CHAPTER 6

SUMMARY & GENERAL DISCUSSION

Marie Orre¹, Jennifer Strong¹, Willem Kamphuis¹, Elly M. Hol¹,²

¹Astrocyte Biology &Neurodegeneration, Netherlands Institute for Neuroscience (NIN), Amsterdam, The Netherlands; ²Swammerdam Institute for Life Sciences, Center for Neuroscience, University of Amsterdam, The Netherlands.
Alzheimer’s disease (AD) is the most prevalent form of dementia in our society and with the elderly population rapidly expanding the number of AD patients is expected to rise dramatically in the coming decades. The pathological hallmarks of AD are the formation of extracellular deposits of aggregated amyloid-β (Aβ) and the intraneuronal accumulation of hyper-phosphorylated tau, known as plaques and tangles, respectively. In addition, Aβ plaques are surrounded by reactive astrocytes and activated microglia, which are part of a neuroinflammatory response considered to be the main feature in AD pathogenesis. Reactive glia are believed to contribute to the disease progression through the release of proinflammatory molecules and reactive oxygen species, leading to neuronal damage and increased Aβ production. These data have mainly been obtained by histological analyses and in vitro experiments. However, it has been a challenge to unravel the exact role of astrocyte and microglia functions from histological analysis, since this method is always biased towards a specific question. Therefore, is still a great deal we do not yet know about their molecular and functional changes in relation to the pathogenesis and progression of AD. In this thesis, by using an unbiased genome-wide approach, we have set out to characterize the molecular phenotype of reactive astrocytes and activated microglia in relation to AD. This has been done by isolation and characterization of these cell populations ex vivo by cell-type-specific isolation and genome-wide characterization, in combination with histology and in vitro experiments. Using this approach we have obtained a more detailed insight into astrocyte and microglia functions and contribution to AD pathogenesis in the APPswePS1dE9 mouse model and in AD patients. In the introductory chapter 1, we briefly describe the phenotypic and functional alterations in astrocytes and microglia in relation to Aβ stimulation and Aβ plaques in the context of AD. Furthermore, we introduce and discuss the role of the proteasome, particularly the immunoproteasome (iPS), in relation to astrocyte and microglia reactivity and neuroinflammation in AD. The ubiquitin proteasome system is the major protein degradation system within the cell and is a natural target for investigation in AD, as accumulation of ubiquitinated proteins is a prominent feature of AD. Over the last decade several studies have focused on the role of the proteasome system and
found this system to be impaired in AD. However, until recently, little attention has been paid to the role of the iPS in neurodegenerative diseases; our understanding of the iPS function is mainly based on studies of peripheral blood cells and tissues. In this chapter, we summarize what is known about the iPS in the brain and in relation to AD. In addition, we discuss the possibilities of targeting its activity to reduce neuroinflammation in AD.

In chapter 2, we aimed to elucidate the role of the proteasome in relation to glia reactivity in AD with a focus on Aβ (plaques). For this, we used novel methods including cell-permeable proteasome-activity probes – enabling readout of proteasome activity in living cells, and new proteasome subunit-specific activity assays. These new methods allowed us to get more detailed information on proteasome activity than the methods we used in previous studies. For the first time, we could analyze the different activities of both types of proteasome present in the cells: the more abundant constitutive proteasome and the immune-induced iPS. In strong contrast to previous studies, we found an increase in proteasome activity, specifically of the iPS, both in human AD tissue and in the APPswePS1dE9 AD mouse model. By means of immunohistochemistry and gene expression analysis we showed that plaque-associated reactive astrocytes and microglia were the main cell types expressing elevated levels of iPS subunits. Moreover, we showed that specific inhibition of the increased β5i iPS activity led to a strong reduction in the expression of proinflammatory molecules, such as Il1b, Tnf in LPS-stimulated microglia from aged AD mice. In this chapter, we thus provide a new insight in the function of the iPS in plaque-related glial cell reactivity and neuroinflammation in AD. We also suggest that the iPS could be a target for reducing neuroinflammation in AD.

Previous data from our lab show that inhibition of the proteasome reduces the expression of GFAP in astrocytes. In chapter 3 we therefore set out to test the hypothesis that proteasome activity is an important regulator of GFAP expression. We also investigated molecular mechanisms linking the proteasome to GFAP regulation. We treated astrocytoma cells with molecules known to increase proteasome activity in other cells and found two molecules that substantially increased proteasome activity in the astrocytes; one agonist for an opioid receptor, and one antagonist for a purinergic receptor. The induction of proteasome activity by these compounds was
paralleled by an increase in GFAP, in line with the hypothesis. Both compounds also increased expression of iPS subunits and molecules involved in Notch signaling. By using specific inhibitors for the iPS (β5i activity) or Notch signaling, we showed that both these pathways are vital for proteasome-mediated regulation of GFAP expression, probably via STAT3 activation. Interestingly, we showed that proteasome activation resulted in a more anti-inflammatory astrocyte phenotype, in contrast with the proinflammatory phenotype of reactive astrocytes isolated from aged AD mice (chapter 5). This highlights the heterogeneity of astrocyte reactivity, a point further discussed in chapter 6.

Research on functional changes of astrocytes and microglia in age-related, neurodegenerative diseases has been held back by the absence of a good method to isolate these glial cell types from the brains of aged mice. In chapter 4, we describe the set-up and optimization of a cell-isolation protocol to obtain viable and pure astrocytes and microglia from the aged mouse cortex based on cell surface markers. Using this optimized method we isolated cortical astrocytes and microglia from young adult (2.5 months) and aging (15-18 months) mice that were later subjected to genome-wide microarray analysis to generate expression profiles. These expression profiles provided valuable information on astrocytes and microglia functions and complemented the transcriptome from the currently available post-natal and young astrocytes. With this information, we could substantiate the concept of astrocytes as an important part of the tri-partite synapse, expressing many genes involved in neuronal signaling, and microglia as the main immune player in the brain. Furthermore, the comparison between young adult and aging populations gave an insight into the molecular alterations of microglia and astrocytes in relation to normal aging, and showed that aged astrocytes have a more pronounced inflammatory phenotype compared to young astrocytes. The inflammatory differences were more ambiguous in the microglia comparison; aging microglia had higher expression of genes within the TNF-ligand family and younger microglia showed higher transcript levels of CC-chemokines.

In chapter 5, we isolated astrocytes and microglia from wild type (WT) controls and AD mice of 15-18 months - an age where the plaque load and glia reactivity are extensive in the AD mice. To analyze the molecular alterations associated
with this Aβ plaque-induced astrocyte and microglia reactivity, we performed a genome-wide expression analysis of the isolated WT and AD glia populations. Both cell types showed an increased expression of proinflammatory genes, together with a parallel reduction of highly cell-type-enriched genes - vital for normal astrocyte and microglia functions, such as genes involved in glutamate uptake and conversion in astrocytes, and genes involved in endocytosis/phagocytosis, in microglia. These data suggest that reactive glia shift their efforts towards defense and repair tasks at the expense of their normal supportive functions, which may further exacerbate neuronal dysfunction and AD pathology. To confirm the mouse data with the expression changes observed in human Alzheimer’s disease, we used a clustering approach to compare the regulated gene clusters obtained from the mouse astrocyte and microglia populations with a gene expression dataset from the prefrontal cortex of human Alzheimer’s disease and control donors. This comparison showed that the plaque induced inflammatory changes in the mouse astrocytes were remarkably similar to the inflammatory changes present in human AD. These expression data revealed strong indications for neuroinflammation and glia dysfunction, both likely to be contributing factors to the neuronal dysfunction and memory impairment observed both in the AD mouse model used here and in human AD pathology, as more extensively discussed in chapter 6.

In chapter 6, we discuss the benefits of cell-type specific analysis in relation to pathological changes observed in AD and the neurological phenotype of the APPswePS1dE9 mouse model, linking it to the change in glial phenotype induced by plaque pathology, as described in this thesis. Finally, we also discuss the role of neuroinflammation and its consequences for the initiation and progression of AD, and its potential as a treatment target for AD.
GENERAL DISCUSSION

1. Cell type-specific gene expression analysis

The use of genome wide expression analysis has increased extensively over the last decade, due to fact that tools, such as the genome-wide microarrays, have become more affordable, more widely available, and sophisticated. This has led to databases with a rich load of information on expression profiles, enabling the comparison between expression datasets from different diseases and disease models. Several genome wide AD datasets are now available, both from human AD brain donors and AD mice [22, 277, 278, 297, 298] and have provided valuable information on general molecular changes underlying AD pathology and progression [299]. However, these datasets are all based on RNA isolated from whole brain tissue, which precludes a determination of the cell type responsible for the observed changes, and on how the disease affects the different cells. Chapters 4 and 5 deal with the glia-specific changes related to aging and AD plaque pathology, by isolating and separating astrocytes and microglia populations, followed by a genome-wide analysis. This approach allowed us to give a cell-type-specific insight into the transcriptional changes in relation to normal aging and AD plaque pathology. Based on this we made several interesting observations that have already been highlighted in the discussion of chapter 4 and 5; (i) astrocytes express many genes involved in neuronal communication, (ii) AD astrocytes show a stronger relative increase of immune-related genes than AD microglia, and (iii) both AD astrocytes and microglia show reduced expression of genes, vital for their normal physiological functions. None of these changes were picked up in the whole tissue analysis, which demonstrates the importance of separate analysis of the different cell types to get a more precise and complete picture of how the brain is affected during AD pathogenesis.

1.2 Cell-type-specific analysis – Increases the molecular resolution

Several genes found in the astrocyte and microglia dataset from AD mice showed great overlap with genes present in a genome-wide study of AD plaque pathology progression in the frontal cortex of the same APPswePS1dE9 mice, especially genes involved in inflammatory processes [278]. Importantly, separate analysis of
astrocyte and microglia changes in AD, as we show in chapter 5, helped us to identify many genes that were regulated at higher fold changes than those found in whole tissue samples. This can be explained by the increase in resolution or sensitivity acquired when analyzing different cell populations separately. An example of this is shown in chapter 5, where a large set of known astrocyte genes were found to be downregulated in AD, such as Fgfr3, Slc1a3 (Glast) and glutamine synthetase (Glul). When looking at their expression in whole tissue of 15-18 month old AD and WT mice no clear difference was found, while their expression was strongly reduced in AD compared to WT astrocytes isolated from 15-18-month-old mice (Fig. 1A-B). The expression of several of these astrocyte genes was also found to be reduced in reactive astrocytes in other disease models [233], as shown in chapter 5, indicating that cell-type-specific characterization provides a more detailed picture of the microenvironment in diseases. There are a number of reasons for this discrepancy between single cell analysis and whole tissue analysis; A, Proliferation of a particular cell type will affect the readout in whole tissue, but not in the analysis of isolated and separated cells, B, “Cell-type specific genes”, such as Gfap, Glast, and Glul – in the case of astrocytes, and CD11b, MHC class II – in the case of microglia, may also be expressed by other cell types at lower levels. C, Subtle fold differences in a

**Figure 1.** Comparison of gene expression of astrocyte genes from whole cortex and isolated astrocytes from AD and WT mice. (A) Shows the expression of Fgfr3, GLAST and Glul assessed by qPCR from the cortex of 15-18-month-old APPswePS1dE9 and wild type mice. (B) Shows normalized array intensity (expression) of the same genes from astrocytes isolated from APPswePS1dE9 and wild type mice at 15-18 months of age. AD = Alzheimer’s disease; WT = wild-type (A: WT, n=21; AD, n = 10 B: WT and AD, n= 4); * p< 0.05, students t-test.
specific cell population may be “diluted” in analyses from whole tissue when other cell types express the same transcripts. D, Other cell types may start to express genes that they normally do not, which will affect the whole tissue analysis, but not a cell-type-specific analysis.

1.3 Aberrant expression of “cell-type-specific” genes

As mentioned in the previous section, cells may start to express genes (and proteins) that they normally do not. Neurons in human AD tissue have been observed to express proteins believed to be “specific” for astrocytes, such as GLUL [300], EAAT2/GLT-1 [301], GFAP [302], and EAAT1/GLAST [303] in advanced stages of the AD pathology. The aberrant neuronal expression of astrocyte genes involved in glutamine uptake and conversion is likely to be a compensatory mechanism used by the neurons to maintain these functions when nearby reactive astrocytes fail to do so, in order to prevent glutamate-mediated neurotoxicity [50, 300]. Conversely, highly reactive astrocytes surrounding amyloid beta plaques can increase their expression of MHC class II molecules [51] that are otherwise mainly expressed by antigen-presenting cells, such as microglia. During our studies on immunoproteasome expression in the human AD brain [chapter 2], we, too, encountered reactive astrocytes expressing MHC class II molecules (HLA-DR, DP, DQ; indicated with an arrow in (Fig. 2)), as well as an increased expression of immunoproteasome subunits (data not shown). These findings provide further evidence for the occurrence of aberrant protein expression, and also provide further evidence for immune-like functions of astrocytes in AD. Moreover, in Chapter 4, we show that both young and aged astrocytes express many “neuronal genes”, such as receptors involved in signaling and genes involved in exocytosis (neurotransmitter release), as discussed in more detail in chapter 4. Taken together, these data provide evidence for both points C and D mentioned in previous section (1.2); that genes may not be as cell-type-specific as initially thought, and that aberrant expression of proteins and genes occur, especially during disease. Thus, these points further strengthen the importance of employing cell-type specific analysis to get a more detailed and realistic picture of normal astrocyte and microglia functions, as well as of their (dys)function in disease.
2. The APPswePS1de9 model as a model for AD with respect to glia reactivity and neuronal dysfunction.

2.1 Neuronal phenotype in APPswePS1dE9 mice

One of the main conclusions of chapter 5 is that the chronically activated astrocytes in the APPswePS1dE9 mouse become proinflammatory and dysfunctional, which may reduce their capacity to support neurons. The APPswePS1dE9 model display several other AD-related features, such as dystrophic neurites [304], cognitive dysfunction [305, 306], reduced neuronal activity [307], impaired cholinergic pre- and post-synaptic transmission [305, 308], and synapse and spine loss [306, 309, 310]. Although there is no large-scale neuronal death according to histological analyses [15, 304], more recent two-photon *in vivo* imaging studies have provided evidence for some degree of neuronal death and dysfunction in relation to Aβ plaques [147, 307, 311]. Xie et al. show that the plaques induce oxidative stress in nearby neurons, leading to neuronal death. Meyer-Luehmann and colleagues show that plaques generate dystrophic neurites 1-2 days after plaque formation and in a follow-up study

![Figure 2. Plaque-bound reactive astrocytes show increased expression of MHC class II molecules in human AD tissue.](image)

Reactive astrocytes are labeled with GFAP (green). MHC class II expression is shown by staining for HLA-DR, DQ, DP (red). A reactive astrocyte expressing both GFAP and MHC class II is indicated with an arrow. Microglia cells positive only for MHC class II are indicated by arrowheads. Blue: DAPI nuclear stain. Donor: NBB 01-141, Braak stage 5, picture: M. Orre, unpublished data.
they show that the neuronal activity in neurons in close proximity to plaques was reduced [307]. Taken together, these data indicate that the neuronal dysfunction emerges in conjugation with the plaques appearance in the APPswePS1dE9 mice. They also indicate that plaques are the initiators for the generation of dystrophic neurites and the oxidative stress that, in turn, may exacerbate the release of inflammatory molecules by activated microglia and astrocytes, as described in chapters 1, 2, and 5 and as shown in (Fig. 3). Not only inflammation, but also the parallel

Figure 3. Schematic overview of astrocytes and microglia in aged AD mice
(1) Both astrocytes and microglia show increased inflammatory signaling in aged AD mice compared to wild-type mice, although microglia produce higher levels of inflammatory molecules and are therefore more likely to be responsible for recruitment of peripheral cells. (2) Astrocytes show a higher fold induction, but lower absolute expression levels of inflammatory molecules, and may be responsible for stimulating nearby cells rather than recruit peripheral cells. (3) Both astrocytes and microglia upregulate MHC class I receptors and antigen processing genes. (4) Microglia show a "primed" phenotype with increased CD45 and CD11c expression. (5) Astrocyte genes involved in neuronal signaling, glutamate uptake and conversion are decreased, which may lead to neuronal dysfunction. Both astrocytes and microglia release neurotoxic factors such as reactive oxygen species and inflammatory molecules, which contribute to the formation of dystrophic neurites and enhanced Aβ production by neurons.
reduction of vital cell-type-specific functions in glia is likely to be a contributing factor to the reduced neuronal function observed in these mice and in AD pathology, as discussed in section 3.

2.2 Modification of glia reactivity and immune signaling in AD mouse models

The APPswePS1dE9 model has been used in several studies involving modulation of the immune response to assess the effect of neuroinflammatory mediators on plaque load and behavior. The effect of reduced astrocyte reactivity was analyzed by overexpressing VIVID - a peptide that inhibits calcineurin/NFAT signaling in astrocytes [312]. After 9 months of treatment, at the age of 16-17 months, these mice show reduced astrocyte reactivity, reduced plaque load, increased synaptic strength and improved memory [48]. Moreover, deficiency in Mrp-14 – a molecule elevated in AD and involved in amplification of inflammation, in APPswePS1dE9 mice reduces the levels of certain proinflammatory molecules such as IL-1β and TNFα, decreases Aβ levels and plaque load, and increases phagocytosis by microglia, at 9 months of age [75]. These results suggest that limiting long-term astrocyte and microglia reactivity may help reduce AD pathogenesis. Another study analyzed the effect of “chronic” neuroinflammation on Aβ plaque load; by crossing an inducible IL1β-overexpressing mouse with the APPswePS1dE9 line. Four weeks after induction of IL1β over-expression these mice showed reduced plaque load and increased microglia density around the plaques, leading the authors to conclude that neuroinflammation is beneficial to the effective removal of Aβ plaques by microglia [231]. However, no histological data from older AD mice was presented, so the effect on long term chronically elevated IL-1β levels remains unclear. Moreover, a short-term (7 days) lipopolysaccharide (LPS) injection in the APPswePS1dE9 mouse model reduced Aβ staining but not congo red staining of plaques in APPswePS1dE9 mice of 5, 11, and 15 months of age [313]. Using another AD model, Herber and colleagues have also shown that short term LPS injections reduce plaque load and soluble Aβ levels [314, 315]. In contrast, long-term LPS injections (for a period of 12 weeks) in APPswePS1dE9 increased the soluble Aβ levels in 11-month-old mice [316] and showed a tendency for an increase in plaque load in 6-month-old mice [317]. However, systemic injection of a TLR4 ligand, which caused a mild and transient
inflammatory response, led to a reduction in plaque load after 12 weeks [317]. In another AD mouse model, 3xTG AD, four weeks of LPS treatment in 4.5-month-old mice resulted in increased levels of intra-neuronal Aβ, which was abrogated when combined with an TNFα inhibitor [76]. It is important to notice that the short-time LPS studies used intrahippocampal injections of LPS, while the long-term studies injected LPS intraperitoneally. Obviously, the intrahippocampal injection disrupts the blood brain barrier, allowing influx of peripheral macrophages that may be responsible for the increased plaque degradation. In the systemically administered mice the blood brain barrier remained intact and the increase in inflammation in the brain was thus solely dependent on the resident cells, such as microglia and astrocytes. The latter represents better what occurs in the APPswePS1dE9 mice naturally, albeit more vigorously, where an increased inflammatory response is apparent without clear signs of monocyte infiltration or blood brain barrier dysfunction [6]. A drastic approach to elucidate the effect of microglia on plaque deposition was taken by Grathwohl and colleagues: they ablated microglia in young and aged AD mice for a period of four weeks. Their finding shows that short-term lack of microglia had little effect on plaque load, but led to an increase in astrocyte reactivity, potentially as a compensatory mechanism. From this study it is hard to draw any conclusions on the long-term effects of the lack of microglia and microglia responses and their relation to AD pathology, although it would be interesting to see what effects an ablation of the proliferating microglia in AD (as shown in [6] and in chapter 2)) would have on the AD pathogenesis, and if such an inhibition of microglia proliferation would be detrimental or preventative.

Taken together, these data further support the notion that sustained chronic immune and glia activation (as described in chapter 5) is likely to exacerbate Aβ accumulation and plaque formation and that inhibition or prevention of this sustained inflammation may be beneficial. Thus, the use of specific modulatory molecules targeting inflammation, such as the targeting of increased immunoproteasome activity with specific inhibitors, as discussed in chapters 1 and 2, can be an attractive strategy to reduce inflammation without severe “off target” effects. However, the data discussed above also show that a quick, transient “burst” of cell activation may help with plaque clearance. As shown in chapter 3, stimulation of astrocytes using
proteasome activators induced an activated, anti-inflammatory astrocyte phenotype and may serve as such a booster. This topic urgently requires further research.

3. The different reactive astrocyte and microglia phenotypes in AD (and other CNS insults)

3.1 Reactive astrocytes are heterogeneous

Thorough work has been done on dissecting the role of scar formation by reactive astrocytes in several acute brain injury models characterized by a compromised blood brain barrier and astrocyte proliferation (reviewed in [274]). From these studies it has been concluded that reactive astrocytes are beneficial to the central nervous system, as they release growth factors and neurotropic factors, have the capacity to repair the blood brain barrier, and isolate the injured areas, thereby protecting the surrounding cells and tissue. Ablation of proliferating, reactive astrocytes worsens the outcome in all kinds of different acute injury models, such as spinal cord injury, and experimental autoimmune encephalomyelitis (EAE; a multiple sclerosis model) [274, 319, 320]. In AD, astrocytes do not proliferate, and show a less severe but long-lasting form of astrogliosis [6]. Due to the different nature of the triggers - an acute and severe injury or in vitro stimulation versus a more subtle, but chronic stimulation - it is not surprising that the characteristics of the subsequent reactive astrocytes is highly divergent. This further strengthens the evidence for the heterogeneity of reactive gliosis (as discussed in chapter 3)[274]. This thesis shows evidence for two forms of reactive astrocytes; chronically activated AD astrocytes displaying increased GFAP expression and elevated levels of proinflammatory molecules (chapter 5) and astrocytes activated by proteasome activators in vitro – also showing increased GFAP expression, together with an increased expression of anti-inflammatory molecules (chapter 3). As described in chapter 1, and chapter 5, and shown in (Fig. 3), chronically activated astrocytes in AD contribute to neuroinflammation by releasing proinflammatory and neurotoxic molecules such as chemokines, cytokines, reactive oxygen species (ROS) and cyclooxygenase (COX) (for reviews see [55, 321]). In chapter 5 we found that this chronic form of reactive gliosis parallels with a reduction of many genes vital for important astrocyte-functions. This indicates that
the efforts of AD astrocytes are redirected towards defensive responses rather than performing their regular tasks, such as neuronal support and signaling.

### 3.2 Chronic reactive astrocytes show a dysfunctional phenotype

The study presented in chapter 5 is one of the first studies that analyses the molecules changes in chronically activated astrocytes. The AD astrocyte phenotype observed in chapter 5, displaying reduced levels of glutamate transporters and receptors, as well as a reduction of Glul and other vital astrocyte genes, is likely to be linked to the sustained chronic activation of astrocytes. A reduction of vital astrocyte genes was also observed in astrocytes isolated from brains of a stroke mouse model ([233] and shown in chapter 5). As mentioned above, experimental in vitro and *in vivo* studies looking at the dynamics of astrocyte reactivity upon chronic stimulation are very few in number. In our lab, astrocytes were chronically stimulated with IFNα for 14 days, followed by 7 days withdrawal. These astrocytes did not reduce their production of inflammatory molecules upon withdrawal [284], indicating that reversal of the inflammatory astrocyte phenotype is a slow process, if total recovery happens at all. However, astrocyte genes involved in neuronal signaling and support, such as Glul, GLAST, GLT-1, were not assessed in this study. As in the AD mouse model, astrocyte reactivity in human AD pathology is extensive and persistent ([35, 198] and chapter 5). This has been linked to a decreased expression of astrocytic Glul, especially near neuronal boutons and blood vessels, suggesting compromised glia-neuron communication and astrocyte dysfunction in AD [322]. Although, this study shows that astrocytes in the direct vicinity of plaques do not appear to have decreased GS levels [322], which differs from our results in chapter 5, where dissected areas from both plaque and non-plaque areas showed decreased expression of Glul. Moreover, both gene and protein expression of GLAST (EAAT1) and GLT-1 (EAAT2) are reduced in the human AD cortex and hippocampus [323–325], providing strong evidence for disrupted glutamate transport and buffering, corroborating our findings discussed in chapter 5. GLT-1 has also been shown to be sensitive to the increased oxidative stress present in AD [326]. Thus, the reduced levels of physiological astrocyte molecules, such as Glul and Glast, may not be solely due to a direct effect of Aβ on astrocytes, but may also indirectly result from the elevated proinflammatory
and oxidative milieu present in the vicinity of plaques in AD (Fig. 3). Taken together, expression data from AD astrocytes show a reduction in the glutamate-related genes, as mentioned above and in chapter 5, and these are also reduced in human AD tissue based on histology, protein, and expression analyses. The array analysis in chapter 5 also provided evidence for a dysfunctional ion homeostasis, with Na\(^{2+}\)/K\(^{+}\) pumps and potassium channels downregulated in AD astrocytes, which would ultimately influence neurotransmitter recycling. Such data provide further evidence that astrocyte-neuron communication may be impaired in AD and thus contribute to neuronal dysfunction. However, further in vitro and in vivo experiments are needed to confirm these findings.

### 3.2.1 Target astrocytes dysfunction – an AD treatment strategy

For years, treatment strategies for AD have been geared towards reducing the effects of excessive neuronal glutamate release by using glutamate receptor antagonists (reviewed in [327]). Based on the findings in this thesis and as presented above, targeting the reduced glutamate uptake in AD astrocytes may provide an additional treatment strategy to reduce glutamate excitotoxicity in AD. Interestingly, a few compounds have the capacity to increase the levels of GLT-1 and lead to a reduction of neurotoxicity and neurodegeneration in an ALS model and in AD astrocytes, such as β-lactam antibiotics [328] and estrogen treatment [329], respectively. Estrogen treatment has been linked to a decreased prevalence and delayed onset of AD, and has a protective effect on Aβ-mediated cell toxicity in vivo and in vitro [reviewed in [330, 331]]. The capacity of estrogens to increase GLT-1 expression may be one of the (many) underlying factors of its protective effect. However, estrogen has also been reported to have anti-inflammatory and anti-oxidative effects as well as the capacity to also increase the expression and activity of astrocytic GLUL in vitro [332]. Interestingly, a predictive activity analysis of regulation molecules was performed on genes regulated in AD astrocytes (from chapter 5) and indicated that the estrogen receptor activity was decreased in AD astrocytes (unpublished observations). These findings suggest that targeting the estrogen receptor pathway may improve astrocyte function in AD, but this needs further investigation. The use of anti-inflammatory molecules to treat AD will be discussed below.
3.2 Chronic microglia responses in AD - priming and senescence

3.2.1 Microglia priming

Aging microglia are proposed to acquire an activated and “primed” phenotype, marked by an increased expression of inflammatory molecules (such as Il1b, IL-6 and TNFα), cell surface receptors (such as Cd68, Cd11b, Cd11c, MHC class II, Cd45), and a reduction of the “Off” receptor Cx3cr1, involved in keeping microglia in a suppressed activation state [225, 333–335]. We provide evidence for a slight phenotypic change in aging microglia in chapter 4, where they express higher levels of innate immune signaling molecules than microglia from younger mice, in addition to a slight reduction in the “Off” receptor, Cx3cr1. In AD microglia, isolated from mice of 15-18 months of age, a clearer shift is observed; the microglia appear to have converted from a “primed” phenotype into a fully activated one, as they release higher levels of proinflammatory molecules and show a reduced expression of Cx3cr1 and Cd200r compared to microglia from WT mice (from array analysis in chapter 5). It has to be noted that they also show elevated levels of Tgfb, but none of the other anti-inflammatory molecules, such as IL-4 and IL-10, were upregulated.

3.2.2 Microglia senescence

Both in aging and in AD, microglia senescence has been proposed, where microglia become dysfunctional and lose supportive functions (reviewed in [336, 337]). Microglia show reduced motility and phagocytic capacity with increased plaque load and disease progression [65, 74]. Moreover, histological analysis of human AD and Down’s syndrome tissue – where plaque load is extensive – shows that microglia acquire a dystrophic phenotype characterized by degeneration of its processes [338]. Such a dystrophic phenotype may be responsible for the impaired function of microglia, in response to the release of neurotropic factors and phagocytosis [336]. Whether this dystrophic microglia phenotype is present in AD mice remains unclear; it may occur at a later age, as we have indeed observed a reduction in microglia proliferation and a reduced number of microglia around plaques in older AD mice (~24 months, unpublished observations). However, the findings we described in chapter 5, where AD microglia show reduced expression of genes involved in endocytosis
and a general reduction of cell-type-enriched microglia genes as defined in chapter 4 could potentially be early signs of senescence. It is likely that microglia priming, initiated by aging, followed by microglial activation, induced by plaque deposition (characterized by a proinflammatory, phagocytic, proliferative phenotype) ultimately lead to a “burn out” of the microglia. This may, then, result in a dystrophic and dysfunctional phenotype that further aggravates plaque load.

4. The role of neuroinflammation in AD pathology and etiology.
As shown and discussed in chapters 1 and 5 of this thesis, neuroinflammation is one of the characteristics of AD and is likely to contribute to the disease progression. Interfering with AD onset and development of the disease, and the use of molecules that inhibit inflammatory signaling has been intensely studied as possible strategies. In this section I will discuss the findings of studies concerning the use of non-steroidal anti-inflammatory drugs (NSAIDs) for treatment and prevention of AD.

4.1 Anti-inflammatory (NSAID) treatment of Alzheimer’s disease
Several epidemiological studies found that people suffering from rheumatoid arthritis have a lower prevalence of AD in later stages of their lives. Rheumatoid arthritis patients are taking daily doses of anti-inflammatory medication, usually NSAIDs, indicating that inflammation may be an initiator of AD pathology, or speed up the onset of the disease. A meta-analysis of several NSAID studies was performed by Szekely and colleagues [339]. This meta-analysis showed that life-long NSAID use reduced the risk of AD with 71%, while at least 2 years of NSAID treatment reduces the prevalence of AD with 48% [286]. This provides strong evidence that chronic, mild, immune inhibition has protective properties against AD. NSAID treatment has also been shown to reduce Aβ 1-42 production in vivo and in vitro [340, 341], therefore, the decreased prevalence of AD in long-term NSAID users may also be partly due to a reduction in Aβ production, and not merely its anti-inflammatory properties. Unfortunately, follow up studies on NSAID use have been disappointing: several trials were done with NSAID treatment in patients with signs of early stage AD (reviewed in [342, 343]) or mild cognitive impairment [344], but they did not show improve-
ments regarding cognitive function or pace of disease progression. A more recent and extended study was performed in the that analyzed both long-term and short-term usage of NSAIDs in both younger and older subjects with, or without current AD pathology (at the beginning of the study) [345]. The conclusions from this study was that NSAID use in already cognitively impaired subjects may have an adverse effect on AD pathogenesis, while healthy subjects treated with common NSAIDs for a longer time (2-3 years) have a reduced chance of developing AD.

To sum up the results obtained from studies on NSAIDs; (i) starting long-term treatment promptly is likely to be beneficial for preventing AD; (ii) treatment at later stages, where underlying AD pathology may already be present, has no positive effect on the outcome of AD or on cognitive function. Our data presented in chapters 4 and 5 provide evidence that, already during normal aging, both astrocytes and microglia acquire slight inflammatory phenotypes, which exacerbate as AD plaque pathology progresses. Our data thus support the fact that early treatment with anti-inflammatory compounds may reduce glia reactivity.

4.2 Aβ vaccination studies in human and mouse

As discussed in chapter 1, the amyloid hypothesis suggests that deposition of Aβ is likely to be an initial event in AD pathogenesis. Therefore, research was focused on removal of Aβ by vaccination. The first to successfully accomplish this were Schenk and colleagues, in a study in which they showed a reduction of Aβ plaque load after active vaccination with Aβ peptides in an AD mouse model [346]. Since then, several studies have shown a reduction in plaque load in different mouse models [reviewed in [347, 348]]. Unfortunately, although plaque levels were reduced [349], vaccination trials in AD patients caused severe encephalitis and death in a few cases and were therefore discontinued. Novel vaccines are now being developed that will not cause a cytotoxic Th1 T-cell response - believed to be the underlying factor for the observed encephalitis [347, 350]. Such a novel vaccine was recently shown to decrease plaque load and levels of soluble and insoluble Aβ, and to lead to an improvement of the cognitive deficits in the APPswePS1dE9 AD model, where vaccination started at 10 months and lasted for four months [351]. These mice also showed a reduced number of activated microglia and a lack of increase
in neuroinflammatory markers. The level of astrogliosis, however, based on GFAP immunostaining, seemed unaltered in vaccinated compared to non-vaccinated AD mice, suggesting that plaque removal reduces microglia reactivity, but has less of an effect on astrocyte reactivity [351]. The latter, implies that the astrocyte activation preceding the point of intervention may be irreversible, and argues for an early intervention to prevent astrocyte reactivity and dysfunction.

5. Conclusions and future directions

5.1 Conclusions

As shown previously as well as in this thesis, reactive astrocytes and activated microglia surround Aβ plaques and the number of activated glia increases with increased plaque load. Initially, these astrocytes may respond with a boost of neuronal support, but after some time the plaque-induced increase in astrocyte reactivity is paralleled by a reduction in physiological astrocyte function, such as a decrease in glutamate buffering and conversion into glutamine, as observed in AD patients (Fig. 4A). AD microglia show a proinflammatory “primed” phenotype, albeit with a lower relative change in the expression of inflammatory genes than in astrocytes. Also signs of microglia dysfunction and senescence are observed during aging and AD and may ultimately lead to reduced responsiveness and phagocytosis (Fig. 4B). Both vaccination and short-term immune activation studies have shown that a transient immune activation can lead to a reduction in, or even the removal of, plaques, probably via increased microglia phagocytosis (Fig. 4C). However, the already present astrocyte reactivity and dysfunction are likely to remain. Moreover, epidemiological studies have shown that early and long-term treatment with anti-inflammatory compounds delay or prevent the onset of AD pathology and plaque accumulation, and tends to dampen astrocyte and microglia reactivity (Fig. 4D). Despite this immune suppressive effect, plaque load may appear at a later stage. Hypothetically, a transient immune activation at this stage – boosting microglia phagocytosis - may prevent and decrease the plaque load and further reduce plaque-associated astrocyte and microglia reactivity and the associated glial dysfunction.
Based on the findings discussed in this thesis, long-lasting early treatment with anti-inflammatory compounds, years before the clinical onset of AD, would be a good strategy to reduce inflammation, oxidative stress, and glial dysfunction within the brain, which in turn may postpone the onset and/ or decrease the severity of the disease. As discussed in chapter 1 and 2, using very specific inhibitors to target the inflammation in the brain, such as immunoproteasome inhibitors, may be a safer strategy to prevent broad “off target” side effects. Also, early Aβ vaccination has shown to have a similar beneficial effect on neuroinflammation with a capacity...
to delay the onset of plaque formation. Nevertheless, long-term, early treatments cannot be initiated without reliable screening methods for early signs of increased AD risk. Therefore, finding very early biomarkers for AD is likely to be an important step to determine if and when to initiate treatments. However, due to the complexity and unknown cause of AD, and strong age-related features in the majority of AD cases, a “cure” for this disease is likely to consist of a combination of treatments targeting different molecular pathways. Early preventive treatment(s) for glia reactivity and plaque formation, in combination with later immune-modulatory treatments that boost the function of microglia and astrocytes, may be a strategy to reduce the glia contribution to AD pathogenesis.