

Master Thesis

# Age, inflammation and A $\beta$ itself influence A $\beta$ degradation in Alzheimer's Disease

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## 1 Abstract

It is well established that AD is caused by accumulation of amyloid-beta (A $\beta$ ) peptides in the brain. The cause of this accumulation in late-onset AD is, however, still uncertain. An increasing amount of evidence on the subject points towards a defective clearance of A $\beta$  peptides from the brain. Furthermore, because both microglia and astrocytes are shown to accumulate around A $\beta$  plaques, multiple studies focused on the role of these cells in A $\beta$  clearance. Interestingly, these cells produce A $\beta$ -degrading enzymes and are thus potential mediators in A $\beta$ -clearance. In this review, various factors are discussed which can influence the expression and activity of A $\beta$ -degrading enzymes in microglia and astrocytes. To provide a concise overview of recent studies, this article is limited to the effects of aging, cytokines, and A $\beta$  itself on the A $\beta$ -degrading enzymes NEP, IDE and MMP-9. By comparing recent studies, it becomes clear that a major influence on the expression of A $\beta$ -degrading enzymes is the production of pro- and anti-inflammatory cytokines. Factors promoting inflammation in the brain generally lower the expression of NEP and IDE, while anti-inflammatory cytokines counter this effect. A $\beta$  itself influences the

expression of A $\beta$ -degrading enzymes by heightening the expression of these enzymes in microglia and astrocytes that are in direct contact with A $\beta$  plaques. Strikingly, recent evidence indicates that AD patients do not show a response of their glial cells under the same conditions, thereby suggesting a defect in this process. Lastly, although age is the most prominent risk factor for late-onset AD, the precise effect of aging on AD progression remains controversial.

## 2 Introduction

Alzheimer's Disease (AD) is an increasingly common form of dementia, which is now estimated to affect approximately 24 million people worldwide (1). Moreover, this number is predicted to have doubled twice by the year 2040. This very serious, ultimately lethal disease is widely studied for its pathology, but a definite cause or treatment is not yet defined. For decades now, it is known that AD is caused by accumulation of amyloid-beta (A $\beta$ ) peptides in the brain. The A $\beta$  protein is a 39 to 42 amino acid long peptide, derived from the amyloid-precursor protein (APP)(2). In the human brain, APP is normally present as a membrane-bound protein until it is cleaved by  $\beta$ - and  $\gamma$ -secretases respectively. In the familial or early-onset form of AD, investigators have identified a few genes that can be responsible for the development of AD when mutated. These genes are APP itself, presenilin-1 and presenilin-2, which are all involved in proper

APP processing. A mutation in one or more of these genes can result in an increased abundance of the 42 amino acid long A $\beta$  peptide (A $\beta$ 1-42) in contrast to the normally more prevalent 40 amino acid long peptide (A $\beta$ 1-40). In the most common form of AD, which is sporadic AD, no such mutation has been found as yet. This form of AD has no clear defect in APP processing, but current findings suggest that the physiological routes of A $\beta$  clearance from the brain might be disturbed (3). Defective clearance of A $\beta$  from the brain subsequently leads to an increase of A $\beta$  in the brain, which gives rise to the same symptoms and pathology as seen in early-onset AD.

A key characteristic of AD pathology is the formation of A $\beta$  aggregates in the brain. These so called plaques consist of self-aggregated insoluble A $\beta$  fibrils (fA $\beta$ ) that accumulate in larger complexes. As excellently reviewed by Ferreira & Klein (4), the general consensus until a decade ago was that these plaques were toxic to neurons, causing neuron death and ultimately cognitive impairment. Because these plaques were commonly surrounded by inflammatory cells of the brain, microglia and astrocytes, the observed neurotoxicity must have been due to an induced neurotoxic inflammatory response. First posed in 1992 (5), this 'amyloid cascade' hypothesis was based firmly upon prior research and continued to gather further support. But despite the numerous studies supplying further evidence in favor of the amyloid cascade theory, some research papers indicated a few serious caveats of the proposed mechanism. One of these publications reported to have measured the effect of A $\beta$  on learning tasks in a mouse model for AD (6). In this study, PDAPP mice were treated with a monoclonal antibody specific to monomeric A $\beta$  and subsequently tested for improvements in cognitive learning. Although treatment with the anti-A $\beta$  antibody was significantly correlated with an improvement in cognitive learning, no apparent decrease in A $\beta$  plaque burden could be measured.

These results clearly indicate that A $\beta$  is indeed related to the development of cognitive decline, but rule out amyloid plaques as being the toxic agent. In 1998, A $\beta$  was again found to correlate with AD pathology, but now in the form of smaller, soluble oligomers (7). These oligomers were shown to be highly neurotoxic, causing loss of long-term potentiation (LTP) and eventually cell death. From here onward, the oligomer theory was formulated (4). This new theory states that soluble A $\beta$  oligomers, rather than amyloid plaques, are responsible for loss of synaptic plasticity and therefore cause cognitive impairment in AD. What remains largely unclear, however, is why levels of A $\beta$  increase with age, and especially in AD.

## **2.1 Amyloid clearance**

In short, sporadic AD seems to be caused by neurotoxic A $\beta$  oligomers that tend to build up in the aging brain. This is most likely caused by a change in the balance between A $\beta$  production and degradation. Further evidence for the pathogenicity of a disturbed amyloid homeostasis is provided by cases of early-onset AD. This form of the disease is only responsible for 5 – 10 % of all AD cases, but is well characterized in terms of etiology. As already mentioned, a mutation in one or more of the genes regulating APP expression causes excessive APP production and thereby increased levels of A $\beta$  in early-onset AD (3,4). As A $\beta$  is now continuously produced in increased levels, one can imagine that clearance mechanisms cannot cope with the excessive amounts of A $\beta$ , thus triggering AD in early life. The same is true for patients with trisomy 21, who tend to develop high levels of neurotoxic A $\beta$  as well. Because the gene encoding APP is located on chromosome 21, these patients will carry an extra copy of this gene and will thus generate excess amounts of A $\beta$ .

However, it is not yet well understood how late-onset AD is caused, as no clear-cut genetic

predispositions can be found for this type of the disease. Although the APOE  $\epsilon$ 4 gene correlates with late-onset AD to some extent, it still only poses a risk factor (8). Nonetheless, a study from 2010 reports a dysfunctional A $\beta$  metabolism in patients suffering from late-onset AD. From the results presented by this study it is apparent that not A $\beta$  production, but A $\beta$  clearance is altered in the diseased brain (9). Evidently, this too would lead to excessive A $\beta$  levels, leading to pathological processes. Further evidence for the involvement of defective clearance mechanisms is provided by genome-wide screens, which identified multiple clearance related genes with the occurrence of late-onset AD (10). In regard to this apparent defect in amyloid metabolism, many studies have been conducted to elucidate the physiologic clearance of amyloid. As is reviewed by Miners *et al.* (3), three distinct routes have been identified by which A $\beta$  can be removed from the brain. These three routes are 1) A $\beta$  transport into the CSF or across blood vessel walls, thereby shuttling A $\beta$  towards the periphery where it can be broken down; 2) internalization of A $\beta$  by astrocytes and microglia, and 3) extracellular enzymatic degradation of A $\beta$ . Of these three routes, this review is focused on the role of enzymes in the degradation of A $\beta$ . In the last few years a lot of research has been carried out to describe how these enzymes degrade A $\beta$ , and with that investigators have described various factors that could influence this route of degradation. These factors will be discussed further on in more detail.

To study if a deregulation of A $\beta$ -degrading enzymes can indeed be an important factor in the development of AD, it is first required to know precisely which enzymes are involved in A $\beta$  degradation. In the same review of Miners *et al.* (3) as above, a comprehensive overview is given of the various enzymes implicated in A $\beta$  degradation. Because some enzymes seem to play a more significant role in the homeostasis of A $\beta$  than others

this review will focus mainly on the enzymes where most current research is focused upon. The enzymes discussed here are: 1) Neprilysin (NEP, or CD10) is a proteolytic enzyme that is expressed in a variety of neurons and glia cells in the human brain. The occurrence of NEP has been shown in pre- and postsynaptic membranes, the endothelium of cortical blood vessels and the surrounding tunica media. Expression on neurons is shown to play a role in regulation of neuropeptide signaling, while the expression around blood vessels plays a role in maintaining the vascular tone (3). Aside from degrading neuropeptides, NEP is also able to cleave A $\beta$ . Moreover, NEP expression is found in microglia and astrocytes that surround fA $\beta$  plaques in AD (11). 2) Insulin-degrading factor (IDE, insulysin) is, like NEP, mostly expressed on neurons, but likewise also in microglia and astrocytes. IDE is mainly a cytosolic protein, but can also be present in peroxisomes or on the plasma membrane. The most common ligand of IDE is insulin, but IDE is capable of degrading a variety of other proteins including A $\beta$  (3). 3) Matrix metalloproteinases (MMP) are zinc- and calcium dependent endopeptidases, also expressed in both microglia and astrocytes. The MMPs implicated in A $\beta$  degradation are MMP types 2, 3 and 9 (3).

## 2.2 Microglia and astrocytes

Because the brain is separated from the peripheral blood circulation by the blood-brain barrier, it can be difficult for some components of the immune system to reach a site of inflammation in the brain. To ensure that a fast immunological response remains possible in these tissues, the brain contains a separate population of immune effector cells. As mentioned in the previous section, some of these cells carry A $\beta$  degrading enzymes as well.

The key players of this population are the microglia, which are monocyte-derived phagocytes analogous to the peripheral macrophages. Under physiological conditions, microglia constitute a prominent role of

the innate immune system. Like tissue macrophages, microglia are scattered throughout the brain, with cellular processes protruding through the brain parenchyma (12,13). This position allows them to react very rapidly to any immunogen that is encountered. When an immunogenic agent presents itself, the microglial protrusions will recognize this trigger and microglia will migrate towards it's the place while giving rise to a proper immune response (13). This immune response includes the release of pro- and/or anti-inflammatory cytokines and receptor-mediated phagocytosis, but also the breakdown of extracellular material by proteolytic enzymes. In the AD affected brain, it is shown that microglia are also activated in this way by A $\beta$  fibrils (14). Thus, these cells are thought to play a role in the clearance of A $\beta$  from the brain.

Besides microglia, astrocytes are found near A $\beta$  plaques as well. This close association between A $\beta$  plaque formation and astrocyte localization suggests a migratory effect of these cells, likely triggered by chemotaxins which are released near the A $\beta$  plaques (15). Under physiological conditions, astrocytes are widely distributed throughout the brain and make up for a considerable fraction of the brain's volume. Such large numbers of astrocytes are vital for maintaining normal brain function, since astrocytes play an important role in neurogenesis, intercellular signaling, inflammation and brain metabolism (16). Of greatest importance for this review are the metabolic and inflammatory processes executed by these cells. Firstly, pathogens can, like in microglia, trigger the production of inflammatory cytokines by astrocytes. In general this trigger will diminish as the pathogen is cleared, but the continuous presence of A $\beta$  in AD seems to evoke a continuous immune response (16). The implications of such a continuous immune response will be discussed later. Secondly, because astrocytes play an important role in the metabolism of

neurotransmitters, these cells carry a variety of peptide degrading enzymes. These enzymes include NEP, IDE and MMP's which, aside from their role in neurotransmitter metabolism and neural inflammation, are also capable of degrading A $\beta$ .

Now that it is established that both microglia and astrocytes are involved in the process of A $\beta$  clearance, the question arises if a deregulation in the expression of proteolytic enzymes contributes to the development of AD. To answer this question, multiple investigators have studied enzyme expression in microglia and astrocyte populations in relation to AD related factors. The next chapter will summarize the key findings in this emerging field of research, to provide an overview of the factors that influence differential activity or expression of A $\beta$ -degrading enzymes in both microglia and astrocytes. Because no studies have been carried out thus far that attempt to unveil all of these important factors, a review of studies focused on the individual factors may provide more insight into the complex network of processes that ultimately contribute to the development of AD. Therefore, through this summary of research, I will try to determine which AD related factors influence the expression or activity of A $\beta$  degrading enzymes in microglia and astrocytes.

Although it is not unimaginable that a complex process as the development of AD involves even more factors than will be discussed below, this review is focused on the role of three factors that seem to play a key part. Firstly, as the most important risk factor for sporadic AD is aging, the effect of age on the expression and activity of A $\beta$ -degrading enzymes will be discussed. Secondly, the following chapter will give an overview of the effects of A $\beta$  itself. Evidently, if an increase in A $\beta$  levels might give rise to further deregulation of A $\beta$ -degrading enzymes this might result in a positive feedback loop. Such positive feedback might in turn

explain why A $\beta$  buildup gradually becomes overwhelming, leaving no way for the brain to restore homeostasis. Lastly, several inflammatory processes play an important role in AD pathogenesis (17). Local upregulation of cytokines, chemokines and other inflammatory substances is found in the AD brain (15) and specifically around amyloid plaques. The effect of these inflammatory substances on amyloid degrading enzymes, with a special focus on IL-4, IL-13, TNF- $\alpha$  and TGF- $\beta$ 1, will be reviewed and summarized. In the following chapter an attempt was made to focus on the effects exhibited by glial cells. Although most studies reported measurements in brain homogenates, and are therefore not cell type specific, the choice was made to continue this focus because both microglia and astrocytes seem to play important roles in A $\beta$  clearance mechanisms. The few studies that do look at a specific cell type are mostly aimed at microglia, and consequently this emphasis is continued below.

### **3 Differential protease activity and expression**

#### **3.1 Age-dependent enzyme expression**

Shortly after neprilysin (NEP) was implicated as being a potential A $\beta$ -degrading enzyme (18,19), J Apelt *et al.* reported the effects of aging on NEP mRNA and protein levels (20). In this study, mRNA expression levels in cortical tissue were shown to be unaffected by aging in mice until the age of 17 months. After this period, at 20 months of age, mRNA levels decreased significantly in both wild-type C57B/6 and Tg2576 mice, the latter used as AD mouse model. These results were, however, not accompanied by a parallel decrease in NEP protein levels. Other studies, however, did report a correlation between aging and a significant

decrease of NEP activity in brain regions commonly associated with AD pathology (21,22). One of these studies measured NEP activity and concentration in the neocortex, hippocampal formation, cerebellum and thalamus/striatum of C57B/6 mice aged 2.5, 20 and 33 months (21). The neocortex and hippocampal formation were considered to be the regions mostly affected by AD, while the cerebellum and thalamus/striatum generally show less plaque formation when NEP activity is disturbed. In line with the first study discussed, the results indicated no decrease of NEP protein levels or activity in the neocortex. However, NEP protein levels were significantly reduced after 33 months in the hippocampal formation. Furthermore, measurements of activity indicate that while NEP levels remain constant in the neocortex and hippocampal region, NEP activity is significantly diminished after 33 months. NEP activity in the thalamus/striatum is significantly increased. NEP activity in the cerebellum remains at a relative low level, and shows no alteration. These results point out that age-dependent decline of NEP expression is only present in those regions where plaque formation is most prominent in AD pathology. Because actual AD pathology was not yet present in the tested mice, the authors conclude that a decrease in NEP levels due to normal aging might be the cause of toxic A $\beta$  deposition in the development of AD.

Notwithstanding these promising results, which seem to explain why increasing age is such a high risk factor for AD, the fact remains that all measurements are obtained from brain tissue homogenates. Therefore, no differentiation is possible between the cell types where NEP expression is decreased. In addition, many immunohistochemical studies focus solely on the expression of A $\beta$ -degrading enzymes in specific brain regions, while the various cell types are not specifically identified. As is mentioned above,

microglial cells and astrocytes are shown to be clustered around fibrillary A $\beta$  plaques and are implicated as major players in the clearance of both soluble and insoluble A $\beta$ . It would therefore be very interesting to determine how aging affects NEP protein levels and enzymatic activity in those cell types. A very comprehensive study which focused on just that topic was published in 2008. This study tested for the expression of A $\beta$  receptors, A $\beta$  degrading enzymes and inflammatory cytokines in microglia (23). The microglia were obtained from whole brains of PS1-APP mice in various stages of aging and A $\beta$  plaque formation and isolated using fluorescence-activated cell sorting. In contrast to the observations in brain homogenates, the results of this study show a significant age-dependent reduction of NEP mRNA levels at an age of 14 months, specific to microglia. In fact, mRNA levels were reduced to only 20% of the concentration in WT control animals. Additionally, a likewise reduction of mRNA levels was shown for the A $\beta$ -degrading enzymes IDE and MMP-9. These results suggest a very dramatic reduction in the microglial production of three major A $\beta$ -degrading enzymes, which could lead to a severe disturbance of A $\beta$  clearance processes. Some reservations regarding this study are in place, however, as only the levels of mRNA are measured without regard to regulation at the level of translation. The results of this study would therefore be much more conclusive if a measurement of protein levels and activity were to be included. Moreover, this study was again performed only in a mouse model, not in human tissue. It is therefore not necessarily so that these results are representative for human AD as well.

These final caveats of the abovementioned studies were partly addressed by other investigators. Specifically, an effort was made to confirm these findings in human brain tissue (24). This study measured mRNA and protein levels in the frontal- and temporal cortex in age-matched AD affected

and non-demented individuals. The results of these measurements indicate significant decrease in NEP protein levels with age in both AD patients and non-demented individuals, without an effect on mRNA levels. Furthermore, the rate by which NEP protein levels decrease are the same for both AD patients and non-demented controls. These results thus confirm the age-related decrease of NEP protein levels in humans. But since NEP levels decrease at a similar rate regardless of cognitive impairment, the results also show that this process is not solely responsible for the development of AD. The authors conclude with the statement that pathological accumulation of A $\beta$  in AD is likely a cumulative effect of various factors, which might be triggered by the age-dependent decline of NEP protein levels. Two years later, however, a study was published with seemingly opposite results. In that study, age-related NEP and IDE protein levels and activities were measured in the human mid-frontal neocortex, next to the same measurements for A $\beta$  generating  $\beta$ -secretase-1 (BACE-1) (25). In contrast to the study from 2008, NEP and IDE protein levels were not found to be significantly different with increased age. The activity of both enzymes, however, significantly increased in patients older than 60 years, and was correlated with age. In addition, the increase of activity was correlated with an increase of BACE-1 activity, possibly suggesting some kind of connection between the expression patterns of both groups of enzymes. Although the authors of the latest article recognize that their findings contradict previous research, they do not provide a clear explanation for this difference. They do, however, conclude that previous notions of decreasing A $\beta$ -degrading enzyme levels as a cause of AD must be incorrect. Further evidence even indicates that differences in enzyme activity could be caused by altered A $\beta$  levels, but this should be further investigated. More details about the effect of A $\beta$  on A $\beta$ -degrading enzymes will be discussed below.

### 3.2 Cytokine-influenced enzyme expression

In 2003, it was observed that the clustering of microglia around fA $\beta$  plaques occurring in AD is mediated by cell surface receptors (26). Although the theory of AD pathogenesis at that time is now outdated, the evidence on the role of receptors in microglial activation holds up. The study performed in 2003 additionally indicated that the receptor CD36 is required for inducing the production of microglial TNF- $\alpha$ , IL-1 $\beta$  and reactive oxygen species (ROS). Thereby, CD36 triggers the major inflammatory response of microglia to fA $\beta$ , which likely makes it an important factor in AD disease progression (26). Because increased levels of TNF- $\alpha$  have been implicated in the early stages of AD (27), Hickman *et al.* studied if these increased cytokine responses are age-related in a similar way to NEP expression. Their results indicated that continuous activation by A $\beta$ 1-42 maintains the microglial pro-inflammatory response during aging, while levels of A $\beta$ -degrading enzymes decrease (23). Specifically, TNF- $\alpha$  and IL-1 $\beta$  were increasingly higher expressed with age. However, this study was performed using A $\beta$  monomers, not with fA $\beta$ . And since fA $\beta$  specifically attracts microglia and astrocytes, it might be that these aggregates cause different effects. Further evidence was provided by a study from the same year, which investigated the response of microglia from PS1<sup>M146L</sup>xAPP<sup>751SL</sup> mice to fA $\beta$  and A $\beta$  oligomers separately (28). Interestingly, this study reported the absence of pro-inflammatory factors, including TNF- $\alpha$ , in the vicinity of microglia and astrocytes surrounding fA $\beta$  plaques. In later stages of AD progression, at 18 months in mice, a general heightened expression of pro-inflammatory factors was found in the brain. These factors were defined as TNF- $\alpha$ , FASL, TRAIL, Cox2 and Nox1. TNF- $\alpha$  expression remained, however, absent in microglia surrounding fA $\beta$  plaques.

Because of the change in environment caused by a variety of inflammatory cytokines, it is a logical assumption that these cytokines might play a role in AD progression. After all, microglia and astrocytes in this environment are shown to play an important role in A $\beta$  clearance, and cytokines might affect gene expression patterns in such a way that they alter the expression levels of A $\beta$ -degrading enzymes as well. For this reason, several studies have investigated the effect of various cytokines on the expression of A $\beta$ -degrading enzymes. In a very comprehensive study performed by Shimizu *et al.* (11), rat microglia were harvested from primary mixed microglial cell cultures and subsequently incubated with anti-inflammatory cytokines IL-4 and IL-13, and pro-inflammatory cytokines TNF- $\alpha$  and TGF- $\beta$ 1. Afterwards, protein levels were measured with an immunoblot assay. In addition, an experiment was included where microglia were incubated with A $\beta$ 1-42 oligomers to confirm the roles of NEP and IDE in A $\beta$  clearance. This experiment was carried out both with and without enzyme-specific inhibitors, in both presence and absence of TNF- $\alpha$ . Because microglia, like macrophages, can exist in different activated states, also a distinction was made between the effects of cytokines on M1 or M2 type microglia. Interestingly, the results of this study indicate that NEP expression can be induced by IL-4 and IL-13 and inhibited by TNF- $\alpha$  and TGF- $\beta$ 1. IDE expression levels were increased by IL-4, TGF- $\beta$ 1 and somewhat less by IL-13 and TNF- $\alpha$ . The additional measurements regarding A $\beta$ 1-42 oligomer clearance show that inhibition of either NEP or IDE leads to decreased oligomer breakdown *in-vitro*, with no significant difference of effect in the presence of TNF- $\alpha$ . Furthermore, this study reports a difference in cytokine induction characteristics between M1 and M2 type microglia. Strikingly, only M2 type cells showed active degradation of A $\beta$  after induction by IL-4, and thereby a considerable greater neuroprotective effect than M1 type cells did. These

findings were in line with earlier findings that M2 type microglia express higher levels of IL-4R on their surface compared with M1 type microglia (11).

Further evidence to support the abovementioned findings is provided in a study which reported the effects of pro- and anti-inflammatory cytokines on the degradation of A $\beta$  by human monocyte-derived macrophages (MDMs) and microglia (29). The investigators found that IL-4, IL-10, and TGF- $\beta$ 1 enhanced degradation of fA $\beta$ 1-40 and fA $\beta$ 1-42 by both MDMs and microglia, while IFN- $\gamma$  inhibited degradation. Additionally, fA $\beta$  degradation was unaffected by specific inhibitors of NEP or the proteasome, but was affected by lysosomal and IDE inhibitors. Further experimentation also indicated IFN- $\gamma$  and TNF- $\alpha$  as direct inhibitors of IDE expression. Subsequent incubation of MDMs with activated T cells indeed resulted in a reduced A $\beta$  clearance compared to the effect of naïve T cells, and this inhibition of A $\beta$  clearance could partially be countered by neutralizing antibodies against pro-inflammatory cytokines. These results suggest, in line with the study above, that A $\beta$  clearance by MDMs and microglia is inhibited by pro-inflammatory cytokines, while certain anti-inflammatory effects counter this inhibition.

### **3.3 Effect of A $\beta$ on the expression and activity of amyloid-beta degrading enzymes**

As is discussed in the previous section, A $\beta$  can trigger the production of certain cytokines, and these cytokines can in turn affect the expression or activity of A $\beta$ -degrading enzymes. It is, however, not unthinkable that A $\beta$  itself can influence the expression or activity of those enzymes directly. An interesting finding which seemingly illustrates this hypothesis is the apparent expression of NEP in astrocytes surrounding fibrillary A $\beta$  plaques (30). Further evidence is provided by a recent study of Wang *et al.*, which included measurements that

indeed seemed to indicate an influence of A $\beta$  on the expression of A $\beta$ -degrading enzymes. This study measured the enzyme expression and activity in the human frontal cortex (31), but again used brain homogenates, not extracted microglia. To differentiate between the different stages of A $\beta$  deposition, and thus clinical features, experiments were done in brain tissue of ten healthy individuals, ten patients suffering from mild cognitive impairment and ten suffering from severe AD. The enzymes that were measured in this study were NEP and IDE. Like earlier studies, mRNA levels were measured using RT-PCR and protein levels were measured by immunoblotting. Enzyme activity was measured using a highly specific fluorescence assay. Furthermore, this study correlated the extent of plaque deposition with changes in enzyme expression and activity. In line with the results obtained in mice, NEP mRNA levels were decreased in patients suffering from AD. This decrease was continued in NEP protein levels and enzyme activity. Furthermore, this decrease in NEP levels was inversely correlated with the increase in A $\beta$  levels as seen in healthy controls and patients suffering from mild cognitive impairment. The same correlation was not found in patients suffering from AD, but that might be due to a lack of information because AD samples always demonstrated high A $\beta$  load with low NEP protein levels. The reducing effect of A $\beta$  on NEP mRNA and protein levels reported by Wang *et al.* is backed up by earlier reports that measured NEP mRNA and protein levels in relation to A $\beta$  load in the brain (32). Although these earlier studies usually appointed decreasing NEP levels as the cause of increasing A $\beta$  load, the measured relationship between A $\beta$ - and NEP levels remains constant. In contrast with the study by Hickman *et al.* in 2008, however, the abovementioned study does not show the same effect on IDE mRNA levels (31). Where IDE mRNA levels were shown to be significantly reduced in whole brain homogenates, Wang *et al.* reports a significant increase in IDE

mRNA levels. Although a similar increase in protein levels and activity was seen, this effect did not reach significance and no correlation was found between A $\beta$  and IDE levels.

Among the direct effects of A $\beta$  is also an upregulation of MMP-9. Although this enzyme is somewhat less studied compared to NEP and IDE, it has been implicated in AD progression very early in the line of research. Moreover, a direct effect of A $\beta$  on the expression of MMP-9 was reported already in 1996 (33–35). In these studies, long A $\beta$ 1-40 fragments were shown to induce increased MMP-9 expression in astrocytes, whereas shorter A $\beta$ 1-40 fragments induce a less strong effect (36). The same mechanism was shown to induce increased MMP-9 expression in rat microglia (37).

Another interesting study that was recently published provides new insights into the influence of A $\beta$  on A $\beta$ -degrading enzymes. This study investigated the effects of fA $\beta$  and oligomeric A $\beta$  in combination with a variety of amyloid associated proteins (AAPs) on the expression of A $\beta$ -degrading enzyme mRNA in astrocytes (38). Reportedly, astrocytic NEP and IDE expression of both non-demented individuals and patients suffering from AD is unaffected by each form of A $\beta$  on itself. Only after addition of certain AAPs do astrocytes of healthy donors demonstrate a heightened expression of NEP and IDE, but this increase is not seen in astrocytes from AD affected brains. Thus it seems that measuring the effects of A $\beta$  on cells *in-vitro* with synthetic A $\beta$  does not yield a representative view of the actual processes. Because AAPs are present in the AD affected brain, any significant influences of these proteins are lost to typical *in-vitro* studies but might contribute to the actual pathological processes.

In the previous chapter, various studies were discussed which investigated the influence of cytokines on the expression of A $\beta$ -degrading

enzymes. The current trend that comes forward in this research is that pro-inflammatory cytokines have a detrimental effect on the effectivity of A $\beta$  clearance, while anti-inflammatory cytokines work to retain a balance in A $\beta$  metabolism. According to a report in 2008, this increase in pro-inflammatory factors correlated closely with oligomeric A $\beta$  complexes, which were present in numbers a 10-fold higher after 18 months compared to 12 months of age (28). This led to the hypothesis that oligomeric A $\beta$  activates microglia into a pro-inflammatory state, while fA $\beta$  activates them into an alternative, anti-inflammatory state. In support of this hypothesis, oligomeric A $\beta$  was shown to induce a strong TNF- $\alpha$  response in microglia (28). Further investigation continued to support these findings, as oligomers were shown to induce a significantly higher expression of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  *in vitro* than A $\beta$  fibrils did (39). Although this last study was performed in microglia of newborn mice and cytokine levels were only measured at mRNA level, the results point towards the more modern idea that microglia in the brain are activated in different states, depending on their localization. Early exposure of microglia to fA $\beta$  may therefore induce a neuroprotective anti-inflammatory response, while the accumulation of A $\beta$  oligomers in later disease stages drive towards a more pro-inflammatory activation state.

## 4 Discussion

The general understanding of AD pathology has made great progress over the last decades. In the past, it was believed that the presence of fA $\beta$  plaques in the brain were sufficient to cause neurotoxic effects. Current evidence, however, points towards a more prominent role of soluble A $\beta$  oligomers. These A $\beta$  oligomers were shown to suppress LTP and ultimately to cause neuron death (40). Although direct neurotoxic effects of fA $\beta$  have thus been invalidated, fA $\beta$  does seem to play a

certain role in mounting an inflammatory response in the brain which, when chronically active, can be detrimental for neurons and the surrounding tissue. In any case, it is well established that increased deposition of A $\beta$  in the brain contributes to AD onset and progression, but the reason for these increased levels of A $\beta$  is not yet clear. In sporadic AD, it is hypothesized that a defective clearance of A $\beta$  may cause an increase of A $\beta$  levels in the brain, which eventually leads to AD (41). As discussed in the previous chapters, A $\beta$ -degrading enzymes contribute to a large extent to A $\beta$  clearance from the brain. It is therefore thought that environmental factors can influence the expression or activity of A $\beta$ -degrading enzymes, thus causing the apparent decrease in A $\beta$  clearance. In this review we discuss key studies regarding the factors that influence expression and activity of A $\beta$ -degrading enzymes, to provide an overview of the current knowledge on this topic. Here we focused on the potential influences of aging, inflammatory factors and A $\beta$  itself on RNA, protein and activity expression of NEP, IDE and MMP-9.

A striking finding concerning the effects of increasing age on the expression levels of A $\beta$ -degrading enzymes in glial cells is that different studies report both a decrease (21,23,24,41) and increase (25,30) of protein activity. Although no direct reason for these paradoxical results is apparent from the studies themselves, an explanation might be found in studies involving cytokines and A $\beta$ . As described above, recent studies that focused on the effects of cytokines on A $\beta$ -degrading enzymes indicate that only microglia in an anti-inflammatory state are capable of A $\beta$  degradation by NEP and IDE (11). Thus, an anti-inflammatory state with typical IL-4 and IL-13 production favors enzymatic break-down of A $\beta$ , while a pro-inflammatory state with TNF- $\alpha$  and IL-1 $\beta$  inhibits A $\beta$  degradation. Based on these findings, two ways can be imagined by which AD could be

induced; either by an increase of pro-inflammatory, or a decrease of anti-inflammatory processes.

As described above, A $\beta$  oligomers have been shown to induce microglial production of pro-inflammatory cytokines (28,42), while fA $\beta$  and monomers may induce a counteracting anti-inflammatory response (38,43). Moreover, the further diminishing cognitive function of AD patients has been correlated with a quite sudden rise in A $\beta$  oligomers (28), which further implicates a dysfunctional balance in pro- and inflammatory cytokines in the progression of AD.

The question remains, however, how this reported imbalance of the inflammatory responses is caused at first onset of AD. A possible explanation for the primary development of this imbalance is provided by the study of Mulder *et al.*, published last year. As reported, this study found that AAPs play a vital role in the direct response to fA $\beta$ , since they are necessary for the direct upregulation of A $\beta$ -degrading enzymes. Both NEP and IDE mRNA expression was found to be increased in astrocytes from non-demented donors, but patients suffering from AD did not demonstrate this same reaction (38). It could therefore be that A $\beta$ -oligomer induced pro-inflammatory processes are kept in balance by the anti-inflammatory effects of fA $\beta$ , thereby maintaining the appropriate expression level of A $\beta$ -degrading enzymes. In patients that ultimately develop AD this regulating reaction to fA $\beta$  is not exhibited properly, and therefore levels of A $\beta$ -degrading enzymes drop. This in turn results in heightened levels of oligomeric A $\beta$ , and subsequently an increase of pro-inflammatory factors. The predominantly pro-inflammatory environment which follows facilitates the further drop in A $\beta$ -degrading enzyme levels, and maintains the imbalance of inflammatory factors.

Although this theory builds on the theory that AD progression is continued by a feedback loop (44,45),

it does not provide an explanation for the primary build-up of  $fA\beta$  in the brain. With that the definite cause of AD is not yet clear, but most experts agree that aging must play a critical role in the onset of the disease. Notwithstanding the various studies that are reviewed in this thesis, many uncertainties remain around the interplay between aging and  $A\beta$ -degrading enzymes. Thus, although various studies implicated some influence, a precise conclusion to the role of these enzymes in disease onset cannot be given. With some caution, however, it can be said that  $A\beta$ -degrading enzymes seem to exert some influence on the development of AD from a very early stage. If it is indeed so that  $A\beta$ -degrading enzyme levels decrease with age, this might lower the number of enzymes to a point where clearance mechanisms are barely in equilibrium with  $A\beta$  production. The body may try to correct for this drop in  $A\beta$  clearance by increasing production of  $A\beta$ -degrading enzymes in response to  $fA\beta$  (31), but for some reason this is not effective in patients that will eventually develop AD (38). Since it has been shown that moderate cases of systemic infection can cause mild cognitive disorders in elderly patients (17), it might just be that a slight increase in pro-inflammatory cytokines in this situation lowers expression of  $A\beta$ -degrading enzymes beneath the threshold. The resulting excess of  $A\beta_{1-42}$  oligomers subsequently initiates a negative feedback loop and prevents the re-establishment of enzyme levels, thus giving rise to AD.

In conclusion, current evidence suggests that levels of  $A\beta$ -degrading enzymes both influence and are influenced by processes concerning AD onset and progression. Because of this intricate interplay between different processes, investigators have yet to elucidate what processes are either causes or consequences during AD progression. In regard to the question asked in the introduction of this paper, this means that it is not always easy to say what factors influence  $A\beta$ -degrading enzyme levels in glial

cells, or what factors are influenced themselves. Because some level of understanding is necessary to direct future research, however, it is important to summarize what is known nowadays about the influences on  $A\beta$ -degrading enzymes. Firstly, the effect of aging on the expression of  $A\beta$ -degrading enzymes is not yet certain. Although an increasing number of studies indicate some influence of aging, more in-depth research is needed to elucidate the exact process. Secondly, the effect of cytokines on the expression of  $A\beta$ -degrading enzymes is better determined. Although cytokines always have a more general influence on tissue than the expression of a few genes, pro-inflammatory cytokines  $TNF-\alpha$  and  $TGF-\beta_1$  lower the expression of  $A\beta$ -degrading enzymes in microglia while anti-inflammatory cytokines IL-4 and IL-13 counteract this effect. Cytokines are therefore considered to be important mediators in AD progression, and further understanding of these molecules and their workings could turn out to be valuable for new therapies. Thirdly,  $A\beta$  itself is shown to have a direct effect on  $A\beta$ -degrading enzyme expression as well. Since microglia and astrocytes migrate towards  $fA\beta$  plaques, these cells are able of direct interaction with these plaques, which heightens their expression of NEP, IDE and MMP-9. Recently, however, it has been shown that astrocytes of AD patients do not exhibit this response to  $fA\beta$ . It might thus be that a dysfunction in this mechanism is responsible for the progression from mild cognitive impairment to degenerative  $A\beta$ . Lastly, a link was discussed between the effects of  $A\beta$  and cytokines on the expression of  $A\beta$ -degrading enzymes. Because  $A\beta$  oligomers were shown to induce a more pro-inflammatory immune response, a further decrease in expression of  $A\beta$  clearance could cause the establishment of a negative feedback loop. Because such a feedback loop gets increasingly more destructive, that would explain the unstoppable degenerative nature of AD,

Since a better understanding of the processes that trigger AD could ultimately lead to a better treatment or even prevention of the disease, it could well be valuable to investigate this hypothesis in further research. As mentioned above, much data regarding the effects of aging on proteolytic enzyme expression is either just available for AD mouse models or studied in human brain homogenates. An extensive study of the effects of aging and AD on the microglial expression and activity of NEP, IDE and MMPs may further insights into the delicate processes that lay at the base of AD development. Furthermore, although Shimizu *et al.* (46) studied the effects of cytokines on enzyme expression extensively, these results remain yet to be verified. In addition, a measurement of localized cytokine expression in the AD affected brain could help to determine what factors are actually present in the environment of microglia, and thus what activation states could arise *in vivo*. Finally, the induction of various microglial activation states by A $\beta$  remains not well characterized. By studying gene expression levels in human microglia specifically located near A $\beta$  plaques or in an A $\beta$  oligomer rich environment, the characteristics of different activation states can be better identified.

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