukemia cells derived from human blood [127], and fibroblasts [128]. A DNA microarray analysis demonstrated that in the aging mouse brain transcriptional suppression was observed of genes related to the ubiquitin-proteasome pathway [129]. Exposure of *Caenorhabditis elegans*, *Drosophila melanogaster* and a mouse embryonic fibroblast cell culture to an oxidant were previously shown to upregulate expression of 20S proteasome in an Nrf2-dependent manner [130,131]. Upon aging, changes are observed in these repair mechanisms. Using *C. elegans* it was recently found that aged animals are less capable of adapting their expression levels of 20S proteasome in response to H₂O₂-induced stress [132]. Further, studies using rats showed that the activity of anti-oxidant enzymes gamma-GCS, GR, GPx, gamma-GTP, GST, catalase and SOD as well as GSH in various brain regions declined with age [133]. A number of clinical studies have attempted to establish this association between aging and oxidative stress in healthy humans but the results are somewhat contradictory. A clinical study that partially supported the free radical theory of aging involved one hundred healthy humans within the age range from twenty to seventy years old. This study demonstrated a positive correlation between aging and lipid peroxidation levels expressed by an increase of malondialdehyde (MDA) levels. MDA is a product of lipid peroxidation and therefore serves as a

marker for lipid peroxidation. Measurement of the activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) demonstrated that only GPx activity was lowered in the 60-69 years age group compared to the 20-29 years age group while CAT and SOD activities were unaffected [134]. Furthermore, studies into the potential association of the process of lipid peroxidation with aging lead to inconsistent reports [135]. One of the reasons for this could be a gender specific variation as was demonstrated upon measurement of the production of thiobarbituric acid reactive substances (TBARS) in rats. Results showed that hepatic lipid peroxidation levels in livers of male rats were increased while female rats showed a decrease in TBAR levels [136]. The underlying mechanism of gender-specific variation and whether these rat-derived results correlate to human biology is not well understood. It appears that, even though animal studies and human clinical investigations both indicate that the general oxidant-quenching capacity of the body declines upon aging while the mechanisms responsible for this observation varies with species and gender.

5.8. AD related oxidative stress

5.8.1. Interconnecting parameters in the Alzheimer brain

A number of studies to date have investigated the oxidative status of AD patients. There generally seems to be an interconnection between AD-related pathophysiological hallmarks including the presence of A β plaques, and tau protein tangles on the one hand and oxidative stress on the other hand [84,107,137–140]. For example, deficiency of MnSOD in APP19959/MnSOD $\pm$ transgenic mice resulted in increased oxidative stress, as evaluated by protein carbonyl levels, and increased levels of A β and amyloid plaque burden in various brain regions compared to APP19959/MnSOD $++$ transgenic mice [141]. Further, oxidative stress was

shown to enhance A β production by decreasing the activity of α -secretase while promoting the expression and activation of β - and γ -secretase, enzymes critical for the generation of A β from APP (see paragraph 5.9.5). Using a transgenic mouse model (Tg mAPP) it was shown that mitochondrial oxidative stress, measured by levels of 4-HNE and H₂O₂ production, increases in these mice and is correlated with accumulation of mitochondrial A β in an age-related manner [115]. Tau is a microtubule associated protein playing a major role in the process of microtubular transport [142–144]. It was reported that overexpression of tau inhibits kinesin-dependent transport of peroxisomes in retinoic acid differentiated N2a cells resulting in neuritic peroxisomal depletion [145]. Mitochondrial depletion was observed in axons of primary rat neurons transfected with HSV-tau showing that microtubular transport of these organelles is hampered in the presence of excess tau [145]. In line with these observations, overexpression of tau made H₂O₂ challenged N2a cells more susceptible to cell death [145]. Accumulation of oxidatively damaged lipids (4-hydroxynonal, malondialdehyde, 2-propenal, F2-isoprostanes and F4-neuroprostanes), proteins (carbonyl and 3-nitrotyrosine), and DNA, and RNA (8-OH-dg and 8-oxoG) have been reported in various regions of AD patient brains which has been elaborately reviewed before [146]. An extensive review on the types of oxidatively damaged cellular molecules in the AD brain has been published before [147].

5.8.2. Disturbed metal ion homeostasis in Alzheimer's disease

Metal ions such as Cu²⁺ and Zn²⁺ play an important role regulating synaptic functioning by inhibiting the excitatory NMDA receptor [148], and rat GABA receptor [148,149]. Fe²⁺ has been documented to regulate synaptic plasticity and synaptogenesis as well as myelination [150] as illustrated by the neuronal expression of iron transporter DMT1 [151–153]. The levels of these metal ions are normally strictly regulated to prevent oxidative stress

resulting from interaction of Fe^{2+} or Cu^{2+} with oxygen to generate radicals such as superoxide ions or hydroxyl radicals. Disruption of metal ion homeostasis has been observed in various neurodegenerative disorders including AD [154]. A patient study using instrumental neutron activation analysis demonstrated that levels of Cu^{2+} were decreased while Zn^{2+} and Fe^{2+} levels were elevated in the hippocampus and amygdala of AD patients which correlated with observed histopathological changes in these regions [155]. On the other hand, serum levels of Cu^{2+} were shown to be increased in AD patients compared to control subjects [156]. Also in preclinical stages and mild cognitive impairment (MCI) Fe^{2+} levels were increased in the cortex and cerebellum and correlated with generation of radicals [157]. Compared with the neuropil of the amygdala of AD patients, senile plaques were observed to contain increased levels of Zn^{2+} , Fe^{2+} , and, selectively in the rim of the plaques, Cu^{2+} [158]. As Zn^{2+} , Fe^{2+} and Cu^{2+} have been shown to interact with $\text{A}\beta$ *in vitro* increasing $\text{A}\beta$ aggregation [159], metal ion dyshomeostasis has been postulated as potential mechanism by which amyloid plaques arise in the AD brain as $\text{A}\beta$ was shown to interact with various metal ions with high affinity which induces oligomer formation of $\text{A}\beta$.

5.8.3. Spatial link between amyloid plaques and cells exhibiting oxidative damage

A multiphoton microscopy-based study using the genetically encoded calcium indicator Yellow Cameleon 3.6 packaged into an adeno-associated virus (AAV2) and expressed in the brains of adult transgenic APP/PS1 mice showed that calcium overloaded neurites in living animals were more likely to be located in close proximity ($< 25 \mu\text{m}$) of a plaque [160]. This observation suggests a direct or spatial link between pathological alterations in neurons and the presence of formation of senile plaques. A second marker that indicates that there is a spatial link between AD-related deposits in the brain and neuronal functioning was the receptor for advanced glycation end

products (RAGE). Paragraph 5.10.9 discusses in more detail how A β may interact with this receptor and how this connects to oxidative stress. Neuronal cells adjacent to senile plaques display increased RAGE expression while little change in expression was demonstrated in brain regions remote from plaques [161]. Two other markers that have been used to topologically differentiate subpopulations of cells affected by oxidative stress include p50, which is a DNA binding subunit of transcription factor NF κ B [162,163], and heme oxygenase type 1 (HO-1). Cellular structures containing accumulations of A β displayed increased levels of oxidative stress as demonstrated by elevated levels of HO-1, and p50 [161]. Inactive NF κ B resides in the cytosol and is bound to inhibitory protein I κ B which prevents nuclear translocation of NF κ B. Phosphorylation, ubiquitination, and degradation of I κ B drives the activation of NF κ B [164]. The redox state regulates activation and nuclear translocation of NF κ B [165], and, as such, ROS was found to induce phosphorylation of I κ B via activation of responsible kinases [166,167]. Using p50 and HO-1, it was observed that the spatial link found between A β deposits and induction of cellular ROS is not limited to CSF residing neurons but this observation extends to endothelial and smooth muscle cells in cerebral blood vessels. The expression of HO-1 was found to be elevated in AD injured neuronal cells, a feature that was more pronounced in regions close to neurofibrillary tangles and A β plaque deposits [168].

5.8.4. Oxidative stress is an early stage pathological feature

A redox proteomics study of the human brain of Down syndrome (DS) patients prior to onset of AD provided insight into the role of oxidative damage in the development of DS related early onset AD [169]. Male and female DS and control brains were analyzed post mortem for carbonylation levels of proteins as hallmark of oxidative stress. DS brains showed increased carbonylation of six proteins including cathepsin D, glial fibrillary acidic

protein and succinyl-CoA:3-ketoacid-coenzyme A transferase 1 mitochondrial protein. This increased carbonylation affected protein functionality, but at the same time, proteasome activity and autophagy activity were decreased [169], potentially leading to increasing levels of non-functional protein. Even though this study was conducted on a small number of subjects, it did provide important insight into the potential role of oxidative stress in early stages of disease. Consistent with this thought, a larger scale study using human peripheral blood mononuclear cells (PBMCs) derived from 104 MCI subjects showed increased oxidative stress markers as detected by the fluorescent probe DCFH2-DA [170]. Also, in MCI and mild AD patient PBMCs homeostasis of ER stress-mediated Ca^{2+} was disturbed and SOD1 levels decreased [170]. Analysis of lymphocytes obtained from MCI subjects and AD patients similarly showed increased ROS levels, detected by 8OHdG, compared to lymphocytes derived from an age-matched control population [171]. The validity of using 8OHdG brain levels as a valid biomarker to detect oxidative stress-related damage to DNA in AD patients has been questioned [172]. However, the detection of increased levels in the frontal cortex of other modified macromolecules such as F2-isoprostanes as well as 3-NT and oxidized glutathione detected in patients with probable AD further corroborates the thought that oxidative stress is an early stage pathological feature of AD [173]. The work by Ansari and Scheff also compared oxidative stress levels in age-matched groups with progressive forms of cognitive disorder, from noncognitive impaired to AD, and showed that oxidative stress progressively worsened with cognitive decline. In addition to this, activities of SOD and catalase in post mitochondrial supernatant, and in mitochondrial and synaptosomal fractions of the frontal cortex were significantly declined already in MCI subjects [173]. Consistent with this, an earlier longitudinal study on autopsied control and patient brains

demonstrated that levels of isoprostane (F2) and F4-neuroprostane were increased in both amnesic MCI and late stage AD patients in various regions of the brain [174].

5.8.5. A vascular component

The microcerebrovascular structure showed age-dependent changes [175] which were more pronounced in cognitive disorders such as dementia [176,177]. For example, the basement membranes of cortical capillaries of patients suffering from cognitive disorders were significantly thicker than those of age-matched controls [177]. Smooth muscle atrophy and general disorganization of these cells was consistently observed in AD subjects although these features seemed unrelated to deposition of A β [178]. These structural changes translate into a decreased capillary flow in aged (16 months old) compared to young (2 months old) mice [179] as well as aggravated loss of blood flow rate in an aged APP^{swe}/PS1 Δ E9 transgenic mouse model [180]. The observed structural and functional changes in the microvascular organization thus lead to hypoperfusion and a general inability of the cerebral vasculature to meet the metabolic needs of the brain while this was partly compensated for by an increased ability to extract oxygen from the remaining blood flow [181]. However, the remaining metabolic deficiency is of sufficient magnitude to result in neural hypoxia [181]. Various conditions have been associated with increased brain oxidative stress and neuronal apoptosis in response to hypoxia, including sleep apnea [139,182], exposure to carbon monoxide [183], and ischemia [184]. Sleep apnea co-occurs frequently with AD [185] while prevalence of sleep apnea positively correlates with aging [186,187], and treatment of sleep apnea slows down the rate of cognitive decline in patients diagnosed with mild-to-moderate AD [185]. Further, hypoxia induced oxidative stress in the brain has been shown to induce cognitive deficits in rats [188]. The mechanisms by which the brain

adapts to hypoperfusion-induced hypoxia have been explored and most proposed mechanisms are centered around the thought that activation of hypoxia-inducible factor 1 α (HIF-1 α) plays an important role. HIF-1 α is a component of a heterodimeric complex with the aryl hydrocarbon nuclear translocator (ARNT or HIF β) [189]. Under normoxic conditions, HIF α is dissociated from this complex and unstable as a result of its hydroxylation which targets it for ubiquitination and proteasomal degradation [190–192]. Hypoxia prevents hydroxylation of HIF α by inhibition of the two hydroxylating enzymes, factor inhibiting HIF-1 and prolyl hydroxylase enzymes [193,194]. This stabilization induces its nuclear localization and heterodimeric complexation with ARNT. Subsequent co-recruitment of this complex with transcription coactivators p300 and CREB binding protein (CBP) initiates gene transcription. Hypoxic conditions are considered to raise cytosolic ROS levels and, in this way, induce the activation of HIF (reviewed in [195]) probably in a mitochondrial complex III-dependent manner [196]. The HIF-dependent hypoxia-inducible genes are generally involved in processes aimed at promotion of cellular survival under hypoxic conditions. A study investigating mRNA expression in adult rat brains upon occlusion of the middle cerebral artery demonstrated that glucose transporter-1 (GLUT-1) and glycolytic enzymes (phosphofructokinase, aldolase, and pyruvate kinase) were upregulated to increase transport of glucose and glycolysis [197].

5.9. Genetic and other factors that correlate oxidative stress to Alzheimer's disease

5.9.1. Clusterin

Apolipoprotein J is a ubiquitously expressed secreted glycoprotein which is also known as clusterin (CLU). Aging induces elevated levels of CLU gene expression [198,199] and plasma CLU levels [200]. In a genome-wide association study CLU has been identified as a genetic determinant in AD

[201] and plasma CLU levels were associated with atrophy of the entorhinal cortex and clinical progression of the disease [202] as well as with longitudinal brain atrophy in MCI patients [203]. Apart from aging, expression of the CLU gene was demonstrated to be sensitive to heat-shock induced changes in the organism or the direct environment of the organism as a result of the presence of activating protein-1 (AP-1) and CLU-specific element regulatory elements in its promotor [204]. Consistent with the idea that CLU plays a role in stress-associated coping of cellular response a study in H9c2 cardiomyocytes revealed that the Akt/GSK-3 β pathway may be involved in the anti-oxidant and anti-apoptotic effect of CLU in a megalin-dependent manner [205]. Multi-ligand receptor megalin has been identified to also act as receptor of clusterin [206]. Various cellular stress stimuli have been shown to regulate transcriptional activity of AP-1 [207]. Differential CLU expression was similarly observed in other oxidative stress-related pathologies including asthma [208], atopic dermatitis [209], diabetes type 2 [210], coronary heart disease [210], and cancer [211]. Oxidative stress increases CLU expression. This was demonstrated in a study in which human diploid fibroblasts were treated with H₂O₂ which resulted in increased mRNA levels of CLU [212]. In human neuroblastoma cell lines SH-SY5Y and IMR-32 both mRNA and protein levels of CLU were found to be upregulated in response to pro-oxidant pair iron-ascorbate [213]. In line with these observations, CLU was originally identified to function as a chaperone protein where its activity was reported to depend on cellular redox state [214]. CLU was shown to protect against oxidative stress in various cellular systems including fibroblasts and prostate cancer cells [205,215] but also *in vivo* in a *Drosophila melanogaster* model [216]. In neuroblastoma N2a and SH-SY5Y cells knockdown of CLU by short hairpin RNA interference was found to down-regulate antioxidant capacity [217]. The precise anti-oxidant

mechanism of CLU is not known although blockage of the sulfhydryl groups contained in the sequence of the protein resulted in abolishment of its oxidative stress preventive activity [216]. A review covering the involvement of CLU in oxidative stress detection and action has been published before [218]. Apart from an antioxidative effect of CLU, an indirect role of CLU actually promoting oxidative stress has been described showing that the presence of CLU induces the formation of slowly sedimenting complexes composed of SDS-resistant synthetic A β assemblies that induce oxidative stress in PC12 cells [219].

5.9.2. Apolipoprotein E and Thioredoxin-1

Apolipoprotein E4 (apoE4) was identified to be one of the major genetic risk factors for AD [220–223]. Apolipoprotein E exists in three isoforms, ϵ 2, ϵ 3 and ϵ 4, which vary in their amino acid composition. Carriers of the ϵ 4-allele have an increased risk of developing AD [220] as well as a decreased age of AD onset [221] compared to non- ϵ 4 carriers. The pathogenic origins of ApoE4 have been studied to great length and indicate that ApoE4 is involved in processes such as aggregation and clearance of A β [224,225], mitochondrial dysfunction, and impairment of calcium [226,227] or cellular iron homeostasis [228], and ApoE4 affects synaptic architecture and functioning [229,230]. A potential connection between the ApoE allele, AD, and oxidative stress was first deduced from the observation that the extent of oxidative stress and anti-oxidant defense is related to ApoE genotype in mice and in patients [14,228,231–233]. ApoE was demonstrated to act, directly or indirectly, as an antioxidant against hydrogen peroxide-induced cytotoxicity in a B12 ApoE expressing cell line [234]. Elevated levels of peroxidized plasma low-density lipoproteins were observed in ApoE-deficient mice [235]. Levels of lipid oxidation were significantly increased in the frontal cortex of AD patients that were homozygous or heterozygous for the ϵ 4-allele of APOE

compared to homozygous $\epsilon 3$ carriers and controls [14]. Upregulation of catalase activity was exclusively observed in frontal cortex tissue of homozygous APOE4 carriers while SOD activity and concentrations of glutathione were not different from that of controls [14]. Additionally levels of HNE were increased in $\epsilon 4$ -carriers [236]. Further, mouse brain synaptosomes with human ApoE4 were more susceptible to $A\beta_{42}$ -associated oxidative stress than synaptosomes from mice with human ApoE2 or ApoE3 [237]. Various experimental findings shed light on the potential molecular mechanism underlying these observations. They show involvement of thioredoxin-1 (Trx1), an endogenous antioxidant with a down-regulating role in apoptosis signal-regulating kinase-1 (Ask-1) [238]. Thioredoxin reductases are reducers of Trx1 [239]. Levels of Trx1 were reduced in AD brains [240,241] depending on ApoE genotype, but also in ApoE4 expressing mouse hippocampi, and human primary cortical neurons and neuroblastoma cells to which ApoE4 was supplemented with the culture medium, compared to ApoE3 [242]. At the same time, Trx1 mRNA levels in ApoE4 TR mouse hippocampi were elevated consistent with findings reporting increased Trx1 expression in conditions of oxidative stress [67]. Persson and colleagues suggested that increased mRNA levels of Trx1 potentially act as a compensatory mechanism for the increased cathepsin D-induced Trx1 turnover as observed in SH-SY5Y neuroblastoma cells [242]. Moreover, $A\beta_{42}$ was demonstrated to cause transient oxidation of Trx1 [240] as well as ApoE4-induced downregulation of this protein which resulted in activation of an apoptotic pathway involving the translocation of Death-Domain Associated Protein-6 [240,242,243] without affecting catalase and GSH activities [242].

5.9.3. Down's Syndrome

DS is characterized by trisomy of chromosome 21 (HSA21). Individuals with DS are prone to develop early-age AD. Comparable to observations in AD patients, mouse models of DS demonstrated deficits in hippocampal learning and memory as well as neurodegeneration of cholinergic basal forebrain neurons [244,245]. DS patients display features of cellular energy impairment [4]. Recent transcriptomic profiling of the skeletal muscle of a DS mouse model showed that among the identified differentially expressed protein-coding genes in this tissue, two, *Sod1* and *Runx1*, were implicated in oxidative stress [246]. Chromosome HSA21 also codes for SOD explaining why expression levels of SOD are increased in DS [247]. Transgenic mice overexpressing SOD1 demonstrate excessive levels of oxidative stress [248] because concentrations of CAT and GPx, two enzymes that act to neutralize hydrogen peroxide, the product of SOD1 activity, do not rise accordingly. Besides SOD1, fifteen other genes on HSA21 were predicted to play a role in mitochondrial energy generation and the metabolism of reactive oxygen species [249]. Levels of various ROS, RNS and aldehyde products of lipid peroxidation were found to be increased in brain [250,251] and urine [252] of DS humans and animals [250] indicating that oxidative stress may play a role in the pathogenesis of DS associated AD. Levels of oxidative stress, *i.e.* high ratio between SOD1 and GPx, correlated with cognitive phenotype [253]. However, a recent study showed that administration of melatonin at the pre- and post-natal stages partially alleviated oxidative stress but did not improve cognitive function in a mouse model [254]. The process of programmed cell death was found to coincide with increased levels of oxidative stress, compared to control cells. Programmed cell death could be rescued by administration of free radical scavengers including vitamin E, and N-tert-butyl-2-sulphophenyl nitron [247] but dietary parameters did not alleviate

oxidative stress biomarkers in young adult DS patients [251]. Similar to AD, the DS brain shows features of oxidative stress at very early stage. For example, the DS fetal brain cortex was observed to show increased levels of thiobarbituric acid reactive substances TBARs, HNE and protein carbonyl groups compared to controls [255]. Also end-products of non enzymatic glycation, pyrraline and pentosidine, were increased in DS fetal tissue [255] and in amniotic fluid of DS pregnancies [256].

5.9.4. Circadian clock genes

Circadian rhythm disturbances and associated disorders of the sleep-wake cycle, *i.e.* fragmentation of sleep and decreased duration of rapid eye movement sleep (REM), occur at various stages of AD-related neurodegeneration and symptoms are generally more severe with increasing A β amyloid beta burden and tau pathology [257–260]. The link between circadian rhythm disturbances and oxidative stress levels has not been investigated in great detail although one report shows that extensive loss of sleep reduced the activity of SOD and the production of ATP in rat hippocampi [261]. In a follow-up report, apart from reduced SOD activity, it was found that the activity of glutathione peroxidase was also decreased while liver malondialdehyde levels were increased with sleep deprivation [262]. On the other hand, brain and peripheral tissues were shown to differ in their peroxiredoxin oxidation rhythms [263] as well as in other clock components [264]. This means that peripheral observations cannot be automatically extrapolated to brain processes. Periods of REM were found to induce a significant reduction in firing rate of wake-active noradrenergic locus ceruleus neurons [265], cells that display high sensitivity to metabolic stress [266–268]. Extensive wakefulness induces loss of locus ceruleus neurons and sirtuin type 3 (SirT3) was observed to be involved in this neurodegenerative process [269]. NAD⁺-dependent deacetylase SirT3 is an important regulator

of energy production and redox response by reduction of glutathione [270,271], mediating the upregulation of SOD2 and catalase [272], and activation of SOD2 [273]. Extensive deprivation of sleep is related to reduced levels of SirT3 in young adult wild type mice locus ceruleus neurons while oxidative stress levels increase presumably as a result of a combined increase in metabolic activity and decline in antioxidant response [269]. Other circadian clock related factors related to redox homeostasis of NAD cofactors include transcriptional activator complex BMAL1 and its binding partners CLOCK and NPAS2 [274]. These clock genes were observed to be involved in glucose metabolism and redox homeostasis in peripheral tissues [274–276]. Expression levels of the master circadian clock regulator genes *Bmal1* and *Clock* are significantly decreased in the cerebral cortex of aged mice [277]. Mice generated with a deletion of *Bmal1* demonstrated increased systemic [276,278] and low levels of brain oxidative stress [279] mediated by a disturbance of its transcriptional targets, including *Per2* and *Dbp* [280] as well as neuropathologies and synaptic degeneration [276,280]. For example, the kinetic occipital region in the brain of these *Bmal1* knock-out mice showed a three-fold increase in level of F4-neuroprostanes when measured indicative of increased lipid peroxidation levels in neuronal cells [280]. The deletion also resulted in increased neurodegeneration caused by mitochondrial 3-nitropropionic acid which was suggested to be as a direct consequence of the decrease in BMAL1 transcription [280]. Both SirT3 and BMAL1 levels are tightly regulated by circadian rhythmicity of oxidoreductase factor nicotinamide adenine dinucleotide (NAD⁺) [281], while, in turn cellular redox status has impacts on the activity of clock transcription factors [274]. Proteasome expression levels and activity was were observed to follow circadian oscillations that correlated with the level of carbonylated proteins

[282] demonstrating that clearance of oxidized proteins are also showing circadian rhythms.

5.9.5. APP processing machinery

5.9.5.1. Exposure to oxidative stress upregulates APP processing into A β

Cells of both neuronal and non-neuronal origin exposed to H₂O₂ or HNE generate increased levels of intracellular and secreted A β [283–286]. The role of oxidative stress in A β generation was further demonstrated in Tg19959 mice, which overexpress a double mutated form of APP. Upon crossing this mouse line with a mouse in which one allele of MnSOD was knocked out, brain A β levels and A β plaque load were significantly increased [141]. Similarly, hypoxia treated transgenic APP23 mice that were subjected to hypoxia conditions demonstrated increased memory deficits and deposition of A β into plaques [287]. Repeated exposure to hypoxia facilitated progress of AD-like pathology in aged APP^{Swe}/PS1^{A246E} transgenic mice [288]. Vascular deposition of A β on the surface of cerebral endothelial cells results in vascular degeneration which has been observed in AD and leads to a condition termed cerebral amyloid angiopathy [289]. Exposure of primary cerebral endothelial cells derived from 2 month old Tg2576 mouse brains to H₂O₂ resulted in upregulation of APP expression and altered APP processing to favor the amyloidogenic pathway [290]. Also in humans it was found that oxidative stress induced by hypoxia due to cardiac arrest increased serum A β levels [291] suggesting that the generation machinery that generates this peptide is upregulated under pro-oxidative stress conditions in a wide range of disease models affecting various regions of the brain.

5.9.6. Oxidative stress has an A β species specific effect

A β is generated as a heterogeneous pool of peptides which vary in the number of C-terminal amino acids. The two most prevalent isoforms of A β are the 40-amino acid A β ₁₋₄₀ and the 42-amino acid A β ₁₋₄₂. It has been demonstrated that

the longer A β_{1-42} peptide is inherently more amyloidogenic than A β_{1-40} . Analysis of the specific species of A β that were generated under HNE-induced oxidative stress conditions using a TN₂ cell culture revealed a 70-80% and 60-140% increase of intracellular A β_{x-40} and A β_{x-42} , respectively. Secreted levels of A β_{x-40} were not affected by oxidative stress while secreted A β_{x-42} was increased by approximately 50% compared to control cells [285]. These findings are potentially pathologically relevant as it was reported previously by our group and others that a marginally increased A β_{1-42} :A β_{1-40} ratio has severe implications for synaptotoxic response [292–294]. A β is generated by sequential cleavage of APP by two enzymes, γ - and β -secretase (BACE), by a process termed amyloidogenic pathway. Alternatively, APP can be cleaved into an N-terminally truncated fragment of the A β peptide, called the p3 peptide, by γ - and α -secretase-mediated processing. Details of APP processing have been extensively covered in a number of reviews [307,308]. This paragraph will further discuss how APP processing and A β generation are affected by exposure to oxidative stress.

5.9.7. Both presenilin and anterior pharynx-defective-1, components of γ -secretase, are upregulated in conditions of oxidative stress

Presenilin (psen1) constitutes the catalytic site of the APP cleaving enzyme γ -secretase. In concerted action with β -secretase psen1 is responsible for the generation of A β [295]. Clinical mutations in psen1 cause familial forms of early onset AD [296] and can affect γ -secretase mediated processing of APP in various ways [297]. Generally mutations in psen1 comprises the composition of the heterogeneous A β mixture by shifting the ratio between the various A β peptides generated [298–300]. In human SK-N-BE neuroblastoma cells, which were exposed to HNE- or H₂O₂-mediated oxidative stress, an increase in A β level was found that could be attributed indirectly to γ -secretase in a c-jun N-terminal kinase (JNK)/c-jun

pathway/BACE1 dependent manner [301]. However, a direct relation between hypoxia-induced oxidative stress and γ -secretase functionality exists. This was later demonstrated in zebrafish by showing that transcription factor hypoxia-inducible factor 1 (HIF-1 α) induces increased mRNA expression levels of zebrafish related PSEN1 [302]. In line with these findings, PSEN1 $-/-$ fibroblasts demonstrated impaired induction of HIF-1 α [303] suggesting an apparent bi-directional interaction between psen1 and HIF-1 α . Importantly, this factor plays a crucial role in the regulation of oxygen homeostasis and the expression and stability of one of the HIF-1 α domains is regulated by oxygen levels [189,193,194]. Anterior pharynx-defective-1 (APH1) is another component of γ -secretase and it was shown that Hela cells express increased levels of APH1 α in response to chemical hypoxia induced activation of HIF-1 α resulting in increased APP and Notch processing [304]. NF- κ B has been identified as important regulator of HIF-1 α expression [305]. NF- κ B was shown to become activated and translocated to the nucleus in response to oxidative stress by addition of metformin, a pro-oxidative biguanide, to LAN5 neuroblastoma cells. This directly induced transcriptional activation of APP and psen1 and ultimately into increased APP cleavage, and intracellular accumulation of A β which promoted A β aggregation [306].

5.9.8. β -secretase is upregulated at a transcriptional level by oxidative stress

As discussed in the previous paragraph, oxidative stress has been reported to induce amyloidogenic APP processing [307]. The enzyme β -secretase (BACE1) is an integral part of the amyloidogenic processing pathway of APP and the functionality of this enzyme was, similar to γ -secretase, reported to be affected by oxidative stress. Low micromolar concentrations of HNE produced by pro-oxidant stimuli ascorbic acid/FeSO₄ or H₂O₂/FeSO₄ or by direct addition to cell medium of NT₂ cells in culture were reported to induce

β -secretase activity. This induction leads to an increased production of APP C-terminal fragments without affecting A β PP synthesis. Pretreatment of these NT₂ cells with α -tocopherol prevented BACE induction and CTF generation demonstrating direct involvement of oxidative stress in inducing BACE activity [308,309]. Also in APP/PS1 mice it was shown that administration of an antioxidant compound, tricyclodecan-9-yl-xanthogenate (D609), which is a glutathione (GSH)-mimetic compound, leads to decreased levels of oxidative stress as well as a reduction in β -secretase levels resulting in decreased APP processing into A β [310]. In line with these observations, various other publications reported that BACE1 protein expression levels were increased in response to oxidative stress [285,301,311–317]. A similar observation has been reproduced in various model systems. For example, a developing (48 h post fertilization) zebrafish animal model exposed to hypoxia showed that the mRNA levels and activity of zebrafish BACE1, the zebrafish orthologue to human BACE1, were affected by oxidative stress. At the same time, the level of CAT, a gene used as a biomarker for oxidative stress, was found to be increased upon exposure of zebrafish to hypoxia [302]. Also, in murine primary cortical neuronal cultures severe and cytotoxic levels of oxidative stress lead to an increased BACE1 expression. Mild oxidative stress conditions were found to result in subcellular redistribution of BACE1 that promoted amyloidogenic processing of APP [318]. Apart from the various animal and cellular models used to study oxidative stress, increased BACE1 levels and activity were also found in brains of sporadic AD patients [319–321]. To understand the cellular signaling pathways involved in oxidative stress-regulated expression of BACE1, an NT₂ cell-based assay was used. In this system, pharmacological based inhibition of C-Jun N-terminal Kinase (JNK) and p38^{MAPK}, involved in the stress activated protein kinase (SAPKs)/JNK signaling pathway, but not Akt signaling, was found to inhibit

transcriptional regulation of BACE1 [285]. P38^{MAPK} was also reported to be active and identified in A β deposits of A β PP tg mice [322]. These observations are consistent with an earlier report that showed that Sp1 regulates transcription of BACE1, where expression levels of Sp1 were positively correlated with the generation of BACE1 and APP processing [323]. In line with this, using a lipofuscin fluorophore A2E-mediated photo-oxidation model to investigate the role of BACE1 in age-related macular degeneration, it was shown that BACE1 expression is competitively regulated by Sp1 and DNA methyltransferase 1 (DNMT1) after photo-oxidation [324]. DNMT1 levels were reportedly decreased resulting in demethylation of specific loci within the BACE1 gene promotor [324]. Moreover, members of the SAPK family were also found to be upregulated in AD patient brains [106,325] and are activated by various stress signals including oxidative stress [326,327]. The oxidative-stress regulated involvement of JNK has been further demonstrated in studies using transgenic mouse models. For example, JNK was found to be significantly activated in mutant A β PP tg mice with extensive oxidative damage but not in mutant A β PP tg mice with little oxidative damage [328].

5.9.9. Downregulation of non-amyloidogenic APP processing

While it was consistently reported that the amyloidogenic processing pathway of APP is upregulated by various direct and indirect mechanisms, the non-amyloidogenic pathway, involving sequential cleavage of APP by α -secretase and γ -secretase, was found to be downregulated under conditions of oxidative stress [290]. Multiple lines of evidence have shown that γ -secretase is upregulated under conditions of oxidative stress (see paragraph 5.9.8). As such, it was anticipated that the net lowering of the non-amyloidogenic processing of APP should be accommodated for by a decrease in α -secretase activity. It was indeed shown that human neuroblastoma cell line SH-SY5Y

exposed to hypoxic conditions decreases the expression of disintegrin and metalloproteinase domain-containing protein 10, or ADAM-10, also called α -secretase, in an O_2 -dose dependent manner [329]. Similarly, human MSN cells exposed to oxidative stress induced by H_2O_2 or $FeCl_2$ were shown to downregulate the active form of ADAM10 [315]. Mechanisms that explain the downregulation of α -secretase under conditions of oxidative stress have not been explored into great detail. One of the hypotheses that has been postulated involves the JNK3-dependent phosphorylation of Thr668 of APP which is considered to be a direct target for BACE1 [315,330].

5.9.10. Klotho, the aging suppressor gene, regulated by oxidative stress

Klotho is a single-pass transmembrane protein hormone containing a long type I transmembrane domain and a short secreted domain [331]. The latter is released into the extracellular space upon insulin-mediated release by A Disintegrin and Metalloproteinases (ADAM) family members ADAM10 and ADAM17 [332]. Mutations in the *Klotho* gene were observed to induce a human aging resembling phenotype in a transgenic mouse model which could be genetically rescued by exogenous expression of klotho cDNA [333]. Consistent with this, aging was found to be suppressed in a klotho overexpressing mouse model [334]. Single nucleotide polymorphisms of *Klotho* affect trafficking and catalytic activity of klotho which was associated to onset of aging in a human population based study [335]. Expression of the protein declines with age as was demonstrated by microarray analysis of the aging brain of a rhesus monkey model [336]. Subsequently, the *Klotho* gene has been dubbed an aging suppressor gene which acts by regulating oxidative stress [337] because it was shown to effectively reduce urinary excretion of 8-OHdG upon renal overexpression in mice [338]. This hypothesis was further supported by the finding that different cell types incubated with Klotho protein were protected from oxidative stress and apoptosis induced by

paraquat [337], hydrogen peroxide [339], glutamate and oligomeric A β [340]. Apart from in the kidney, the protein is also expressed in the choroid plexus in the brain with low levels of expression in the hippocampus [333,341]. In this brain region Klotho plays a role in hippocampus-dependent memory by regulating adult hippocampal neurogenesis [342]. Mutation of klotho in a transgenic mouse model demonstrated increased levels of 8-OHdG and malondialdehyde in the hippocampus in an age-dependent manner [343]. One of the molecular models by which secreted Klotho has been suggested to regulate oxidative stress includes the insulin/IGF-1 growth signaling pathway [344]. Klotho inhibits the insulin/IGF-1 growth signaling pathway leading to activation of Forkhead box O (FOXO) transcription factor. This, in turn, enhances the expression of ROS scavenging enzyme mitochondrial manganese SOD2 [337] indicating that Klotho may perhaps lend its anti-aging capacity by indirectly regulating the generation of an antioxidant enzyme.

5.10. Putative molecular mechanisms of Alzheimer's disease-related oxidative stress

In this paragraph putative mechanisms that may explain how oxidative stress arises in the AD brain will be discussed. One of the striking observations in this context is that microglia in close proximity to amyloid plaques are often found to be activated and release O $_2^{\cdot-}$ and H $_2$ O $_2$ [345]. Clearly, the multicellular organization of the brain may be a relevant determinant in outcome of oxidative stress in AD, and potentially also other, neurodegenerative diseases. Characteristic histochemical hallmarks of AD are the deposition of the proteins A β and tau. For both proteins a number of potential contributory as well as inhibitory pathways in the process of oxidative stress generation have been proposed. For example, A β can reduce

Cu^{2+} to Cu^+ in a catalytic cycle that uses O_2 and biological reducing agents as substrates while generating neurotoxic H_2O_2 [346,347] and a peptide radical inducing oxidation and dityrosine cross-linking of $\text{A}\beta$ were observed [348,349]. This paragraph will discuss the current state of the art knowledge on the contribution of tau and $\text{A}\beta$ to AD related oxidative stress.

5.10.1. A protective or advancing effect of $\text{A}\beta$ on oxidative stress may be subtly defined

Perhaps one of the most prominent features of $\text{A}\beta$ is its affinity for metal ions such as Zn^{2+} , Fe^{3+} and in particular Cu^{2+} [348,350], which approximates the reported femtomolar affinity of SOD1 for these ions [351,352]. A potential anti-oxidant effect of $\text{A}\beta$ has been attributed to the reported ability of $\text{A}\beta$ to sequester these redox-active metals [348,350,353–358]. Copper is an abundant metal ion in the brain and accumulates in plaques to reach concentrations up to 400 μM [158,359,360]. Cu^{2+} , along with iron and zinc ions are involved in regulation of synaptic function highlighting the physiological relevance of a tightly regulated metal homeostasis [361]. Cu^{2+} concentrations of up to 15 μM have been measured in the synaptic cleft [362]. Published experimental data support the possibility of Cu^{2+} to directly interact with $\text{A}\beta$ involving tyrosine at position 10 and three histidine residues at positions 7, 13 and 14 acting as a metal chelator [349,350]. As a result of this binding, it has been proposed that $\text{A}\beta$ may quench Cu^{2+} preventing Cu^{2+} from generating H_2O_2 . In contrast, other reports have shown that the binding of $\text{A}\beta$ to such transition metal ions actually results in enhanced formation of ROS [363] by the reported ability of $\text{A}\beta$ to reduce Cu^{2+} , upon binding, to Cu^{1+} [364]. A subsequent publication questioned the role of Cu^{2+} binding in generation of ROS as it was shown that rodent $\text{A}\beta_{1-42}$, in which Cu^{2+} binding is ameliorated, results in similar levels of oxidative stress as human $\text{A}\beta_{1-42}$ [364]. Heme *a*, an essential component of mitochondrial complex IV, was shown to interact with $\text{A}\beta_{1-40}$, resulting in decreased assembly of this complex

into a functional electron transport chain complex [365]. These *in vitro* observations are supported by the finding that mitochondrial whole brain levels of cytochrome-*a* are decreased with 25% in the AD brain [366,367]. Both these protective and toxic roles of A β -metal complexation in oxidative stress receive ample support in the scientific field and perhaps the actual outcome whether metal ion binding is toxic is more subtly defined by factors such as A β level, A β aggregation state, co-occurring factors such as the availability of a reducing agent [346], redox kinetics [368], or disease stage. Supportive of such a hypothesis is the observation that A β was found to act as a neurotrophic agent selectively at low nM concentrations while at higher peptide concentrations neurotrophic functionality was abolished [354,369,370]. Even though the functional role of A β has been heavily debated, it has been argued that antioxidant activity may be the primary role of this peptide in the brain [370]. However, a compelling finding arguing against an anti-oxidant function as primary role for A β was that oligomeric human A β , but not rodent A β , can bind two molecules of heme with sub μ M affinity which results in the generation of a peroxidase [353]. Moreover, rat A β_{1-40} was reported to interact with zinc with lower affinity than human A β_{1-40} . Such species specificity directly argues against a primary anti-oxidant role of A β although it does not rule out the potential of the peptide to also, next to its yet to identify primary role, demonstrate anti-oxidant activity via indirect routes.

5.10.2. Tau plays an indirect role in oxidative stress via organelle transport

Next to A β , tau is also considered to play a role in oxidative stress, albeit indirectly: upon overexpression tau inhibits kinesin-dependent transport of mitochondria and peroxisomes into neuronal processes while the microtubular network remained intact [145]. Lack of transport of these two organelles was shown to deplete neurites from ATP and protection

mechanisms against oxidative stress as was illustrated by an increased vulnerability of tau overexpressing differentiated N2a cells upon exposure to H_2O_2 [145]. Another parameter proposed to play a role in tau-induced oxidative stress includes tau aggregation into paired helical filaments [371]. Addition of synthesized HNE at micromolar concentrations to retinoic acid differentiated P19 embryonal carcinoma cells induced tau to crosslink into high molecular weight species [371]. Crosslinking of tau into paired helical filaments was shown to be driven by abnormal levels of phosphorylation, or hyperphosphorylation, of tau, which renders the protein insoluble and dysfunctional [372,373]. The role of phosphorylation of tau in this process and the linked to oxidative stress was highlighted by three subsequent publications showing that i. phosphorylation of tau is partly regulated by the extracellular signal-regulated protein kinase (ERK2) which becomes activated upon exposure to H_2O_2 [374], and also, ii. by rapid and potent activation of transcription factor NF κ B by reactive oxygen intermediate-mediated release of inhibitory factor I κ B from NF κ B [166], and iii. acrolein, a peroxidation product of arachidonic acid, induced p38 stress-kinase-mediated tau phosphorylation [375]. An *in vitro* follow up study demonstrated that co-incubation of a pseudo-phosphorylation mimicking form of tau with acrolein and methylglyoxal induced the formation of tau dimers and high molecular weight oligomers [376]. Other than phosphorylation, also the glycation of tau was observed to connect tau tangle formation with oxidative stress. Using SH-SY5Y cells, advanced glycation end product-recognizing antibodies, increased heme oxygenase and malondialdehyde reactivity were shown to colocalize with tau paired helical filaments [377].

5.10.3. Direct generation of ROS by A β fragments

A pioneering publication in 1994 used mass spectrometry and electron paramagnetic resonance spin trapping to demonstrate that A β *in vitro* under cell free conditions itself can fragment into free radical peptides in an oxygen-dependent but metal-independent manner [378]. The generated A β_{25-35} fragment was capable of inactivating the enzymes glutamine synthetase and creatine kinase. The authors suggested that methionine 35 may be capable of reacting with oxygen to produce sulfoxide, which, in turn, can result in radical generation [378,379] although this hypothesis has not been verified.

5.10.4. A β aggregation state

A β was demonstrated to accumulate into various aggregation states ranging from monomeric to larger assemblies into amyloid plaques found upon post-mortem analysis of AD patient brains. In-between these two states an apparent continuum oligomeric aggregates with different aggregation numbers, *e.g.* the number of monomers per oligomer, different n-number aggregates exist, and many studies have attempted at identifying and characterize the most toxic species within this range. Detailed reviews have been published on this specific topic highlighting the potential toxic role of these intermediates and their relation with the clinicopathological features of AD [380–383].

5.10.5. The dynamic nature of A β assembly hampers studies into oxidative stress

Generally experimental studies indicate that particularly the intermediate soluble aggregated forms of A β are highly toxic and these species are commonly referred to as oligomers or pre- or protofibrils [384,385]. More specifically, soluble SDS-stable dimers, extracted from AD brains [386], up to 56 kDa soluble A β assemblies that are capable of inducing cognitive impairment in Tg2576 mice [387] have been identified as potentially toxic species acting upon AD progress. While most of these studies merely

investigate the association between assembly state and loss of cellular viability, synaptic function, or cognition, consistent with these observations, a study investigating the effects of different A β aggregate species on oxidative stress showed that specifically prefibrillar and oligomeric A β_{1-42} potentially increased levels of oxidative stress in NT2 cells as detected by HNE and H₂O₂ generation [388]. Using a combination of electron spin resonance spectroscopy coupled to spin trapping, a short burst of H₂O₂ generation was observed during early aggregation stages of A β_{1-40} [389]. Recently, incubation of A β with Cu²⁺ and H₂O₂ were shown to modulate A β self-assembly. Depending on the Cu²⁺ to H₂O₂ ratio, up to hexameric species of A β_{1-42} could be detected using SDS-PAGE with little propensity to develop into ThT positive fibrils upon prolonged incubation [390]. These A β_{1-40} and A β_{1-42} oligomers demonstrated prolonged disruption of phospholipid vesicles which is one of the proposed cytotoxic mechanisms of A β oligomers [390]. However, the precise aggregation number of such A β oligomers is difficult to pin down using most standard biophysical and biochemical techniques as a result of their heterogeneous, dynamic, and interconverting nature.

5.10.6. Interaction of A β with mitochondrial factors

Several mechanistically indirect pathways have been suggested by which means A β can influence mitochondrial function.

5.10.7. By interaction of A β with mitochondrial alcohol dehydrogenase

Yeast-two-hybrid based screening of the human brain and a HeLa cell model demonstrated that A β and mitochondrial alcohol dehydrogenase may interact [391]. This interaction was shown to be specific involving residues 12-24 of A β_{1-40} and disturbs the NAD-binding pocket and the catalytic triad of the enzyme leading to functional inhibition of nicotinamide dinucleotide binding required for the function of the enzyme. [139,391–393], and resulting in oxidative stress and neurodegeneration [391]. Given the fact that A β is

primarily an extracellularly generated peptide it is of course questionable whether A β and mitochondrial alcohol dehydrogenase may ever reside in close proximity of each other. However, by means of co-immunoprecipitation assays, immunogold electron microscopy and confocal microscopy it was demonstrated that A β can be detected in the mitochondria within the cerebral cortex of AD patients and mutant APP (mAPP) as well as mAPP/ABAD transgenic mice. Also, in these *in vivo* animal models A β was reported to co-localize with the N-terminal (residues 98-101 and 108-110) of alcohol dehydrogenase while mutations within this site abrogated A β binding to alcohol dehydrogenase [139]. Strengthening the suggestion that alcohol dehydrogenase may play a role in AD, degenerating neurons in the brains of AD patients were found to express up-regulated levels of alcohol dehydrogenase [139], particularly in close proximity to A β deposits [391]. Although it cannot be ruled out that this observation represents an A β -unrelated compensatory mechanism to counteract the impaired energy homeostasis generally observed in AD neurons [394,395]. The role of alcohol dehydrogenase in AD has been more extensively covered in a review [396].

5.10.8. By disruption of energy generation from mitochondria

The brain requires ATP and its intermediates for the formation of the neurotransmitter acetylcholine [397], and the critical membrane component cholesterol [370]. To accommodate these requirements it was recognized more than a century ago that the vascular system exerts some degree of plasticity to ensure sustained local activity of the neuronal network [398]. Further, astrocytes, expressing GLUT1 type glucose transporters [399], play a central role in neuronal energy supply [400] supporting the notion that the multicellular context of the brain is highly supportive of neuronal energy-consuming activities. Current PET and fMRI functional brain imaging techniques are based on the assumption that brain function is associated with

brain energy consumption and from such techniques a wealth of information has been acquired over recent years on brain energy homeostasis in a variety of neurodegenerative disorders. The association between oxidative stress and energy production becomes apparent when considering that the regeneration of GSH from GSSG is an NADPH dependent process, where NADPH is mainly obtained through glucose metabolism. In various neurodegenerative disorders, including AD, a decrease in brain ATP generation is observed. Membrane fluidity changes observed upon iodoacetic acid-induced inhibition of ATP production could be rescued by treatment with anti-oxidants tirilazad and gossypol [401] suggesting that reduced ATP availability may result in oxidative stress and membrane damage. Upon aging, glucose metabolism derails progressively as a result of changes in brain insulin [402], and cortisol [403] levels. Also, activity of synaptic ATPases were significantly decreased in rat frontal cerebral cortex upon aging [404]. This condition seems to worsen in AD patients, as it was shown in patients with incipient late-onset AD type dementia, that cerebral glucose utilization had reduced with 45% which progressed to 55% in advanced stages of the disease [405]. Oxidative stress, induced by NO was shown to transiently reduce ATP generation in rat astrocytes while increasing glycolysis rate in an F_1F_0 -ATPase and adenine nucleotide translocase dependent manner specifically in primary astrocytes to rescue this ATP-depleted phenotype [406]. At the same time, rat primary neurons exposed to NO were shown to progressively become ATP depleted which ultimately lead to cell death [406]. A number of metabolism-related enzymes have been identified to be affected in a progressive manner in AD, including pyruvate dehydrogenase, ATP-citrate lyase and acetoacetyl-CoA thiolase [407]. Activities of glycolysis and citric acid cycle related enzymes aldolase, triose phosphate isomerase, phosphoglycerate kinase, and phosphoglycerate mutase are affected in AD as a result of oxidation or

nitration [408–412]. Inconsistent results have been published on phosphofructokinase activity in AD brains, with some researchers suggesting no significant reductions [413]. Subcortical regions of the brain further displayed increased activities of hexokinase, an enzyme involved in the initiating step of the glycolysis, and lactate dehydrogenase in AD patient brains while activities of these enzymes in cortical regions were unaffected [414]. As the authors already suggest [414], increased activity of lactate dehydrogenase is suggestive of a metabolic need for anaerobic respiration to compensate for lost ability to generate ATP via aerobic metabolic routes. Taken together, oxidation and nitration processes of many of the enzymes involved in cellular metabolism may reduce activity of such enzymes sufficiently to explain the significantly reduced ATP generation observed in AD brains while the bioenergetic adaptation of the cell towards anaerobic routes for obtaining sufficient quantities of ATP to sustain high levels of ATP generation cannot sufficiently be compensated for. The question remains whether tau or A β only play an indirect role in activity reduction by inducing oxidation or nitration of these enzymes or whether a direct role, for example by activity-reducing interaction such as demonstrated for ABAD (see paragraph 5.10.7) causes loss of metabolic rate. A study using co-immunoprecipitation assays combined with tandem mass tag multiplexed quantitative mass spectrometry identified glycolysis enriched proteins such as pyruvate kinase and aldolase as potential interactors with au [415]. Using ELISA and gel filtration assays, it was reported that synthesized A β_{42} and A β_{1-28} can interact *in vitro* with a K_D of 5 nM with rat phosphofructokinase, but not lactate dehydrogenase, to which reduces its activity [416]. It is still debatable what the triad of factors oxidative stress, A β or tau and cellular metabolism exactly comprises in terms of molecular interactions and how they reciprocally interact with each other. One attempt to address this

question was published by Casley and colleagues who incubated isolated rat brain mitochondria either with A β ₂₅₋₃₅ or A β ₁₋₄₂ with or without NO to determine the relative impact of each of these factors on mitochondrial respiration using oxygen sensitive electrodes [417]. Both A β peptides significantly inhibited mitochondrial respiration by specifically affecting the activity of complex IV, while exposure of the mitochondria to NO substantially worsened respirational outcome.

5.10.9. Interaction with RAGE

Receptor for advanced glycation end products, or RAGE, is a transmembrane receptor widely expressed in all tissue types including the brain [418,419]. One diverse group of ligands known to interact with this receptor are advanced glycation end products, AGEs. AGEs are the product of non-enzymatic aldose-mediated glycation or oxidized proteins [420], and accumulation of AGEs was shown to be aging-related [421,422] and accelerates in conditions such as diabetes [423]. Potential clinical relevance of RAGE to AD was demonstrated using ELISA of AD brain homogenates showing a 2.5 fold increase in expression of RAGE compared to age-matched control subjects [161]. The roles of RAGE and AGEs in AD pathogenesis have been covered in a number of reviews [424,425]. The offspring of a transgenic RAGE overexpressing mouse model crossed with Tg APP animals showed neuronal perturbation already at 3-4 months of age, astrogliosis and reactive microglia at 14-18 months [426]. One of the general observations is that A β can interact with RAGE. For example, Yan and co-workers have shown that endothelial or PC12 RAGE provides a binding site for ¹²⁵I-labeled synthetic A β on cellular surfaces. This interaction was found to result in cellular perturbation and dose-dependent generation of thiobarbituric acid-reactive substances (TBARS), NF- κ B-mediated microglial activation, and cytotoxicity. TBAR generation could be blocked by pre-treatment of cultures

with antioxidants probucol or N-acetylcysteine [161] demonstrating a direct or indirect association with oxidative stress. Indeed, interaction of A β with RAGE on the cell-surface exerted localized oxidant activity. The authors further observed that stimulation of RAGE by other ligands which do not themselves generate ROS induces intracellular generation of oxidants in target cells [161] suggesting that it is the interaction between RAGE and its ligand that induces the oxidation effect and not the ligand by itself. Not only A β as AD hallmark peptide appeared to show an association with RAGE-induced oxidative stress: using immunostaining it was shown that tau paired helical filaments colocalize with AGEs in AD temporal cortex tissue [377]. Also, tau was shown to be amenable to ribose-mediated glycation and exposure of SH-SY5Y cells to these glycated tau species resulted in oxidative stress without affecting cell viability [377]. It appears that AGE-RAGE interaction with AD hallmark partners results in a variety of cellular responses that can mediate oxidative stress.

5.10.10. Disruption of calcium homeostasis

5.10.10.1. ROS generation and Ca²⁺ signaling – a bidirectional paradigm

One important step in the process of synaptic transmission is the Ca²⁺-regulated fusion of neurotransmitter containing synaptic vesicles with the pre-synaptic membrane which results in the release of neurotransmitters in the synaptic cleft. Apart from neurotransmitter release, a plethora of other cellular mechanisms are regulated by Ca²⁺-dependent signaling, for example regulation of membrane excitability, neuronal growth and differentiation as well as expression of a wide range of genes that are activity-induced and apoptotic processes. Extracellular and endoplasmic reticular Ca²⁺ levels largely exceed intracellular levels and this sustained Ca²⁺ homeostasis is therefore an important factor required for intact cellular functioning. A number of reviews that extensively cover neuronal Ca²⁺ homeostasis

mechanisms have been published [427–430]. To aid Ca^{2+} homeostasis, Ca^{2+} levels in neuronal cells are strictly regulated by controlled release from various intracellular and extracellular pools. Release of Ca^{2+} from the extracellular pools into the cell is mediated by factors such as voltage-gated Ca^{2+} channels, N-methyl-D-aspartate (NMDA) receptors and transient receptor potential channels while intracellular Ca^{2+} , stored in the endoplasmic reticulum, is released via the inositol triphosphate receptor (Ins[1,4,5]P3R) and ryanodine receptor (RyRs) [427]. These factors act in a concerted manner to regulate intracellular Ca^{2+} levels in temporal and spatial manner. Reports show that dyshomeostasis of Ca^{2+} levels and oxidative stress amplify each other in a bidirectional manner. For example, ROS species superoxide anion, hydrogen peroxide and hydroxyl radicals are known to regulate signaling pathways involving Ca^{2+} . Mitochondrial Ca^{2+} mediates activity of a number of mitochondrial enzymes involved in the tricarboxylic acid cycle and oxidative phosphorylation and, as such, increased Ca^{2+} levels were reported to elevate cellular metabolic rate [431–433]. In turn, cellular metabolic rate was shown to be directly proportional to ROS generation in rat and porcine lung [434], and hepatomas [435]. Bidirectionality of the ROS-generation/ Ca^{2+} signaling paradigm was observed by functional impairment of membrane-bound receptors and channels that regulate influx or efflux of Ca^{2+} induced by oxidative stress-induced lipid peroxidation. Ca^{2+} dyshomeostasis was reported in neurodegenerative disorders in general, including AD. For example, whereas wild type presenilin was reported to form Ca^{2+} permeable channels allowing Ca^{2+} leakage from the endoplasmic reticulum [436,437], some, but not all FAD related mutations in presenilin 1 and 2, show deficiencies in Ca^{2+} leak function in a mouse embryonic fibroblast model, primary fibroblasts obtained from patients and a planar lipid bilayer [437,438]. Apart from neurons, astrocytes also play an ion regulating

role supporting neuronal activity. Astrocytes act in a concerted effort to regulate Ca^{2+} levels via release of ATP which subsequently binds to purine receptors on the membrane of adjacent astrocytes [439]. ATP binding then results in release of intracellular Ca^{2+} via the phospholipase C beta/inositol triphosphate (IP3) pathway [440]. Using a microarray analysis, it was reported that the expression of 32 Ca^{2+} signaling pathway-related genes were altered in astrocytes at various stages of AD pathology [441]. $\text{A}\beta$ was reported to perturb astrocytic Ca^{2+} regulation indirectly by inducing oxidative stress which impairs membrane Ca^{2+} pumps and enhances Ca^{2+} influx through voltage-dependent channels and ionotropic glutamate receptors [442–444].

5.10.10.2. By potentiating mitochondrial permeability transition pore formation

The mitochondrial permeability transition pore (mPTP) plays an important role in the mitochondrial Ca^{2+} homeostasis [445]. Its molecular composition and mechanism of action have been under debate in the last years. According to initial studies, the mPTP consists of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane and Cyclophilin D (CypD) in the matrix. When CypD translocates to the inner mitochondrial membrane to interact with ANT and VDAC, [446] the mPTP opens allowing non-selective exchange of calcium [447–449]. On the contrary, more recent findings dismiss the original idea and indicate that VDAC is not a key component of the mPTP [450,451] and ANT has only a regulatory function rather than being a core unit of the mPTP [452]. Only CypD, a peptidyl-prolyl isomerase F located in the mitochondrial matrix, remains critical molecule in the mPTP in both postulations. In fact, studies in animal models have shown that the mPTP formation can be efficiently blocked by the addition of a cyclophilin D inhibitor, cyclosporine A (CSA) or by depletion of Cyp D [446,453,454]. Short and transient mPTP opening has been shown to occur under physiological conditions, allowing

rapid passage of protons, Ca^{2+} and other substances of a size up to 1.5 kDa [455,456]. However, prolonged mPTP leaking may results in mitochondria disruption of mitochondria. A part from high concentration of Ca^{2+} in the mitochondrial matrix [457], ROS itself can lead to mPTP opening. In fact, ROS present in the cytosol could induce signals leading to mPTP opening with further ROS release and this phenomenon is referred to as “ROS-induced ROS release (RIRR)” [458]. Moreover, several lines of investigation have proposed that A β -induced ROS and increased Ca^{2+} levels could well link with mPTP formation. In this regard, increased CypD expression has been identified in neurons of the hippocampus and temporal lobe of AD patients [459]. Consistent with this, CypD was shown to specifically bind to A β oligomers both *in vitro* and *in vivo* in a dose-dependent manner facilitating permeability transition and ROS formation causing mitochondrial dysfunction [459–461]. Removal of CypD improved the cognitive and synaptic function in a mouse model for AD [459], protects cells from Ca^{2+} , oxidative stress or A β -induced cell death [446,462,463], and restores oligomeric A β_{42} -mediated loss of axonal mitochondrial transport in neurons. The role of ROS was highlighted by demonstrating rescue of A β -induced loss of axonal mitochondrial movement upon administration of Probucol, an anti-oxidant agent [464]. As a result of these observations, various studies embarked targeting CypD/mPTP inhibition as potential treatment for neurodegenerative diseases [465,466].

5.11. Oxidation of tau and A β

While A β and tau themselves were found to induce the production of ROS by inhibition of key mitochondrial enzymes, dysfunctional mitochondria in turn were shown to affect A β and tau by forming a number of oxidative stress-

induced A β and tau adducts. This paragraph will discuss the various types of modifications that have been reported in literature.

5.11.1. Oxidation of A β

A β has been reported to undergo a number of modifications as a function of oxidative stress. Particularly the methionine, tyrosine and histidine residues of A β were demonstrated to be vulnerable to modification mediated by oxidation reactions [349,360,467–470].

5.11.2. Copper-induced dityrosine cross-linking

A specific type of A β assembly involves dityrosine cross-linking which has been connected with clinical markers of oxidative stress in AD but also other neurodegenerative diseases [471]. Increased levels of oxidative stress in the brain are reflected by increased brain content of copper and zinc, specifically in the neuropil and in AD plaques [158] [360]. Copper was shown to catalyze dityrosine cross-linking [467] in monomeric and, at a lower rate, fibrillar A β_{40} [472] which is induced by oxidizing agents including hydroxyl radical, peroxynitrite, nitrosoperoxycarbonate and lipid hydroperoxides [473] in a concentration-dependent manner [472]. The precise mechanism of crosslinking has been subject of study [474] and it was shown that the picomolar affinity complexation of A β with copper [475] results in H₂O₂, which, in turn, promotes the formation of SDS-resistant dityrosine cross-linked A β_{1-28} , A β_{1-40} and A β_{1-42} [350,467]. It has also been shown that A β_{1-42} , the 42-residue more amyloidogenic version of A β , has higher affinity to bind Cu²⁺ than A β_{1-40} , the 40-residue version of A β [476]. One of the hypotheses by which binding of A β to Cu²⁺ can induce the H₂O₂ required for crosslinking is by its ability to undergo Fenton redox cycling [477]. Consistent with this thought, histidines 6, 13 and 14 in A β that were identified to be involved in the redox cycling of bound Cu²⁺ [475] are located in close proximity to tyrosine 10. Density functional theory calculations and tyrosine-to-alanine mutational studies experimentally demonstrated that indeed tyrosine residue

10 in A β critically determines the generation of H₂O₂ mediated by A β -Cu²⁺ interaction [478]. The resulting crosslinked species were shown to accumulate in the AD brain, and were shown to exert high levels of toxicity to neuronal cells [350,479,480]. Also, *in vitro* these dityrosine crosslinked forms of A β , can be generated under conditions of oxidative stress as demonstrated using enzymatic peroxidation detected by tandem mass spectrometry [481]. A recent paper showed that exposure of *in vitro* generated A β ₁₋₄₀ fibrils to Cu²⁺ significantly reduced fibril length as a result of fibril fragmentation [472]. Even though exposure of A β ₁₋₄₀ to Cu²⁺ was shown to induce thioflavin T positive fibril assembly [472,482,483], the addition of H₂O₂ inhibited the assembly process [472].

5.11.3. Methionine-35 oxidation

A second commonly detected Cu²⁺-induced modification of A β in plaques is the reversible modification of oxidation-sensitive methionine 35 to its sulfoxide [360,484] or its further irreversible oxidation product methionine sulfone. APP23 transgenic mice show methionine oxidized forms of A β ₁₋₄₀ [485] and methionine oxidized A β is also abundantly detected in AD patient brains [360,484,485]. The sulfoxide intermediate can be reduced by the action of peptide-methionine sulfoxide reductase [486], although levels of this enzyme in the AD brain were reportedly reduced [487]. In line with this observation, upon knock-out of methionine sulfoxide reductase A in an human APP mouse model, levels of soluble methionine sulfoxide A β are increased and associated with defects in mitochondrial respiration and cytochrome c oxidase activity [488]. Inconsistent results have been published on the effect of methionine-35 oxidation on A β aggregation. For example, it was shown that H₂O₂/Cu²⁺-induced methionine-35 oxidation of commercially derived A β ₁₋₄₀ and A β ₁₋₄₂ slows down ThT-positive A β fibril formation compared to wild type A β without affecting morphological features of A β

fibrils as observed by TEM microscopy [489]. Marked differences in response to H_2O_2 -induced methionine oxidation of $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ were observed in a study showing that oxidation of $\text{A}\beta_{1-40}$ increases fibril formation kinetics while slowing down fibril formation of $\text{A}\beta_{1-42}$ [490], suggesting an isoform dependency. Imaging of the resulting fibers using TEM showed that fibers generated by oxidized $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ were both highly fragmented compared to unoxidized peptide [490]. In another study, methionine 35 of synthesized $\text{A}\beta_{1-42}$ was oxidized by exposure to H_2O_2 and oxidation was validated using mass spectrometry. Subsequent atomic force microscopy (AFM) and circular dichroism spectroscopy assays showed that methionine oxidation in this way hindered the typical random coil to β -sheet conversion and filamentous morphology characteristic for $\text{A}\beta$ fibril formation [491]. Early aggregate formation of methionine oxidized $\text{A}\beta_{1-40}$ was studied using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry showing that trimer formation was inhibited without affecting dimer assembly [492]. One of the mechanisms proposed to affect the decreased aggregation propensity of $\text{A}\beta$ upon oxidation of methionine 35 was that oxidation results in a reduced hydrophobicity of $\text{A}\beta$ [491,493]. This hydrophobicity is one of the main driving forces for $\text{A}\beta$ self-assembly. That hydrophobicity changes upon oxidation could indeed play a role was illustrated for apolipoprotein A-I, which, upon oxidation, affected the ability of this protein to interact with lipids [493]. A functional role for reversible methionine oxidation of $\text{A}\beta$ was suggested in a study showing that, the activity of the enzyme methionine sulfoxide reductase type A increased in rat neuroblastoma cell line IMR-32, in response to exposure to methionine-oxidized $\text{A}\beta_{1-42}$ [494]. It is difficult to delineate the precise origin of the diversity in results that have been obtained in cell viability and aggregation

kinetics studies in response to methionine oxidation, but it is likely that variations in sample preparations may contribute.

5.11.4. 4-hydroxynonenal modification

A third type of oxidative stress related feature that is prominent in the AD disease brain is the accumulation of 4-hydroxynonenal (HNE) [104,495,496]. HNE generation has been detected both *in vitro* and *in vivo* as a result of lipid peroxidation [497,498]. A 1990 hypothesis paper proposed several multi-step iron-catalyzed chemical routes for the generation of HNE through the oxidation of n-6 polyunsaturated fatty acids, particularly linoleic acid, γ -linoleate acid, and arachidonic acid [499]. Further, the presence of A β was shown to induce Cu²⁺-mediated production of HNE from lipids [500], and that, in turn, the released HNE can conjugate with A β and induce the generation of high molecular weight species and the increase the generation of A β by modulating BACE activity [301,308,501] (see also paragraph 5.9.5 for a more extensive outline on the effect of oxidative stress on APP processing). Collectively, these data suggest that a number of in-brain factors interrelate to generate a downward spiral that is possibly associated with the observed pathogenic progress of AD. When studying factors modulating HNE-conjugation to A β , it was shown that the presence of metals both *in vivo* and *in vitro* inhibit HNE modification of A β [502]. For example, it was observed that *in vitro* HNE modification of A β can be achieved by means of coincubation of A β with HNE upon overnight incubation only in PBS that is free from magnesium and calcium [502]. Another study showed that physiological levels of calcium effectively inhibit HNE modification of A β [501]. Of interest then was the observation that HNE conjugation of A β is a ROS-induced modification often encountered in amyloid plaques [2,103,236,503], while in plaque levels of calcium and copper were sufficiently high to prevent such A β modification. A study mimicking *in vivo*

plaque conditions, involving physiological levels of calcium and high levels of copper, demonstrated indeed that, apart from HNE-adducts and A β both being recognized, though not colocalized, in cerebral vessels [501], increased levels of lipid oxidation could be demonstrated surrounding amyloid plaques obtained from a PS/APP mouse model, the formed HNE adducts did not colocalize with the plaques [501] demonstrating that perhaps the raised levels of metals in plaques specifically inhibits HNE conjugation to A β in these plaques although lack of penetration by HNE-recognizing antibodies could not be ruled out [501]. *In vitro* mass spectrometry assays further underlined that calcium and copper prevented the oxidation of lipids and reduce conjugation of HNE to A β ₁₋₁₆ [501]. Lysine, histidine and cysteine seem to be the most reactive residues toward HNE adduct formation, although A β lacks cysteine residues and it was suggested that the microenvironment of a specific residue determines the actual reactivity to react with HNE [504]. Two chemical reactions were identified that dictate the HNE-A β adduct formation: via formation of a Schiff's base or by Michael addition [504]. The conjugation reaction can reportedly be quenched by addition of azide, primary amines, ammonia, Tris, DTT [502] or trifluoroacetic acid [505] to the reaction mixture. Addition of an antioxidant, such as hydralazine [502] or 3,5-di-*tert*-butylhydroxytoluene (BHT), or copper chelator diethylenetriaminepentaacetic acid (DTPA) [505], were reported to inhibit HNE-modification of A β .

5.11.5. Heme-complex formation

Heme-complexed A β adducts have been postulated to affect cytochrome c oxidase (COX) activity, part of the mitochondrial electron transport chain, which is significantly decreased in the AD brain [506]. COX requires heme-*a*, of which regulatory heme is a precursor, to assemble and perform its function [507,508]. In turn, heme-*a* levels were observed to be significantly

decreased in the temporal lobes of AD patient brains compared to age-matched controls [365]. A potential A β -mediated role in the availability of heme in the AD brain came to light when it was shown that the presence of heme dose-dependently inhibits oligomer formation of both A β_{1-40} and A β_{1-42} in an immunoassay and prevented loss of cellular viability upon addition of the complex to human neuroblastoma cell line IMR32 [509]. It was thought that, by competitive binding to heme, A β could deplete the availability of regulatory heme leading to deprived COX functionality and energy deficiency in AD. Similarly, the presence of heme was found to inhibit activation of A β -induced inflammatory response in primary mouse astrocytes [510]. At the same time, A β and heme, in the presence of H₂O₂, was reported to increase tyrosyl radical formation in A β_{1-16} and mediate its dimerization through 3,3'-dityrosine cross-linking [349,511,512], a reaction that was observed to be competitively inhibited in the presence of NaNO₂ [511]. A rapid interaction between heme and A β was shown upon addition of heme-*a* or heme-*b* to A β_{1-40} or a mixture of A β_{1-40} and A β_{1-42} which resulted in an immediate spectral shift of heme [365]. A β histidine residues were speculated as potential binding site via involvement of the π -electrons of the histidine imidazole rings, as addition of copper and zinc ions competitively inhibited the interaction of A β with heme, but only when heme was added to the reaction mixture after copper and zinc [365,509]. In a subsequent site-directed mutagenesis study, using voltammetry, histidines 13 and 14 were specifically identified as heme binding sites in A β [513]. In addition to this, a NMR-based spectroscopic study showed that heme *b* binds to A β_{1-16} with higher affinity compared to free histidine or other histidine-containing peptides indicating that other parts of the A β peptide contribute to the interaction with heme *b* [512]. Heme-A β conjugates have also been found in AD plaques and were shown to inhibit aggregate formation in a cell-free system and to dissociate

existing A β aggregates [514]. Spectroscopic studies shed more light on the structural implications of A β_{1-16} interacting with heme *b* [512]. This study showed that two complexes can be formed that exist in equilibrium, a low spin six-coordinated 1:2 heme/A β_{1-16} stoichiometry and a high-spin heme-(A β_{1-16}) species. A β -heme adducts were found to act as a peroxidase [515] although *in vivo* relevance of this catalytic activity was questioned as a result of the reported very low k_{cat} value of the complex of 0.016 s⁻¹ at 278 K compared with a value of 45.5 s⁻¹ for horse radish peroxidase [512]. However, a substantial effort has since gone into understanding the structural basis of this peroxide activity. One study showed that mutation of either histidine 13 or 14, but not both, does not affect peroxidase activity of the A β_{1-16} -heme complex [511]. Free histidine, similar to the unmutated A β_{1-16} -heme complex, induced peroxidase activity as observed using an 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) oxidation assay [511]. Apart from a regulating role by histidines, peroxidase activity of the A β -heme complex was shown to involve arginine 5 as proton donating residue cleaving the O-O bond of the peroxide [511,513,515]. At the same time, the addition of free arginine to heme failed to induce peroxidase activity demonstrating that the structural incorporation of arginine 5 within a protein environment somehow is relevant for its action [511,512]. Peroxidase activity of the complex was reported to depend on the heme-A β ratio and temperature, with increasing A β_{1-16} to heme and temperature inducing peroxidase activity more potently [512,516].

5.11.6. Oxidation of tau

The protein tau plays an important role in microtubule organization by dynamically interacting with the formed microtubule [517]. Intracellular dynamics of microtubule organization were observed to be disrupted in AD patients [518]. Various cell lines, including ventricular myocytes, rat

pheochromocytoma PC12, and pancreatic epithelial cell line AR42J, when exposed to H_2O_2 are characterized by a decreased growth of the microtubular network as a result of increased microtubular catastrophe rate [519–523]. The possible role of oxidative stress in this pathological feature was investigated by showing that exposure of neuro-2A cells to HNE resulted in rapid disruption of microtubuli and decrease of neurite outgrowth [524]. This interaction was shown to be largely mediated by Michael addition [524].

5.11.7. Oxidative damage colocalizes with neurofibrillary tangles

Oxidative damage was found to colocalize with tau enriched neurofibrillary tangles [328]. In this study, hippocampal tissue from AD patients was subjected to postmortem analysis investigating the localization of the enzyme dimethylarginase. This enzyme acts as regulator of nitric oxide synthase [525]. In AD hippocampal tissue, neurons that contained neurofibrillary tangles also stained positive for dimethylargininase providing a first indication that nitric oxide is generated in close proximity to the tau that makes up the neurofibrillary tangles. In line with these observations, an antibody that recognizes an HNE-lysine adduct was found to colocalize with endogenously obtained paired helical tau filaments from AD brains [104]. Also, acrolein, which is an aldehyde product of lipid peroxidation, was observed to colocalize with neurofibrillary tangles in AD patient brains [526]. Further, the antibody Alz50 [527], which recognizes a conformational change in tau [528], coincides with heme oxygenase-1 (HO-1), which is an antioxidant enzyme [104], levels of which are strongly increased in the AD and MCI brain [529]. Whether HO-1 activity is beneficial in terms of alleviating oxidative stress or in fact can induce neurotoxicity in the MCI and AD brain has been subject of debate as increased HO-1 levels were also correlated with increased phosphorylation of tau serine residues [529].

5.11.8. Oxidation of tau affects filament assembly

Ascorbate/Fe(III)/O₂-induced oxidation of tau, albeit of bovine origin, was shown to induce the *in vitro* generation of tau into filamentous assemblies [530]. Specifically, oxidation of one of the cysteine residues was found to be involved in the induction of the assembly of a recombinant fetal isoform of tau into paired helical filaments and neurofibrillary tangles [531]. These data suggested that the ability to form disulfide bridges critically determines generation of tau aggregates while oxidation modulates the ability to self-assemble. Consistent with this thought it was recently shown that Zn²⁺ interacts with the cysteine residue of a truncated version of tau containing only the third repeat unit of the microtubule-binding domain accelerating its aggregation rate and toxicity in a Neuro-2A cell line [532]. A role for oxidative stress in tau assembly was further demonstrated upon administration of the anti-oxidants 2,4-disulfonyl α -phenyl tertiary butyl nitron and N-acetylcysteine which reduced immunoreactivity against tau oligomers [533]. Of interest was the observation that peroxynitrite treatment of tau induced nitration, S-nitrosylation and oxidation of methionine, as observed by HPLC-electrospray ionization tandem mass spectrometry while markedly reducing aggregation, as analyzed by light scattering and electron microscopy [534]. Collectively, these observations suggest that oxidation of tau may modulate aggregation by either inducing or inhibiting the self-assembly process and that the specific outcome may depend on the type of oxidant and the specific amino acid residue involved. Phosphorylation was shown to importantly regulate HNE-induced assembly of tau as exposure of phosphorylated tau, opposed to unmodified tau, induced misfolding of tau, recognized by antibody Alz50, and the formation of tau aggregates [104]. In line with this, the self-assembly of a tau fragment including the first and third tubulin-binding domains showed that the presence of HNE mediated

polymerization of phosphorylated tau [535]. A more mechanistic link between phosphorylation and oxidative stress was elucidated when a study showed that the activity of alkaline phosphatase was inhibited in the presence of HNE. Exposure of tau to HNE hence resulted in the generating a tau species resistant against dephosphorylation [105].

5.11.9. AGE-associated tau is associated with oxidative stress markers

Advanced glycation end products (AGEs) are the oxidation product of sugars that interact with proteins and their accumulation has been related to amyloid deposition in AD [536]. Tau assembled into paired helical filaments has been shown to be immunoreactive against N ϵ -(carboxymethyl)lysine, one of the major AGEs [537]. Interaction of recombinantly produced tau with ribose-derived advanced glycation end (AGE) products was shown to result in the generation of reactive oxygen intermediates, which, in turn, activate NF κ B to induce amyloidogenic processing of APP [538]. Uptake of AGE-glycated tau into SH-SY5Y neuroblastoma cells was associated with malondialdehyde and heme oxygenase 1 detection which was prevented by the exposure of these cells to N-acetylcysteine and probucol, two antioxidant compounds [377]. Diffuse cytosolic immunoreactivity against AGE was shown in many neurons of post-mortem AD brains that also contain tau that is hyperphosphorylated [539]. Astrocytes residing in the temporal cortex of medium to severely affected AD subjects were found to be immunoreactive for iNOS as well as AGEs [540]. Thus far it is unclear whether AGE-glycation of tau has implications for the physiological role of this tubulin binding protein in the cytoskeletal organization or what the hierarchical correlation is between AGE formation and tau assembly into filaments.

Collectively, a clinical link between mitochondrial dysfunction and AD has been firmly established, with a central role for the A β peptide. While ROS-

mediated modifications of A β and tau play a potential role, the precise implications of these species on disease progress have not been investigated.

5.12. Oxidative stress as biomarker

A series of biomarkers have been clinically validated to allow for differential diagnosis of AD. Examples of those currently used in clinical applications are CSF levels of total tau, phosphorylated tau and A β_{1-42} [541–543]. None of these typical AD-related biomarkers target conditions of oxidative stress although oxidative stress related biomarkers do exist and two extensive reviews covering these have been published [544,545]. Peroxidation and oxidation of biomolecules, including lipids, proteins, DNA and RNA, caused by oxidative stress generates products that can be detected in plasma, serum or cerebral spinal fluid (CSF) and may act as biomarkers although their specificity towards AD may be questioned as oxidative stress commonly occurs in many diseases. A number of biomarkers has been validated for use in clinical diagnostics and are commonly used as output measure for oxidative stress in experimental research and clinical trials. This paragraph will discuss features of oxidative stress that show significant correlation with AD and their potential for use in diagnosis of AD.

5.12.1. F2-isoprostanes

Isoprostane generation is mediated by peroxidation of polyunsaturated fatty acids (PUFAs) which are a major component of cellular membrane phospholipids. Based on gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICMS) analysis, F2-isoprostanes, and, more specifically, 8, 12,-iso-iPF $_{2\alpha}$ VI, were found at elevated levels in CSF of AD but also MCI patients suggesting that oxidation of PUFAs occurs early in AD pathogenesis [546–548]. CSF isoprostane levels were demonstrated in some studies to predict progress of MCI into AD [547,548] although reports

are contradicting. F2-isoprostane levels in urine were also reportedly increased in urine or plasma from AD and MCI patients compared to healthy controls [548–550], although low numbers of subjects were included in this study [548], and various other reports were unable to reproduce this observation [551,552]. Further, F2-isoprostane levels in plasma and urine did not appear to correlate with brain oxidative status [13,551]. F2-isoprostane levels seem to lack the clear correlation and distinguishing power to accurately assess oxidative status in AD brains.

5.12.2. 8-hydroxydeoxyguanosine

A widely used biomarker to assess levels of oxidative stress is 8-OHdG, a ROS-induced oxidation product of DNA bases that are subject to high mutagenic potential [13,553]. Increased levels of 8-OHdG have been detected in AD post-mortem brains tissue including cerebral cortex, and cerebellum, as well peripheral cells [171,554]. CSF levels of 8-OHdG were found to be increased up to five times in CSF of AD patients compared to healthy age-matched controls [555,556]. Also levels in lymphocytes were found to be increased in AD patients compared to controls and these higher levels were shown to correlate well with low plasma antioxidants, including vitamin E, vitamin A, and carotenoids [553]. In a small scale study including 30 patients and their sex- and age-matched controls, plasma levels of 8-OHdG were significantly higher in AD patient samples compared to a control group [557]. However, the specificity of this biomarker for AD may be questioned as hydroxylation of guanosine was found to occur in response to both normal aging, a wide range of pathological conditions, as well as a variety of environmental factors.

5.12.3. Carbonylated proteins

Carbonylated proteins present a heterogeneous pool of oxidatively modified proteins induced, either directly or indirectly, by various sources and reactants. Reactive carbonyl compounds generated under conditions of

oxidative stress can target the amino acids Lys, Arg, Pro, Thr, Cys, His or Lys to generate protein carbonyl derivatives including aldehydes and ketones [558]. A number of proteins were found to be readily carbonylated, in response to oxidative stress conditions, in the hippocampus of MCI subjects [4,412,559]. In the AD brain it was shown that glutamine synthase is extensively carbonylated compared to healthy brain [560], which lead to partial inactivation of the enzyme which was used as read-out to demonstrate that activity of this enzyme was selective for the frontal pole compared to the occipital pole [561]. A decrease in activity of glutamine synthase, and also of creatine kinase, was further observed in a different study but activities of both enzymes were globally decreased throughout the entire brain without any brain-region specific effects [562]. However, brain regional differences in protein carbonyl levels were reported in a different study by comparing hippocampus, inferior parietal lobule and cerebellum, where hippocampal carbonyl content was observed to be significantly higher in AD patients compared to healthy age-matched controls [562]. Specific targeted carbonylation was observed in 12-month old senescence accelerated AD mouse model, SAMP8, of α -enolase, collapsing response mediator protein-2, creatine kinase, lactate dehydrogenase and α -spectrin [563]. An ELISA-based study on clearance of oxidative stress markers showed that, in patients undergoing coronary heart surgery, protein carbonyl levels were cleared from the serum at a slower rate than lipid peroxidation products suggesting that the detection of protein carbonyls provides a relatively stable read-out for oxidative stress status [564]. Various other methods were developed to detect protein carbonyl levels, including a proteomics approach based on 2D fingerprinting with immunological detection coupled with mass spectrometry detection of proteins [560]. Other reported methods to detect levels of protein carbonylation are dinitrophenylhydrazine-coupled assays including western

blots, ELISA, immunocytochemistry, HPLC and spectroscopic absorption at a wavelength of 370 nm [565–569]. As many other diseases have demonstrated increased levels of carbonylated proteins, including amyotrophic lateral sclerosis [570], Parkinson's disease [571], and dementia with Lewy bodies [572], it is difficult to assess the differential power of this oxidative stress related marker, although perhaps the reported specific proteins involved and brain-region specific differences can be used to establish the extent of oxidative stress as factor in to map progress of AD as carbonyl levels were found to correlate well with the detection of neurofibrillary tau tangles [573] and severity of histopathological alterations [562].

5.12.4. Total antioxidant capacity (TAC)

Total antioxidant capacity (TAC) is a quantitative output of antioxidant activity contained in a sample. Biological fluid samples such as plasma, CSF or urine can be used to measure their TAC. TAC is commonly evaluated by detection of 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) that converts into of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTSS+) upon oxidation induced by ferrylmyoglobin [574,575]. The presence of antioxidants inhibits the oxidation process, which can be measured as a delay in radical formation spectroscopically at 600 nm or 734 nm. Another method, involving ferric reducing ability in plasma (FRAP) assay, is based on transfer of single electrons from the oxidant, Fe (III), to antioxidants in the sample. The measurements are taken as a change in absorbance of the Fe (III) tripyridyltriazine complex as it reacts with antioxidant [576]. Two other assays include the total radical trapping antioxidant potential, or TRAP [577], and the Trolox equivalent antioxidant capacity (TEAC) [578]. TAC read-outs were used as a plasma marker in a clinical trial where AD patients, diagnosed according to neuropsychological and functional cognitive tests, were

compared with healthy subjects. The AD group was marked by a significant decrease in TAC compared to healthy controls [579]. Similarly, plasma TAC assays observed significantly decreased TAC levels in mild, intermediate and advanced AD compared to controls, suggesting that a decreased capacity to neutralize oxidative species can already be observed in early forms of AD. However, in another study, TAC was only found to be significantly lower in cases of severe AD, leaving those suffering from mild or early forms of AD unaffected [580]. Genotype specific differences have also been reported, where TAC levels measured by the FRAP method, were significantly lower in subjects carrying the alleles $\epsilon 4/\epsilon 4$ of AD-related genetic risk factor APOE compared to $\epsilon 4/\epsilon 3$ and $\epsilon 3/\epsilon 3$ carriers [581]. Measurement of TAC seems to provide some level of insight into levels of oxidative stress in AD patients. However, the usability of the test seems to be limited to a selective AD subgroup.

5.12.5. Heme oxygenase type 1 (HO-1)

HO-1 is an oxidative stress-induced enzyme which catalyzes the oxidation of heme into biliverdin, free iron and carbon monoxide. It is also known to be induced due to a stress response as the protein activity acts in mediating protection against oxidant injury by oxygen free radicals to attain homeostasis [582]. The potential of HO-1 to act as a biomarker to diagnose AD and MCI has been reviewed before [583]. HO-1 was found to colocalize with astrocytes, neurofibrillary tangles, and senile plaques in the human AD brain [584–586]. Already in the MCI stage, glial expression levels of HO-1 in temporal cortex and hippocampus were found to significantly exceed those of non-demented patients [587], suggesting that HO-1 expression is an early stage event in AD pathology. Moreover, probable AD patients, compared with healthy elderly controls, show decreased plasma HO-1 protein, determined by ELISA, lymphocyte mRNA levels and CSF protein levels

[587]. Suppressed mRNA levels of HO-1, detected by RT-qPCR, in lymphocytes was confirmed in a small-scale study involving twelve AD patients and seven age-matched controls [588]. ELISA-determined protein levels in plasma of probable AD subjects did not differ significantly from non-AD related dementia patients, Parkinson's disease or ALS patients demonstrating that distinguishing power of HO-1 as biomarker for AD may be shared with a range of other neurodegenerative disorders. In line with this, it was shown that expression of HO-1 is similarly increased in Parkinson's disease patients where expression levels are localized with affected nigral astroglia and dopaminergic neuronal Lewy bodies [589].

5.12.6. Malondialdehyde lysine epitopes

Malondialdehyde (MDA) is an aldehyde generated upon peroxidation of lipids. MDA levels can be detected by a number of assays based on spectrophotometry or fluorimetry, HPLC, gas chromatography as well as immunological methods [590–592]. In a case control study, plasma levels of MDA in AD patients were observed to be significantly increased, and inversely correlated with levels of retinol and α -Tocopherol, compared to healthy controls [593]. At the same time, another study established that malondialdehyde levels did not correlate with increased oxidative stress in AD patients [594] questioning the ability of MDA to reliably reflect on the oxidative stress status in patient material. Moreover, plasma MDA levels were also found to be significantly increased in diabetes patients that undergo hemodialysis, particularly those patients suffering from cardiovascular complications [595] showing that MDA levels are non-specific to the AD brain. Even though MDA levels are often employed to study levels of oxidative stress in various types of systems, including clinical assays, and cell biological applications, the use of MDA as a clinical biomarker is further limited by virtue of its short half-life [596].

5.12.7. Non-invasive detection

Molecular imaging with PET and SPECT has been employed to provide quantitative and spatial *in vivo* assessments of A β and tau accumulations in AD patients [597]. A novel imaging method of PET has been developed using [^{62}Cu]-diacetyl-bis (N4-methylthiosemicarbazone) (^{62}Cu -ATSM) as a tag that crosses the BBB and accumulates in areas with excess electrons allowing for regional visualization of oxidative stress in AD patients [598]. The tag used has shown minimal toxic effects on research studies subjects but within a narrow dose range [599] limiting its use as diagnostic tool for regular examination of oxidative stress status of patients.

5.12.8. Oxidative stress as AD specific biomarker.

The occurrence of oxidative stress at prodromal and early stages of AD [3–7] raised the possibility that the oxidized biomolecules that are generated in response to oxidative stress may act as biomarker indicative of early disease onset. A model to characterize specific biomarkers related to AD has been developed [601,602], which includes increased levels of A β_{1-42} and tau in CSF, along with their structural imaging by magnetic resonance imaging (MRI), amyloid PET and its hypo-metabolism in fluorodeoxyglucose (FDG)-PET [602], but excludes the use of oxidative stress biomarkers. The model has demonstrated its value by indicating the dynamics of the development of the various markers in relation to progress of AD. Even though oxidative stress levels can be measured in AD patients by means of the measurement of oxidized molecules or remaining antioxidant capacity, it should be questioned if inclusion of an oxidative stress biomarker would enhance the objective measure of the pathogenic process of AD by the existing model. Published data, as discussed in the paragraphs in this section, demonstrate that, even though oxidative stress is a highly relevant pathogenic parameter in AD and somewhat correlates with disease progress and can also be determined at early stages of disease, it does not provide sufficient distinguishing power from

other aging related diseases that are also often accompanied by increased levels of oxidative stress.

5.13. New biomarkers

To identify potential new biomarkers that can report on early stage AD, we performed transcriptomics analysis to identify previously unreported but oxidative stress related genes in AD patient brains. mRNA expression levels were assessed in the publicly available transcriptome datasets of brain from patients with various stages of AD (GSE1297; reference PMID: 14769913) obtained from the National Center for Biotechnology Information Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>). Patients were categorized into four groups: normal individuals (n=9), incipient AD patient (n=7), moderate AD patients (n=8) and severe AD patients (n=7), and data was analyzed using online database GEO2R.

Table I: Heatmap of significant genes being up (red), down (green), or unaltered (black) regulated in various stages of AD.

The heatmap provided in table I shows that expression levels of some genes become differentially regulated only at later stages of disease. For example, DRD4 is a dopamine D4 receptor that has been reported to induce apomorphine-mediated protection against oxidative-stress-induced cell death [603]. The density of dopamine D4 receptors is also significantly increased in patients suffering from schizophrenia [604], suggesting that oxidative-stress induced upregulation of DRD4 may not be specific to AD. In this

paragraph we will address only those markers that show up- or down regulation in incipient AD.

5.13.1. Ets-2 repressor factor (ERF)

ERF is significantly upregulated in all phases of AD compared to healthy control subjects. ERF encodes for the Ets-2 repressor factor protein that is ubiquitously expressed in the various organs and tissues. Ets2 is a member of the Ets family of transcription factors that is characterized by an evolutionary conserved characteristic Ets domain that aids the binding to DNA sequences rich in purine with a GGAA/T consensus core with flanking nucleotides [605]. In a 3T3 fibroblast cell model, exposure to H₂O₂ was found to induce mRNA expression of Ets-2 and that, in turn, this induced Ets-2 expression sensitizes cells to apoptosis mediated by H₂O₂ [606]. Another study demonstrating a relation between Ets-2 and oxidative stress similarly showed induction of Ets-2 by oxidative stress related to an amyotrophic lateral sclerosis model [607]. While co-activator CBP/p300 has been described to activate Ets2-mediated transcription [608,609], Ets-2 repressor factor was observed to bind to the c-ets-2 promoter and is involved in the regulation of cell proliferation [610]. While ERF was identified previously in a study that investigated the use of Logistic Regression Ensemble to predict AD [611], no other studies to date are known to report on an association between ERF, oxidative stress and AD specifically.

5.13.2. ATPase phospholipid transporting 11A (ATP11A)

The gene ATP11A encodes for the protein ATPase phospholipid transporting 11A, which is an class 4 P4-ATPase plasma membrane localized phosphatidylserine and phosphatidylethanolamine translocase [612]. In this way, ATP11A actively contributes to the asymmetrical distribution of phospholipids in the plasma membrane. AD is characterized by upregulation of ATP11A throughout disease progression. An increased intracellular Ca²⁺ concentration was reported to inhibit ATP11A activity, presumably by direct

binding of Ca^{2+} to ATP11A [612]. The sequence of ATP11A contains two evolutionary conserved caspase cleavage sites that act as target for caspase-mediated apoptosis [613]. Oxidative stress is characterized by dysregulation of intracellular calcium homeostasis, and, as such, oxidative stress provides an indirect means of inducing ATP11A mRNA expression. ATP11A gene expression upregulation has been identified as valuable marker to evaluate the prognosis of patients suffering from colorectal cancer [614]. No publications to date discuss the relevance of ATP11A to AD progression.

5.13.3. Fatty acyl-CoA reductase 2 (FAR2)

Expression levels of FAR2 are downregulated throughout the progress of AD and this gene has also been identified in a gene-based genome-wide association study into resilience of executive functioning [615]. Further, FAR2 was found to be downregulated in patients with HIV associated dementia, AD and Parkinson's disease [616] and the gene was identified to affect the hippocampus in AD in a bioinformatic analysis [617]. FAR2 encodes the peroxisomal protein fatty acyl CoA reductase 2, an enzyme that reduces saturated fatty acids of 16 or 18 carbons into fatty alcohols [618]. The observed localization of FAR2 in the peroxisome and the relation of this cellular compartment with oxidative stress suggests in indirect association of oxidative stress with FAR2.

5.13.4. Dimethyl arginine dimethyl aminohydrolase 2 (DDAH2)

DDAH2 is a gene coding for the cytosolic [619] protein dimethyl arginine dimethyl aminohydrolase 2. This enzyme functions in the metabolism of nitric oxide [620] and is therefore potentially directly correlated with the increased levels of oxidative stress observed in the AD brain. Our results show that DDAH2 gene expression is upregulated throughout the entire progress of AD. Analysis of DDAH2 levels in post mortem AD brain samples showed regional differences, where the occipital lobe showed reduced and the frontal lobe increased levels of DDAH2. Inhibition of DDAH2 activity was

reported in cardiovascular and the renal system as a result of its high sensitivity to oxidative stress [621,622]. It is plausible that inhibition of activity of DDAH2 is compensated for by upregulation of DDAH2 gene expression. These suggestions were substantiated by the observation that neuroblastoma SH-SY5Y cells treated with IL18 showed a robust increase of DDAH2 production [623]. Another study, exposing HUVEC to 4-HNE, showed that expression of intracellular asymmetric dimethylarginine (ADMA), inhibitor of nitric oxide synthase, was increased concurrent with a decrease in expression of DDAH2, both at mRNA and protein level [624]. DDAH2 was previously observed to metabolize ADMA into citrulline and dimethylamine [625]. Nevertheless, the notion that AD is correlated with an increased generation of DDAH2 seems to be contradictory to the observation that exposure of young rats to ROS with bile-duct-ligation showed downregulation of DDAH2. This same study showed that *in vitro* expression and activity of DDAH were inhibited by exposure to superoxide and H₂O₂ [626]. Precisely how levels of DDAH2 expression and activity are regulated in the brain is perhaps difficult to predict from studies that focused on peripheral systems.

5.13.5. Hemoglobin subunit Alpha 2/1 (HBA2/HBA1)

The genes HBA1 and HBA2 encode for two subunits of the heme scavenging protein hemoglobin. Free hemoglobin was reported to induce damage to cells because the heme moiety is able to catalyze the peroxidation of lipids [627–630]. Various reports describe that hemoglobin levels are affected as a function of AD. For example, a rat based study observed that expression levels of hemoglobin and cognitive impairment were positively corrected [631], and a prospective cohort analysis showed that both higher and lower levels of hemoglobin were associated with the risk to develop AD [632]. This notion was further underlined by the finding that both anemic participants as

well as those with high hemoglobin levels in this study showed an increased risk for developing AD which also correlated with the rate of cognitive decline [632]. An association between anemia and AD was also observed in a cross-sectional cohort study including 211 participants with AD [633]. These data were partially reproduced in a study among an Indian 55+ year old population showing that low levels of hemoglobin could be linked with risk for AD [634]. CSF [635] and sensorimotor cortex [636] consistently showed an increased level of hemoglobin β chain levels in AD patients. Further, mRNA levels of hemoglobin were found to be increased in the inferior temporal gyri of AD patients compared with non-affected subjects [637] while hemoglobin protein concentrations were highest in the hippocampal formation and the parietal gray matter in AD patient [637]. Interestingly, it was found that the *in vitro* deposition of synthetic $A\beta_{1-40}$ is promoted by high hemoglobin levels [637–639], and that hemoglobin is co-localized with $A\beta$ -containing senile plaques as well as cerebral amyloid angiopathy in human brain material [637].

5.13.6. Cytochrome p450 family 4 subfamily F member 12 (CYP4F12)

CYP4F12 is a member of the p450 cytochrome family [640,641] and expression of this protein is induced by activation of the p53 pathway [642]. The p450 superfamily is a series of proteins involved in oxidative metabolism of substrates of various origin. The CYP4F family is specifically involved in ω -hydroxylation of eicosanoids [643], and CYP4F12 metabolizes ebastine [644]. Expression of CYP4F12 is induced by activation of the p53 pathway [642]. P53 signaling pathways have been implicating in oxidative stress on various occasions. For example, p53 was found to be involved, together with p66Shc, a protein that induces apoptosis based on oxidative signals, to genetically regulate steady state levels of intracellular oxidants [645]. CYP4F12 was originally identified in the human liver [640,646] and intestine.

Involvement of CYP4F12 in AD has not been reported yet while we found that mRNA levels of this protein are upregulated throughout AD progression.

5.13.7. Protein phosphatase 3 catalytic subunit alpha/Calcineurin A alpha (PPP3CA)

Our transcriptomic analysis indicated that mRNA levels of the gene PPP3CA, encoding the protein calcineurin A α , are downregulated in incipient, moderate and severe cases of AD. Calcineurin 3 A α , is one out of three different isoforms of calcineurin A, which interacts to form a heterodimer with calcineurin B forming a calmodulin-regulated protein phosphatase [647]. Within this complex, calcineurin B forms the Ca^{2+} -binding regulatory subunit, while calcineurin A consists of the catalytic site. In T lymphocytes, calcineurin was shown to act, when Ca^{2+} levels rise, by dephosphorylating the DNA-binding protein Nuclear factor of activated T cells (NF-AT) inducing its translocation from the cytosol to the nucleus [648]. Truncated forms of calcineurin found in the brains of AD patients [649]. Various functions of this protein have been ascribed to its abundant presence in the brain [650] and various tissues containing neuroendocrine cells [651]. N-methyl-D-aspartate receptor-mediated increase of intracellular Ca^{2+} levels was suggested to activate calcineurin [652]. Calcineurin-related signaling pathways have been implicated in AD in various occasions. For example, neuronal damage was observed to irreversibly activate calcineurin [653] and that soluble oligomeric forms of A β are directly responsible for this process. As Ca^{2+} signaling events, apoptosis, and oxidative stress are interlinked, as was demonstrated in a cardiomyocyte system showing that apoptosis is induced by an increase in intracellular Ca^{2+} level [654], the role of calcineurin in AD-related oxidative stress becomes palpable. A study using SH-SY5Y cells showed that pharmacological inhibition of calcineurin activity decreased the expression of APP, while activation of calcineurin-NFAT signaling reduced the expression of tau and NEP [655]. Levels of NFAT, the target of calcineurin, show

differential expression and nuclear localization in MCI and AD hippocampi in an NFAT isoform-dependent manner [656].

5.13.8. Transmembrane protein 70 (TMEM70)

Our transcriptomic analysis suggests that TMEM70 mRNA levels are downregulated in all stages of AD. This gene encodes for mitochondrial localized transmembrane protein 70 [657], which is thought to be involved in the biogenesis of mitochondrial ATP synthase [658], in particular F1 assembly or F1-F0-subunit interaction. TMEM70 deficiency, as a result of mutations in the TMEM70 gene, was first identified to result in deficiency of ATP synthase activity [658]. Patients are characterized by abnormally shaped mitochondria and nucleoid disorganization [659]. No earlier publications reported an association between AD and TMEM70. However, the generation of reactive oxygen species has been observed as a result of upregulated mitochondrial membrane potential in response to ATP synthase activity modulation [660–663]. In line with the suggested role of TMEM70 in ATP synthase biogenesis, *Tmem70*^{-/-} mouse embryos showed upregulation of SOD2 but without significantly affecting SOD1 levels [664].

5.14. Oxidative stress therapeutics

To date, there is no medication available to treat AD [1] although disease-alleviating therapeutics exist. These currently include galantamine, rivastigmine and donepezil which target cholinergic loss observed in AD patients by inhibiting degradation of the neurotransmitter acetylcholine [665] and memantine that prevents the activation of NMDA-receptors which become inaccessible to glutamate [666]. These AD management strategies are often complemented with symptom-alleviating drugs including antidepressants [667,668] and antipsychotics [668]. One of the challenges in effective treatment is that AD is often left undiagnosed at early pre-clinical

stages and by the time clinical symptoms are recognized, the disease may already have been developing over a decade [597] leaving a potential window of treatment opportunity unutilized.

The current therapeutic strategy to target oxidative stress involves either exogenous supplementation of antioxidants or drugs that aim at augmenting endogenous antioxidant defense. Other strategies linked to management of oxidative stress involve mitochondrial targeted therapy, modulation of cellular calcium homeostasis, nitric oxide synthase modulation and metal chelation.

5.14.1. Nutrition-based therapeutics

Exogenous antioxidants are potentially complementary to the endogenous antioxidant defense [669]. The majority of the exogenous antioxidants originates from a dietary source and include ascorbate (vitamin C), α -tocopherol (vitamin E), resveratrol, curcumin and many other co-factors with antioxidant reducing capacity such as copper, zinc, manganese, iron and selenium. They delay or prevent oxidative damage by breaking the chain reaction caused by ROS and RNS [5]. Based on data derived from several observational and epidemiological studies, several compounds with antioxidant activity have been proposed to support delay AD-related cognitive decline and these compounds have therefore been tested in clinical trials. Mecocci and Polidori have previously reported therapeutics involved in clinical studies up to 2002 [5]. In table II, we show a summary of recent clinical trials implicating antioxidants in AD. The data were collected from www.clinicaltrials.gov, with “Alzheimer’s disease” and “antioxidants” or “compound name” as keywords.

Table II: Summary of recent clinical trials

Compound	Phase	Number of subjects	Intervention	Main result(s)	Reference
Vitamin E, Vitamin C, and Alpha-lipoic Acid	I	75 patients with mild to moderate AD	800 IU/d of vitamin E plus 500 mg/d of vitamin C plus 900 mg/d of α -lipoic acid (E/C/ALA); 400 mg of CoQ 3 times/d; or placebo 3 times per day for 16 weeks	Decrease in markers of lipid peroxidation in CSF. No changes in CSF biomarkers related to amyloid or tau pathology.	[670] Clinicaltrials.gov NCT00117403
Vitamin E	III	613 patients with mild to moderate AD	2,000 IU per day of vitamin E plus placebo for memantine for 48 months	Slower functional decline.	[671] Clinicaltrials.gov NCT00235716
Vitamin/ Nutriceutical Formulation	II	135 participants with mild to moderate AD	folic acid (400 μ g), Vitamin B12 (6 μ g), Vitamin E (as alpha-tocopherol; 30 IU), S-adenosylmethionine (SAM; 400mg), N-acetyl cysteine (NAC; 600mg) and Acetyl-L-carnitine (ALCAR; 500mg). Taken once daily for 1 year	Ongoing	Clinicaltrials.gov NCT01320527
Vitamin E	III	349 participants with AD	1,000 International Units, two times a day for 36 months	Ongoing	Clinicaltrials.gov NCT01594346
Vitamin E and Selenium (PREADVIS E)	I	4246 healthy subjects	Participants were randomized to 400 IU vitamin E, 200mcg selenium, vitamin E and selenium, or 1 placebo pill daily	Neither supplement prevented dementia	[672] Clinicaltrials.gov NCT00040378.

5.14.1.1. Vitamin E

Vitamin E, also known as α -tocopherol, can be found in unprocessed vegetable oils, whole grains and legumes [673]. It is a fat-soluble antioxidant of 430 Da which plays a role within the lipid membrane defense system where it protects the polyunsaturated fatty acids (PUFAs) that are present in the cellular membranes from oxidative damage, inhibits the peroxidation of low-density lipoproteins (LDL) and captures the superoxide anion in order to convert it into hydrogen peroxide, a less reactive form. Due to its lipophilic nature and its small dimension, vitamin E has the ability to cross the BBB [674] and to accumulate in the central nervous system [5]. Vitamin E exerts antioxidant activity by donating an H atom to free radicals. The O-H bond, present in the structure of vitamin E, is sufficiently weak to dissociate allowing the hydrogen to neutralize the peroxy radical and prevent advancement of the chain reaction of lipid peroxidation [675]. However, when vitamin E reacts with a free radical it becomes a radical itself, which, in turn, is neutralized by ascorbic acid. Insufficient availability of ascorbic acid for regeneration of vitamin E was shown to promote the autoxidation of linoleic acid [676].

5.14.1.2. Vitamin C

Vitamin C, also called ascorbate, is a small water-soluble molecule with antioxidant capacity. The human body is unable to produce vitamin C itself due to deficiency of the enzyme L-gulonolactone oxidase that is required for its synthesis. Dietary sources of vitamin C include citrus fruit and vegetables [39]. Following ingestion, vitamin C is absorbed in the body by both active transport and simple diffusion [677]. Upon absorption, the ascorbic acid is converted into an ascorbyl radical followed by the formation of dehydroascorbate. However, the antioxidant activity is primarily assigned to the ascorbate form and it has been shown that ascorbate is able to reach

microglia of the CNS, by means of the Sodium Vitamin C co-Transporter 2 (SVCT2) transporter [678]. Vitamin C is also associated with the reactivation of vitamin E by reduction of α -tocopheryl to α -tocopherol [675]. Ascorbic acid deficiency was observed to compromise the antioxidative efficacy of vitamin E and promote LDL oxidation [679].

5.14.1.3. Copper

Depletion of copper (Cu) has been observed in brains of AD patients and APP transgenic mice and is linked to amyloidogenic processing of APP, accumulation of A β and poor SOD-1 activity [680,681]. These conditions were reversed with Cu supplementation which normalized SOD-1 levels in the aged APP23 brain [682]. Bayer and co-workers have suggested that when N-terminal domain of APP is bound to reduced Cu (CuI) [683], it is primarily cleaved by α -secretase, preventing A β generation and that the complex APP-CuI is able to neutralize hydrogen peroxide. Moreover, free extracellular copper is able to bind A β , the product of APP processing. In turn, the complexation of CuII with A β prevents the metal ion from generating detrimental ROS [684,685]. Encouraged by these findings, a recent clinical trial was initiated to investigate the effect of dietary Cu intake on cognitive function, A β levels in the CSF and volumetric changes in the brain in 70 AD patients of which the results are not yet published (Clinicaltrials.gov NCT00608946).

5.14.1.4. Selenium

Human studies suggest a protective role for the antioxidant selenium and selenoproteins against cognitive decline [686]. A recent study showed a significant cognitive improvement upon administration of Se-methylselenocysteine (SMC), a Se-enriched compound, in triple transgenic AD mice (3 x Tg-AD), a widely-used model for AD containing three mutations: APP Swedish, MAPT P301L and PSEN1 M146V [687].

In another study, the effect of sodium selenate, an inorganic form of selenium, was studied on the proteomic profile in the cortex derived from 3 x Tg-AD mice. Proteomic expression levels, measured by Isobaric tags for relative and absolute quantitation (iTRAQ) technology, showed that 96 proteins were down-regulated while 46 proteins were upregulated in AD mice [688]. However, after 4 months of sodium selenate supplementation, 41 of the previously down-regulated proteins were now up-regulated in the treated group while 8 up-regulated proteins in AD mice showed lower expression level in selenate-treated group. Further analysis identified the protein Platelet-activating factor acetylhydrolase type-II (PAFAH-II) to have a reverse expression, being up-regulated in the selenate-treated AD mice. PAFAH-II, present in tissues and plasma, is proposed to protect the cell against oxidative stress, most probably by hydrolyzing oxidized phospholipids [689]. Moreover, peroxiredoxin-1 (Prx-1) which was also shown to be down-regulated in AD mice, was instead found to be up-regulated upon Se treatment. Peroxiredoxins (Prxs) are antioxidant enzymes able to reduce H_2O_2 levels, protecting the cells from the oxidative damage [690,691]. These findings suggest that the reversed levels of specific proteins in selenate treated AD mice are involved in the reduction of oxidative stress and that Selenate plays a role in regulating the expression of those proteins [688]. In line with this thought, another study investigated the oral administration of selenium nanoparticles-loaded chitosan/citrate complex (SeNPs-C/C) in a D-galactose (D-gal)-induced aging mouse model. SeNPs were shown to slow down the d-gal-induced aging process in mice by enhancing the activity of antioxidantase, as evidenced by higher levels of SOD, CAT and GSH and lower level of TBARS in Se-treated mice compared to the control group [692].

5.14.1.5. Polyphenolic compounds

Polyphenolic compounds are plant based antioxidants and represent an important constituent of human diet. A phenolic compound is characterized by a ring structure and a hydroxyl (OH) group attached to it. These diverse chemicals can transfer their H atom to a free radical, exerting an antioxidant effect. In a clinical trial with 24 early AD patients, 24 moderate AD patients and 52 controls, 200 ml/day of polyphenolic antioxidant rich beverages were given to each subject. The trial investigated plasma total homocysteine (tHcy) levels which are directly proportional to oxidative stress as homocysteine undergoes auto-oxidation generating ROS. Following the treatment, patients with moderate AD showed significant lower increase of tHcy concentrations than that observed in the moderate AD subjects taking placebo. The same tendency was observed in the healthy group and in the early AD patients, but a significant difference was not reached [693].

5.14.1.6. Epigallocatechin gallate

Flavonoids, and particularly its subclass epigallocatechin gallate (EGCG) has demonstrated antioxidant activity. It is abundant in green tea and it has been shown that EGCG consumption can be beneficial to health [694]. EGCG acts by reducing superoxide anions and hydroxyl radicals and possesses the ability to up-regulate SOD, GSH and tocopherol. An animal study used young and old rat groups, that were orally administered 2 mg/kg body weight of EGCG per day, showed alleviation of oxidative stress by increasing levels of endogenous antioxidants SOD and GPx while decreasing markers of brain lipid and protein oxidation [695]. Since EGCG has shown neuroprotective and antioxidant effects both *in vitro* and in animal models, it has been investigated in a clinical trial involving 21 AD patients who were administered with increasing doses of EGCG (ranging from 200 mg to 800 mg per day) over a period of 18 months. Even though the investigators expect

EGCG to ameliorate the course of AD, results are not available yet (ClinicalTrials.gov NCT00951834).

5.14.1.7. Curcumin

Curcumin is the main active ingredient of the spice turmeric. In the recent years there have been multiple clinical trials which utilized supplementation of curcumin and the results showed antimicrobial [696], anticancer [697], anti-inflammatory and antioxidant properties [698].

In particular, curcumin was found to reduce oxidative stress *in vitro* as well as in animal models which carry a mutation in APP (Tg2576 APPSw), by preventing the oxidation of proteins and reducing levels of IL-1 β . Administration of low (160 ppm) and high doses of curcumin (5000 ppm) reduced the concentration of carbonyl groups on oxidized proteins in the residual cortex and piriform cortex of the AD mouse model, by 46.3% and 61.5%, respectively, compared to the untreated control group [699]. Another study showed that administration of curcumin lead to significantly lower levels of lipid peroxidation and increased the activities of SOD, GPx in the cortex, hippocampus, cerebellum and medulla of 6 and 24-month-old rats. All together, these data indicate a significant antioxidative action of curcumin [700]. However, one of the major limitations of curcumin is its poor bioavailability and its difficulty to reach the bloodstream. The specific mechanism by which curcumin is inducing its anti-oxidant effect remains to be established yet [701].

5.14.1.8. Resveratrol

Resveratrol, is a substance with antioxidant and neuroprotective properties that can be found in red wine [702]. Several observational studies have shown that moderate consumption of wine is associated with a lower incidence of AD [703,704] triggering interest in the red wine constituents possibly responsible for this observation. A randomized clinical trial conducted on 39 AD patients, aimed at testing the effects of a 15 mL mixture containing 5 g

glucose, 5 g malate and 5 mg resveratrol which was administered once a day for one year. Glucose and malate were included to aid the regeneration of reduced resveratrol. Slower disease progression was observed in the group that was treated with the oral preparation. However, this reduction was not significant, possibly due to the small number of participants [705].

5.14.1.9. l-Carnitine and acetyl-l-carnitine

l-Carnitine is a compound biosynthesized in the human body by the amino acids lysine and methionine. It acts as a shuttle to transport activated long fatty acids from the cytosol to the mitochondria for energy production and for their subsequent degradation through the β -oxidation process [706]. l-Carnitine deficiency therefore interferes with normal mitochondrial function [707]. Studies show that l-carnitine and its ester, acetyl-l-carnitine (ALC), can act as antioxidants. l-Carnitine exerts its antioxidant activity by protecting antioxidant enzymes from oxidative damage by neutralizing free radicals [708]. During a 4-week experiment, water with 0.15% of ALC or l-Carnitine were administered to aged rats. Although both l-carnitine and ALC were found to equally contribute to enhance the levels of carnitine in plasma and brain, only ALC effectively contributed to decreased levels of lipid peroxidation, nucleotide oxidation and nitrotyrosine [709].

A randomized controlled clinical trial of ALC demonstrated improved cognitive dysfunction in patients with early and mild AD. The study had durations ranging from 3 to 12 months and the dosages varied between from 1.5–3 g ALC per day. Even though there was no dose-dependent effect, patients treated with ALC showed amelioration of the disease symptoms [710].

5.14.1.10. DL-3-n-butylphthalide

DL-3-n-butylphthalide (NBP) is a synthetic chiral compound composed of L- and D-isomers of butylphthalide and is present in Chinese celery. Studies demonstrated that NBP aids in alleviating oxidative damage and

mitochondrial dysfunction [711]. In experimental studies, micromolar concentrations of NBP were able to promote cell survival the viability of SHSY-5Y cells, previously treated with H₂O₂ to induce oxidative stress and subsequent cell death. Also in rats suffering from chronic cerebral ischemia, a condition that is known to co-occur with oxidative stress as a pathological feature, a dose of 60 mg/kg NBP improved impaired cognitive function as assessed with a Morris water maze behavioral test [712]. Currently, NBP is being tested in mild to moderate AD patients treated with donepezil ([Clinicaltrials.gov NCT02711683](https://clinicaltrials.gov/ct2/show/study/NCT02711683)), and it is currently included in a phase-II clinical trial for acute ischemic stroke ([Clinicaltrials.gov NCT02905565](https://clinicaltrials.gov/ct2/show/study/NCT02905565)).

5.14.2. Non-nutrition based therapeutics

Non-nutrition based therapeutics comprise the substances which are not components of human alimentation.

5.14.2.1. Coenzyme Q

Coenzyme Q (CoQ) is ubiquitously expressed in the body, e.g. heart, kidney, and liver. CoQ possess antioxidant activity as well as can aid the regeneration of other antioxidants [713]. At a cellular level, it plays a role in the mitochondrial inner membrane where it neutralizes the peroxide produced by the electron transport chain. Even though no support for CoQ antioxidant capacity was observed upon oral administration of 400 mg CoQ to AD patients in clinical trials [670], animal studies showed its potential to scavenge and reduce reactive oxygen species. Rats with cognitive deficits and biochemical and structural changes in their brain due to generation of free radicals and glucose energy alteration, were injected with CoQ (10 mg/kg; i.p.) directly into the cerebrospinal fluid in cerebral ventricles. After 3 weeks of treatment, the rats showed improved memory and learning capabilities [714]. While many clinical trials have been shown to fail upon translation from an animal model to human patients, the precise origin of these differences in outcome are still unclear.

5.14.2.2. AntiOxBENs

AntiOxBENs are protocatechuic and gallic acids-derived compounds that have been designed as mitochondria-targeted antioxidants [715]. AntiOxBENs, upon administration, localize inside the rat liver mitochondria preventing lipid peroxidation, enhancing radical scavenging and iron chelation processes. These compounds are non-toxic to several cell lines up to a concentration of 100 μ M while exerting an antioxidant action already at 10 μ M. Most of the observed effects resulted from an increase of proton leakage through the mitochondrial inner membrane, which has been reported to decrease ROS formation [716].

5.14.2.3. Metal chelators: Clioquinol, Deferasirox, Deferoxamine and Deferiprone

Metal chelators generally act by making complexes with metal ions, preventing their ability to react with other elements or to precipitate. As metal homeostasis is impaired in AD, metal ion chelators have been suggested as potential therapeutics. Clioquinol is a chemical compound that acts as an antioxidant by reducing copper, iron and zinc metals. This might explain its beneficial effect in memory observed in prions-infected hamsters that were treated with a daily dose of Clioquinol equal to 50 mg/kg/animal. Subsequent behavioral and biochemical analysis of the animals showed that lipid peroxidation was reduced simultaneously with an improvement in memory, while no effect was observed in non-treated animals [717]. Moreover, Clioquinol reduces the deposition of A β in the brain [718,719].

Deferasirox, deferoxamine, deferiprone are iron chelators which are orally administered and are used as combine therapy with antioxidant to relief oxidative stress and restore mitochondrial function [720].

5.14.3. Current clinical trials into antioxidant therapeutics

Apart from dietary sources of antioxidants, organic chemicals have been developed to target oxidative stress reduction. The most recent tested drugs

targeting oxidative stress have been selected from [ClinicalTrials.gov](https://clinicaltrials.gov) and are listed in the paragraphs below.

5.14.3.1. Laptrepirdine

Laptrepirdine (Dimebon) supplied from Medivation Inc. and Pfizer Inc. was commercially available as an antihistamine [721]. Because of its ability to increase the level of acetylcholine by inhibition of either acetylcholine esterase or histamine receptors and based on a small clinical study of 14 mild to moderate AD patients [722], Dimebon was thought to have a potential as a new medication to prevent the progression of the disease. Therefore, it was investigated in a Russian II phase clinical study in which 155 AD patients were showing improvements of cognition over 6 and 12 months after intaking 20 mg Dimebon 3 times a day (ClinicalTrials.gov NCT00377715) [723]. Given the positive results of this initial trial, Dimebon was included in a larger-scale clinical study (phase III) including 598 patients diagnosed with mild to moderate AD. The latter were treated with 20 mg of Dimebon, 3 times a day but it failed in showing cognitive performance enhancement. (ClinicalTrials.gov NCT00838110) [724]. The discrepancies in the results, though disappointing, could be explained due to: i) lack of homogeneity in subjects' characteristics between the two trials, ii) the fact that the results of the trials are mainly based on cognitive outcomes and lack established biomarkers, iii) the unknown target of Dimebon. It was shown to primarily have a stabilizing effect on the mitochondrial membrane potentially by acting as an anti-uncoupling agent decreasing proton leakage into the mitochondrial matrix resulting in lower potential. An attempt to beneficially affect mitochondrial damage due to oxidative stress [725].

5.14.3.2. Idebenone

Idebenone, a synthetic analogue to Q10 but with a more hydrophilic character, acts as a potent antioxidant compound by scavenging-glycerophosphate (GP) and succinate-dependent ROS production [726] and protecting against

peroxidation of the mitochondrial membrane lipids. First, it was shown to significantly improve behavioral, attention and memory in AD randomized clinical trials on 102 patients with mild to moderate AD, after 4 months of receiving two doses of 45 mg per day [727]. On the other hand, Idebenone failed to rescue AD-associated cognitive impairment in a larger multicenter, double-blind, placebo-controlled trial [728].

5.14.3.3. Mitoquinol

Mitoquinol (MitoQ) is an improved synthetic version of coenzyme Q10 coupled to methyltriphenylphosphonium (TPMP), which is positively charged and allows the compound to cross the negatively charged double membrane and enter the mitochondria [729].

Lu and coworkers have shown that MitoQ prevents cognitive decline in AD transgenic mouse model [730]. A new ongoing clinical trial, involving patients with early AD, aims to examine the effects of MitoQ supplementation on carotid artery vasodilatory function and cerebrovascular blood flow ([ClinicalTrials.gov NCT03514875](https://clinicaltrials.gov/ct2/show/study/NCT03514875)). MitoQ is a mitochondria-targeting antioxidant that can normalize the nitric oxide production in the blood vessel, which should improve endothelial function in aging, and thus cerebrovascular blood flow [731].

5.14.3.4. Cerebrolysin

“Cerebrolysin™ (CBL) is a cocktail of low molecular weight neuropeptides and free amino acids that possess neuroprotective and neurotrophic properties. In this regards, CLB was shown to reduce APP APP phosphorylation as well as Tau pathology by reducing the activity of glycogen synthase kinase-3beta (GSK3β) and cyclin-dependent kinase-5 (CDK5) in models of AD [732]. In an another *in-vivo* study, 14 APP tg mice showed significant performance deficits in the water maze test compared to the wild type mice. Upon injection of 5 ml/kg CBL, the APP tg mice showed improved

learning ability compared to the non-treated group [733]. Moreover, CLB is able to cross the blood-brain barrier due to the presence of lipid-soluble factors. It has been shown to exert a protective effect against oxidative stress-induced apoptosis in neurons upon glutamate exposure [734]. CLB has been extensively investigated in 217 patients diagnosed with probable mild-to-moderate AD with promising clinical results ([clinicaltrials.gov NCT00911807](https://clinicaltrials.gov/NCT00911807)). In this clinical study, patients were treated with 10 mL Cerebrolysin, 10 mg donepezil and a combination of both drugs (n=67). Brain functioning, assessed by Alzheimer's Disease Assessment Scale Cognitive Subpart (ADAS-COG+) and Clinician's Interview-Based Impression of Change plus caregiver input (CIBIC+), resulted improved in all treated groups with greater amelioration in the combined therapy.

5.14.4. Current challenges in the development of antioxidant therapeutics

Utilization of antioxidants in most clinical trials generally aimed at prevention or treatment of the disease in early stages. The efficacy of antioxidants is mostly investigated in preclinical form of the disease (mild to moderate) as oxidative stress is considered an early occurring condition in AD [600]. The development of therapeutics is hampered by the fact that oxidative stress does not follow a specific pathway, e.g. receptor-mediated pathway or metabolic pathway, but is instead a diffuse phenomenon in the cell. Even though mitochondria are considered to be the main sources of oxidative reactions, increased levels of ROS have been identified in the cytosol as well. Thus, it is unclear whether to target cytosolic or mitochondrial oxidation [735]. Many attempts to neutralize ROS production consist of boosting the endogenous antioxidant system with high concentrations of dietary supplements. However, concerns regard the very high required dosage to attain any effect and the limited bioavailability of nutrition-based compounds. For example, an overdose of vitamins can lead to conditions such as gastrointestinal

disturbances or kidney failure [736]. A major obstacle of other orally administered substances, e.g. curcumin, is the poor absorption which limits the antioxidant effect [701]. Even though the bioavailability of a drug depends on several factors including dissolution rate and route of administration, poor solubility of the drug itself plays a major role [737,738]. In order to be absorbed and thereby exert the pharmacological response, drugs need to be sufficiently soluble in biological fluids. Nowadays, the main challenge that the pharmaceutical industries face is the improvement of the pharmacokinetic properties of drugs (absorption, distribution, metabolism and excretion) to guarantee the clinical success [737]. Strategies, currently employed by companies, to enhance water solubility of drugs include both physical and chemical modifications. Physical methods consist in reducing the size of the molecule by micronization [739] or nanosuspension [740]. In case of chemical modifications, change of pH, complexation and use of buffer are highly useful [741]. Despite the substantial literature on the improvements of drug bioavailability, the number of drugs approved by the Food and Drug Administration (FDA) is still limited.

Another important aspect to take into consideration when developing drugs for the treatment of neurodegenerative diseases, is the fact that these chemicals are often not able to reach the brain due to the limited permeability of the blood brain barrier (BBB) to these compounds. The blood-brain barrier is a dense layer of specialized epithelial cells, which dictates the rules for the passage of substances into the brain, preventing the drug to act at the right site [742]. Nowadays, researchers are employing several strategies to increase BBB permeability, such as nanoparticles-based approach [743], engineering of proteins and receptor-mediated transport [744].

Finally, it must be considered that AD is a multifactorial complex disorder, characterized by several pathological features and oxidative stress is only one

of them. Therefore, interventional studies should investigate possibilities for a more beneficial treatment by targeting simultaneously the hallmarks of the disease rather than a single marker.

5.15. Conclusion

In conclusion, the most relevant reactive species causing oxidative stress in the early AD brain are ROS that is mainly produced in the mitochondria. Lipids and proteins in the brain seem to be firstly affected by oxidative stress since the markers of their peroxidation and oxidation is detectable in early stage. While DNA and RNA oxidation is mostly in late stages but some experiments suggest that it might be occurring in early stages therefore further experimental investigations are required for confirmation. Spatial and quantitative assessment for oxidative stress in early stages will be gravely essential. ^{62}Cu -ATSM-PET as a diagnostic tool is already showing great potential since it will indicate the specific location of the oxidative stress in the brain making it possible to characterize optimal therapeutic windows and thus more effective design of drug and clinical trials. Yet there is a main drawback of toxicity due to accumulation of the tag. Antioxidants used in clinical trials reduce and can prevent oxidative stress when used solely or in combinations. Their effectiveness is more reliant on their transportability and bioavailability at targeted site of the oxidative stress since a better response was noticed when the antioxidant could pass the through BBB.

5.16. References

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PART 4:

Neuroinflammation

Chapter 6

IFN γ and A β act in a concerted manner to induce microglial IL-1 β secretion and loss of synchronous neuronal firing activity

Neuronal loss, neuroinflammation, and deposition of amyloid beta (A β) peptide are collectively observed in the AD brain. How these three hallmark characteristics interact is unclear and we addressed this question by studying the interaction between the pathways involved, and investigating neuronal activity in response to this. We found that A β and IFN γ together potently activate microglia into a pro-inflammatory phenotype and we identified that this resulted into microglial NLRP3 inflammasome assembly. In response to this, IL-1 β secretion levels were raised, which, in turn, resulted in loss of synchronous firing activity in a rat primary neuronal culture.

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6.1. Introduction

The AD brain is characterized by the presence of aggregated A β species surrounded by a corona of pro-inflammatory activated microglia [1]. The precise nature of the preference of such microglial phenotype to cluster around A β deposits is unknown but the implicated generation and secretion of cytokines and reactive oxygen species by pro-inflammatory microglia suggests that the processes of A β deposition and neuroinflammation are somehow associated [2]. In the healthy brain, microglia are maintained primarily in a quiescent state by the coordinated suppressing action of neurons and astrocytes [3]. However, following an insult, microglia are quickly activated to adopt a pro-inflammatory phenotype mediated by the protein IFN γ [4]. This is a highly functional response as activated microglia proliferate at the site of damage to promote repair of the injured tissue. A short but potent pro-inflammatory response aids the restoration of homeostasis after which microglia return to their resting immunosurveillance activities once the damage is reconciled [5,6]. In AD the proinflammatory activation of microglia is a sustained condition which is associated with neuronal dysfunction and ultimately degeneration [7–9]. A direct relation between A β and onset of neuroinflammation was demonstrated upon injection of A β oligomers into mice; the presence of A β oligomers raised the Interleukin-1 β (IL-1 β) mRNA expression levels [10]. Similarly, Tg2576 mice, overexpressing a mutant form of amyloid precursor protein (APP), showed higher mRNA levels for TNF α in the hippocampus and cerebellums of AD mice compared to wild type mice [11].

Inflammation of the brain is a feature of other neurodegenerative diseases and is thought to be a result of many factors such as protein misfolding, aggregation and oxidative stress [12]. Microglial abnormalities were also reported in Huntington's Disease (HD). In HD patients, higher pro-

inflammatory markers such as IL-6, IL-8 and TNF- α are found in patient CSF and in the striatum and cortex [13,14]. Additionally, microglia were found associated with plaques in the prion diseases CJD, Kuru and Gerstmann-Sträussler-Scheinker syndrome (GSS) [15]. Prion-infected mouse brains show up-regulation of many cytokines, including IL-1 β and TNF- α [16].

In the current study, we show that IFN γ together with A β induce a microglial proinflammatory phenotype, resulting in NLRP3 inflammasome assembly, and increased IL-1 β secretion. We further show that exposure of a primary neuronal culture to such increased levels of IL-1 β results in loss of synchronous firing activity and ultimately degeneration.

6.2. Materials and Methods

6.2.1. Caspase-1 and IL-1 β expression in the human cohort from the public database

Caspase-1 and IL-1 β mRNA expression were assessed in the publicly available transcriptome data sets of entorhinal cortex from AD patients and healthy subjects (GSE5281).

6.2.2. Electrophysiology

We obtained cortical cells from newborn Wistar rats at post-natal day 1. After trypsin treatment cells were dissociated by trituration. About 200,000 dissociated neurons (200 μ l suspension) were plated on a micro electrode arrays (MEAs; MultiChannel Systems, Reutlingen, Germany), precoated with polyethylene imine (PEI). We used MEA's containing 12 (pitch 300 μ m) or 60 electrodes (pitch 200 μ m) of 30 μ m diameter. 12 electrode MEAs were part of a multi-well system. Culture chambers was filled with 500 μ l (12 electrode MEA) or 1000 μ l (60 electrode MEA) of R12 medium [17] MEAs were stored in an incubator, under standard conditions of 36°C, high humidity, and 5% CO₂ in air. For recording, we closed the culture chambers

to avoid bacterial infections and evaporation (while still facilitating the entrance of O_2 and CO_2 into the chambers), and placed the cultures in a measurement setup outside the incubator. During recording we maintained the CO_2 level of the environment at 5% and we moisturized the air. All cultures generated at least 250 spikes/min during base line (before administration of IL-1 β), and their firing patterns included network bursts.

We used 6 neuronal cultures for 6 experiments, which were performed in the fourth week after plating of the cells. All research involving animals has been conducted according to Dutch law (as stated in “Wet op de dierproeven”), and approved by DEC, the Dutch Committee on Animal Use (AVD110002016802).

6.2.3. IL-1 β administration

After a baseline recording IL-1 β was added to the culture medium in three steps, to achieve cumulative concentrations of 16 ng/ml, 32 ng/ml, and 64 ng/ml. After recording activity at the highest concentration, five cultures were washed twice with fresh R12 and then placed in conditioned medium. In these cultures we also recorded activity one day later.

6.2.4. Analysis

To evaluate the effect of IL-1 β we observed total activity and the burstiness index of recorded activity. Total activity was calculated as the summed activity of all electrodes in 20 min recordings. We applied a method introduced by Wagenaar and co-workers [18] to estimate the burstiness of a culture, which is a measure of synaptic connectivity. In short, 5 min recordings were divided into 300 bins of 1s each, and the total number of spikes in each bin was counted. We then computed the fraction of spikes accounted for by the 15% of all bins containing the largest spike counts (f_{15}). Should most of the spikes occur in bursts, f_{15} will be close to 1; tonic firing should lead to a $f_{15} \approx 0.15$. Burstiness index (BI) was then defined as $BI = (f_{15} -$

0.15)/0.85, so that BI is normalized between 0 (no bursts) and 1 (burst dominated). For averaging across cultures, total activity and BI were normalized to their baseline values.

6.2.5. A β peptide solubilization

A β_{42} (rPeptide), were dissolved according to the standard procedure developed and validated in our laboratory [19]. In short, A β peptides were dissolved in hexafluoroisopropanol (HFIP). HFIP was evaporated using nitrogen gas and the peptide film was redissolved using dimethyl sulfoxide (DMSO). The peptide was separated from DMSO by elution from a HiTrapTM desalting column (GE Healthcare) equilibrated in phosphate-buffered saline (PBS) pH 7.4. Peptide concentration was determined using the DCTM Protein Assay kit (BIO-RAD) and diluted to the required final concentration. Aggregation of A β peptides occurred at 37 °C under quiescent conditions.

6.2.6. IFN γ preparation

IFN γ (cat. no. SRP3058) was commercially obtained from Sigma and was dissolved in 10 mM potassium phosphate pH 7.4. Concentration was measured by means of absorption at 280 nm using a Nanodrop® ND-1000 UV-Vis spectrophotometer.

6.2.7. Cell culture differentiation

Human leukemia monocytes (THP-1 cells) were routinely cultured in RPMI-1640 supplemented with 2mM L-Glutamine, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco). Differentiation of THP-1 into macrophage-like cells was induced upon incubation with 50 ng/ml PMA (phorbol 12-myristate 13-acetate; Sigma-Aldrich) for 48 h. Cell culturing was performed at 37°C and 5% CO₂ in a humidified atmosphere.

6.2.8. Enzyme-linked immunosorbent assay (ELISA)

Medium from PMA-differentiated THP-1 was collected and analyzed for IL-1 β release, using the PeliPairTM reagent set and according to the manufacturer's instructions (Sanquin, catalog: M9334).

6.2.9. RNA isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) from THP-1 cells

Total RNA was isolated according to the protocol supplied with an RNA isolation kit (Sigma) according to the manufacturer's instructions. The concentration and the purity of the RNA samples were determined using a Nanodrop® ND-1000 UV-Vis spectrophotometer. Total RNA (1000 ng) was reverse transcribed with the use of iScript Reverse Transcriptase (BioRad). Primers used for quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) were for the human proteins: Interleukins 1 β and 6 (IL-1 β and IL-6) and Tumor Necrosis Factor α (TNF α). qRT-PCR was conducted in BioRad Real-Time detection system (CFX 384, C1000 Touch) in 384-well plates with 20 ng of cDNA, 750 nM forward and 750 nM reverse primers and 4 μ l of Fluorescein and SYBR mix (BioLine) in total volume of 8 μ l per well with 2 technical and 3 biological replicas.

6.2.10. SDS-PAGE and Western blot

Lysates were prepared from stimulated THP-1 cells using SDS-lysis-loading buffer (45 mg/ml SDS, 35 mg/ml DTT, 10 μ g/ml BPB, 20% v/v glycerol and 2.5% v/v 0.5 M Tris pH 6.8). Prior loading onto 10% running and 4% stacking polyacrylamide gel, lysates were treated with a short burst of ultrasound and heated to 95°C for 5 minutes. As a molecular weight marker, SDS-PAGE ruler (ThermoFisher, cat. 26616) was loaded next to the lysates. PAGE was conducted with a constant voltage (100V) for approximately 1 hour in SDS-Tris-glycine running (1x) buffer. Protein extracts, separated by SDS-PAGE and transferred onto PVDF membranes, were probed with antibodies against pSTAT1 α (58D6 monoclonal, Cell Signaling) or β -actin (AC-15 monoclonal, Sigma, cat. A5441). Proteins were detected with HRP Immobilon (Merck, cat. WBKLS050) and visualized with a Westburg Imager. Subsequently, the images were analysed with ImageJ.

6.3. Results

6.3.1. mRNA level of IL-1 β is upregulated in AD entorhinal cortex.

Overexpression of IL-1 β is observed in many neurodegenerative diseases [20]. To investigate whether a similar observation holds for AD, we performed a transcriptome analysis experiment on database GSE5281. We observed significant ($*P < 0.05$) induction of IL-1 β mRNA expression (Fig. 6.1) compared to healthy subjects (Controls: 86.72 ± 17.39 N=13, AD patients: 190.9 ± 36.65 N=10).

Figure 6.1. mRNA expression levels of IL-1 β in AD patients are significantly elevated. IL-1 β mRNA expression levels in the entorhinal cortex from publicly available transcriptome profiling data sets (www.ncbi.nlm.nih.gov/geo). GEO accession number: GSE5281. Statistical significance levels were $*P < 0.05$.

Increased mRNA levels seem to translate well to protein expression levels as it has been reported previously that IL-1 β protein concentrations in the post-mortem AD hippocampus are significantly raised as compared to age-matched controls [21].

6.3.2. IL-1 β induces dose-dependent loss of primary rat cortical neuronal synchronicity

Upregulation of p38 MAPK activity in the AD brain [22] and involvement of the p38 MAPK signaling pathway in synaptic plasticity regulation in the central nervous system [23] suggests that AD-associated raised levels in IL-1 β may promote loss of activity and neuronal degeneration. To assess the effect of raised IL-1 β protein levels on neuronal activity, electrophysiological activity of rat primary cortical neurons (PCN), upon exposure to increasing concentrations of IL-1 β (ranging from 0 to 64 ng/ml) was measured using microelectrode arrays. The firing rate (Fig. 6.2A) and the burstiness index (Fig. 6.2B) were derived based on the obtained measurements.

Figure 6.2. IL-1 β decreases synchronicity in a dose-dependent manner without affecting the firing rate of rat primary cortical neurons. Statistical significance of the results was established by P-values Kruskal-Wallis test followed by a Tukey test. Values represent results of six independent replicates. Statistical significance levels were * $P < 0.05$ and ** $P < 0.01$.

The burstiness index provides an estimate of the neuronal synchronicity [24] which is significantly decreased in presence of IL-1 β indicating that synaptic propagation is lost. The firing rate is not affected by IL-1 β up to a concentration of 64 ng/mL.

6.3.3. Upregulation of IL-1 β mRNA levels can be recapitulated in a THP-1 cell line exposed to A β ₄₂

Oligomeric A β , particularly the oligomeric A β ₄₂ isoform, plays a key role in AD pathogenesis while [25] IFN γ acts as a hub protein that mediates microglial activation [4]. To establish whether raised IL-1 β mRNA expression levels are induced by A β and IFN γ , PMA-differentiated THP-1 cells were exposed to A β , IFN γ and mixtures thereof. First, the sub-toxic concentration of A β and IFN γ were determined using CellTiter-Blue® (Promega) viability activity assay (Fig. S1) and 82 nM A β ₄₂ and 1.64 nM IFN γ were selected to perform qRT-PCR assays. Pro-inflammatory response was monitored by means of mRNA expression levels for IL-1 β and other inflammation-related proteins. Stimulation of THP-1 cells for 24 and 48 h with a pre-incubated (1.5 h, 37 °C) combination of oligomeric A β ₄₂ and IFN γ (“A β ₄₂xIFN γ “), as well as separately pre-incubated proteins (“A β ₄₂+IFN γ “) resulted in a significantly enhanced pro-inflammatory response, as assayed by qRT-PCR, compared to the addition of A β ₄₂ or IFN γ separately (Fig. 6.3A-C).

Figure 6.3. Collective action of A β ₄₂ and IFN γ induce a pro-inflammatory response by THP-1 cells. PMA-differentiated THP-1 cells were stimulated with A β ₄₂ (82 nM), IFN γ (1.64 nM), pre-mixed A β ₄₂ and IFN γ (“A β ₄₂ x IFN γ ”), or both proteins separately pre-incubated (“A β ₄₂ + IFN γ ”). Controls are represented by treatment with anti-inflammatory IL-4 and IL-3 (20 ng/ml) and PBS. mRNA expression levels on a log-10 scale of (A) IL-1 β (B) TNF α , and (C) IL-6 cytokines were detected. Experiments were repeated three times (N=3). Results are presented as mean $\pm$ standard error of the mean (SEM).

6.3.4. JAK2/STAT1 signaling is induced by IFN γ and by combined treatment with IFN γ and A β ₄₂

As cytokine expression is mediated through the JAK2/STAT1 signaling pathway [26–28], the involvement of this pathway in A β and IFN γ -mediated induction of pro-inflammatory response was studied. Phosphorylation of STAT1 (p-STAT1), one of the first steps in activation of this pathway [29],

was strongly induced after 6 h of stimulation with pre-incubated (for 1.5 h at 37 °C) IFN γ and both IFN γ /A β_{42} combinations, but not in the presence of A β_{42} alone. After 24 h, only the combined stimulation with A β_{42} and IFN γ results in a synergistic effect of JAK2-STAT1 activation (Fig. 6.4A-B) suggesting a stabilizing and sustained mechanism to induce JAK2/STAT1 signaling.

Figure 6.4. A β_{42} and IFN γ induce JAK2/STAT1 activation. **A)** pSTAT1 α western blot immunostaining of cell lysates after 6 h and 24 h of stimulation with IFN γ (1.64 nM), A β_{42} (82 nM), co-stimulation with separately pre-incubated A β_{42} and IFN γ (82 nM + 1.64 nM), and with co-incubated A β_{42} and IFN γ (82 nM $\times$ 1.64 nM) for 1.5 h at 37 °C. Protein levels were normalized to actin. **B)** The intensities of p-STAT1-positive bands were determined with ImageJ software relative to vehicle (PBS)-treated cells.

6.3.5. IL-1 β generation in AD is upregulated via NLRP3 assembly and A β_{42} stimulates IL-1 β secretion.

IL-1 β is generated as the precursor pro-IL1 β which requires caspase-1-mediated proteolytic processing to attain its active form [30]. Transcriptome analysis of the entorhinal cortex of AD patients showed a significant induction of caspase-1 mRNA expression levels (Fig. 6.5) compared to

controls (GEO accession number: GSE5281) indicative of involvement of the NLRP3 inflammasome in AD progression.

Figure 6.5. Caspase-1 mRNA expression levels are increased in the AD brain. Caspase-1 mRNA expression levels in the entorhinal cortex from publicly available transcriptome profiling data sets (www.ncbi.nlm.nih.gov/geo). GEO accession number: GSE5281. Statistical significance levels were $**P < 0.01$.

The observed increased mRNA levels in AD brains raised the possibility that NLRP3 assembly is responsible for the increased generation of IL-1 β in AD [20]. To address this question, PMA-differentiated THP-1 cells were treated with A β_{42} , IFN γ , a pre-incubated combination of A β_{42} and IFN γ (“A β_{42} xIFN γ ”), as well as separately pre-incubated proteins (“A β_{42} +IFN γ ”) in presence or absence of the NLRP3 inhibitor CRID3 [31]. Nigericin was used as a positive control since it damages the cell membrane by inducing the formation of pores [32], and the release of IL-1 β in response to nigericin has been demonstrated to be inflammasome-dependent [33,34]. After 24 h of incubation, medium was collected and subjected to ELISA in order to determine levels of secreted IL-1 β . Secretion of IL-1 β was substantially increased upon exposure of THP-1 cells to A β_{42} compared to untreated cells (Fig. 6.6). Addition of CRID3 resulted in inhibition of IL-

1 β release, confirming the involvement of the NLRP3 inflammasome assembly in mediating the IL-1 β secretion

Figure 6.6. Collective action by IFN γ and A β ₄₂ reinforce NLRP3-inflammasome-mediated secretion of IL-1 β from THP-1. Medium from THP-1 cells was collected after 24 h of stimulation with Nigericin (10 μ M), A β ₄₂ (82 nM), IFN γ (1.64 nM), co-stimulation with separately pre-incubated A β ₄₂ and IFN γ , and with co-incubated A β ₄₂ and IFN γ for 1.5 h at 37 °C. Statistical significance of the results was established by P-values using two-tailed t-tests (GraphPad Software). Values represent results of three independent replicates. Statistical significance levels were *P < 0.05, **P < 0.01 and ***P < 0.001.

6.4. Discussion and conclusions

We addressed the question how neuroinflammatory response and neuronal degeneration are associated in Alzheimer's disease (AD). Our data show that A β ₄₂ and IFN γ co-act to induce microglial activation into a sustained pro-inflammatory phenotype secreting increased levels of the pro-inflammatory

cytokine IL-1 β , similar to that observed in the brains of AD patients [35,36], while both proteins individually induce short-term activation. We observed that increased IL-1 β levels induced the synchronicity of rat cortical neurons. Whereas physiological levels of IL-1 β regulate LTP [37], high concentrations of IL-1 β were shown to inhibit long-term potentiation and synaptic strength [38,39]. Neurons are known to express IL-1 β receptor [40], and pre-treatment of neurons with an antagonist prevented IL-1 β -mediated neuronal responses [38]. The sustained inflammatory activity of microglia in response to simultaneous exposure to A β and IFN γ raised the question whether alternative signaling pathways had been activated as to the well-characterized IFN γ -inducible JAK2/STAT1 signaling pathway. However, we observed that the increased IL-1 β generation is mediated by activation of the JAK2/STAT1 signaling pathway and that IFN γ and A β together induce subsequent NLRP3 inflammasome assembly which is an important component of the immune system [41].

The synergistic effect of A β and IFN γ thus involves signaling via similar pathways as those that have been identified before. Upon binding to IFNGR1, a cascade of signals is initiated, ultimately leading to upregulation of transcription of pro-inflammatory factors [42,43]. Our data possibly suggest that A β_{42} may be internalized together with IFN γ upon interaction with IFNGR1. A similar process was shown for Lipocalin-2 mediated transportation of A β_{42} into the cell [44]. Alternatively, based on an earlier observation that caspase-1, apart from IL-1 β , is also able to process pro-interleukin-18 into active interleukin-18, which, in turn, can stimulate IFN γ [45], it is possible that our observations can be explained by a positive feedback loop in which presentation of IFN γ to cells generates a preserved and prolonged generation of IFN γ . The prolonged and stabilized action of IFN γ in combination with A β_{42} , as we observed in our experiments, may

strengthen this effect. In addition to this, A β was observed to induce microglial p38 MAPK signaling leading to upregulated generation of IL-1 β , which in turn, was found to stimulate p38 MAPK signaling in cells of neuronal origin [46].

A stabilizing interaction between A β and IFN γ may however be questioned as we observed similar effects between the pre-mixed A β and IFN γ (“A β_{42} xIFN γ “) and the separately pre-incubated proteins that were added together to the cells (“A β_{42} +IFN γ “). Moreover, as reported in chapter 7, no direct interaction between IFN γ and A β could be identified apart from a potent IFN γ -mediated A β aggregation inhibitory effect.

In conclusion, the present data reveal that monocyte-derived microglia-like cells respond to A β and IFN γ in a highly synergistic manner to induce IL-1 β secretion by involving the JAK2-STAT1 signaling pathway and the activation of the NLRP3 inflammasome. These processes lead to an increased production of pro-inflammatory IL-1 β , which, in turn, mediates loss of synchronous firing activity in a neuronal network. The observed additive effect of A β and IFN γ together suggest that these two proteins may undergo an interaction that serves as prime hub connecting neurodegenerative and neuroinflammatory processes as clinically observed in AD.

6.5. Acknowledgments

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6.6. Supplementary Information

Figure S1. Determination of sub-toxic A β_{42} and IFN γ concentration. IFN γ (0.6 and 6 nM) and A β_{42} (1 – 10 -100 and 1000 nM) are non-toxic to differentiated THP-1 cells. Viability normalized against medium-treated cells: error bars show SD, n=5. Controls include a 2 μ M solution of staurosporine.

6.7. References

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Chapter 7

Characterization of the interaction between Amyloid- β and Interferon- γ

Neuroinflammation is associated with Alzheimer's disease (AD) and it is observed to simultaneously occur with AD hallmarks amyloid-beta ($A\beta$) deposition and neuronal cell death. The neuroinflammatory response is driven by interferon-gamma ($IFN\gamma$)-mediated microglia proinflammatory activation but how neuroinflammation and neurodegeneration are connected is unclear. In the previous chapter we have shown that $A\beta$ and $IFN\gamma$ co-act to induce IL-1 β secretion, which, in turn, generates an asynchronous firing profile in rat primary cortical neurons. In this study we set out to identify how $IFN\gamma$ and $A\beta$ co-act by characterizing their potential interaction. We used biophysical techniques to show, for the first time, that $IFN\gamma$ can modulate the aggregation behaviour of $A\beta$.

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7.1. Introduction

Alzheimer's disease (AD) is a widespread neurodegenerative disorder that primarily affects elderly people [1]. One of the characteristic histopathological features observed in the AD brain is the extracellular deposition of amyloid-beta peptide ($A\beta$) into so-called amyloid plaques. Strikingly, this observation was described to occur simultaneously with the pro-inflammatory activation of immune cells of the brain followed by neuronal degeneration [2]. Dissemination of the precise sequence of events or their interaction regulating the complex pathological hallmarks observed in AD turned out to be a challenging question but assembly of $A\beta$ into oligomers was demonstrated to play a key role in this process [3–5]. $A\beta$ oligomers were shown to act as external stimuli that induce mitochondrial activation through various identified pathways [6] upon which a wave of chemical mediators is released that can induce neurodegeneration [7,8]. While it is recognized that a short period of inflammation is highly functional to clear the brain from damaging factors and restore neuronal functionality and homeostasis, a prolonged response, as experienced in AD, causes a chronic overproduction of reactive oxygen species (ROS), nitric oxide (NO), and the expression of other small proteins that can be detrimental to neurons [9,10]. Knowing this, it is appealing to hypothesize that $A\beta$ oligomerization and microglial activation act together to induce neuronal cell death. Such hypothesis would be in line with the previous observation that oligomeric $A\beta$ -mediated neuronal cell death is exacerbated in the presence of microglia [11]. Specific molecular mechanisms that drive this interaction have yet to be identified, but it is known that the process of neuroinflammation is driven by the potent immune-mediator Interferon- γ ($IFN\gamma$) [12] which can be produced and released by astrocytes following a traumatic and metabolic injury [13]. $IFN\gamma$ itself can promote the secretion of other cytokines and signaling molecules by modulating the activation of

macrophages in the central nervous system (CNS) [14]. Consistent with this view, IFN γ is overexpressed in J20 mice [15], an established mouse model for AD, while activated microglia in the AD brain preferentially reside in close proximity of A β deposits [16]. We hypothesized that IFN γ may play a role linking A β deposition and chronic microglial activation. In the previous chapter we report how IFN γ and A β co-act to induce JAK2/STAT1 signaling to mediate NLRP3 inflammasome assembly and subsequent IL-1 β processing and secretion. Even though the interaction between IFN γ and A β cannot be detected with standard biochemical/biophysical methods, the presence of IFN γ significantly delays A β aggregation.

7.2. Materials and Methods

7.2.1. A β peptide solubilization

A β_{42} (rPeptide) was dissolved according to the standard procedure developed and validated in our laboratory [17]. In short, A β was sequentially dissolved in hexafluoroisopropanol (HFIP) and dimethyl sulfoxide (DMSO) after which the peptide was separated from DMSO by elution from a HiTrapTM desalting column (GE Healthcare). Peptide concentration was determined using the Coomassie (Bradford) Protein Assay kit (for NMR, the peptide concentration was estimated) and diluted to the required final concentration. A β aggregation was studied in PBS at 37 °C under quiescent conditions.

7.2.2. IFN γ preparation

IFN γ (cat. no. SRP3058) was commercially obtained from Sigma and dissolved in 10 mM potassium phosphate pH 7.4.

7.2.3. Thioflavin-T assay

Aggregation was followed using a Thioflavin-T (ThT) fluorescence assay. A β_{42} peptide concentration was adjusted to 25 μ M using PBS buffer, pH 7.4 and a final concentration of 12 μ M ThT in a 96-well plate (Greiner flat bottom

transparent black, Sigma – cat. M9685). Fluorescence intensity was measured at 37 °C using an automated well-plate reader (Tecan® Saffire2) with an excitation wavelength of 450 nm and emission detection from 480 to 600 nm. ThT curves were prepared by plotting the emission intensity readings at 500 nm versus aggregation time. Triplicate values were averaged and blank subtracted. The rate of A β fibril elongation (k) was determined by curve fitting (fig. S1A-E) a Boltzmann function (1), using the OriginLab software:

$$y = \frac{A_1 - A_2}{1 + e^{(t - t_0) \times k}} + A_2 \quad (1)$$

A₁ and A₂ represent the fluorescence levels at the beginning of the measurement and at the maximal fibrillization level, respectively, t₀ is the time at the midpoint between A₂ and A₁ [18].

7.2.4. Solution NMR spectroscopy

Prior to incubation, 5% D₂O was added to A β ₄₂ (estimated 50 μ M final concentration) in presence or absence of IFN γ (0.5 μ M final concentration). The 2D ¹H-¹⁵N heteronuclear single quantum correlation spectroscopy (HSQC) experiments were conducted at the University of Utrecht at 25 °C with a 900 MHz Bruker Avance III system equipped with TCI cryo-probe. Data were processed and analyzed using Topspin 3.2. Water was suppressed by excitation sculpting [19]. A spectral width of 12 ppm was used, and 512 scans were accumulated with an acquisition time of 750 ms. Spectra were zero-filled once and processed with exponential multiplication using 5 Hz line-broadening. After baseline correction, proton signal was integrated over the entire region from 9-6 ppm.

7.2.5. Circular dichroism (CD)

A β ₄₂ in the absence and presence of IFN γ was placed in a quartz cuvette with an optical path of 1 mm (Hellma). Far-UV circular dichroism (CD) spectra were recorded in a Jasco J-1500 spectrometer. The wavelength range was set from 190 to 260 nm with 0.5 nm data pitch, 8.0 s response time, and 1.0 nm

bandwidth. Data were collected as averages of 8 scans at a scanning speed of 50 nm/min. Spectra were corrected by subtracting the buffer baseline. Measurements were performed as independent triplicates. Data are presented as the mean residue ellipticity (MRE, in $\text{deg cm}^2 \text{dmol}^{-1}$).

7.2.6. Atomic force microscopy (AFM)

A β fibrils were visualized with AFM imaging using a BioScope Catalyst atomic force microscope, operated in tapping mode in air by using silicon cantilevers with a resonance frequency of 325 kHz, a spring constant of 46 Nm^{-1} , and a tip radius of 10 nm (μMASCH , NSC15/no Al). Images were collected at a scan rate of 0.1 Hz. Each fibrillar sample was deposited for 15 min onto freshly cleaved mica surfaces (Muscovite Mica V-1 quality, Electron Microscopy Sciences, cat. 71855) to enable adsorption. The samples were rinsed with ultrapure water (5 x 200 μL) and left to dry in air before imaging.

7.2.7. Total Internal Reflection Fluorescence Microscopy (TIRFM)

Co-localization of A β_{42} and IFN γ -A647 co-aggregated samples was performed by means of Total Internal Reflection Fluorescence Microscopy (TIRFM). Samples were prepared from IFN γ -A647 and A β_{42} (2:1) as described in 7.2.1 and 7.2.2. The resultant mixtures were incubated for 72 h at 37 °C. Samples were then mixed with 50 μM ThT in a 3:1 v/v ratio and placed on a clean microscope glass, immediately covered with a cover-slip and sealed with nail-polish. The resultant sample was placed upside-down overnight in the dark to allow protein assemblies to sediment. Images were made with a Nikon Eclipse Ti microscope with Apo 100x/1.45 oil TIRFM objective (Olympus) in TIRF mode. ThT and A647 were excited with a 457 nm or 640 nm lasers (TIRF) and emission was collected using 455/10 or 620/20 ex.

7.3. Results

7.3.1. IFN γ delays A β aggregate formation

In the absence of IFN γ , A β_{42} aggregation at 37 °C is marked by a strong and sigmoidal increase in ThT fluorescence (fig. 7.1A), indicative of intermolecular β -sheet structure formation [20]. Addition of substoichiometric IFN γ concentrations (0.125 – 1.5 μ M) to monomeric A β_{42} at a concentration of 25 μ M resulted in a dose-dependent change of the kinetic profile of fibril formation (fig. 7.1A) within the time frame of the measurement (72 h). In particular, the presence of IFN γ induces an increase in the length of the lag (fig. 7.1B-C) and elongation phases (fig. 7.1D) compared to A β_{42} alone. Interestingly, already very low concentrations of IFN γ were capable of modulating A β_{42} fibril formation to a significant extent, suggesting that IFN γ is a very potent inhibitor of A β_{42} aggregate assembly.

Figure 7.1. IFN γ affects A β aggregation in a dose-dependent manner. **A)** Aggregation kinetics of 25 μ M A β_{42} incubated at 37 $^{\circ}$ C for 72 h was monitored by ThT fluorescence. IFN γ (concentrations at 0.125 μ M to 1.5 μ M) was added to A β prior to incubation of A β_{42} . **B)** Zoom in of the lag phase. **C)** Overview of time points at different IFN γ concentrations where polymerization trace starts to deviate from linearity. **D)** Kinetics of A β fibrillization determined according to Boltzmann equation. Statistical significance of the results was established by P-values using paired two-tailed t-tests. Statistical significance levels were *P < 0.05, **P < 0.01 and ***P < 0.001.

Similar to our ThT fluorescence assay, the aggregation of 15 N-labeled A β_{42} , was delayed in the presence of low (approximately 100-fold) concentrations

of $\text{IFN}\gamma$ when monitored using NMR spectroscopy. A gradual decline of $\text{A}\beta_{42}$ integral signal intensity was observed over time. Such gradual loss of integral signal intensity is typically associated with the formation of assemblies of increasing size, *i.e.* constituting higher ordered structures of $\text{A}\beta_{42}$ [19]. Signal intensity dropped at a slower rate in presence of $\text{IFN}\gamma$ suggesting that (i) $\text{IFN}\gamma$ reduced the rate of aggregation, or (ii) early in the $\text{A}\beta$ aggregation process smaller NMR visible assemblies were stabilized by the presence of $\text{IFN}\gamma$ that contribute to overall signal intensity (fig. 7.2).

Figure 7.2. $\text{IFN}\gamma$ delays aggregation $\text{A}\beta_{42}$ monitored by solution ^1H NMR spectroscopy. Comparison of normalized ^1H signal integrals between 6 and 9 ppm for $\text{A}\beta_{42}$ (50 μM) in the presence and absence of $\text{IFN}\gamma$ (0.5 μM) as function of time. Replicated twice, representative sample shown.

Consistent with the data obtained using NMR and ThT fluorescence, circular dichroism (CD) spectroscopy showed that $\text{IFN}\gamma$ affected $\text{A}\beta_{42}$ secondary structure content upon incubation of $\text{A}\beta_{42}$ at 37°C for 72 h. In the absence of

IFN γ , substantial β -sheet signal was observed at a wavelength of 218 nm (fig. 7.3A). Co-incubation of A β_{42} with different concentrations of IFN γ (ranging from 0.125 μ M to 1.5 μ M) for 72 h resulted in a dose-dependent reduction of the β -sheet signal (fig. 7.3B). The contribution of IFN γ to the CD signal was found to be minimal as assessed by measuring the spectra of IFN γ in the absence of A β_{42} at different concentrations after 72 h of incubation at 37°C (fig. S2A-B).

Figure 7.3. A β β -sheet formation is inhibited in an IFN γ dose-dependent manner. **(A)** The secondary structure content of A β_{42} and IFN γ (concentrations ranging from 0.125 μ M to 1.5 μ M) was studied by CD after 72 h of incubation. Spectra reveal a predominant β -sheet structural signature for all samples characterized by a minimum intensity at a wavelength around 218 nm. **(B)** Intensity of β -sheet signal at 218 nm plotted as a function of IFN γ concentration. Data are averages of three independent measurements. Error bars represent standard deviations. Statistical significance of the results was established by P-values using paired two-tailed t-tests. Statistical significance levels were *P < 0.05 and **P < 0.01.

As CD, NMR and ThT fluorescence assays collectively show that IFN γ delays, but not fully inhibits A β_{42} aggregation, it is of interest to investigate the effect of IFN γ on A β_{42} aggregate morphology. The dose-dependent effect of IFN γ on A β_{42} aggregate morphology after incubation for 72 h was assessed

using AFM imaging. In presence of IFN γ , A β ₄₂ was found to form highly clustered aggregates and formation of such structures positively correlated with increasing concentration of IFN γ (fig. 7.4).

Figure 7.4. IFN γ affects A β fibril morphology. AFM characterization of A β ₄₂ in presence or absence of IFN γ (concentrations ranging from 0.125 μ M to 1.5 μ M) upon incubation at 37 °C for 72 h.

As final A β ₄₂ fibril morphologies were found to be affected by the presence of IFN γ , we hypothesized that IFN γ may adhere in the fibrils. To test this hypothesis, a fluorescently labelled IFN γ (A647-IFN γ) and A β ₄₂ were co-incubated in conditions analogous to those used for AFM and stained with ThT, to detect A β aggregates, prior to TIRF microscopy evaluation.

In fig 7.5 spatially concentrated ThT positive structures, composed of multi-fibrillar A β assemblies, can be observed. The A647 signal of IFN γ colocalizes

with the ThT signal suggesting that IFN γ is present in the final A β fibril structure.

Figure 7.5. A β fibrils colocalize with IFN γ . TIRF microscopy images of: Left panel: 25 μ M A β_{42} stained with ThT; middle panel: 25 μ M A β_{42} incubated with 1 μ M A647-IFN γ ; right panel: merged signal. CFI Apo TIRF 100X Oil, NA = 1.49.

7.3.2. IFN γ inhibits early stage A β aggregation

To assess at which stage IFN γ interferes with the A β_{42} aggregation process, A β_{42} was pre-aggregated for various amounts of time after which IFN γ was added. Based on the ThT kinetic curves (fig. 7.1A), A β_{42} was pre-aggregated at 37°C for 0 ('monomeric'), 1.5 (before onset exponential phase), 6 (mid-exponential phase), and 24 h (plateau phase). A concentration of 1 μ M IFN γ was added to the pre-formed A β_{42} assemblies at these specific time points and A β_{42} /IFN γ mixtures were further incubated to a total endpoint of 72 h (fig. 7.6A). Secondary structure of the resulting samples was assessed by CD spectroscopy. IFN γ exerted the strongest effect in reducing β -sheet formation when added to monomeric A β_{42} (t = 0 h) or early aggregates (t = 1.5 h). The aggregation inhibitory effect of IFN γ tended to diminish when added to more mature A β_{42} aggregates (t = 6 h), and no effect was detected for A β_{42} aggregates that were incubated for 24 h (fig. 7.6B). These results suggest that IFN γ primarily affects A β_{42} aggregation by stabilizing monomers or small,

early-stage aggregates preventing their assembly into mature fibrils. This observation is consistent with the increase in lag phase observed in ThT fluorescence assays (fig. 7.1).

Figure 7.6. IFN γ selectively interferes with early-stage aggregation of A β_{42} . **(A-B)** CD spectra of 25 μ M monomeric or pre-incubated (0 h, 1.5 h, 6 h and 24 h) A β_{42} in presence or absence of 1 μ M IFN γ . Samples were incubated at 37 °C for a total of 72 h and then measured. Data are average of three independent measurements (N=3).

7.4. Discussion

The contribution of neuroinflammation to AD pathogenesis is currently recognized and is described as a multicellular process, coordinated by neuronal, glial, and immune components [21]. It is yet unclear how the neuroinflammatory response and AD hallmark neurodegeneration interact. In the previous chapter we identified that IFN γ and A β co-act to induce IL-1 β secretion to a neurodegenerative extent suggesting that the interaction between neuronal degeneration and neuroinflammation may be fostered by

an interaction between these two proteins. In this chapter, we showed that specifically the monomeric or early aggregated A β species are stabilized by IFN γ delaying their maturation into fibrillar aggregates. Although already very low concentrations of IFN γ were shown to be capable of partially inhibiting the A β_{42} aggregation process, IFN γ did not fully prevent the formation of mature fibrils but results in morphologically deviant A β_{42} fibrils that colocalize with IFN γ . Aggregate morphology is primarily determined by the aggregation pathway [22] so it would be straight forward to interpret this observation from an interaction point of view, where IFN γ binds to A β_{42} preventing its conversion into mature amyloid fibrils. However, various biophysical assays were performed to establish the precise mode of interaction between IFN γ and A β_{42} , but without exception it was found that the affinity of IFN γ for A β_{42} is non-detectable (data not shown). Based on the findings described in this paper, it could be possible that IFN γ transiently interacts with monomeric A β_{42} or early aggregated forms, or off-pathway oligomeric intermediates as was previously observed for polyphenolic compounds and aggregation of islet amyloid polypeptide [23], potentially altering their structure and stabilizing toxic A β_{42} aggregates of higher molecular weight. This hypothesis is in agreement with our data showing a ThT kinetic profile with extended lag and elongation phase upon addition of IFN γ . Co-localization of IFN γ with A β_{42} aggregates, as observed by TIRFM could be the result of non-specific interaction between the hydrophobic surface of the A β deposit and IFN γ . The build-up of A β_{42} oligomers has been proposed as the primary neurotoxic event in AD [24–26]. The IFN γ -mediated accumulation of these assemblies of low numbers could play an important role in the clinically observed sustained neuroinflammatory process in the AD brain which is also consistent with our observation that phosphorylation of

STAT is sustained for a prolonged period of time in the presence of IFN γ and A β compared to either of these two proteins individually.

7.5. Acknowledgments

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7.6. Supplementary Information

Figure S1

Figure S1. Curve fitting with Boltzmann function. **A)** 25 μM $A\beta_{42}$. **B)** 25 μM $A\beta_{42}$ + 0.125 μM $IFN\gamma$. **C)** 25 μM $A\beta_{42}$ + 0.5 μM $IFN\gamma$. **D)** 25 μM $A\beta_{42}$ + 1 μM $IFN\gamma$. **E)** 25 μM $A\beta_{42}$ + 1.5 μM $IFN\gamma$.

Figure S2

Figure S2. CD signals of $IFN\gamma$ controls agree with the protein being largely helical. **A)** CD spectra of $IFN\gamma$ at different concentrations after 72h of incubation at 37°C. **B)** Zoom in of the α -helical region (positive absorbance at 193 nm and negative absorbance at 208 and 222 nm).

7.7. References

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Chapter 8

General discussion

8.1. Overall insights on AD

AD is a devastating neurodegenerative disorder, leading cause of dementia worldwide [1–3]. Brain changes such as A β accumulation, phosphorylated tau formation, chronic neuroinflammation and neuronal loss are believed to be the underlying pathology of AD. However, the factors driving these alterations have not been completely elucidated [4].

More than hundred years have passed since the first patient, Auguste D, was diagnosed with this brain disease by Dr. Alois Alzheimer [1].

In spite of all advances in the study of AD, the drugs currently available on the market (donepezil, rivastigmine, galantamine and memantine) are mainly able to slow down the disease progression and provide patients with symptomatic relief, without a complete cure.

To date, AD is one of the biggest medical and social challenges of our generation with massive implications for our social and economic system [5]. Moreover, as the world's population is rapidly aging and no viable treatment insights are available, the necessity for the development of new effective intervention strategies has been the main focus of AD research in the recent years [6,7].

8.2. The multifactorial character of AD

In this thesis, we have investigated the molecular complexity of AD. In particular, we have evaluated four early pathological features of AD (summarized in figure 8.1) including: A β aggregation [8] and accumulation [9,10], oxidative stress [11,12] and inflammation [13], which have been speculated to influence each other's progression.

Fig. 8.1. Four early AD markers. Aβ aggregation: Aβ monomers aggregate into oligomers and fibrils, which eventually form senile plaques. Excessive Aβ deposition: An imbalance between Aβ production and clearance leads to abnormal accumulation of Aβ which could be targeted with specific proteolytic enzymes. Oxidative stress: Mitochondria are the primary site for ATP production, a multi-step process that can lead to the formation of threatening substances, such as reactive oxygen species (ROS). Neuroinflammation: Chronic activated microglia release cytokines which induce an inflammatory response and neuronal damage.

Inhibition of Aβ aggregation

In chapter 2 and 3 we described *in vitro* studies aimed at investigating the potential of small molecules to reduce Aβ aggregation or blocking the toxic assemblies forming during the aggregation process. Inhibition of Aβ aggregation is a common strategy that has resulted in the development and testing of several compounds [14,15]. We have shown that small peptides [16] and benzodifuran compounds [17] interact with Aβ monomer preventing its conversion to an aggregation-prone conformation. Further understanding of the mechanism of Aβ aggregation and its kinetics are essential for developing inhibiting compounds [18]. Also, even though the tested compounds show promise on their potential to partially inhibit the formation of Aβ aggregates, many aspects of relevance that would allow translation into the clinic remain

to be investigated. These include pharmacokinetics and pharmacodynamics properties, blood brain barrier (BBB) permeability and specific target toward the brain. One of the main criticisms in the field of A β -targeted therapeutics is the question if the A β aggregation is the key to AD pathogenesis. Many previous clinical trials targeting A β have failed raising the question if other components of the disease hold more promise for therapeutic development.

A β proteolytic cleavage

Enzymatic removal of soluble A β , could provide a means to maintain brain A β homeostasis. Since the A β proteolytic cleavage apparatus is hampered in the AD brain [9], in chapter 4 we investigated the ability of a known A β degrading enzyme, named IDE, to cleave A β at different aggregation stages. Our findings show that A β monomers, either alone in solution or in equilibrium with higher aggregates, are cleaved at multiple sites by IDE. Moreover, we report a new A β cleavage site between Met₃₅ and Val₃₆, and show that the resulting A β fragments form non-toxic amorphous aggregates that are not or less sensitive for ThT staining. While IDE is reported to have a relatively small catalytic chamber which allows the hydrolysis of proteins with less than 75 aa [19], other A β proteases are known to specifically target more assembled species such as oligomers or fibrils [10].

Future proteases-based therapeutic strategies for AD should focus on the simultaneous administration of different A β targeting enzymes which could be better capable of contributing to regulation of A β levels and reduce the amyloid plaque load in the AD brain.

Oxidative stress

In chapter 5, we reviewed the available literature on molecular mechanisms that drive the contribution of oxidative stress to AD and describe factors that may be causative of oxidative stress *in vitro*, *in vivo* and in clinical studies. Moreover, anti-oxidants molecules are examined as ancillary treatment for AD. Even though oxidative stress is commonly observed in AD at all stages, it is also observed during normal aging as well as a wide range of other neurodegenerative diseases. Biomarkers for oxidative stress may thus be not AD specific but rather represent a general state of the formation of ROS. Together with more AD specific markers, including A β oligomer and plaque formation, tau tangles and hyperphosphorylation of tau, oxidative stress biomarkers may be informative on the disease state and potential use of antioxidant administration.

Neuroinflammation

The last part, which includes chapters 6 and 7, describes a potential link between neuroinflammation and neurodegeneration in AD. Neuronal loss, observed in AD coincides with neuroinflammation and deposition of A β peptide in the brain. We found that A β and IFN γ , which plays a major role in inflammation, together potently activate microglia into a pro-inflammatory phenotype and we identified that this resulted into microglial NLRP3 inflammasome assembly. In response to this, IL-1 β secretion levels were raised, which, in turn, resulted in loss of synchronous firing activity in a rat primary neuronal culture. Moreover, we showed, using biophysical techniques, that IFN γ can modulate the aggregation behaviour of A β , even though they do not seem to strongly interact. The obtained results created an understanding of processes that underlie AD pathogenesis involving

interactions of A β ₄₂ with other extra- and intracellular partners and formed a potential link between neuroinflammation concepts and AD.

8.3. Towards a multi-target treatment of AD

Most current strategies to develop treatment focus on isolated targets which present only part of the disease spectrum. Recent examples of such targets, which failed under investigation in phase III clinical trials include Solanezumab (Eli Lilly), a humanized monoclonal antibody aimed at scavenging of soluble forms of A β , a target relevant to the amyloid cascade hypothesis [1]. Further examples of recently evaluated single target therapeutics that failed in Phase III randomized, placebo controlled double-blind clinical trials include Atorvastatin (Pfizer) [20] and Simvastatin (Merck & Co) [21], both HMG CoA reductases, Latrepirdine (Pfizer) [22], an antihistamine, NSAIDs to impact on neuroinflammation and Xaliproden, a serotonin antagonist and modulation of γ -secretase activity by Semagacestat (Eli Lilly and Élan) [23]. Currently, failure of compounds in clinical trials can be ascribed to lack of efficacy and toxicity as most common reasons for Phase III failure. As a result, only symptom alleviating medication is available at the moment, despite substantial research effort in this field and aging becoming a pressing issue of social and economic impact. The fact that therapeutic development to effectively treat AD is not a simple task is further demonstrated by the finding that genetic factors play a role, in combination with environmental factors, as well as perhaps even unknown factors [24]. Next to this, AD is a multifactorial disease implicating pathogenic processes including deposition of A β , hyperphosphorylation and assembly of tau into tangles, oxidative stress and neuroinflammation while knowledge on sequence of events is lacking. These observations clearly call for a multi-target approach in which various contributing factors are addressed simultaneously. Another

complicating factor is that AD proves to be very heterogeneous and the generally aged population of patients suffers from multiple co-morbidities of which their contribution to the pathogenesis cannot really be distinguished from other complicating factors.

Another major issue in fighting neurodegenerative disease, including AD, is the BBB permeability. Many of the generated drugs are not able to cross this barrier which separate the circulating blood from the brain and extracellular fluid in the central nervous system [25]. To aid BBB permeability, researchers have been proposing polymer-based nanoparticles strategies to deliver the drugs at the right site [26,27]. For example, negatively charged particles have been shown to display enhanced uptake in the brain as compared to neutral and cationically charged particles while receptor-mediated transport was proven crucial to dictate delivery across the BBB [28]. Apart from the surface type of the nanoparticle, the size of these particles plays an important role. Investigations into differently sized gold nanoparticles revealed substantial uptake of the particles in the brain [29]. Interestingly, the smallest gold nanoparticles, 15 nm in diameter, passed the BBB significantly more readily than 100 or 200 nm nanoparticles, displaying up to 70-fold higher concentrations. Although the size and size distribution of inorganic nanoparticles can be accurately controlled, abilities for incorporation of drug molecules are limited and should be further subject of investigation.

8.4. Conclusive remarks and future perspectives

The results of this thesis point out that in order to develop therapeutics that effectively halt AD, the multifactorial complex character of this disease should be acknowledged. Therefore AD should be addressed using a multi-target approach rather than being treated as a single event-disease [30]. A

multi-target treatment should simultaneously modulate various features of AD pathology [31,32].

These drugs may then serve as leads for further development and as a platform to understand the complexity of a disease and all the molecular pathways involved [33].

8.5. References

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Appendix

List of abbreviations

aa	Amino acid
AD	Alzheimer's disease
AFM	Atomic force microscopy
ANT	adenine nucleotide translocase
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	adenosine triphosphate
A β	Amyloid-beta
BBB	Blood brain barrier
BI	Burstiness index
CD	Circular dichroism
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTB	CellTiter-Blue
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ETC	Electron-transport chain
FDA	Food and Drug Administration
GPx	glutathione peroxidases
GSH	glutathione
GSSG	Glutathione disulphide
HFIP	hexafluoroisopropanol
HIF-1 α	Hypoxia-inducible factor 1
HRP	Horseradish peroxidase
IDE	Insulin degrading enzyme
IDP	Intrinsic disorder proteins
IFN γ	Interferon- γ
IL-1 β	Interleukin-1 β
JAKs	Janus kinases
MAPKs	Mitogen-activated protein kinases
MCI	Mild Cognitive Impairment
MD	Molecular dynamics
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NEP	Neprilysin
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NFT	Neurofibrillary tangles

NLRP3	NLR Family Pyrin Domain Containing 3
NMR	Nuclear magnetic resonance
ODNs	Oligodeoxyribonucleotides
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
RAGE	Receptor for advanced glycation end products
REMD	Replica Exchange Molecular Dynamics
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	sodium dodecyl sulfate
SOD	Superoxide dismutase
STATs	Signal Transducer and Activator of Transcription proteins
ThT	Thioflavin T
TNF α	Tumor necrosis factor α
UCP	Uncoupling protein
WT	Wild type

Summary

Of all the diseases that lead to dementia, Alzheimer's disease (AD) is the most common, affecting millions of elderly people worldwide. Although a lot of research has been carried out to understand the cellular and molecular events responsible for brain dysfunction and AD pathogenesis, no effective therapy has been identified yet. In the research described in this doctoral thesis we tried to obtain a more detailed understanding of four early hallmarks of AD: Amyloid-beta (A β) aggregation, A β accumulation, oxidative stress and neuroinflammation.

In chapter 2 and 3 we described *in vitro* studies aimed at investigating the potential of small molecules to reduce A β aggregation or blocking the toxic assemblies forming during the aggregation process. Inhibition of A β aggregation is a common strategy that has resulted in the development and testing of several compounds. We have shown that small peptides and benzodifuran compounds interact with A β monomer preventing its conversion to an aggregation-prone conformation.

Enzymatic removal of soluble A β , could provide a mean to maintain brain A β homeostasis. Since the A β proteolytic cleavage apparatus is hampered in the AD brain, in chapter 4 we investigated the ability of a known A β degrading enzyme, named Insulin-degrading enzyme (IDE), to cleave A β at different aggregation stages. Our findings show that A β monomers, either alone in solution or in equilibrium with higher aggregates, are cleaved at multiple sites by IDE. Moreover, we report a new A β cleavage site between Met₃₅ and Val₃₆, and show that the resulting A β fragments form non-toxic amorphous aggregates that are not or less sensitive for Thioflavin T (ThT) staining. While IDE is reported to have a relatively small catalytic chamber which allows the

hydrolysis of proteins with less than 75 aa, other A β proteases are known to specifically target more assembled species such as oligomers or fibrils.

In chapter 5, we reviewed the available literature on molecular mechanisms that drive the contribution of oxidative stress to AD and describe factors that may be causative of oxidative stress *in vitro*, *in vivo* and in clinical studies. Moreover, anti-oxidants molecules are examined as ancillary treatment for AD. Even though oxidative stress is commonly observed in AD at all stages, it is also observed during normal aging as well as a wide range of other neurodegenerative diseases. Biomarkers for oxidative stress may thus be not AD specific but rather represent a general state of the formation of reactive oxygen species (ROS). Together with more AD specific markers, including A β oligomer and plaque formation, tau tangles and hyperphosphorylation of tau, oxidative stress biomarkers may be informative on the disease state and potential use of antioxidant administration.

The last part, which includes chapters 6 and 7, describes a potential link between neuroinflammation and neurodegeneration in AD. Neuronal loss, observed in AD coincides with neuroinflammation and deposition of A β peptide in the brain. We found that A β and Interferon- γ (IFN γ), which plays a major role in inflammation, together potently activate microglia into a pro-inflammatory phenotype and we observed that this resulted into microglial NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome assembly. In response to this, Interleukin-1 β (IL-1 β) secretion levels were raised, which, in turn, resulted in loss of synchronous firing activity in a rat primary neuronal culture. Moreover, we showed, using biophysical techniques, that IFN γ can modulate the aggregation behaviour of A β , even though they do not seem to strongly interact.

Summarizing, we have attempted to understand and obtain better insights into the molecular complexity of AD. This thesis highlights the multifactorial and

complex character of AD and suggests that a multi-target approach, most likely, will contribute to the development of an effective AD therapy, halting the progression of this devastating disease.

Nederlandse samenvatting

Van alle ziektes die dementie kunnen veroorzaken, is de ziekte van Alzheimer de meest voorkomende oorzaak, met wereldwijd miljoenen patiënten. Ondanks intensief onderzoek om de cellulaire en moleculaire processen die verantwoordelijk zijn voor hersendysfunctie en Alzheimer pathogenese beter te begrijpen, is er nog geen effectieve therapie voor deze ziekte gevonden.

In het onderzoek dat wordt beschreven in deze PhD thesis, hebben we geprobeerd meer inzicht te verkrijgen in vier vroege kenmerken van de ziekte van Alzheimer: A β aggregatie, gedeeltelijk verlies van enzymen die A β kunnen degraderen, oxidatieve stress en neuroinflammatie.

In hoofdstuk 2 en 3 beschreven we *in vitro* studies die gericht waren op het bestuderen van kleine moleculen, en hun potentie om A β aggregatie te verminderen of de formatie van toxische conformaties tijdens het aggregatieproces te blokkeren. Het remmen van A β aggregatie is een gebruikelijke strategie, die heeft geresulteerd in de ontwikkeling en onderzoek van verschillende stoffen. We hebben aangetoond dat kleine peptides en benzodifuranen interacteren met A β monomeren, en hun omzetting naar meer aggregerende conformaties voorkomen.

Enzymatische verwijdering van A β zou een manier kunnen bieden om A β homeostase in het brein te behouden. Het is bekend dat de proteolytische capaciteit om A β te in stukken te knippen verstoord is in het Alzheimer brein. Om deze reden onderzochten we in hoofdstuk 4 de mogelijkheid van een bekend A β -knippend enzym, genaamd IDE, om A β te knippen in verschillende stadia van aggregatie. Onze resultaten laten zien dat A β monomeren, zowel wanneer alleen in oplossing als wanneer in evenwicht met grotere aggregaten, op meerdere plekken door IDE geknipt worden. Verder vonden we een nieuwe knip-positie in A β tussen Met₃₅ en Val₃₆, en toonden

we aan dat de resulterende A β fragmenten non-toxische amorfe aggregaten vormen die niet of minder gevoelig zijn voor ThT staining. Terwijl IDE een relatief klein katalytisch centrum heeft, die de hydrolyse van eiwitten kleiner dan 75 aa mogelijk maakt, zijn er andere A β proteases bekend die specifiek gericht zijn op meer geaggregeerde vormen zoals oligomeren of fibrillen.

In hoofdstuk 5 gaven we een overzicht van de beschikbare literatuur, aangaande de moleculaire mechanismes die verantwoordelijk zijn voor de bijdrage van oxidatieve stress aan de ziekte van Alzheimer, en beschreven we factoren die oxidatieve stress kunnen veroorzaken in *in vitro*, *in vivo* en klinische studies. Daarnaast onderzochten we anti-oxidanten als ondersteunende behandeling voor Alzheimer. Oxidatieve stress is aanwezig in alle stadia van de ziekte van Alzheimer, maar treedt ook op tijdens normale veroudering en in een breed scala aan andere neurodegeneratieve ziektes. Biomarkers voor oxidatieve stress zijn daarom waarschijnlijk niet specifiek voor Alzheimer, maar geven eerder een algemene staat van ROS formatie weer. In combinatie met meer Alzheimer-specifieke markers, waaronder formatie van A β oligomeren en plaques, tau tangles en tau hyperfosforylatie, zouden oxidatieve stress biomarkers inzicht kunnen geven in het ziektestadium en het mogelijke voordeel van behandeling met anti-oxidanten. Het laatste deel, met daarin hoofdstuk 6 en 7, beschrijft een mogelijke link tussen neuroinflammatie en neurodegeneratie in Alzheimer. Dood van neuronen, te zien in het Alzheimer brein, valt samen met neuroinflammatie en opstapeling van A β in de hersenen. We vonden dat A β en IFN γ , die een belangrijke rol in inflammatie speelt, samen een sterke stimulus vormen voor de activatie van microglia naar een pro-inflammatoir fenotype, en we constateerden dat dit resulteerde in NLRP3 inflammasoom activatie in microglia. In reactie hierop nam het niveau van uitgescheiden IL-1 β toe, wat leidde tot het verlies van synchrone vuur-activiteit in gekweekte primaire

neuronen van ratten. Daarnaast toonden we met biofysische technieken aan dat IFN γ het aggregatiegedrag van A β kan veranderen, ook al lijken A β en IFN γ niet sterk met elkaar te interacteren.

Samenvattend hebben we geprobeerd om een beter begrip van, en meer inzicht in, de moleculaire complexiteit van Alzheimer te verkrijgen. Deze thesis benadrukt het multifactoriële en complexe karakter van Alzheimer, en suggereert dat een multi-target aanpak hoogstwaarschijnlijk zal bijdragen aan de ontwikkeling van een effectieve therapie tegen de ziekte van Alzheimer, waarmee het verder voortschrijden van deze verwoestende ziekte kan worden stopgezet.

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About the author

Federica Cioffi was born in Caserta, Italy on the 18th October 1989. In 2008 she began a bachelor degree in Biotechnology at the University of Napoli "Federico II". After obtaining her bachelor degree in 2012, she was accepted at the University of Trieste to study for a masters in Medical Biotechnology. During this period Federica was also granted a EU scholarship to study for six

months at the University of Uppsala in Sweden. In 2014, upon completion of her research on the characterisation of proteins interacting with telomeric repeat-containing RNA (TERRA), Federica was awarded a master degree. Always following her passion for science, Federica began her PhD studies in January 2015 at the department of Nanobiophysics, faculty of Science and Technology (TNW) at the University of Twente.

During her research, Federica gained further insight into the molecular complexity of Alzheimer's disease. These findings are described in the present PhD thesis. Alongside her PhD work, Federica also joined the PhD network at the University of Twente (P-NUT) in the role of secretary, which helps organise social and informative events. Between October and November 2018, Federica attended the lab of Professor Randy Schekman at the University of Berkeley, California, as a visiting researcher to investigate the link between neuroinflammation and neurodegeneration in Alzheimer's disease. Since June 2019, Federica has been working as Field Application Scientist at Cell Signaling Technology, Leiden.

List of publications

Journal articles

Gera J, Szögi T, Bozsó Z, Fülöp L, Barrera EE, Rodriguez AM, Méndez L, Delpiccolo CML, Mata EG, Cioffi F, Broersen K, Paragi G, Enriz RD. (2018). Searching for improved mimetic peptides inhibitors preventing conformational transition of amyloid- β 42 monomer. *Bioorganic chemistry*, 81, 211-221.

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Conference contributions (oral presentation)

“A protein-protein interaction connecting neuroinflammation and neurodegeneration in Alzheimer’s disease” (2017). CHAINS, Eindhoven, The Netherlands.

“The effect of simultaneous administration of two proteolytic enzymes on the clearance of Amyloid-beta” (2017). The 13th International Conference on Alzheimer's and Parkinson's Diseases and Related Neurological Disorders, AD/PD™, Vienna, Austria.

“Enzyme-mediated changes in plaque deposition” (2016). MIRA day, Institute for Biomedical Technology and Technical Medicine, Enschede, The Netherlands.

Conference contributions (poster presentation)

“Characterization of insulin-degrading enzyme-mediated cleavage of A β in distinct aggregation states” (2016). Alzheimer’s Research UK Conference, Manchester, United Kingdom.

“Encapsulation of Amyloid-beta scavenging compounds into BBB permeable nanoparticles” (2016). Alzheimer's Association International Conference (AAIC), Toronto, Canada.

“The multifactorial character of Alzheimer’s disease” (2015). Summer school “Ageing brain”, University of Groningen, The Netherlands

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