Glia in Alzheimer's disease and aging: Molecular mechanisms underlying astrocyte and microglia reactivity
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Chapter 1

General Introduction: Reactive Glia in Alzheimer’s Disease – Focus on the Immunoproteasome and Immune Signaling

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**ABSTRACT**

Alzheimer’s disease (AD) is an incurable form of dementia that mainly affects elderly people. Two of its hallmarks are amyloid-β (Aβ) plaques and neuroinflammation. Closely associated with Aβ plaques in the AD brain are reactive astrocytes and microglia; these reactive glia cells show an increase in their immune response and immune-related pathways. The proteasome is a major protein degradation system within the cell. Previously it has been postulated that the proteasome activity is impaired in AD. However, the increase in inflammatory signaling in AD suggests the involvement of an inducible proteasome variant called the immunoproteasome (iPS), whose proteolytic properties differ slightly from those of the constitutive proteasome. The iPS has predominantly been studied in the peripheral immune system, where it was shown to be involved in antigen presentation on major histocompatibility complex I molecules (MHC I), T-cell activation and maturation, and regulation of proinflammatory signaling. Although, most of what is known about the iPS today we have learned from non-neuronal cells, tissues, and diseases; the first studies on the role of the iPS in relation to AD pathology and glia reactivity are currently emerging. In this chapter we will give an overview of the role of astrocytes and microglia in relation to AD-related neuroinflammation. Additionally, we will discuss the current data on the role of the iPS in AD, on the functional consequences of changes in its activity, on its role in AD related immune signaling, and its possibility to serve as a therapeutic target in AD.
1. Alzheimer’s Disease

Alzheimer’s disease (AD) is the most common cause of dementia among elderly people and the number of AD patients is expected to rise dramatically in the coming decades. The prevalence of this progressive disorder increases with age; nearly 50% of individuals over the age of 85 suffers from AD [1]. One of the major hallmarks of AD is the formation of extracellular plaques, composed of aggregated amyloid-β (Aβ). Other hallmarks of AD are the presence of intra-neuronal aggregates of hyper-phosphorylated tau (tangles) [2, 3], activated microglia [4], and reactive astrocytes [5–7][chapter 2]. Our focus is Aβ pathology in relation to glia activation. Although the exact trigger for the cascade of events leading to symptomology of AD remains unclear to this day, the “Amyloid Cascade Hypothesis” [8] has been a focus of attention since 1991. This hypothesis postulates that the Aβ accumulation is the trigger for the disease and is caused by an increased production of Aβ and/or by impaired clearance [9].

1.1 Amyloid β and the “Amyloid Cascade Hypothesis”

The Aβ peptide is a cleavage product of the amyloid precursor protein (APP), a transmembrane protein. The cleavage of APP by two protease complexes, the γ-secretase and the β-secretase, results in the Aβ cleavage product. The γ-secretase complex consists of four integral proteins, of which presenilin 1 or 2 (PSEN1 and PSEN2) forms a catalytic subunit. This γ-secretase complex cleaves the APP protein in the intramembrane part, while the β-secretase cleaves the intracellular part of the APP protein. This dual cleavage of APP results in three cleavage products: a C-terminal APP intracellular domain (IACD), an N-terminal extracellular secretory APP, and a range of slightly different Aβ peptides - ranging in length between 36 and 43 amino acids. The peptide sequences (Aβ)1-40 and (Aβ)1-42 are the most abundant, with the latter being especially prone to aggregate and to form higher weight Aβ multimeric structures, such as oligomers and fibrils. The role of Aβ in AD is supported by the identification of disease-causing mutations in the APP, PSEN1 and PSEN2 genes in familial early onset AD (reviewed in [10, 11]). All these mutations lead to either increased production of Aβ or to an increased propensity for Aβ to aggregate and support the “Amyloid Cascade Hypothesis”, which identifies the accumulation
and aggregation of Aβ (either monomeric, oligomeric or fibrillar) and Aβ plaque formation as the initial event of the disease etiology. A polymorphism in the apolipoprotein (ApoE) gene is also associated with AD; the presence of an ApoE 4 allele increases the risk of early onset of the disease, while the ApoE allele 2 protects, to some degree, against development of the disease [12]. This genetic variation is also connected to the “Amyloid Cascade Hypothesis”, since ApoE is believed to play a crucial role in uptake and removal of Aβ by glial cells [13, 14].

Several AD mouse models have been generated by introducing mutations in APP alone or in combination with additional mutations in PSEN and tau; all these mice show a presence of Aβ plaques, at different levels. The APPswePS1dE9 mouse model, used by our group, has a transgenic expression of human/ mouse chimeric APP containing the Swedish (K594M/N595L) mutation, in combination with the human PS1 gene, with a deletion of exon 9, both driven by the mouse prion promoter. These mutations increase the level of Aβ 1-42, while the Aβ 1-40 level remains stable [15]. Such a shift in Aβ 1-42/ Aβ 1-40 ratio results in Aβ plaque deposition before 6 months, in these mice, that steadily increases further with age [6, 15]. Apart from plaque deposition, this AD model mimics several other characteristics of AD pathology, such as the presence of reactive astrocytes and activated microglia, neuroinflammation, memory deficits, impaired learning, and some degree of neuronal loss [6, 7, 16–18][chapter 2]. The presence of all these disease hallmarks in a mouse model only carrying mutations in genes involved in Aβ generation supports the “Amyloid Cascade Hypothesis” as the instigator of AD.

1.2 Immune activation of glia cells in relation to Aβ

Pro-inflammatory cytokines have been detected in the brain of AD patients [19], and inflammatory pathways resulting from toll-like receptor (TLR) signaling, Fc-receptor signaling and complement activation are all associated with AD plaque pathology [20–22]. Aβ plaques activate microglia to release several pro-inflammatory molecules, together with reactive oxygen species, all of which are involved in creating a neurotoxic environment [20, 23, 24]. A major activating pathway of glial cells in response to Aβ is believed to be TLR signaling, where TLR2, TLR4, and CD14 have all been found to interact with Aβ leading to an activation of glia [25–27]. Also other
routes, via the Scavenger receptor A1 (Scara1) and CD36 [28, 29], of Aβ-induced immune activation have been identified. Binding of fibrillar Aβ to these receptors leads to phosphorylation of IκB and, subsequently, an NfκB-mediated transcription of innate pro-inflammatory genes [25, 26, 30]. Microglia and astrocytes stimulated with Aβ in vitro showed an increased release of pro-inflammatory molecules via activation of NfκB [31, 32]. The NfκB pathway is a proposed target for treatment of AD [33, 34].

2. Glia cells in relation to Alzheimer’s pathology

2.1 Astrocytes

Astrocytes are indispensable for maintaining a healthy central nervous system (CNS). They are the most numerous cell-type in the human brain. Although there are probably several subtypes of astrocytes present in cortical gray and white matter areas of the brain, only two morphological subtypes are well described: the protoplasmic astrocytes residing in the cortical gray matter and fibrous astrocytes present mainly in the white matter [35, 36]. The ratio of astrocytes per neuron is approximately 1.4 in humans and 0.4 in mice [37]. Each gray matter astrocyte covers a large number of neuronal synapses, adding up to 2 million in humans and to around 140.000 in mice [37, 38]. Astrocytes are the main cell type to take up and buffer glutamate from the synaptic cleft that are released by neurons upon signaling. The uptake of glutamate by astrocytes occurs through two types of glutamate transporters rather specifically expressed by astrocytes, GLT-1 (EAAT2, Slc1a2) and GLAST (EAAT1, Slc1a3)(Fig. 1). Intracellularly, glutamine synthetase converts glutamate into glutamine, which is then stored or released by astrocytes and subsequently taken up by neurons for further generation of the excitatory neurotransmitter glutamate and of the inhibitory neurotransmitter GABA (reviewed in [39]). This whole process is called the glutamate-glutamine cycle and is one of the pivotal functions of astrocytes (depicted in Fig. 1). Moreover, neurotransmitters, such as glutamate, GABA and ATP, can elicit receptor-mediated astrocyte activation, resulting in an elevation of intracellular Ca\(^{2+}\) and Ca\(^{2+}\) transients through astrocyte networks that, in turn, lead to astrocytic release of neurotransmitters such as glutamate [40, 41]. Thus, astrocytes
have an active role in neuronal signaling and together with the pre- and postsynaptic compartments of neurons, they form the "tripartite synapse" [37, 42–44]. Astrocytes are also responsible for the metabolic support of neurons, by providing them with lactate produced by astrocytic glycolysis [45]. Other vital functions carried out by astrocytes are keeping the blood brain barrier in shape, and monitoring ion, water, and pH balance via an array of transporters and channels, such as connexins, and gap junctions [36, 46, 47].

2.2 Amyloid β and astrocytes

Astrogliosis is a neuropathological hallmark of AD. The increased expression of glial fibrillary acidic protein (GFAP) and vimentin characterizes the reactive astrocyte phenotype. The expression of GFAP is strongly associated with AD disease severity and cognitive decline (reviewed in [35]). Astrocytes cluster around Aβ plaques and appear hypertrophic when stained for GFAP, although GFAP does not label the cell membrane of the astrocytes, and is therefore not suitable for estimation of cell size and overall morphology. One aspect of neurotoxicity of Aβ in the cascade of events leading to AD is probably its capacity to induce and chronically maintain glial cell activation [20, 48–50][chapter 5]. Astrocytes are considered to be immune competent cells [51–53] and have been studied for decades in terms of their response to insults and diseases of the brain. They have been shown to express several receptors involved in Aβ binding and recognition, such as different TLR receptors, scavenger receptors, complement receptors, and the RAGE receptor [53, 54], depicted in (Fig. 1). Activation via these receptors results in the release of several molecules involved in innate immune signaling, such as IL-1b, IL6, TNFα, CCL2, CCL3 - all involved in evoking a pro-inflammatory response in nearby cells (reviewed in [54, 55]). These molecules are contributing to sustained neuronal toxicity and further activation of glia, which in turn leads to increased APP expression and generation of Aβ itself, creating a vicious circle [55]. However, reactive astrocytes can also release anti-inflammatory and neuroprotective molecules, such as TGFβ, S100b, and different types of growth factors [52, 54, 55] (Fig. 1). Moreover, exogenous Aβ treatment leads to long-lasting sporadic elevations of intracellular Ca^{2+} in astrocytes, which results in a depletion of astrocytic glutathione, an anti-oxidant important for neuroprotection [56].
A disruption of intracellular astrocytic Ca\textsuperscript{2+} concentration has also been observed in AD mice, and is likely to affect astrocyte functions and, subsequently, neuronal networks [57]. This deranged astrocytic Ca\textsuperscript{2+} homeostasis, induced by A\textbeta, has been linked to increased NF\kappaB activation in astrocytes, and improved upon inhibition of NF\kappaB [58]. Activation of NF\kappaB also increase GFAP expression in astrocytes, which can be inhibited by treatment with aspirin, a known NF\kappaB inhibitor [59]. Although \textit{in vitro} experiments have increased our understanding of astrocyte function in relation to A\beta and AD pathology, a major drawback of some of these experiments has been the presence of contaminating microglia [60]. Such a contamination can have a major effect on the immune related molecules released from the culture. Either the actual readout molecules are produced by the microglia alone, or an initial microglia response to the stimuli makes them release factors that, in turn, activate astrocytes, which gives a skewed picture of the astrocyte response.

2.3 Microglia

Microglia are the tissue macrophages of the brain and act as surveyors. They make up approximately 10% of the cells within the CNS, and have extensive processes that are constantly scanning the nearby microenvironment for insults and imbalances in brain homeostasis. Microglia are highly phagocytic cells; they quickly clear up apoptotic cells and debris in the brain [4, 61, 62]. They are also involved in pruning synapses during development and in the adult brain, a function believed to play an important role in circuit formation during learning and memory [63]. In their inactivated state, microglia have a ramified morphology and upon activation they gradually acquire a more rounded, amoeboid phenotype. The activated microglia upregulate various cell-surface receptors, such as complement receptors, pattern recognition receptors, and major histocompatibility complexes. They also express an array of pro- and anti-inflammatory signaling molecules, as well as of growth factors, compliment factors, and molecules such as reactive oxygen and nitric oxygen (NO) (thoroughly reviewed in [4, 61]) and depicted in (Fig. 1). Microglia activation is crucial for removal of cellular debris within the CNS, although in the aged or diseased brain the microglia are believed to acquire a dysfunctional and dystrophic phenotype [64], and display a reduced phagocytic capacity [65].
2.4 Aβ and microglia

Upon Aβ stimulation, microglia acquire an activated and inflammatory [61, 66] phenotype with enhanced phagocytic capacity [26, 67]. Activated microglia play an important role in clearing away Aβ and Aβ plaques (reviewed in [68]); several studies have shown that they are capable of clearing away both fibrillar and soluble Aβ [69, 70]. This clearance is done by both phagocytosis of Aβ and by secretion of proteolytic enzymes that can cleave Aβ, such as insulin degrading enzyme (IDE), Matrix metallopeptidase 9 (MMP9) and neprilysin (NEP) [71–73] (Fig. 1). Despite this ability to degrade Aβ, in the end microglia fail to successfully clear away all the Aβ accu-

![Image: Schematic figure showing glia functions - normal and in response to AD pathology.](image-url)

**Figure 1. Schematic figure showing glia functions - normal and in response to AD pathology.** Normal astrocyte functions are e.g. neurotransmitter buffering and recycling, ion homeostasis, maintenance of water balance and neurotropic support. Microglia are constantly scanning the environment with their extensions, phagocyte proteins and cellular debris, and respond swiftly to homeostatic changes. AD is characterized by Aβ plaque depositions and intraneuronal tangles with reactive astrocytes and microglia surrounding the plaques. Several Aβ recognition receptors are present on both astrocytes and microglia. In response to Aβ, astrocytes can release both pro-inflammatory (IL-1β, IL-6, TNFα etc.) and neurotropic factors, such as growth factors (EGF, FGF). Aβ can cause microglia to release nitric oxide, both pro- and anti-inflammatory (IL-4, IL-10) molecules, and proteolytic enzymes capable of degrading Aβ (NEP, IDE, MMP9).
mulating in an AD brain. Microglia from AD mice show a reduced expression of Aβ phagocytosis receptors and degradation enzymes, together with an impaired Aβ uptake, which undermines their ability to cope with Aβ levels in the brain as their age advances [65, 74]. A growing number of studies show that a chronic pro-inflammatory microglia phenotype is linked to a suppression of their phagocytic capacity [65, 68, 75–77]. More information can be found in recent reviews centered on Aβ-induced microglia activation (see [4, 49, 78]).

3. The proteasome in AD and its relation to glia reactivity

The presence of intraneuronal accumulation of aberrant tau proteins and extracellular deposits of aggregated Aβ in AD brains imply a dysregulation in protein degradation. The proteasome is an evolutionarily conserved enzymatic complex and is the main protein degradation system within the cell; it is also the major contributor of peptide production for antigen presentation on MHC I molecules [79–83]. The observation of an accumulation of ubiquitinated proteins and protein aggregates in AD tissue has led to the interpretation that proteasomal protein degradation is impaired. These findings led researchers to analyze the proteolytic capacity of the proteasome in AD mice and in human AD tissue, resulting in several studies concluding that the proteolytic capacity of the proteasome is reduced in AD (as discussed below). The sections below will introduce and discuss the role of the proteasome in relation to glia activation and inflammatory signaling, with a focus on AD.

3.1 The constitutive proteasome

A large proportion of the function of the ubiquitin-proteasome pathway is to degrade short-lived and regulatory proteins along a two-step process, namely tagging proteins with multiple ubiquitin molecules by ubiquitin-ligases, followed by the actual degradation of these poly-ubiquitinated proteins by the proteasome complex [84]. Several forms of the constitutive proteasome (cPS) are present in a dynamic equilibrium in the brain, involved in modulation of nascent protein folding, transport, degradation, signal transduction, and turnover of short lived proteins [85]. The proteolytic complex of the proteasome is a barrel-shaped unit, the 20S core. The 20S core is a dimeric
complex composed of two rings of seven, non-identical, β subunits sandwiched between two rings of seven, again non-identical, α subunits [86]. The inner β rings harbor three to seven catalytic sites formed by β1 (caspase-like), β2 (trypsin-like), and β5 (chymotrypsin-like) subunits. Each subunit preferentially cleaves proteins after a specific residue: β1 subunits cleave preferentially after acidic residues, β2 subunits after basic residues, and β5 subunits after hydrophobic residues [87, 88]. Although the 20S core can exist independently within the cell, it is often capped at either end by regulatory protein complexes that modify its function. In order to degrade ubiquitinated proteins, the 20S connects with 19S (PA700) complexes at either end to form the 26S proteasome. The lid of the 19S is responsible for recognizing peptides that exhibit lysine-48-linked polyubiquitin chains, which specifically signal cPS-mediated degradation. The base binds the 20S α subunits in an ATP-dependent manner and contains six ATPases whose function it is to unfold ubiquitinated substrates and thread them through the interlacing N-terminals of the 20S α rings, thus enabling access to the proteolytic core and subsequent substrate degradation [89, 90] (Fig. 2A)

### 3.2 The immunoproteasome

The release of the cytokines IFNγ and TNFα stimulates the expression of an inducible form of the proteasome, the immunoproteasome (iPS) [91, 92] (see Fig. 2 and Fig. 3A). The general structure of the iPS 20S core is almost identical to that of the constitutive 20S. Only the three catalytic β subunits are replaced by the inducible homologues: Low Molecular weight Protein 2 (LMP2, β1i, PSMB9), Multicatalytic Endopeptidase Complex subunit 1 (MECL1, β2i, PSMB10), and Low Molecular weight Protein 7 (LMP7, β5i, PSMB8). The β1i subunit attributes the bulk of the chymotrypsin-like activity to the iPS, with β5i providing the remainder [93]. Both these inducible subunits also increase trypsin-like activity, β5i to a greater extent than β1i [94]. The proteolytic activity of β2i remains trypsin-like, similar to the β2 of the constitutive proteasome [95]. As a result, the trypsin and chymotrypsin-like activities are enhanced while the caspase-like activity is eliminated, giving the iPS catalytic properties that are optimally tuned for MHC class I antigen generation [94, 96]. The incorporation of each iPS β subunit is mediated by the proteasome maturation protein POMP, which makes use of a small amount of available and constitutively expressed β5i pro-pep-
This POMP mediated activation of β5i, via its pro-peptide, stimulates the transcription and posttranslational processing of the other iPS subunits, which are interdependent for incorporation into the iPS [86, 98]. The immunoproteasome can be capped by either the 19S lid or by an activator lid (PA28αβ or 11S). The latter induces conformational changes that open up the 20S core to facilitate protein degradation [95] (Fig. 2B).

3.3 The properties of the iPS
Knock-out and inhibition studies of the different iPS subunits have provided informa-
tion about their specific functions. The iPS is involved in: (i) antigen presentation and the generation of peptides (a different repertoire than the cPS) for MHC class I presentation, (ii) regulation of the expression of inflammatory mediators, (iii) modulation of proteins by partial degradation, leading to activation of transcription factors, (iv) degradation of inhibitory proteins, leading to an activation of signaling pathways, and (v) degradation of oxidized proteins [reviewed in [99]] see (Fig. 3B). These functions suggest that the iPS has an integrated role in the initiation and modulation of both the adaptive and the innate immune responses and makes it an interesting target for modulation in diseases where inflammation plays a central role.

3.4 iPS and antigen presentation

The peptides generated by the cPS- and iPS-mediated degradation differ substantially. The iPS is involved in degradation of newly generated proteins, defective proteins (called defective ribosomal products (DRiPS)), and other endogenous proteins via enhanced trypsin-like and chymotrypsin-like proteolysis. This displays basic or hydrophobic C-terminal tails, conferring immunodominance to the resulting peptides [95, 96]. Peptides cleaved by cPS often display acidic C-terminal tails, which are not recognized as antigenic [95]. After degradation by the iPS, the antigen peptides are transported from the iPS via TAP1 and TAP2 carriers to the endoplasmic reticulum, where they associate with MHC class I molecules. At the MHC class I molecules, on the cell surface, the peptides are presented to CD8+ T-cells, resulting in an activation of the adaptive immune response [99–102]. Mice totally devoid of iPS subunits show a reduced expression of MHC class I molecules on the cell surface, which is strong evidence for their involvement in antigen presentation [103]. However, mice with totally impaired iPS function can still show strong T-cell activation [104] and indicates that the cPS and/or other proteolytic complexes may compensate for a total loss of iPS function.

3.5 Variation in proteasome content

The proteasome component in brain tissue is dominated by the cPS [105]. Low levels of β2i and activator lid PA28αβ/11S are present in healthy neurons, along
Figure 3. Schematic representation of induction and function of the immunoproteasome in AD. (A) Molecules and signaling pathways leading to the induction of immunoproteasome (iPS) in AD, such as oxidative stress (ROS), Aβ mediated signalling, and IFNγ signalling. (B) Functions of the iPS in AD. Binding of AD related molecules to their receptors activates signalling cascades resulting in degradation of the inhibitory protein IκB by the iPS, facilitating NFκB translocation to the nucleus allowing transcription of additional iPS subunits but also of proinflammatory and neurotoxic genes, such as iNOS (ROS) and innate immune molecules. The iPS is also involved in regulation of innate immune signalling in additional (non NFκB related) ways, not fully understood - as indicated with dashed lines. Moreover, the iPS facilitates degradation of accumulated, oxidatively damaged proteins and generates peptides for MHC class I presentation.
with a small pool of β5i pro-peptides used for initial iPS assembly [97, 105, 106]. In normal mouse brain, a low basal gene expression of all iPS subunits is present, predominantly in microglia [107][chapter 4]. Upregulation of iPS subunits occurs after several types of CNS injuries and during CNS diseases, such as traumatic brain injury, cytotoxic T-cell mediated injury, epilepsy, and AD [7, 108–110, 110, 111][chapter 2 and 5]. *In vitro* studies and cytotoxic T-cell mediated CNS injury shows that this response is tightly regulated and that transcription levels return to normal and the balance of cPS and iPS subunits is restored upon removal of the offending stimulus [95, 104]. It has been proposed that the persistent presence of iPS subunits “hijack” and impair the function of the cPS [112]. However, the existence of mixed proteasomes that express both cPS and iPS proteolytic subunits has also been observed [113, 114] see (Fig. 2C).

4. iPS, glia reactivity and neuroinflammation in relation to AD

4.1 Glial expression of iPS

Most of the iPS induction occurs within microglia and macrophages, although astrocytes, Purkinje cells, and some cortical neurons are also capable of producing inducible subunits [109]. Microglia are efficient antigen-presenting cells and constitutively express detectable levels of all three proteolytic iPS subunits (β1i, β2i, β5i) [107][chapter 4] and LPS or IFNγ stimulation increases this expression further [7, 115][chapter 2]. Astrocytes, however, do not constitutively express high levels of iPS subunits, although they, too, are capable of producing inducible subunits in response to injury. Reactive astrocytes express inducible subunits in response to traumatic brain injury and AD plaque pathology, where changes in iPS expression correlate with astrocyte reactivity [7, 109, 116, 117][chapter 2]. Plaque-bound reactive astrocytes predominantly express β1i, while activated microglia mainly show elevated expression of β5i [7][chapter 2]. Such a difference in subunit expression pattern is further evidence that a cell-type specific iPS composition is likely.

4.2 Neuropathology affects iPS function

The iPS is not only induced by the release of IFNγ, but also by a general inflamma-
tory environment caused by a myriad of events, including oxidative stress, protein misfolding and aggregation, and viral infection. AD glia are known to upregulate several innate immune molecules and release reactive oxygen species (Fig. 3A), which in turn increase the expression and transcription of iPS subunits. Such an iPS induction is likely to both prevent and contribute to AD-related alterations, by preventing build-up of oxidatively damaged proteins and a contribute to the release of pro-inflammatory molecules, as described below and depicted in (Fig. 3B).

4.3 iPS in Alzheimer’s disease
AD brain tissue is neuropathologically characterized by extracellular Aβ plaques, intraneuronal neurofibrillary tangles, and neuroinflammation (reactive glia). The cPS is ubiquitously expressed in both the healthy and the AD diseased brain. The iPS, on the other hand, is strongly induced in hippocampal neurons and astrocytes, as well as in cortical astrocytes and microglia [7, 116, 118] [chapter 2]. The ratio of cPS to iPS varies depending on the degree of pathology exhibited by each area: iPS (β1i) expression predominates in the hippocampus - an area highly affected by AD pathology, while cPS (β1) is favored in unaffected areas, such as the AD cerebellum and hippocampus of non-demented controls [116]. Several studies have reported a reduced proteasome activity in relation to Aβ and AD, both in human post-mortem tissue, and in AD mouse models [116, 118–124]. Many of these studies found a decrease in the chymotryptic and tryptic-like activity of the proteasome. The induction of the iPS should hypothetically lead to an increase in these activities, but as this was not observed [118] it was suggested that either the β1i or the β5i subunit incorporation or function was disturbed in AD [118]. We recently challenged this view. By applying a new method to assess subunit-specific activity in brain tissue, we showed that the activity of all three iPS activities was increased in total brain homogenates of AD patients and of an AD mouse model. We were also able to show increased mRNA expression of the β1i and β5i subunits [7][chapter 2]. The increase in activity did not occur at the expense of the cPS, since we observed no decrease in cPS activity or subunit expression in relation to AD pathology [7][chapter 2]. These data demonstrate an activation of the iPS function in relation to AD pathology, which is in line with the increased expression levels of the iPS previously reported [116, 118].
Moreover, as mentioned above, an increased expression of the β1i and β5i subunits was predominantly found in plaque-bound reactive astrocytes and microglia, respectively, although some degree of nuclear expression of β5i was observed in astrocytes and microglia. This increase in iPS expression in the reactive astrocyte and microglia surrounding the plaques provides strong evidence that the increase in iPS activity is linked to the increased glial reactivity.

4.4 iPS and immune signaling

In professional immune tissues and during systemic disease, the iPS is involved in generating antigens from bacterial and viral surface proteins, as well as from infected cells. In the brain, however, the blood brain barrier prevents the majority of these threats from entering the CNS. However, both Aβ and Aβ plaques have the capacity to elicit activation of the immune response by activating pattern recognition receptors. For example, Aβ can activate microglia via TLR and CD14 receptors [7, 25, 30][chapter 2]. Activation of TLR- and CD14-mediated signaling pathways results in NFκB-dependent transcription of pro-inflammatory cytokines, such as IL6, IL1b, and TNF. Proteasome-mediated degradation of the IκB protein is necessary for NFκB to enter the nucleus and initiate transcription [125] (Fig. 2B). Studies in different cell types obtained from LMP2 (β1i−/−) knockout mice, show an alteration of NFκB-mediated signaling, as a consequence of reduced IκB degradation [126] or via a more indirect regulation of NFκB-dependent genes [127]. The specific inhibitor ONX0914 (previously PR-957) targeting the β5i iPS activity, reduced the expression of LPS-stimulated microglia isolated from AD and control mice [7][chapter 2]. Application of the same β5i inhibitor also reduced proinflammatory molecule levels and improved the clinical outcome in mouse models for rheumatoid arthritis, inflammatory bowel disease and lupus [128–130]. Data from iPS subunit knock outs and pharmacological inhibition of the iPS provide strong evidence for the involvement of iPS in the regulation of proinflammatory signaling and support the idea that iPS can serve as a target to modulate the innate immune responses in the brain [95, 131]. However, the precise underlying molecular pathway for this immune-regulatory function of the iPS remains unclear, and may differ between different tissues and cell types.
4.5 iPS antigen presentation in the AD brain
Activated microglia and astrocytes increase their expression of MHC class I molecules in AD mice [chapter 5], which suggests an enhanced antigen presentation to T-cells, possibly initiated by Aβ plaque pathology. Indeed, infiltrating T-cells are present in higher numbers in the brains of AD mice, as well as in human AD brain tissue, compared to controls [132, 133]. As the main producer of MHC class I antigens, the iPS is likely to be a main player in T-cell activation in the CNS, either via MHC class I antigen-mediated CD8⁺ T-cell activation or by cross-presentation to CD4⁺ T-cells [99]. What is as yet unknown is whether Aβ antigens are presented on MHC class I molecules. In all likelihood the iPS creates antigens both from pathogenic Aβ and damaged or oxidized proteins that accumulate within the reactive glia cells. This, in turn, would attract CD8⁺ T-cells to destroy the offending cell. However, a direct link between CD8⁺ T-cells and iPS-mediated Aβ degeneration has not yet been established.

5. Glia, oxidative stress, and the iPS
Several oxidatively damaged proteins and lipids are present in AD and this results in the upregulation of anti-oxidants, protein aggregation and neurotoxicity [134, 135]. In AD mice, expression of markers indicating increased oxidative stress was found in plaque bound microglia cells [136]. Activated microglia and astrocytes, releasing pro-inflammatory cytokines, are likely to be the initiators of the oxidative stress [137, 138]. Binding or recognition of Aβ to CD36 [139] or RAGE – a multi ligand immunoglobulin receptor [140] leads to production of reactive oxygen species (ROS) that will activate many downstream signaling pathways propagating an inflammatory response (Fig 2B). Moreover, the increased production of reactive oxygen species does increase the production of Aβ itself, and the interplay of oxidative stress, glia reactivity and neuroinflammation thus forms a vicious circle, leading to an exacerbation of AD pathology [141]. Aging also induces oxidative stress, which is correlated with an age-related increase in iPS and their respective activities [7, 142, 143][chapter 2]. Chronic oxidative stress correlates with an increase in iPS, although high levels of oxidation result in apoptosis, regardless of iPS induction [143, 144].
5.1 Prevention of protein aggregation by the iPS

The iPS is capable of quickly degrading oxidatively damaged proteins with or without an ubiquitin tag, which accumulate in the presence of oxidative stress [145]. Removal of iPS subunits, particularly LMP2 and LMP7, impairs degradation of oxidized protein and consequently stimulates protein aggregation within the cells [143, 145, 146]. Aβ can induce release of reactive oxygen species in glia cells. Indeed, we found increased levels of hypoxia inducible factor α (HIF1α) co-localizing with β5i staining around Aβ plaques in AD mice (Fig. 4A-A’), providing further evidence that these cells are under oxidative stress. Recent data from Xie et al. has shown that Aβ plaques strongly induce levels of oxidative stress that lead to the rapid death of nearby neurons, starting with oxidative damage in the neurites close to Aβ plaques that then propagates to the neuronal cell body [147]. Interestingly, we have observed
an increase in ubiquitin staining in such dystrophic neurites (identified with an APP C-terminus antibody) surrounding Aβ plaques in AD mice (Fig. 4B-B'), providing further evidence that oxidative stress in neurons leads to protein accumulation. No accumulation of ubiquitinated proteins was observed in microglia or astrocytes surrounding Aβ plaques (Fig. 4C-D).

The increased load of oxidative stress and inflammation around the Aβ plaque is likely to increase iPS expression, in order to deal with the protein damage and accumulation that goes with it. A few studies conclude that the underlying factor for the accumulation of aberrant and damaged proteins in AD may result, partly, from a decrease in proteasome activity and a failure of the iPS to degrade these proteins [118]. However, we recently showed that the iPS is highly induced in AD, together with more than doubling its activity at later stages of the pathology [7][chapter 2].

Based on this, we argue that this increase in iPS activity is an attempt to reduce the increased levels of damaged proteins induced by the oxidative and inflammatory environment around plaques. Due to the failure to reduce Aβ plaque load and Aβ generation, the glial activation becomes persistent and chronic. Consequently, the iPS response becomes sustained and chronic rather than transient and due to its important role in pro-inflammatory signaling, this sustained activation of iPS may further exacerbate inflammation in AD.

6. The iPS as a therapeutic target in AD

Natural compounds such as curcumin, used in Indian cuisine, resveratrol, a substance in red wine, and EGCG, a compound found in green tea, have all been suggested to have neuroprotective capacities and to have a beneficial effect on
cognitive function and aging. Interestingly, all three compounds have been shown to have an inhibitory effect on proteasome activity, and have a capacity to reduce pro-inflammatory signaling [148–150]. Both resveratrol and EGCG are considered potential targets for AD and are currently being investigated in clinical trials [151] [trials: NCT00951834, NCT01504854]. These compounds are so-called unspecific proteasome inhibitors that are likely to also have an effect on other cellular pathways [131] and may therefore assert their beneficial effect also through those. Moreover, there is no data available on whether these compounds inhibit the cPS, the iPS, or both. Nevertheless, their anti-inflammatory capacity, together with their positive effect on cognitive function suggests that a chronic mild dampening of inflammatory signaling may be an attractive strategy to slow the course of AD.

Using a specific inhibitor targeting β5i iPS activity, ONX0914, we showed that inhibition of iPS activity led to strong reduction of not only pro-inflammatory molecules, such as IL1β and TNF, but also of the receptors responsible for the Aβ-induced glia activation, TLR2 and CD14, in activated microglia isolated for AD mice [7][chapter 2]. As the iPS is highly involved in the degradation of oxidized and damaged proteins, inhibition of its activity may also affect this capacity. It is likely that a total block of iPS activity would lead to accumulation of damaged intracellular proteins, therefore a constant but mild inhibition of specific subunits of the iPS would be a more favorable strategy. A study using the general proteasome inhibitor MG132 showed that mild proteasome inhibition does indeed have the capacity to increase the expression of anti-oxidative molecules in cardiac myocytes and in a rat model of diabetic nephropathy [152, 153] although it is unknown which was responsible: the inhibition of the cPS or of that of the iPS. Nevertheless, it shows that inhibition of the proteasome may also benefit the targeting of the increase in oxidative stress observed in AD. The low activity of the iPS in normal tissue, together with the increase in AD tissue, makes the application of specific inhibitors targeting the iPS highly attractive, since these will leave the vital cPS unaffected and only target chronically induced iPS activity. Future studies using such specific compounds in in-vivo models of AD will be necessary and will provide further information concerning the role of iPS in AD pathology.
**SCOPE AND OUTLINE**

Alzheimer’s disease (AD) is the most common form of dementia in our society. The disease is characterized by pathological hallmarks such as Amyloid beta (Aβ) plaques and neurofibrillary tangles. These pathological changes are associated with neuronal dysfunction and severe cognitive impairment. It has long been recognized that both reactive astrocytes and activated microglia are closely surrounding Aβ plaques and their presence can be considered an additional hallmark of the disease. The general aim of this thesis is to elucidate the molecular changes underlying the reactive phenotype of astrocytes and microglia in relation to AD plaque pathology. In addition, we would like to understand the functional consequences of the phenotypic changes in AD glia.

Understanding the altered functions in reactive astrocyte and microglia is essential, since, in the healthy brain, glial cells are important players in neuronal support and communication. Thus, a change in their function is likely to contribute to, or even initiate the neuronal dysfunction in AD. Despite their putative impact on the AD pathogenesis, little is known about the molecular alterations paralleled with these reactive phenotypes. The increase in expression of the intermediate filament GFAP is the most commonly used marker for reactive astrocytes. How an upregulation of GFAP is orchestrated and what downstream consequences of enhanced GFAP expression on astrocyte function are is unclear. Our lab has a long-standing interest in the function of GFAP. Previous data from our group show that GFAP expression is tightly regulated by proteasome activity *in vitro* and *in vivo*.

In chapter 1, we briefly review what is known concerning astrocyte and microglia reactivity in relation to Aβ and AD plaque pathology. We furthermore discuss what role the ubiquitin proteasome system plays in AD pathogenesis and glia reactivity. In chapter 2, we move on to investigate the role of the ubiquitin proteasome system in relation to astrocyte reactivity in AD. For this we studied the APPswe/PS1dE9 AD mouse model and human post-mortem tissue from AD and control donors. These experiments showed that the expression and activity of the immunoproteasome, an inducible form of the proteasome that is involved...
in innate immune signaling and antigen presentation, was increased in reactive
glia and correlated to the extent of AD pathology, both in the mouse and human
brain. Importantly, inhibition of this elevated immunoproteasome activity suppressed
inflammatory responses in microglia isolated from AD mice brains. These findings link
an increased immunoproteasome activity to activation of innate immune signaling
in plaque-associated reactive astrocytes and microglia. An activation of immune
signaling has previously been described in AD, however, the exact contribution of
the different glia cell population to these inflammatory changes remains unclear. Also,
how the sustained neuroinflammation, in turn, affects the astrocytes and microglia
and their functions remains to be elucidated.

Based on the AD induced increase in proteasome activity shown in chapter
2, we set out to analyze the transcriptional regulation of GFAP in relation to en-
hanced proteasome activity in chapter 3. We found that two different small molecu-
lar compounds were able to increase the immunoproteasome activity in astrocytes,
which in turn increased the expression of GFAP. These results further strengthen the
functional link between proteasome activity and GFAP regulation. Moreover, we also
found that Notch activation, which emerges as an important regulator of astrocyte
reactivity, was involved in the proteasome-mediated regulation of GFAP expression.
A detailed insight into changes related to cell reactivity, in adult/aged astrocytes and
microglia, requires a separate analysis of these cell populations. To this end, we
optimized existing cell isolation protocols to allow for a simultaneous isolation of
pure astrocyte and microglia populations from the cortex of aged mice, described in
chapter 4. By isolating separate glial cell populations from the cortex of both young
adult and aged mice, followed by genome wide expression analysis, we identified
the basal transcriptional profile of both astrocytes and microglia and their age-relat-
ed changes. These cell-type specific expression profiles together with bioinformatics
tools also allowed us to identify important cellular and molecular functions of adult/
aged astrocytes and microglia.

The optimized single cell isolation method was further applied in chapter 5
where we analyzed the genome wide transcriptional changes of astrocytes and mi-
croglia in response to Aβ plaque pathology. Astrocytes and microglia from APPswe/
PS1dE9 transgenic AD mice were compared with control littermates at an age where
the AD mice display a severe plaque load and extensive activation of astrocytes and microglia. Our findings showed that both astrocytes and microglia acquire an inflammatory phenotype, accompanied by a strong reduction in support functions, such as glutamate recycling and neuronal signalling/support in astrocytes and a reduction in endocytosis in microglia. These results suggest that reactive astrocytes, foremost, but also microglia, shift their efforts towards prolonged inflammatory/defence tasks at the expense of their normal neurosupportive functions. Such a functional shift may further exacerbate neuronal dysfunction and AD pathology.

In chapter 6, the main findings from the experimental chapters within this thesis are summarized and discussed in a broader perspective stressing the importance of cell-type specific analysis to obtain vital information on neurological diseases. Furthermore the role of the inflammatory glia phenotypes and their contribution to the behavioral and pathological features of the APPswePS1dE9 mouse model, and to AD pathogenesis is discussed. Lastly, the possible treatment strategies to modulate glia function and neuroinflammation in AD are presented.