Neuroinflammation in Early, Pre-Clinical Stages of Alzheimer's Disease: Evidence from a New Transgenic Model of Alzheimer's Disease-like Amyloid Pathology

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This thesis is dedicated to my mother

Abstract

Despite much advancement in our understanding of its pathobiology, there is no cure for Alzheimer's disease (AD), a devastating neurodegenerative disorder affecting more than 35 million people world-wide. At diagnosis, the brains of AD patients are already severely damaged and display massive accumulation of intraneuronal neurofibrillary tangles and extracellular amyloid plaques. Amyloid plaques are composed of aggregated, insoluble amyloid beta peptide (Aβ), which is known to be highly neurotoxic. At this stage, AD drugs can only delay, but not arrest, the progression of the disease, likely because the neuronal damage is already beyond rescue.

It is currently accepted that, for any pharmacological agent to achieve efficacy, the treatment should be initiated prior to extensive central nervous system (CNS) damage, possibly in pre-clinical stages. A better understanding of the earliest events occurring in the pre-clinical phase of the disease is therefore a priority. Unfortunately, the study of pre-clinical stages in AD is complicated by the lack of biomarkers signalling the conversion from non cognitive impairment to AD. On the other hand, transgenic (Tg) models of the AD-like amyloid pathology represent a very suitable model to investigate the progression of the disease.

In these studies, we took advantage of our newly generated Tg model of AD-like amyloid pathology, coded McGill-Thy1-APP mice to identify early pathological events preceding plaque deposition. We first examined the cognitive status of the animals and demonstrated that cognitive impairments occur prior to plaque deposition. Such deficits were associated with a paradoxical up-regulation of cholinergic pre-synaptic boutons. Prior to plaque deposition we also described the occurrence of intraneuronal Aβ-immunoreactive material. Furthermore, using oligomeric specific antibodies, we found that the intraneuronal Aβ material was in large part composed of Aβ-oligomers.

To further elucidate the mechanisms involved in the early neuronal dysfunction, we characterized the occurrence of inflammation in young, pre-plaque mice. We started by confirming the occurrence of the well known, plaque-associated microglial activation in old, 13-14 months old Tg mice. In young, pre-plaque mice we observed a moderate, but significant up-regulation of inflammatory enzymes (iNOS, inducible nitric oxide synthase) and membrane bound receptors (CD40 and MHC-II); the neuronal marker COX-2 was also found to be up-regulated. Furthermore, we observed that microglial cells at this age display an activated morphology and are specifically associated with Aβ-oligomers burdened neurons.

Finally, the anti-inflammatory drug minocycline was administered to 2-month old mice for one month, till the age of 3 months, thus in the absence of any amyloid plaque deposition. This protocol allowed us to investigate the role of inflammation in the early, pre-plaque stage of the disease. We found that, besides correcting neuroinflammation, minocycline significantly reduced the levels of amyloid precursor protein and APP-immunoreactive fragments, including C-terminus fragments (CTF). Furthermore, we observed that minocycline reduced the levels and activity of BACE, which were up-regulated in Tg animals compared to Non Tg.

Taken together, our results showed that intracellular accumulation of Aβ-oligomers *per se*, prior to plaque deposition, is sufficient to trigger a number of CNS alterations. Amongst these, microglial activation appears to be, at least in its earliest manifestation, a key player in APP processing. We suggest that neuroinflammation in pre-clinical stages of AD might represent a suitable target for the discovery of novel preventive agents and/or early diagnostic markers.

Résumé

Bien que la compréhension des mécanismes pathologiques de la maladie de Alzheimer (MA) a grandement progressé, il n'existe aucun remède pour cette maladie neurodégénérative dévastatrice, qui attaque plus de 35 millions de personnes dans le monde entier. Lorsque cette condition est diagnostiquée, le cerveau des patients atteints de MA est déjà gravement endommagé et montre l'accumulation d'enchevêtrements neurofibrillaires intracellulaires et des plaques amyloïdes extracellulaires. Les plaques amyloïdes sont composées d'agrégats insolubles de la protéine beta amyloïde (Aβ) qui est neurotoxique. À ce stade de la pathologie, le dommage neuronal est tellement élevé que les médicaments ne peuvent pas arrêter sa progression, mais seulement la ralentir.

Il est actuellement admis que, pour obtenir un résultat thérapeutique, il faudrait commencer le traitement pharmacologique dans les premiers stades de la maladie, lors de la période pré-clinique, avant que les dommages neuronaux deviennent irrécupérables. Une meilleure compréhension des événements survenant dans ces premiers stades (pré-cliniques) de la maladie est donc une priorité. Malheureusement, l'étude de ces stades pré-cliniques est compliquée par l'absence de biomarqueurs indiquant la conversion du stade sans troubles cognitifs à MA. Ainsi, les modèles transgéniques (Tg) de la pathologie amyloïde similaires à MA représentent des modèles très appropriés pour étudier l'évolution de la pathologie amyloïde.

Afin d'étudier les premiers événements pathologiques survenant avant l'accumulation des plaques, nous avons profité d'un modèle Tg nouvellement généré dans notre laboratoire et nommé McGill-Thy1-APP. Nous avons d'abord examiné l'état cognitif des souris Tg et nous avons démontré que les troubles cognitifs précèdent l'accumulation des plaques. Nous avons aussi démontré qu'une augmentation paradoxale de boutons cholinergiques corticaux

accompagnait les troubles cognitifs chez ces animaux. Avant l'apparition des plaques, nous avons décrit la survenance de matériel intraneuronal immunoréactif pour l'Aβ. Par ailleurs, en utilisant des anticorps spécifiques pour les formes oligomériques, nous avons constaté que le matériel intraneuronal est en grande partie composé d'oligomères de l'Aβ.

Pour élucider si les mécanismes impliqués dans la dysfonction neuronale précèdent l'accumulation de plaques, nous avons caractérisé l'apparition de l'inflammation chez les jeunes souris Tg, avant qu'elles montrent des plaques (pré-plaque). Nous avons d'abord confirmé l'apparition de l'activation microgliale associée avec les plaques amyloïdes chez les vieilles souris Tg de 13-14 mois. Chez les jeunes souris pré-plaques, nous avons observé une augmentation modérée mais significative des enzymes (iNOS, synthase inductible de l'oxyde nitrique) et des récepteurs de membranaires (CD40 et CMH-II) inflammatoires; le marqueur neuronal COX-2 a également été trouvé élevé. Par ailleurs, nous avons observé que les cellules microglies à cet âge affichent une morphologie activée et sont spécifiquement associées à des neurones contenant des oligomères de l'Aβ.

Enfin, la minocycline (un médicament anti-inflammatoire) a été administrée à des souris âgées de deux mois pour un mois (jusqu'à l'âge de 3 mois) donc en l'absence de tout dépôt de plaques amyloïde. Ce protocole nous a permis d'étudier le rôle de l'inflammation dans les premiers stades de la maladie, avant le dépôt de plaques. Nous avons constaté que, outre la correction de la neuroinflammation, la minocycline réduit considérablement les niveaux de la protéine précurseur de l'amyloïde (APP) et les fragments connexes avec l'APP, y compris les fragments du carboxy terminaux. Par ailleurs, nous avons observé que la minocycline réduit les niveaux et l'activité de BACE.

Ensemble, nos résultats montrent que l'accumulation intracellulaire des oligomères de l'Aβ en soi, avant le dépôt de plaques, est suffisante pour

déclencher des modifications du SNC. Parmi ces derniers, l'activation microgliale semble avoir, du moins dans sa première manifestation, un rôle fondamental dans le métabolism de l'APP. Nous suggérons que la neuroinflammation dans les stades pré-cliniques de la MA pourrait représenter une cible appropriée pour la découverte de nouveaux agents préventifs et/ou de marqueurs diagnostiques.

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List of Abbreviations

Acetylcholine (ACh)

APP intracellular domain (AICD)

Alzheimer's disease diffusible ligands (ADDLs)

Alzheimer's disease (AD)

Amyloid precursor protein (APP)

Amyloid-β peptide (Aβ)

Amyotrophic Lateral Sclerosis (ALS)

Beta amyloid converting enzyme (BACE)

Blood brain barrier (BBB)

Central Nervous System (CNS)

Cerebrospinal fluid (CSF)

Cholinesterase inhibitors (ChEI)

C-terminus fragment (CTF)

Cyclic AMP activated element (CRE)

Cyclooxygenase 2 (COX-2)

Dentate gyrus (DG)

Down's syndrome (DS)

Experimental autoimmune encephalomyelitis (EAE)

Familial Alzheimer's disease (FAD)

Frontotemporal Dementia (FTD)

Full length amyloid precursor protein (flAPP)

Functional magnetic resonance imaging (fMRI)

Glial fibrillar acidic protein (GFAP)

Immunoblot (IB)

Immunohistochemistry (IHC)

Immunoreactivity (IR)

Inducible nitric oxide synthase (i-NOS)

Inhibitor of NFkB (IkB)

Insulin degrading enzyme (IDE)

Interleukin-1 beta (IL-1β)

Intracellular amyloid-β peptide (i-Aβ)

Intraperitoneal (i.p.)

Ionized calcium binding adaptor molecule 1 (Iba-1)

Lipopolysaccharide (LPS)

Long term potentiation (LTP)

Major histocompatibility complex class II (MHC-II)

Matrix metalloprotease 9 (MMP9)

Mild Cognitive Impairment (MCI)

Morris water maze (MWM)

Multiple sclerosis (MS)

Neprilysin (NEP)

Neurofibrillary tangles (NFT)

Nitric oxide (NO)

Non steroidal anti-inflammatory drugs (NSAIDs

Non-cognitively impaired (NCI)

Noradrenaline (NA)

Novel object recognition (NOR)

Nuclear factor kappa B (NFkB)

Polymerase chain reaction (PCR)

Pattern recognition receptors (PRR)

Peripheral nervous system (PNS)

Peroxisome Proliferator-Activated Receptor gamma (PPARγ)

Positron emission tomography (PET)

Presenilin (PS)

Receptor for advanced glycation end products (RAGE)

Sporadic Alzheimer's disease (SAD)

Transmission electron microscopy (TEM)

T helper lymphocytes (Th)

Trans Golgi network (TGN)

Transforming growth factor beta (TGF-β)

Transgenic (Tg)

Tumor necrosis factor alpha (TNFα)

Vesicular acetylcholine transporter (VAChT)

Western blot (WB)

Contribution of Authors

The Chapters 2-4 of this doctoral thesis describe work that was used for 3 individual publications.

Dr A. Claudio Cuello was the supervisor of my doctoral thesis and mentor throughout my graduate studies. He provided me with training and original ideas that were fundamental for the completion of the experimental work. For all publications, he offered intellectual guidance and edited the writing of the manuscripts.

Dr Martin A. Bruno contributed to the original ideas behind these studies

Adriana Ducatenzeiler offered technical assistance for immunohistochemical and biochemical assays and performed all the genotyping.

CHAPTER 2

"Transgenic mice as models of pre-clinical Alzheimer's disease"
Published in "Current Alzheimer Research" on February 1st 2011, by: Ferretti MT, Partridge V, Leon WC, Canneva F, Allard S, Arvanitis DN, Vercauteren F, Houle D, Ducatenzeiler A, Klein WL, Glabe CG, Szyf M, Cuello AC

Vanessa Partridge: provided technical assistance and training in perfusion, tissue processing and immunohistochemical stainings. Generated the preliminary observations on the temporal evolution of the amyloid pathology in the McGill-Thy1-APP mice; performed some of the immunohistochemical stainings with oligomeric specific antibodies.

Dr Wanda Leon: offered technical assistance and training in behavioral techniques and Western blotting; performed the Morris Water Maze test on young, 3 month old Tg mice.

Dr Fabio Canneva: performed the gene copy number analysis and contributed to interpretation of results.

Dr Simon Allard: performed the control experiments of Nu1 immunostaining in the absence of fixatives, offered assistance with confocal microscopy and provided helpful discussions.

Dr Dina Arvanitis: performed the quantification of APP over-expression in Tg animals compared to Non Tg.

Dr Freya Vercauteren: generated the original Thy1-APP construct under Drs Cuello and Szyf guidance.

Daniel Houle: was responsible of the microinjection for the generation of the transgenic animals.

Dr William Klein: provided us with the monoclonal antibody Nu1 and helpful discussions on intracellular Aβ-oligomers.

Dr Charles Glabe: provided us with the polyclonal antibody OC and helpful discussions on intracellular Aβ-oligomers.

Dr Moshe Szyf: contributed to the generation of the construct and provided intellectual guidance.

CHAPTER 3

"Intracellular Aβ-oligomers and early inflammation in a model of Alzheimer's disease"

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Dr William Klein: provided us with the monoclonal antibody Nu1 and helpful discussions on intracellular Aβ-oligomers.

CHAPTER 4

"Minocycline corrects neuroinflammation and inhibits BACE in a transgenic model of AD-like amyloid pathology"

In preparation, by: Ferretti MT, Allard S, Partridge V, Ducatenzeiler A, Cuello AC

Dr Simon Allard: provided technical assistance in dosing the animals and tissue processing; contributed significantly to data interpretation and discussion.

Vanessa Partridge: provided technical assistance in dosing the animals and performed the perfusions.

CHAPTER 1

General Introduction

1.1 Statement of the problem and purpose of the investigation

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the elderly and accounts for most cases of dementia diagnosed after the age of 60 (Holtzman, 2010). It is characterized by gradual cognitive dysfunction which eventually results in severe dementia and stupor. Its neuropathological hallmarks are the deposition of extracellular amyloid plaques and intracellular neurofibrillary tangles (Selkoe, 2001b).

Despite intense efforts from the scientific community and much advancement in our understanding of its biology and neuropathological implications, there is no cure for AD. Pharmacological treatment available to AD patients remains merely symptomatic and can only delay, but not arrest, the progression of the disease (Cuello, 2007). It is starting to be acknowledged that the limited therapeutic effect of current AD drugs could be due to the late clinical diagnosis of the disease, which is achieved when the neuronal function is irreversibly compromised and beyond therapeutic rescue (Becker et al., 2008).

Recent imaging and biochemical findings support the view that neuropathological events might precede clinical diagnosis and be disconnected from plaque deposition (for a review: Perrin et al., 2009).

Over the past 5 years imaging studies using positron emission tomography (PET) scanning and functional magnetic resonance imaging (fMRI) have cumulatively revealed that amyloid deposition (Morris et al., 2009), inflammation (Okello et al., 2009) and functional network alterations (Sperling et al., 2009) can be detected even decades before the appearance of clinically diagnosable dementia.

Similarly, careful biochemical analysis of the evolution of biomarkers indicated that the disease has a latent, pre-clinical, phase lasting for several years before AD onset (Jack, Jr. et al., 2010).

Seminal, biochemical studies have further indicated that neuronal dysfunction might be independent from plaque deposition. In particular, the discovery that soluble levels of amyloid beta peptide (Aβ), rather than plaque load, correlate with the severity of dementia (McLean et al., 1999) and that oligomeric forms of Aβ are neurotoxic species (Lambert et al., 1998), have opened a new, fertile field of investigation.

Since the accumulation of soluble Aβ precede plaque formation in time, these biochemical findings would concur with the *in vivo* imaging and biomarker findings in indicating that a pre-clinical, pre-plaque phase of AD pathology should exist.

Unfortunately, such an early, pre-plaque stage of the pathology cannot be easily investigated in humans, as there is no biomarker yet available to predict the conversion from normal, non-cognitively impaired individuals to AD. However, transgenic (Tg) mice -engineered to generate human Aβ peptide and that will invariably develop the full AD-like amyloid pathology- represent a very suitable model to study early events in the progression of the disease.

Accordingly, we investigated the early pathological events occurring in pre-plaque Tg mice, with a particular focus on the role of oligomers and inflammation in the progression of the disease. Thus, we characterized a novel human amyloid precursor protein (APP) Tg mouse model, generated in our laboratory, the McGill-Thy1-APP mouse. We then used this model to investigate the occurrence and the features of a pro-inflammatory reaction preceding plaques deposition but associated with Aβ-oligomers. Finally, to better understand the role of inflammation in early stages of the disease, we carried out a therapeutic experiment with the tetracyclic anti-inflammatory and neuroprotective drug minocycline.

1.2 Introduction to AD neuropathology and current therapeutics

1.2.1 Clinical progression of AD

Criteria for the clinical diagnosis of dementia and AD-related dementia have been recently reviewed (McKhann et al., 2011); briefly, dementia is established based on the presence of impairments in memory and an additional domain in cognitive function, to the extent that it interferes with one's occupational or social function. Delirium or major psychiatric disorders must be excluded. Furthermore, since dementia can have several, non-AD causes, the diagnosis of *probable* AD-related dementia is made by exclusion of other possible conditions (dementia with Lewy bodies, vascular dementia, behavioral variants of frontotemporal dementia and primary progressive aphasia). It is important to know that the definitive diagnosis of AD is made only *post-mortem*, based on neuropathological examination (see below).

However, prior to clinical diagnosis, the disease onset is insidious and evolves over a period of many years (Mayeux, 2010). The clinical progression of AD, from its earlier stages to the death of the patient is depicted in Fig 1-1.

Incipient AD patients are affected by only minor, retrograde amnesia, and mostly complain of loss or lowering of memory and executive function (Albert et al., 2001). Classically, this is considered a pre-symptomatic phase of the pathology, as the impairments are below the threshold of standard diagnostic tests used to detect dementia. At this stage the patients are often depressed, and the degree of depression strongly correlates with the self-reported subjective

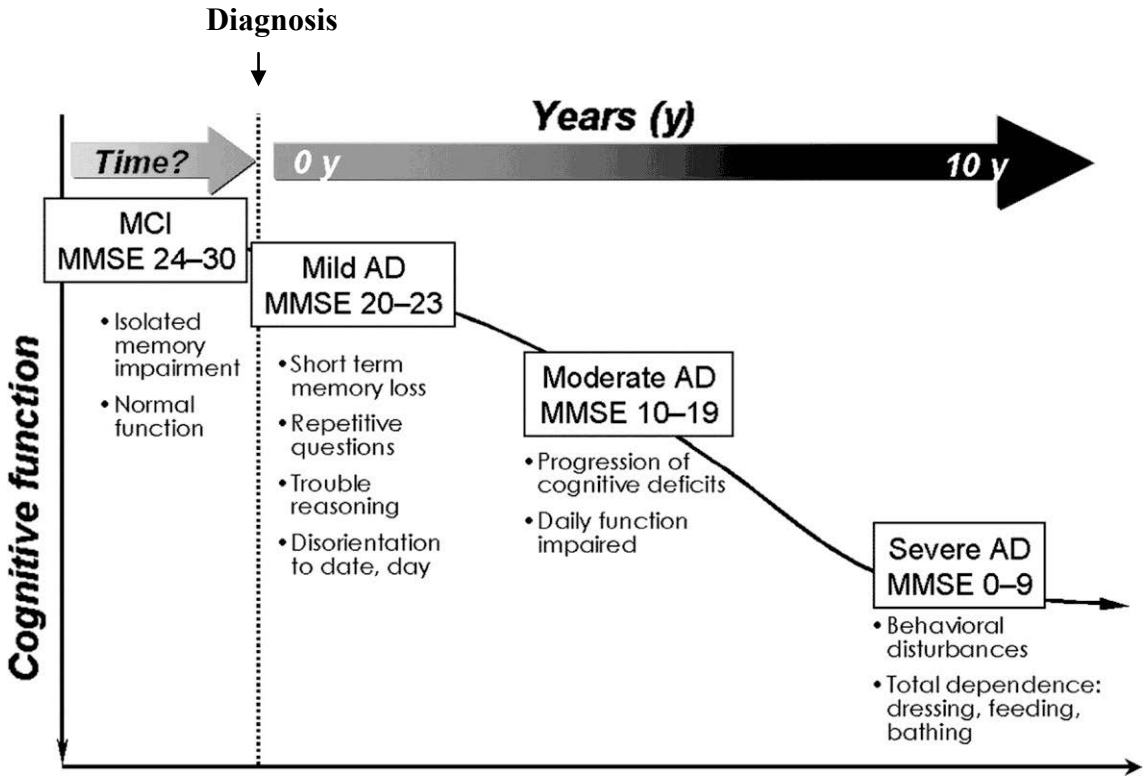

Figure 1-1 Clinical progression of the AD pathology

MMSE= mini-mental status examination

(Modified from: Petrella et al, 2003)

complaints (called ‘subjective memory complaints’). Furthermore, subjective memory complaints are good indicators of future cognitive decline (Reid and Maclullich, 2006). These findings suggest that such early, pre-symptomatic stages of the pathology are already characterized by neuropathological events, but these still escape our diagnostic tools.

As the disease progresses and the first cognitive symptoms are detectable, patients enter in the MCI phase (mild cognitive impairment, Albert et al., 2011). Here, objective deficits in one or more cognitive domains can be detected and quantified, but they are considered mild as they do not interfere with daily function as measured with the ADL –activities of daily living- scale (Robert et al., 2010). Four different MCI subtypes have been proposed: (1) amnestic MCI (isolated memory impairment, memory complaint); (2) single non memory MCI (isolated impairment of a cognitive domain other than memory); (3) multiple domain amnestic (slight impairment of multiple cognitive domains including memory); (4) multiple domains non amnestic MCI (slight impairment of multiple cognitive domains but without memory deficits; see Petersen et al., 1999; Mariani et al., 2007). Of these, amnestic MCI patients showed a higher rate of conversion to AD (a conversion to AD of up to 80% during approximately 6 years; Petersen, 2006). Based on the likelihood of conversion to AD, MCI of the amnestic type is currently considered the earliest stage of AD.

With disease progression, impairments in multiple areas of cognition (e.g., language, abstract reasoning and executive function or decision making) occur to varying degrees and coincide with increasing difficulty at work, in social situations or household activities. Mild AD patients usually need support with a variety of organizational matters but they may still be able to live independently (Forstl and Kurz, 1999).

As they enter in the moderate AD state, the patients gradually lose contact with the present due to progressive retrograde amnesia (they appear to “live in the

past", Beatty et al., 1988). Their severe impairments in reasoning, planning and organizing skills result in the incapacity to survive without close supervision. In its advanced phase, AD is characterized by loss of almost all cognitive functions. Remote and even biographical memories cannot be retrieved. Motor functional incapacity renders advanced AD patients completely dependent on comprehensive nursing care. Very often psychotic symptoms develop at this stage, including hallucinations, paranoia and aggressive behavior (Doody et al., 1995). Death occurs usually between 5-8 years after diagnosis (Walsh et al., 1990), and finds the patient completely unaware and unable to master his mind and body.

1.2.2 Neuropathology of AD

In striking contrast to the dynamic changes in behavior and cognition that accompany the disease progression, our understanding of the neuropathology of AD relies mostly on the somehow 'static' analysis of *post-mortem* AD brains. This approach offers only a snap-shot of the status of the brain at the moment of death, and leaves very little space for a natural history of the pathology. However, even with such limitations, the neuropathological observation of AD brains has prompted pivotal discoveries and it is still of fundamental value.

The search for anatomical correlates of mental disease started at the beginning of the 20th century with the introduction of the Bielschowsky silver staining technique. It was using this technique that Alzheimer reported the peculiar case of his patient, Auguste Deter, a 56-year old woman affected by cognitive and language deficits, auditory hallucinations, delusions, paranoia and aggressive behaviour (Alzheimer, 1907). *Post-mortem* examination of her brain allowed Alzheimer to observe and link the two main hallmarks of the pathology: foci of extracellular deposition of a "special substance" (amyloid plaques) and tangles of cytoskeleton proteins (neurofibrillary tangles, NFT) inside sick or dead neurons.

While he did not consider plaques as pathological, he identified in the tangles a possible cause for brain atrophy and dementia.

At about the same time, a Czech doctor, Fischer, was making similar observations. He completed a comprehensive study on the occurrence of plaques in senile dementia and their relationship to the mental status of the patients. The presence of plaques in brains from elderly or neurological patients was not *per se* a fundamental finding, as they had been already noted by several authors using a variety of techniques. Blocq and Marinesco first described 'amas rond', round heaps in the brain from an elderly individual with epilepsy (Blocq and Marinesco, 1892, in Goedert, 2009) using haematoxylin–eosin technique. Later, Redlich observed several 'miliare Sklerose' (miliary sclerosis) intensely stained with the dye carmine red in the brain of a demented 75-years old woman (Redlich, 1898, *ibidem*). According to these authors the plaques derived from abnormally proliferating glial cells. Fischer, on the opposite, correctly identified that plaque formation is the result of the extracellular deposition of a (yet) unknown amyloidaceous product of brain metabolism (Goedert, 2009).

Furthermore, he predicted that such deposition would be able to induce a local inflammatory reaction followed by a regenerative response of the surrounding nerve fibers. While he could not find evidence for positive inflammation for lack of appropriate staining technique (''Aber–wo bleibt dann die entzundliche Reaktion?'' (''However, where is then the inflammatory reaction'', see: Eikelenboom et al., 2006), he studied the neuritic response to plaques extensively. He was the first to describe the swelling and tortuous morphology of neurites in proximity to plaques (what we call today 'dystrophic neurites') and the presence of a subpopulation of plaques that are decorated by such altered neurites (neuritic plaques). Dystrophic neurites were associated by Fischer with a proliferative changes of what were called 'neurofibrils', and reminded him of structures that had been described before, mostly in the developing nervous system.

Taken together, Alzheimer's and Fischer's observations and predictions were impressive for their insight and they are still perfectly valid. Neurodegeneration (as in neuronal loss, neurofibrillar pathology, dystrophic neurites), deposition of amyloid plaques, inflammation and aberrant tissue proliferation are indeed cardinal aspects of the AD pathology which are still actively investigated. In particular, NFT and neuritic plaques represent the fundamental histological hallmarks that are still universally accepted to identify AD.

Nowadays, in fact, Alzheimer's disease-caused dementia is diagnosed *post-mortem* based on the presence of both neuritic amyloid plaques (composed of aggregated Aβ peptide; for a review see: Selkoe, 2000b) and NFT, composed by paired helical filaments of hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986; for a review: Iqbal et al., 2005).

In an effort to standardize procedures for the evaluation and diagnosis of patients with AD the National Institute on Aging created the 'Consortium to Establish a Registry for Alzheimer's disease (CERAD) in 1986. These criteria have since been updated to take into account the developments in the scientific community. In particular, together with quantification of neuritic plaques, it soon became clear that the presence and distribution of NFT was of high diagnostic value. Braak and Braak in the early 90s noticed a regional progression of tau pathology that paralleled neuropsychological data, and identified six stages based on number and localization of NFT: from Stage I-II, corresponding to early AD, where the lesions are limited to the transentorhinal stage, to Stage V and VI, with widespread isocortical pathology corresponding to late AD (Braak and Braak, 1991).

Therefore, the required criteria currently in use for AD diagnosis are a 'frequent' neuritic plaque score according to CERAD and a Stage V/VI according to Braak and Braak (for a review: Hyman and Trojanowski, 1997).

1.2.3 The amyloid hypothesis: APP processing

The original observation of the deposition of amyloid plaques in the brain of AD patients remained a consistent, but only as qualitative datum for almost a century. It was at the end of the 20th century, when Glenner and Wong managed to purify the protein composing the amyloid plaques, that major breakthrough were made possible in our understanding of the neuropathology of AD (Glenner and Wong, 1984). The main component of amyloid plaques was found to be a peptide initially referred as A4 and more recently known as the Aβ peptide (Masters et al., 1985). In subsequent years a great deal of evidence has accumulated, indicating that the biology of Aβ is crucial in the development of the disease.

It is now well established that Aβ is endoproteolitically produced by neurons from a precursor protein called amyloid precursor protein (APP) (Simons et al., 1996; Kang et al., 1987). APP is a large type I membrane protein whose function is still undefined, but has been implicated in neuritic outgrowth, cell adhesion and axonal transport (for a review: Koo, 2002). According to the protease type APP can undergo two kinds of processing: the amyloidogenic processing, which leads to the formation of the Aβ peptide, and the non-amyloidogenic processing, which in contrast prevents Aβ formation (see Fig. 1-2) (for a review: Selkoe, 2002).

In the non-amyloidogenic processing, a first fragment is shed from the membrane upon the action of an alpha secretase (ADAM-10, TACE). The remaining membrane bound fragment can be further cut by a peculiar intramembrane-cleaving protease called gamma (γ)-secretase. Selkoe and Wolfe identified presenilin (PS), an aspartyl protease, as the catalytic subunit of γ-secretase (Wolfe et al., 1999). More recently it was discovered that γ-secretase is a protein complex composed of 4 integral membrane proteins: PS, nicastrin, Aph-1, and Pen-2 (Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). Gamma secretase cut will yield p3 and AICD (APP intracellular domain). The

non-amyloidogenic processing accounts for most of the metabolism of APP in the cell in normal, physiological conditions.

Alternatively, a small percentage of APP can undergo the amyloidogenic processing, whereby it is cleaved by a membrane-anchored aspartyl protease called β-secretase (BACE1, reviewed by Vassar, 2004). This scission releases a soluble fragment, called sAPPβ, and a membrane bound fragment, C99 (or beta C terminus fragment, β-CTF). Gamma secretase cleavage of C99 will lead to the formation of Aβ and AICD (Fig. 1-2).

1.2.4 The amyloid hypothesis: genetic evidence

In the course of the 20th century it has become increasingly clear that the APP metabolism is crucially involved in the etiology of AD. The most convincing evidence in favor of the amyloid hypothesis came from the field of genetics.

It was well known that a low percentage (less than 10%) of cases of AD are familial forms of the disease, with an early onset and very aggressive pathology, but otherwise indistinguishable from the sporadic cases. On the other hand, the vast majority of AD patients are presented with the sporadic form of AD, i.e. do not show any clear evidence of Mendelian transmission. As soon as the APP gene was cloned (Kang et al., 1987; Tanzi et al., 1987) and the new technology of PCR became available, researchers were able to analyze the APP sequence in familial and sporadic cases of AD. Starting with the discovery of the London mutation (Goate et al., 1991), it was soon found that many cases of AD are autosomal dominant diseases. Mutations in the APP gene were all proved to lead to altered processing of the precursor and increased production of Aβ (Citron et al., 1992; Suzuki et al., 1994). Besides mutations in the APP gene, a second set of mutations was linked to familial AD: mutation of the presenilins (PS1 and PS2), which are the catalytic portion of the γ-secretase complex. In both cases, point mutation

result in modified APP processing and enhanced generation of Aβ42 (for a review: Coulson et al., 2000).

The role of APP processing also explained the observation that Down syndrome (DS) patients develop an invariable AD-like pathology as early as at 40 years of age (Schweber, 1989). DS is in fact caused by trisomy of the chromosome 21, where the APP gene lies: DS patients are therefore natural over-expressors of APP. This finding strongly indicated that the APP over-expression can mediate the dementia characteristic of the DS. Supporting this notion, a rare case of DS has been reported, in which the patient was diploid for the APP gene and showed no signs of dementia (Prasher et al., 1998).

Even in the sporadic cases where no autosomal cause is present, several risks factors have been identified. The strongest genetic risk factor that has so far been identified in non familial, sporadic cases of AD is the genotype of the lipoprotein APOE (Poirier et al., 1993). Carriers of the e4 allele have a highest risk (from 20 to 90%) of developing the disease and lower (from 84 to 68 years) age of onset (Corder et al., 1993). Although the mechanism by which APOE isoforms affect risk of AD is not entirely understood, there is strong evidence that APOE isoforms differentially modulate Aβ metabolism and in particular glial-mediated Aβ degradation (Jiang et al., 2008) with the e4 allele being the least effective variant.

Taken together, these findings definitely proved that an abnormal processing of APP and excessive formation of Aβ are key events involved in the etiopathology of the disease.

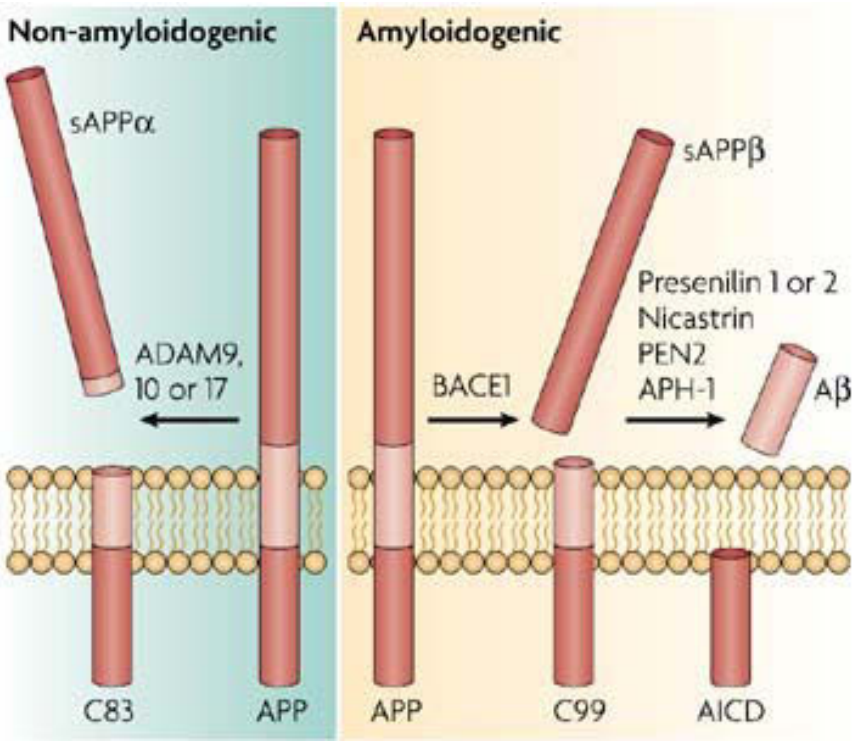

Figure 1-2: APP processing

Modified from: LaFerla et al., 2007)

1.2.5 The amyloid hypothesis: the past and the new

Following this evidence, Hardy and Higgins (Hardy and Higgins, 1992) formulated the so-called amyloid hypothesis. According to this hypothesis, abnormal production and or reduced clearance of Aβ leads to accumulation of fibrillar Aβ peptide, which eventually will produce amyloid plaques and cause neuronal death and hence dementia. All the neuropathological features of AD (dystrophic neurites, synaptic deficits, inflammation, oxidative stress) were seen in this model as mere consequences of the amyloid deposition.

More recently this model has been questioned. It is now well established that there is no correlation between the number of plaques or neuronal death and the cognitive status of a given patient (Terry et al., 1991). In fact, the best correlate with the dementia status appears to be synaptic counts (*ibidem*). Furthermore, the therapeutic intervention that stemmed from the original amyloid cascade hypothesis (achieving Aβ reduction via direct or indirect immunization) has so far failed to show cognitive improvement, even when plaques are cleared (Holmes et al., 2008). A possible explanation for this failure is that these strategies, while clearing plaques, did not affect soluble, oligomeric forms of Aβ that are now regarded as the most neurotoxic agents in AD (see below).

Therefore a novel version of the hypothesis is now currently considered (Hardy and Selkoe, 2002) which includes two significant corrections: early memory loss is attributed to synapse failure, not neuron death, and synapse failure is attributed to Aβ-oligomers, not amyloid fibrils. Aβ-oligomers have proven to be neurotoxic *in vivo*, *ex vivo* and *in vitro*. However, the exact mechanism of their neurotoxicity is still poorly understood.

1.2.6 Current therapeutics: too little, too late

Even though the amyloid hypothesis is well supported and rather compelling, there is no current effective treatment based on such hypothesis in the clinical practice. Instead, symptomatic treatment with cholinesterase inhibitors (ChEI) and memantine are currently the only licensed drugs for the treatment of AD.

The use of ChEI is based on the so called "cholinergic hypothesis of aging', originally formulated by Bartus (Bartus et al., 1982). According to this hypothesis, a serious loss of cholinergic function in the CNS underlies the cognitive symptoms associated with AD and advanced age. Indeed, cholinergic markers are dramatically reduced in AD (Davies and Maloney, 1976; Etienne et al., 1986; Nordberg and Winblad, 1986) and senile dementia (Bowen et al., 1976). Whitehouse and collaborators (Whitehouse et al., 1982) further demonstrated a loss of nucleus basalis neurons in *post-mortem* samples of AD patients.

Furthermore, supporting the cholinergic hypothesis of aging, pharmacological experiments indicated that anticholinergic drug scopolamine would induce amnestic syndromes in humans (Drachman and Leavitt, 1974). Importantly, it was noted that such amnestic syndrome could be reverted by administering ChEI (Drachman and Leavitt, 1974).

On the grounds of such evidence, anticholinesterase agents were expected to reduce the AD-related dementia. Indeed early clinical trials demonstrated some efficacy of these drugs in late dementia and tacrine was officially approved for AD in 1993. Old generation ChEI, including tacrine and physostigmine, displayed however significant adverse effects (gastrointestinal events, hepatic toxicity). This led to the development of second-generation ChEI: donepezil, galantamine and rivastigmine, with better tolerability and safety profiles. It must be noticed that clinical trials with ChEI demonstrated only minor statistical differences between placebo groups and treated, and mainly due to reduced deterioration, rather that

recovery of cognitive function. Furthermore, only 50% of patients appear to benefit from the drug (for a review see: Giacobini, 2004).

On a different note, memantine, an NMDA antagonist with selectivity for extrasynaptic receptors, is believed to act by reducing excitotoxicity (Xia et al., 2010). It is licensed for moderate-to-severe AD. As per ChEI, the treatment is only symptomatic, and is not capable of modifying the course of the disease.

It is starting to be acknowledged that the limited therapeutic efficacy of the current AD-drugs is due to the late clinical diagnosis of AD, which arrives when the neuronal health is irreversibly compromised. Highest efficacy would be indeed reached if the treatment was started at the very first stages of the pathology (Becker et al., 2008).

This prompts the following question: when does AD start? It is still impossible to answer it; however, a deeper understanding of the progression of the amyloid pathology and its natural history might offer us a key to solve the problem.

1.3 Aβ natural history

1.3.1 Aβ generation: a specific brain peptide

To understand the pathology of Aβ one has first to acknowledge that the peptide is not necessarily a pathological product of the brain. Aβ is in fact a normal product of cell metabolism (Haass et al., 1992). Its precursor protein, APP, is widely expressed in virtually all tissues (Selkoe, 1994), although the highest levels of expression appear to be in brain and kidney.

While APP is ubiquitous, the enzymes processing it are not. BACE, in particular, is specific to the brain and is constitutively produced by neurons (Vassar et al., 1999). The regional specificity of BACE expression is one of the few elements we have to explain the brain selectivity of the AD pathology. Gamma secretase, on the other hand, is ubiquitously expressed (Lee et al., 1996).

Several variants of Aβ are known which derive from slightly different proteolytic cleavage of the γ-secretase. The two most common forms are Aβ40 and 42: in a normal cell they are present in a ratio of 8:1 (Gravina et al., 1995), but Aβ42 has a higher propensity to from fibrils (Jarrett et al., 1993). Picomolar concentrations of Aβ (ng/ml) are detectable in human fluids such as cerebrospinal fluid (CSF), plasma (Seubert et al., 1992) and microdialysates (Brody et al., 2008) under normal conditions, suggesting that indeed Aβ has a physiological role.

1.3.2 Physiological role of Aβ

The concept of Aβ as a physiological product specific of neuronal cell metabolism raised the question of its function. While this is still an extremely controversial issue, several hypothesis have been put forward.

Synaptic modulator. Early studies with Aβ peptide and its fragments revealed that in some conditions Aβ could have trophic effects on neurons in culture (Whitson et al., 1989; Whitson et al., 1990). It soon became clear that neurotrophic or neurotoxic effect could both be elicited, and that the final effect would depend on Aβ concentration and on the differentiation state of the neurons used (Yankner et al., 1990). Similarly, our laboratory has noticed that Aβ can activate cyclic AMP activated element (CRE)-regulated gene expression that is fundamental for synaptic plasticity, via phosphorylation of ERK40/42 and CREB (Echeverria et al., 2004b). Interestingly, this effect was highly dependent on Aβ levels: high concentrations of Aβ exerted opposite effects and disrupted ERK-CREB signalling (Arvanitis et al., 2007).

Supporting a role for Aβ in synaptic plasticity, Kamenetz (Kamenetz et al., 2003) showed that Aβ is released during long term potentiation (LTP) formation. While Kamenetz believed that Aβ had a negative regulatory function preventing excitotoxicity, Arancio et al. showed that picomolar (considered physiological) concentration of Aβ can positively modulate and facilitate LTP (Puzzo et al., 2008). More recently, Holtzmann and Cirrito have shown that Aβ levels in the brain are proportional to awakeness (Kang et al., 2009), neurological health status (Brody et al., 2008) and synaptic activity (Bero et al., 2011).
Taken together these evidence indicate that Aβ, released during synaptic activity, might have a role in either facilitating and strengthening synaptic transmission or in offering negative feedback that could keep neuronal hyperactivity in check.

Stress response and infection. It is well known that various stressors (such as oxidative stress, concussion, ischemia, energy inhibition) can increase BACE activity and Aβ levels in the brain (Tamagno et al., 2002; Blasko et al., 2004a; Wen et al., 2004; Velliquette et al., 2005). It appears that APP and BACE are part of a stress-response specific to neuronal cell; however, the role of Aβ in such scenario has not been elucidated yet. Recent evidence showed that Aβ monomers can be neuroprotective in the context of trophic deprivation (Giuffrida et al., 2009). Alternatively, some authors have proposed that Aβ might be involved in the response to bacterial infection. Tanzi and collaborators (Soscia et al., 2010) in fact demonstrated that Aβ can effectively exert bacteriostatic effects on at least eight common pathogens, including the bacteria S.pneumoniae and C. albicans.

Blood homeostasis. On a similar note, some authors have observed that Aβ has metal chelation, anti-oxidant and sealant properties that might be involved in maintaining the structural integrity of the blood brain barrier and parenchymal structures under stress conditions (Atwood et al., 1998). Aβ has the physicochemical properties to act as a sealant and APP metabolism has been involved in the blood clotting cascade (Van Nostrand et al., 1990; Hardy, 2007).

This hypothesis might explain the observation that during acute head trauma, which heavily impacts the brain vasculature, Aβ is deposited in form of non congophilic Aβ plaques (Roberts et al., 1991). Furthermore, such hypothesis is supported by the results of passive Aβ immunotherapy, in which decreased levels of Aβ were accompanied by an increase in cerebral microhemorragies (Pfeifer et al., 2002).

Taken together, the data presently available support the notion that Aβ is a physiological, brain-specific peptide involved in the normal synaptic activity of default brain areas and transiently up-regulated in stress conditions.

1.3.3 Aβ localization

The exact localization of Aβ is still controversial. The most accepted model proposes that Aβ is found and exerts most of its neurotoxic effects in the extracellular space, where it is released after intracellular APP processing. This view is substantiated by the fact that Aβ can be easily detected in the medium of APP over-expressing cells, and that plaques are extracellular deposits. However, there is strong evidence that extracellular Aβ represents only a small fraction of the total peptide and that most of it is produced and stored intracellularly. There are at least 4 arguments in favour of the intracellular production of Aβ:

1. Only a small percentage of APP is processed at the plasma membrane following the secretory pathway. In fact, within minutes of arriving at the plasma membrane, surface-bound APP is largely (70%) internalized and following endocytosis, APP is delivered to late endosomes (Thinakaran and Koo, 2008).

2. Inhibiting or increasing endocytosis respectively reduces or increases the production of Aβ (Grbovic et al., 2003; Carey et al., 2005; Schneider et al., 2008; Cirrito et al., 2008).

3. At basal states the majority of BACE is located on Golgi, trans Golgi network (TGN) and endosomes (Koo and Squazzo, 1994). The optimal pH for BACE catalytic activity is acidic, thus making the acidic environment of the endosomes ideal for APP-processing.

4. The presence of functional *γ*-secretase complex has been shown on multiple compartments including the ER, late-Golgi/TGN, endosomes, and the plasma membrane (Xu et al., 1997; Greenfield et al., 1999; Kaether et al., 2002; Vetrivel et al., 2004). Furthermore, recent estimates suggest only a minor *γ* -secretase activity at the cell-surface, whereas the majority of the mature components of *γ*-secretase complex are found, and shown to be enzymatically active, in intracellular organelles such as ER, Golgi apparatus, the TGN, and late endosomes (Thinakaran and Koo, 2008).

Taken together this evidence strongly indicates that most Aβ is produced within intracellular vesicles.

1.3.4 Aβ degradation and clearance

Once produced, Aβ has a very high turnover rate (Savage et al., 1998), suggesting the existence of a very efficient clearance machinery. At the present day, at least three distinct mechanisms of Aβ degradation have been identified, including enzymatic degradation, active phagocytosis and transport through the blood brain barrier (BBB).

Enzymatic degradation. The rapid turnover of Aβ led the scientist to hypothesize the existence of an efficient, enzymatic-based clearance mechanism. Several enzymes have been shown to be able to degrade Aβ *in vitro* (for a review: Eckman and Eckman, 2005), but only a few of these have been proved to have a biological role *in vivo*. Amongst these insulin-degrading enzyme and neprilysin are the most relevant.

Insulin degrading enzyme (IDE, insulase) is a 110-kDa metalloendopeptidase located mostly in the cytosol as a soluble form, while a small fraction is membrane-bound on peroxisomes, endosomes and cell surface (Farris et al., 2003). It cleaves several small proteins (such as Aβ, insulin, glucagon, amylin, atrial natriuretic factor and calcitonin) which have diverse sequence but share a propensity to form beta-pleated sheet-rich fibrils. IDE, which can be of both glial and neuronal origin, has been shown to regulate extracellular (Vekrellis et al., 2000) and intracellular (Sudoh et al., 2002) levels of Aβ, but is capable of cleaving only monomeric Aβ.

Neprilysin (NEP, also known as neutral endopeptidase, enkephalinase, neutrophil cluster-differentiation antigen or common acute lymphoblastic leukemia antigen) is a 90–110 kDa glycoprotein (Miners et al., 2008). As IDE, it belongs to the zinc metalloendopeptidase family. It occurs mostly bound to the membrane, where it is in an ideal position to hydrolyze extracellular Aβ peptides, including oligomers. The pivotal role of IDE and NEP in Aβ degradation has been highlighted by studies in genetically modified mice: mice deficient in NEP and IDE have higher Aβ levels (Farris et al., 2003; Iwata et al., 2001). Conversely, over-expression of these Aβ degrading enzymes resulted in reduced amyloid pathology (Leissring et al., 2003).

Phagocytosis. Microglial cells are the resident immune cells of the CNS and are capable of turning into tissue macrophages and act as phagocytic cells. Phagocytosis involves the internalization of large particles upon interaction with specific cell surface receptors. Aβ fibrils can bind to several receptors on the

surface of microglial cells (a composite of class A scavenger receptor, B-class scavenger receptor CD36, the integrin-associated protein/CD47, the α6β1-integrin, the Toll Like Receptor 2,6 and 9 and their co-receptor CD14). This process results in the stimulation of intracellular signaling cascades and eventually activation of the phagocytotic process (see Lee and Landreth, 2010).Internalization of soluble Aβ can also be mediated by constitutive fluid phase macropinocytosis (Mandrekar et al. 2009). There is vast evidence that embryonic and pups microglial cells are able to phagocytose Aβ *in vitro* (for a review: Rogers et al., 2002). Some evidence exist that Aβ is phagocytosed *in vivo* by microglia, but most of this relies on the use of irradiation and chimeric systems (Simard et al., 2006). While very likely, the exact contribution of microglia-macrophages phagocytosis to Aβ clearance *in vivo* is still unclear.

On the other hand, astrocytes have also been shown to be able to phagocytose Aβ, even though less efficiently than microglial cells.Intracellular Aβ has been detected in endosomes and lysosomal granules of astrocytic cells, suggesting phagocytic and lysosomal activity (see Lee and Landreth, 2010). In particular, APOE expression in astrocytes has been linked to their ability of degrading Aβ (Koistinaho et al., 2004).

Vascular clearance. The CNS parenchyma, the CSF and blood levels of Aβ are known to be in a dynamic balance. As the BBB would not allow for the free movement of a polar peptide as Aβ, a carrier system would be required. Several studies have demonstrated the existence of a transport exchange across the BBB of soluble Aβ in the CNS. Transport of Aβ across the BBB and through vessels seems to be regulated by a number of receptors, including receptor for advanced glycation end products (RAGE) (Deane et al., 2003), the low-density lipoprotein receptor related protein-1 (LRP1) (Shibata et al., 2000), megalin (LRP2) (Zlokovic et al., 1996) and P-glycoprotein (Cirrito et al., 2005). Aβ-binding proteins such as α2-macroglobulin, apoE and apoJ, could also influence transport of Aβ at the BBB (for a review: Zlokovic, 2005).

1.3.5 Aβ accumulation mechanisms

Genetic and sporadic cases of AD all share the common feature of increased Aβ levels and the occurrence of amyloid plaques. The mechanisms underlying the accumulation of the physiological Aβ peptide into misfolded, toxic forms and eventually amyloid plaques are not fully understood and appear to differ from case to case.

In this regard, familial AD and DS are the best characterized systems. In these genetically driven neurodegenerative conditions, in fact, the increase of Aβ levels is clearly due to augmented and pathological processing of APP (Coulson et al., 2000).

In non-genetic cases of AD but carriers of APOE4 allele there is no evidence for an altered production of APP or Aβ peptide; however, several lines of research would substantiate that the mechanism responsible for Aβ degradation (the vascular clearance, the enzymatic cleavage and the phagocytosis) are defective (for a review: Kim et al., 2009).

In sporadic AD, the causes underlying increased levels of Aβ and Aβ deposition are still not fully explained. They could be due to increased Aβ production via increased levels and activity of BACE (Yang et al., 2003). On the other hand, evidence exists that CNS clearance of Aβ is impaired in AD (Mawuenyega et al., 2010). Furthermore, lower levels and activity of IDE and NEP were reported (Cook et al., 2003; Wang et al., 2005). Microglial cells for AD patients appear to be less capable of phagocytose Aβ than control (Fiala et al., 2005). Finally, several instances of microvascular degeneration have been reported in AD (Farkas et al., 2000, for a review: Bailey et al., 2004), and the levels of LRP proteins have been shown to be down-regulated in AD (Jeynes and Provias, 2008; Donahue et al., 2006).

In sum, a combination of increased production and decreased clearance of Aβ is thought to cause the rise in Aβ levels in the brain which will eventually cause AD. Regardless of the causes of its aggregation, Aβ accumulation is known to be toxic and is currently the main focus of research in the field.

1.4 Aβ-oligomers

Uncontrolled accumulation of Aβ eventually results in its deposition in the form of insoluble plaques. Prior to plaque generation, however, Aβ aggregates into intermediates of different sizes, called oligomers. Aβ-oligomers are currently regarded as the culprit of the AD neuropathology; however, the exact process of Aβ aggregation and the mechanism by which they exert their neurotoxic effects *in vivo* are under investigation.

1.4.1 Aβ biochemistry: why does Aβ aggregate?

Aβ42 spans with its C terminus in the membrane, while its N terminus is free in the lumen/extracellular space (Fig 1-2). Accordingly, its sequence shows a lypophilic and a hydrophilic portion: it is therefore an amphipathic peptide with the tendency to self aggregate and capable of forming micelles. Interestingly, while there is a very high (90%) homology in the overall APP sequence between human and rodents (Yamada et al., 1987; Shivers et al., 1988), the Aβ sequence differs for 3 aminoacids between rodents and human. These substitutions confer higher lipophilicity, possibly accounting for the inability of murine Aβ to form plaques (Otvos Jr. et al., 1993).

Unfortunately we do not have a resolved structure of Aβ. Aβ in fact does not show a defined tertiary structure and it tends to form amyloid fibrils rather than crystals (for a review: Roychaudhuri et al., 2009). Most of our knowledge on the

structure of Aβ comes from NMR and molecular dynamics, but also these analyses have been complicated by the propensity of the Aβ peptides to aggregate (Roher et al., 2000). The models available show that in water-SDS micelle medium buffer the Aβ peptide displays a random coil conformation characterized by a hairpin shape, with two strands separated by a turn that form a small alpha-helix (Fig. 1-3). The arms of two the hairpins can then align to form a dimer (Fig. 1-3a). In this conformation, the C-termini form beta-sheet fibrils with an antiparallel symmetry, stabilized by intermolecular hydrogen bonding and hydrophobic interactions (Fig. 1-3a). The successive stacking of dimers creates a helical ribbon or protofilament; aggregation of several protofilaments results in mature fibrils (Fig. 1-3b-e).

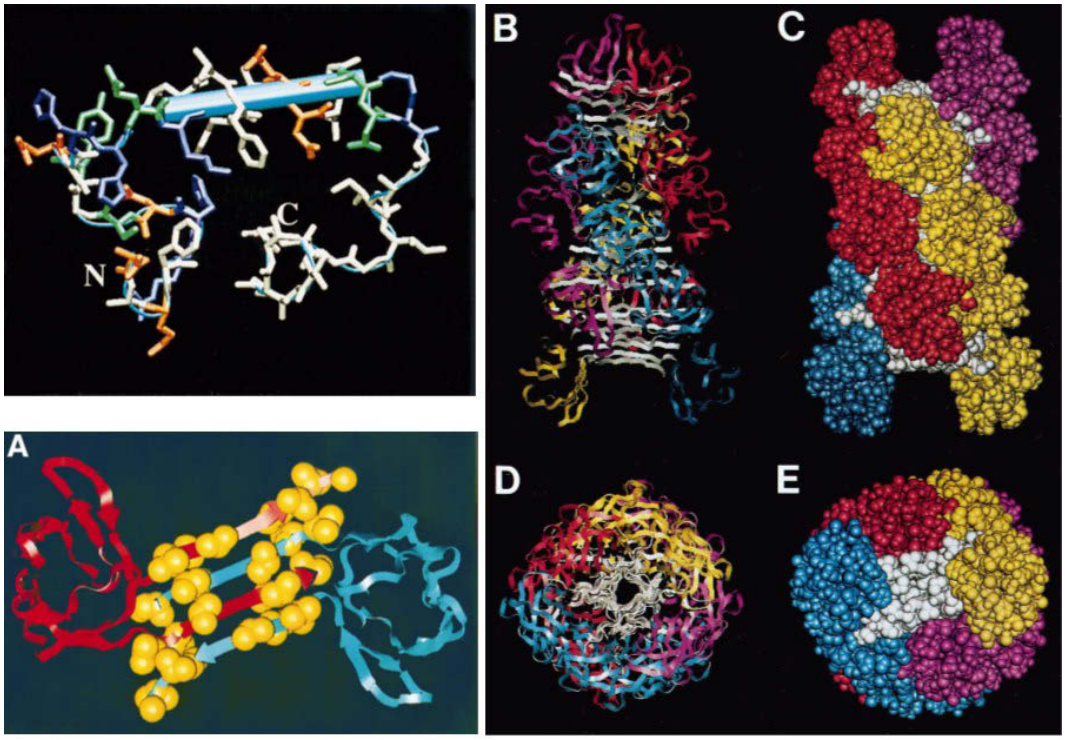

Figure 1-3: Aβ structure and oligomerization

(A) Two Aβ dimers with extended C-termini forming an antiparallel β-sheet stabilized by intermolecular hydrogen bonding and hydrophobic interactions. The β-sheet is flanked by the N-terminal globular domains of the dimers (represented in red and blue ribbons). The successive stacking of dimers creates a helical ribbon or protofilament.

A model of a two-protofilament strand is shown in ribbons and space filling displays viewed in a normal to the filament axis (B and C) and along the filament axis (D and E), respectively. Each protofilament resembles a helical `ladder' in which each of the `railings' is formed by the consecutive superimposition of the N-terminal domains (residues 1-28) of AL dimers (shown in yellow and blue, and red and purple), and the `rungs' formed by the extended C-termini antiparallel β-strands (residues 30-42) of opposing Aβ dimers (depicted in white). Two protofilaments intertwined in parallel against each other generate a core of two helical β-sheets (white ribbons and balls) with their hydrogen bonds parallel to the protofilament main axis. In this structure, the hydrophobic C-termini of the Aβ peptides are shielded from the aqueous environment by the mostly polar N-terminal Aβ residues.

(Modified from: Roher et al., 2000).

Besides being the result of aggregation of two monomers in solution, Aβ dimers might be the result of the processing of dimeric APP, as suggested by Dr Multhaup's group (Scheuermann et al., 2001).

1.4.2 Studies with synthetic Aβ: toxic gain of function parallels gain of structure

As soon as Masters and collaborators (Masters et al., 1985) identified Aβ as the building block of the amyloid plaques, researchers started to use synthetic Aβ preparations to study the biology of Aβ fibrils. Synthetic Aβ peptide proved to aggregate readily *in vitro* (for reviews see: Caughey and Lansbury, 2003; Teplow, 1998; Lomakin et al., 1997). Incubation of Aβ peptide for hours/days at room temperature/37°C would generate Aβ fibrils indistinguishable from the ones extracted from AD brains, thus allowing the study of the aggregation process. In general, the Aβ aggregation process appeared to be a nucleation-dependent polymerization, where the nucleation time (lag phase) is the rate-limiting step; after the seed formation a fast elongation phase occurs (exponential phase) and fibrils are formed.

It became soon apparent that the aggregation of Aβ and the formation of true, Thioflavin-S positive structures were highly dependent on the experimental conditions such as concentration, salts, pH and temperature. Concentrations in the order of uM are required for the formation of fibrils. Also, the presence of salts could seed and accelerate Aβ fibril formation. Importantly, a lower pH would favour the aggregation process, and so did a higher temperature (Stine Jr. et al., 2003). In general, Aβ fibrils can be reliably obtained with incubation at 37°C for a few days: following ultra-centrifugation, one can separate the pellet, containing insoluble fibrils, and a supernatant, containing what was defined as 'soluble Aβ'. Fibrillar Aβ prepared in this fashion has been shown to be highly toxic to neurons

(Lorenzo and Yankner, 1994), to elicit microglia activation (Meda et al., 1995) and to induce behavioural impairment in naive rats (O'Hare et al., 1999).

Importantly, it was Pike' and Lorenzo and Yankner's merit to establish that Aβ is not toxic per se, and it is innocuous until it undergoes structural reorganization (Pike et al., 1991; Lorenzo and Yankner, 1994). In fact, freshly prepared solutions of monomeric Aβ were found to be non-toxic or even, as demonstrated recently, neuroprotective (Ono et al., 2009; Giuffrida et al., 2009). However, the same solutions tested 24 hours later, after aggregation has occurred, exert their full toxic effect (Pike et al., 1991; Lorenzo and Yankner, 1994). Aβ therefore displays a toxic gain-of-function which parallels a specific structural reorganization or 'gain-of-structure'. The question raised quickly on what specific structure would confer to Aβ its toxicity, and in the 2000s investigators moved their attention from fully mature fibrils to intermediate species.

1.4.3 Studies with synthetic Aβ: the challenge of Aβ-oligomers

When the field started to focus on intermediate species of aggregation, the preparation of reliable, reproducible 'soluble' Aβ proved to be more challenging. The nature and morphology of the soluble aggregates formed were soon found to differ enormously from preparation to preparation in terms of structure and size. Transmission electron microscopy (TEM) studies revealed different morphological species, from amorphous aggregates to globular aggregates, amylospheroids, paranuclei, protofibrils annular protofibrils and micelle-like oligomers. As per their size, aggregates ranging from dimers up to particles of one million Da or greater have been reported *in vitro* (for a review: Walsh and Selkoe, 2007).

In the literature, 'soluble Aβ-oligomers' is an extremely vague term that has been used to describe any of the above species, plus the more recently discovered

ADDLs (Alzheimer disease diffusible ligands, Lambert et al., 1998) and Aβ*56 (Lesne et al., 2006) aggregates.

The respective relevance of such aggregates is still object of debate. Some authors (Ono et al., 2009) have proposed a structural-functional correlation, where tetramers>trimers> dimers>fibrils> monomers in terms of toxicity. Others have shown that oligomeric preparations of different nature can be all toxic, but via distinct mechanisms and dynamics (Deshpande et al., 2006). However there is no definitive consensus on this, and suffice here to say that different oligomeric species have been all shown to be active neurotoxins, induce LTP deficits and behavioural impairments (for a review: Walsh and Selkoe, 2007).

Despite of their biological relevance and toxic effects, a rigorous classification of Aβ-oligomers is still needed.

1.4.4 Oligomeric-specific antibodies

A significant contribution to our understanding of the biology of Aβ-oligomers came from the generation of oligomeric, conformational-specific antibodies. It is well known in fact that in the course of antibody response usually the antigen is processed by macrophages and presented to T-cells to trigger the immune response. There is evidence, however, that most of the antibodies generated against a given protein recognize the native (unprocessed) protein in its original conformation (Fujio et al., 1985). It was realized in the 70s that the folding of a protein in a specific conformation exposes a new epitope, usually sequence-independent, that can be highly immunogenic (Sachs et al., 1972). Based on this observation, conformational specific antibodies have been generated and they have been widely used in biochemistry as immunological tools to study the conformational equilibria of polypeptides.

In 2001 Dr Klein's group applied this approach to the generation of Aβ-oligomeric specific antibodies (Lambert et al., 2001), by immunizing animals with preparations of low-n oligomers called ADDLs (ranging from dimers to 24-mers). These studies allowed the group to publish the first evidence of Aβ-oligomeric specific antibodies (Lambert et al., 2007). Soon after, Glabe's group applied a different approach to obtain conformational specific antibody: to use oligomers coupled to gold particles. Immunization with such compounds has generated oligomer-selective antibodies with the ability to recognize pre-fibrillar oligomers (A11 sera) or fibrillar (OC sera) oligomers of multiple fibrillogenic proteins besides Aβ (e.g., alpha synuclein and islet amyloid polypeptide, Kayed et al., 2007). The use of these antibodies has so far allowed two major discoveries:

1) brains from AD and Tg models display a specific immunoreactivity to oligomeric-specific antibodies, suggesting that oligomers are indeed present *in vivo*;

2) the existence of different pools of oligomers reactive with OC or A11 has allowed Glabe to propose an immunological classification of Aβ-oligomers, which is to date the best attempt made to put order in the field (Glabe, 2008).

According to this classification, misfolding of Aβ monomers results in Aβ oligomerization and fiber formation via two distinct pathways: a 'direct' and an 'indirect' pathway.

In the first, direct pathway, misfolded monomers of Aβ aggregate to form 'soluble' fibrils. The term 'soluble' indicates that these fibrils are not pelleted with ultra-centrifugation. Even though they do not qualify yet to be defined 'mature' based on their solubility, such soluble fibrils would share the same structural organization as mature, insoluble fibers. In fact, this population of fibrils is OC positive, indicating that they acquired a conformational change that is common to

mature fibers. Such Aβ aggregates are therefore named "soluble fibrillar oligomers"; operationally, they are defined by being OC positive but A11 negative. Examples of this type of oligomers include Aβ56* and globulomers. Mature fibrils formation will take place from such fibrillar oligomers by addition of further misfolded monomer.

In the second, indirect pathway, misfolded monomers aggregate into fibrillar species that are still soluble, but are not reactive with OC. Instead, they are specifically recognized by the A11 antibody. Such immunological profile suggests that they have not undergone the conformational change necessary for mature fibril formation, and they represent therefore an intermediate step in the aggregation process. These Aβ aggregates are therefore named 'pre-fibrillar' oligomers; operationally, they are identified as being OC negative but A11 positive. They may (or may not) further proceed in the aggregation process. To continue in the process, they undergo an en bloc conformational change and become fibrillar (i.e. A11 negative but OC positive).

1.4.5 Studies with cell-derived Aβ-oligomers

While the early studies using synthetic Aβ increased our understanding of the Aβ structure and toxicity enormously, it was still to be proven that such models would be relevant in a physiological environment. An important unanswered question was whether SDS-resistant oligomers would form *in vivo* and whether they would display the same neurotoxic profile.

In terms of structure, an important insight came when conditioned media from APP-transfected Chinese Hamster Ovary cells was shown to contain metastable Aβ-oligomers, even at nanomolar concentration (Podlisny et al., 1995). While confirming the physiological relevance of studies using synthetic Aβ, this first report has suggested that the aggregation properties of natural Aβ differ

significantly from that of synthetic Aβ (Podlisny et al., 1995). Supporting the notion of potential differences in aggregation properties between synthetic and natural Aβ, more recent data demonstrated that the recombinant bacterial Aβ42 aggregates faster than synthetic Aβ42 (Finder et al., 2010).

The toxicity of such naturally-derived Aβ has also been tested and compared with synthetic preparations. Cell-derived trimers from conditioned media of cells over-expressing APP were indeed able to inhibit hippocampal synaptic plasticity ex *vivo* (Walsh et al., 2002; Townsend et al., 2006b), and induce cognitive impairment when applied *in vivo* in naïve animals (Cleary et al., 2005; Townsend et al., 2006a). These results confirmed that natural secreted Aβ-oligomers are at least as toxic as synthetic preparations. Studies comparing side-to side synthetic and naturally derives oligomers showed that naturally derived oligomers are more potent neurotoxins than synthetic peptides (Jin et al., 2011).

With the notion that Aβ-oligomers are not artifacts of synthetic *in vitro* preparations, but physiologically relevant entities that do occur *in vivo*, the interest slowly shifted towards a better characterization of oligomers in *in vivo* settings such as Tg animal models and in *post-mortem* AD brains.

1.4.6 Aβ-oligomers in Tg models

Numerous over-expressing Tg models have been developed that mimic some aspects of the AD-like amyloid pathology (for review see: Gotz and Ittner, 2008). As these mice recapitulate plaque pathology, inflammation, dystrophic neurites and cognitive impairments, they are valuable tools in studying AD-related events.

A number of methodological approaches has been exploited to analyze the oligomeric burden in these models. Several labs used a combination of Western blot and immunoprecipitation to show the occurrence of SDS-stable Aβ-oligomers

in brain homogenates from different APP Tg mice models. While most of these studies agree in showing abundant oligomeric material, the nature of the oligomers seems to differ according to Tg models and protocols used. Ashe and collaborators were the first to pin-point a specific molecular species of Aβ which migrated as a 56KDa band in the extracellular portion of the brain homogenates from Tg 2576. This species coincided with behavioural impairments, and its injection in naive rats also induced cognitive deficits (Lesne et al., 2006). McLaurin and collaborators have instead identified a 12KDa band in the Tg CNRD8, which was considered as trimers (McLaurin et al., 2006). A recent study from Walsh's group demonstrated the simultaneous presence of several oligomeric species in the J20 mouse model, with an age-dependent increase in Aβ monomer and SDS-stable dimer, trimers and tetramers (Shankar et al., 2009).

On the other hand, recently, the occurrence of oligomeric immunoreactive (IR) material has been demonstrated using the oligomeric-specific antibody A11 (Oddo et al., 2006; Shankar et al., 2009).

1.4.7 Aβ-oligomers in human tissue

Early evidence of soluble Aβ material in AD brains appeared in the 90s (Kuo et al., 1996; Frackowiak et al., 1994). Soon after Funato and collaborators identified SDS-stable Aβ dimers as the prominent soluble Aβ species in the CA1 region of the hippocampus of AD patients, before the appearance of plaques (Funato et al., 1999). This observation remained somehow neglected, and only recently researchers have started to look into this issue again. Confirming and extending Funato's results, Selkoe's group has demonstrated the presence of Aβ-dimers from AD brains, and furthermore has proved that such oligomers impair synaptic plasticity and memory (Shankar et al., 2008).

More evidence of Aβ-oligomers in human brain came from oligomeric-specific antibodies. Klein's group elegantly showed the presence of ADDLs in

AD brains and CSF (Gong et al., 2003), and they confirmed that the material seen was akin to synthetic preparations via 2d electrophoresis. Similarly, Glabe's group showed that several deposits are OC-IR in AD brains (Sarsoza et al., 2009).

Taken together, the evidence collected so far strongly indicate that Aβ oligomers are pathologically relevant species in AD and AD-like pathology in Tg. However, the dynamic, localization and consequences of the Aβ-oligomerization process *in vivo* remain to be elucidated.

1.4.8 Molecular mechanisms of Aβ-oligomers-toxicity

While there is wide consensus on Aβ-oligomers being neurologically potent toxins, the exact mechanism responsible for their neurotoxicity is far from being established. The picture is considerably complicated by the fact that, according to the concentration and the cellular model used, Aβ-oligomers can interfere with the neuronal function at different levels, from synaptotoxicity to frank neuronal death. Neuronal death, however, is normally achieved with extremely high concentrations of Aβ (uM) that most likely do not apply to the clinical conditions. Furthermore, in the progression of AD neuronal death is known to happen relatively late, and it is not thought to be causative of the initial cognitive impairments. On the other hand, AD is prominently a memory disease, and memory formation begins at synapses. It is therefore very likely that the disruption of memory formation in Alzheimer's disease itself begins at synapses. Here we will therefore review only the mechanism involved in synaptic plasticity.

The ability of Aβ-oligomers to interfere with normal synaptic transmission *in vitro* and *in vivo* has been extensively documented. There are structural (Lacor et al., 2007; Shankar et al., 2007) and functional (Townsend et al., 2006b; Wang et al., 2002) data to prove that Aβ-oligomers selectively target on synaptically active sites of cells. Several molecular mechanisms have been proposed to explain the

synaptotoxicity of Aβ-oligomers; some of the most relevant are briefly reviewed here.

Aβ synaptic targeting via NMDA receptors. When applied to primary neuronal cell cultures, ADDLs appeared to specifically target synaptic sites especially at post-synaptic, NMDA rich spines (Lacor et al., 2004). Spines are particularly interesting because they contain the post-synaptic signal transduction components of excitatory synapses responsible for LTP formation. Many studies have tried to elucidate the interaction between Aβ-oligomers and glutamatergic receptors. It appears that most of the neurotoxic effects of Aβ-oligomers including spines abnormalities (Shankar et al., 2007) and oxidative stress (De Felice et al., 2007) are mediated by NMDA receptors (Decker et al., 2010). While there is no evidence to indicate a direct binding of Aβ to NMDA receptors, a vast body of evidence indicates that Aβ-oligomers interfere with the glutamatergic signaling in several, indirect ways. Early studies showed that Aβ-oligomers affect the trafficking of NR2B and NR1 subunits to the plasma membrane, and that this would explain the decrease in LTP (Snyder et al., 2005). Selkoe's group has gathered consistent evidence that the disruption of glutamate re-uptake underlies both the facilitation of long term depression (LTD) and the inhibition of LTP (Li et al., 2009; Li et al., 2011).

Aβ binding to nicotinic receptors. While binding to the NMDA receptor has been excluded, it is well established that Aβ and Aβ-oligomers bind to the α-7 containing nicotinic receptor with picomolar concentrations (Wang et al., 2000; Dineley et al., 2001). The question as to whether this association is functional is still a matter of debate. Nordberg's group has shown that Aβ-oligomers act as agonists of the receptors, whereas Aβ fibrils act as antagonists (Lilja et al., 2011). Other authors (Dougherty et al., 2003) demonstrated that pre-synaptic increase in calcium influx induced by Aβ can be prevented by blocking the α-7 containing nicotinic receptors. More recently, Snyder et al. showed that the effects of Aβ-

oligomers on NMDA receptor trafficking could be inhibited by antagonizing this nicotinic receptor (Snyder et al., 2005).

Aβ forming membrane pores. Several authors maintain that Aβ is a sticky protein that will be found to interact with many receptors but is not a true ligand of any. Its stickiness is on the other hand responsible for a direct interaction with lipid bilayers, disruption of membrane organization and facilitation of calcium entry in the cell. Aβ40 oligomers form cation-sensitive ion channels in neuronal plasma membranes (Arispe et al., 1993). Atomic force microscopy images of Aβ-treated reconstituted bilayers have revealed disk-like structures with pore-like concavities of 8–12-nm outside diameter and 1–2-nm inside diameter. However, pore formation remains a contentious issue, and some authors support the notion that oligomers alter membrane conductivity without forming discrete pores (Sokolov et al., 2006).

Calcium deregulation. Whether due to membrane thinning, pore formation or modulation of membrane calcium-permeable receptors, intracellular calcium is known to be dysregulated in AD (for a review: Stutzmann, 2007). Under resting conditions, cytosolic calcium is maintained at low nanomolar concentrations by an array of pumps, buffers, and transport mechanisms. Application of Aβ-oligomers triggers unregulated flux of calcium through the plasma membrane and from intracellular storage, and these events are accompanied by a number of dramatic changes in the cytoarchitecture. Dendrites and spines seem to be particularly vulnerable to the calcium dysregulation (Shankar et al., 2007; Zempel et al., 2010). Calcium dysregulation is, most likely, responsible for the majority of the neurotoxic effects of Aβ-oligomers, particularly through excitotoxicity mechanism and-or cell signalling perturbation (for a review: Demuro et al., 2010).

Cell signalling dysregulation. The interference with calcium is tightly linked to a number of cell signalling pathways that have been shown to be altered following application of Aβ-oligomers. In general, several kinases have been

found to be hyper-activated in AD and following Aβ-oligomers application. Mitogen activated ERK1/2 for instance are up-regulated in AD (Pei et al., 2002) and the activity of one of its targets, cAMP-response element-binding protein (CREB), a ubiquitous transcription factor, is known to be impaired in AD (Yamamoto-Sasaki et al., 1999) and in Tg models (Ma et al., 2007). This is relevant to synaptic toxicity since the ERK1/2-CREB cascade is the best characterized signalling pathway involved in synaptic strengthening and LTP in the CNS (Kandel, 1991). Recently, it has been shown that oligomers of Aβ are responsible for the ERK1/2-CREB dysregulation in the 3xTg model of AD (Caccamo et al., 2010).

Besides ERK1/2, many other kinases are known to be hyper-activated by Aβ-oligomers, such as GSK3-β (Hoshi et al., 2003) and JNK (Ma et al., 2009). Since these kinases have been shown to induce tau phosphorylation (Goedert et al., 1997; Singh et al., 1995), it has been suggested that the synaptotoxic effect of Aβ is mediated by tau. Supporting this idea, tau KO is thought to abolish Aβ-oligomers toxicity (Jin et al., 2011).

Interference with the brain insulin system and Wnt signaling. The above mentioned mechanisms involve pathways that are actively operated on by Aβ-oligomers. However, recent studies have shown that Aβ-oligomers pathophysiology can be also linked to down-regulation of protective pathways. Examples of protective pathways that are down-regulated by Aβ are the insulin and the Wnt cascades.

ADDLs were shown to down-regulate plasma membrane insulin receptors (IRs), via a mechanism sensitive to calcium calmodulin-dependent kinase II (CaMKII) and casein kinase II (CK2) inhibition. Most significantly, application of insulin completely prevented this loss of surface IRs, and ADDL-induced oxidative stress and synaptic spine deterioration (Zhao et al., 2008; De Felice et al., 2009). These data suggest that Aβ-oligomers might exert some of their toxicity by interference with the insulin brain pathway.

On the same lines, Inestrosa and collaborators have gathered considerable evidence that the activation of Wnt signaling protects against the Aβ neurotoxicity. Aβ-oligomers in fact induced a decrease in the levels of cytosolic β-catenin, a member of the Wnt signalling pathway, similar to what observed in AD patients. Importantly, Wnt agonists block the behavioural impairments induced by hippocampal injection of Aβ (Inestrosa et al., 2002; Cerpa et al., 2010).

It is very likely that in an *in vivo* setting, Aβ-oligomers exert their synaptotoxic effects both through direct mechanisms (as seen above) and indirect mechanisms involving the immune system. Recent papers from our and other groups have demonstrated that *in vivo* application of Aβ-oligomers can induce microglia activation (Bruno et al., 2009; Maezawa et al., 2011). Microglia activation and neuroinflammation are known to be involved in several CNS neurodegenerative diseases, where are thought to have a detrimental effect in the progression of the pathology (Glass et al., 2010). However, neuroinflammation is a term that refers to a wide range of events and can differ extremely according to the cause, the timing and the duration of the insult. The general mechanisms described for peripheral inflammation do not always apply in the CNS, an organ with limited reparative capabilities. Therefore the features and the role of such oligomers-induced inflammation in AD remained to be elucidated.

1.5 Inflammation and Neuroinflammation

1.5.1 General definition of inflammation: innate vs. acquired immunity

Inflammation is a physiological response that organism developed against threat. The first breakthrough in our understanding of the immune system was made in the late 19th century by Mechnikow while studying the starfish response to small

thorns from tangerine trees. The Russian doctor described the process whereby mobile cells serve as a defense mechanism by attacking and engulfing invading parasites, a process that he called phagocytosis. This response, which is characteristic of lower creatures, is called ‘‘innate’’ and is based on macrophage as the central cell type. We now know that the ability of macrophages to recognize specific harmful molecules derives from the existence of a series of pattern recognition receptors (PRR), such as Toll like receptors (for a review: Gordon, 2002). Remarkably, the availability of PRR and a variety of small antimicrobial molecules is sufficient to protect invertebrates from all type of pathogens. This type of response is conserved in higher organism and represents the first line of defense against threats.

Starting with teleostan (jawed vertebrates) a far more complex system has been developed in addition to the innate response, called adaptive response (for a review: Litman et al., 2010). Here, a selected population of cells from the innate response became professional antigen presenting cells. Repertoires of clonally distributed lymphocytes can be generated, each of them capable of recognizing a defined antigenic determinant. B cells, in particular, will learn to develop antibodies against the given antigen, so that, when a second exposure should happen, a much stronger and more efficacious response, from professional killer cells, can take place. This response is virtually capable of covering the entire spectrum of possible antigens.

1.5.2 The cardinal signs of inflammation

In the periphery, the classical inflammatory response involves four cardinal signs which were originally described by Celsus in the 1st century a.D. as: ‘dolor’ (pain), ‘calor’ (heat), ‘rubor’ (redness) and ’tumor‘(swelling). Modern medicine has identified a complex cascade of signal transduction responsible for these signs.

It is now known that following insult, macrophages, neutrophils and mast-cells will be recruited to the tissue and, upon activation, will release a number of soluble factors directed at different targets: nerve endings, blood vessels, endothelial cells and other inflammatory cells (for a review: Nathan, 2002). Release of bradykinin will activate receptors for the transduction of pain signal on nerve endings, causing pain. Bio-active amines (such as histamine and serotonin), eicosanoids and tryptases will cause vasodilatation (responsible for the heat and redness). Following stimulation by inflammatory cytokines (such as tumor necrosis factor alpha, TNFα) and bradikynin, endothelial cells lining the vessels will express adhesion molecules that increase vessel permeability and allow exudation of plasma factors (edema) and extravasation of leukocytes: these phenomena are responsible for the 'tumor' (swelling).

The activation of macrophages and neutrophils is also accompanied by a massive respiratory burst, resulting in release of proteinases, hydrolases, antibiotic proteins and oxidants (such as hydrogen peroxide, hypohalites and chloramines). The oxidants are responsible for the overall antibacterial, tissue-damaging effects of inflammation. If on one hand this is a beneficial process, that allows elimination of tissue threat, on the other it is responsible for significant tissue breakdown and loss of function ('Functio laesa', a fifth cardinal sign of inflammation).

Since acute inflammation is a homeostatic response of the organism, as soon as the triggering agent has been removed the inflammation will remit. However, whenever the microbial and/or host pro-inflammatory stimuli cannot be removed, the inflammation will be sustained and become chronic.

1.5.3 Damage vs. regeneration

While the main role of inflammation is to solve as quick as possible a potential threat, its development often involves some damage in the tissue and its resolution is accompanied by a process of regeneration. In order to do so, a crucial commitment has to be made to convert the response from the antibacterial, tissue-damaging mode to a mode that promotes tissue repair (for a review: Serhan et al., 2008).

In terms of signalling, relatively little is known about those factors that mediate resolution and thereby prevent chronic inflammatory diseases from occurring. A number of endogenous anti-inflammatory mediators have been identified, including the cytokines interleukin (IL)-10 and transforming growth factor beta (TGF-β), and lipid mediators, such as lipoxins and cyclopentenone prostaglandins (Lawrence et al., 2002).

In cellular terms, healing begins right after the acute response and partly overlaps with it. It is started by migration of fibroblasts to the injured site, where they form a temporary extracellular matrix by secreting collagen and fibronectin. This layer provides structural support for cellular attachment and subsequent cellular proliferation. Growth factors such as EGF, PVGF, VEGF, FGF, released by mast cells and macrophages are responsible for tissue regeneration in the periphery (for a review: Werner and Grose, 2003).

The balance damage-regeneration is extremely delicate and severe pathologies occur when such balance is lost. Excessive and continuous damage, with no capability of the system to block it, results in chronic inflammation which is accompanied by tissue fibrosis and persistent loss of function. Several common conditions are caused by a chronic inflammation component, such as celiac disease, vasculitis, lupus, chronic obstructive pulmonary disease, irritable bowel

disease, atherosclerosis, arthritis, and psoriasis. Chronic subclinical inflammation has been implicated in the onset of less obvious diseases, such as diabetes (Haffner, 2003) and cardiovascular events (Luxembourg et al., 2009).

On the other hand, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-damage-promoting agents potentiates and/or promotes neoplastic risk. It is in fact well known the link between chronic inflammation and cancer (Coussens and Werb, 2002).

In sum, inflammation in the periphery is characterized by a complex and delicately balanced cascade of events involving recruitment of inflammatory cells from the blood stream, release of antibacterial-tissue damaging factors and concomitant regenerative process.

1.5.4 Special features of neuroinflammation in the CNS

An example of a stereotypical, robust inflammatory response can be obtained in the periphery (such as in the skin) following implantation of tumor or ectopic tissue. This procedure results in a violent reaction from the host tissue, massive infiltration of white blood cells and eventually rejection of the engraft.

In a milestone experiment in the 40s, Medawar et al. noted that the same procedure had different outcome in the brain. In fact, implanted tumors appeared to survive better in the brain than in other tissues (Medawar, 1948). It was therefore concluded that the brain has an ‘immune privilege’, by which no classical inflammation can be seen in this tissue. Based on this assumption, for many years the idea of ‘neuroinflammation’ was strongly argued against and neglected in the neuroscience field.

However, in recent years, the immune privilege of the brain has been challenged.

Widner et al., for example, found that once the host brain was sensitized, immunological rejection of the grafted tissue was almost certain (Widner et al., 1988). Studies using facial nerve injury revealed that Wallerian degeneration, as seen in the peripheral nervous system (PNS), is associated with microglial activation (Streit and Graeber, 1993). Interestingly, CNS inflammation appeared to have peculiar characteristic, with minimal leukocyte infiltration and a slow and delayed increase in the number of macrophages. Similarly, excitotoxic-induced injury elicited a moderate inflammatory response, with lack of neutrophils infiltration and delay in macrophage response (Coffey et al., 1990; Andersson et al., 1991). Some leukocytes invasion does occur in extreme conditions such as CNS infection, autoimmune disease (i.e. multiple sclerosis, MS) and in injuries or strokes where the BBB is breached (Lassmann, 1983; Holmin and Mathiesen, 1999; for a review see: Ransohoff et al., 2003).

It is now acknowledged that the immune privilege of the CNS does not mean absence of immune response, but rather a controlled and carefully counter-balanced reaction. Overall, it appears that the inflammation in the CNS displays unusual properties, especially in terms of its down-graded myelomonocytic response to injury. This is to be expected considering that edema and tissue damage, which normally accompany acute inflammation, cannot be afforded in the brain, an organ with limited (if any) regenerative resources (Galea et al., 2007). Evolution has therefore developed an extremely regulated (and still not much understood) mechanism of protection of the brain from insult. This mechanism includes a very tight physical barrier (the BBB), professional cells testing the environment to detect even the smallest alteration and a complex signalling system able to confine the damage to few localized areas.

1.5.5 The blood brain barrier and CNS inflammation

The term ‘blood–brain barrier’ (BBB) covers a range of passive and active features of the brain endothelium, which ensure controlled exchange of substances between the blood and the brain (for a review: Abbott et al., 2006).

The main physical, ‘active’ barrier is constituted by CNS endothelial cells that line cerebral microvessels. Adjacent endothelial cells are linked by tight junctions that impede paracellular movement of most molecular traffic, and rather force the molecules to take a transcellular route across the BBB. Only small gaseous molecules (such as O2 and CO2) and small lypophilic agents (such as ethanol) diffuse freely through the lipid membranes, while small, hydrophilic molecules reach the brain parenchyma via a specific transport systems on the luminal and abluminal membranes.

Furthermore, transport of hydrophilic molecules through the membrane is further controlled by a ‘passive’ feature of the brain endothelium, which has been shown to have a much lower degree of endocytosis/transcytosis activity than the peripheral endothelium. In addition, specialized enzymes such as γ-glutamyl transpeptidase, alkaline phosphatase, and aromatic acid decarboxylase, are present at much higher concentration in the cerebral endothelium, and they significantly contribute to the metabolism of drugs and nutrients (the so called ‘enzymatic barrier’; see Pardridge, 2005).

As a result, the BBB shields the brain from toxic substances in the blood, supplies brain tissues with nutrients, filters harmful compounds from the brain back to the bloodstream and produces a brain interstitial fluid that provides an optimal medium for neuronal function.

In the context of neuroinflammation, the BBB normally impedes the entrance of blood molecules including antibodies or complement factors and limits leukocytes recruitment to the brain. It should be noted that the BBB is not an absolute barrier to immunoglobulins or to leukocytes, and surely allows the passage of cytokines, thus mediating the peripheral-central inflammation communication (Bechmann et al., 2007). Well-controlled leukocyte migration is a key physiologic event in immune surveillance, acute self-limiting inflammation, and antigen recognition. Only under chronic pathologic inflammatory conditions, disruption of the BBB is accompanied by uninhibited transendothelial migration and tissue accumulation of leukocyte (i.e., MS and encephalitis).

1.5.6 Cellular players: microglia, astroglia, neurons

The next level of protection that the brain possesses against pathogens is represented by the cellular response of professional (and not) immune cells. At least three different cell types can exert some type of immune function in the brain: microglia, astrocytes and neurons.

Microglial cells are the innate immune cells of the brain, thus representing the first and most professional line of defense against threat. Adult microglia derives from primitive myeloid progenitors (different from monocytic origin) that arise before embryonic day 8 in the yolk sac and subsequently migrate into the CNS in embryonic stages (Ginhoux et al., 2010). Here, they maintain an ameboid, monocytic phenotype until 14 days, when the morphology of the cells changes, assuming a more ramified, resting phenotype. *In vivo* imaging elegantly showed the morphology and behaviour of microglial cells in basal and damage condition (Nimmerjahn et al., 2005). In the absence of threat, microglial cells display a uniform distribution in the gray matter, where each cell explores and survey the surrounding microdomain with its processes. Upon stimulation (usually attack by pathogens, ischemic or traumatic injury, cancer invasion) microglial cells become

activated: they retract and thicken the processes, increase their soma size and eventually re-acquire the ameboid phenotype (Haynes et al., 2006; see Fig 1-4). This process is accompanied by their proliferation, migration to damaged site and the local release of soluble factors meant to neutralize the pathogen (such as NO and cytokines; Hanisch and Kettenmann, 2007).

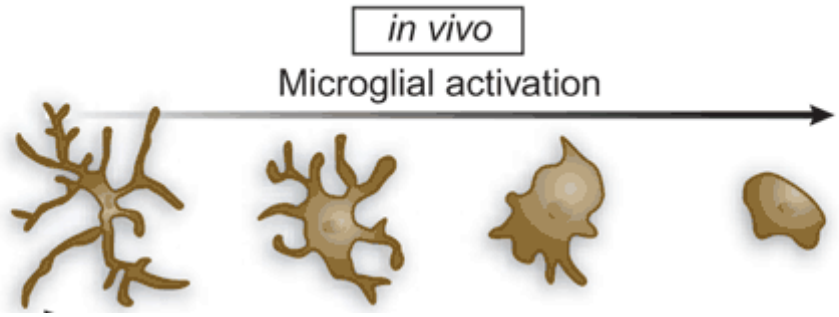

Figure 1-4: Morphological changes accompanying microglia activation
(Modified from: Haynes et al., 2006)

Usually the phagocytosis of debris and dead cells concludes the activation process. Besides their role in infection, microglial cells are known to have physiological roles in synaptic stripping. Microglia have been shown to contact frequently specific synapses (Wake et al., 2009) and the rate of contact depends on neuronal activity. These results indicated that the basal state of neuronal cells can be sensed by the microglial cells and affect their activity. There is now a vast body of evidence indicating that neurotransmitters, ions, ATP and cytokines released for neurons can signal to the microglia, at least *in vitro* (Biber et al., 2007). However the relevance of these signalling *in vivo* and their role in AD is still to be elucidated.

Astrocytes are the main type of glia in the CNS, and by far the most numerous cell type. They are generated from neuronal precursors, and they populate both the gray and white matter in the brain. Each astrocyte occupies a specific microdomain, where the arborization of one cell does not overlap with that of another (Bushong et al., 2002). However, astrocytes are functionally bound through gap junctions to form a functional syncytium. The existence of such system allows for intercellular exchange of ions and transmitters and long distance communication within the glial network (Houades et al., 2008). Importantly, astrocytic processes reach neighboring blood vessels and form endfeet that wrap the capillaries, thus taking part in the formation of the BBB (for a review see: Kettenmann and Verkhratsky, 2008).

In basal, unchallenged conditions astrocytes are responsible for the brain homeostasis, ensuring metabolic support, nutrition, controlling ion and neurotransmitters concentrations, and the regulation of the BBB. Following CNS damage, astrocytes immediately respond in the attempt to isolate the damage, help clearing the pathogen and facilitate regeneration. Typically, astrocytotic activation involves well-described morphological alterations involving hypertrophy and up-regulation of glial fibrillar acidic protein (GFAP, Panickar and Norenberg, 2005); however activation of astrocytes can also occur in an isomorphic fashion (Wells et

al., 1992). The functional consequences of astrocyte reactivity are less understood, but they clearly depend on the molecular pathway involved. Astrogliosis may result in the enhancement of several neuroprotective and neurotrophic functions, as well as in the release of neurotoxic factors (NO, cytokines).

Neurons are not professional immune cells, but in challenging times can come to the help of the innate immune system. iNOS expression has been demonstrated in neurons (Heneka et al., 2001). Several authors reported complement-gene expression by neurons and neuroblastoma cells (for a review see: Gasque et al., 2000), and the expression was shown to be up-regulated in AD (Terai et al., 1997). In experimental autoimmune encephalomyelitis (EAE) neurons were shown to be capable of instructing regulatory T cells and modulating the CNS inflammation (Liu et al., 2006). It is now starting to be investigated the role of specific neurotransmitters in the regulation of the immune system: a role as immunomodulators has been suggested for noradrenaline (NA; Heneka et al., 2010), GABA (Bjurstom et al., 2008), acetylcholine (ACh; De Simone et al., 2005) and endocannabinoids (Walter et al., 2003).

1.6 Neuroinflammation and AD

1.6.1 Amyloid-plaque associated inflammation

Initially proposed by Fischer in 1910 as a process involved in plaque-induced neurotoxicity, inflammation escaped the investigations of the time due to lack of proper techniques (for a review see: Eikelenboom et al., 2011). Plaque-dependent inflammation could be clearly demonstrated only later, when the development of monoclonal antibodies allowed the identification of early complement factors (C1q, C3 and C4) within senile plaques (Eikelenboom and Stam, 1982; Ishii and Haga, 1984) and clusters of activated microglia surrounding them (McGeer et al.,

1987; Rogers et al., 1988). The concept of an AD-associated inflammatory reaction was strongly argued against initially, given the prevailing idea of the brain having an 'immune privilege' at the time. As discussed above, it is now clear that the 'privilege' in the CNS does not relate to the complete absence of immunological components, but rather their elaborate regulation, indispensable in order to limit cellular damage in an organ with limited regenerative capacity (for a review see: Galea et al., 2007).

In agreement with this view, over the last 15 years a plethora of inflammatory mediators has been found to be up-regulated in AD brains, including complement factors, cytokines and chemokines, and cyclooxygenases (for a review: Akiyama et al., 2000). Furthermore, hallmarks of oxidative damage, a likely result of reactive oxygen species and nitrogen species secreted by activated microglia, are present in AD brains (Ando et al., 1998; Good et al., 1996; Smith et al., 1997).

Since then, the occurrence of a plaque-induced inflammatory reaction has been confirmed and substantiated by additional *in vitro* and *in vivo* studies. Transgenic mice over-expressing the human APP with AD-causing mutations demonstrated microglial activation around amyloid plaques that closely resembles the condition found in AD brains (for a review: Morgan et al., 2005). The microglial reaction observed in association with plaques could be mechanistically explained when *in vitro* studies showed the ability of fibrillar Aβ to induce microglial activation and complement activation (Rogers et al., 1992; Yan et al., 1996; Meda et al., 1995; Combs et al., 2001; Klegeris et al., 1997; Tan et al., 1999).

1.6.2 Evidence of early, pre-clinical involvement of innate immunity in AD

Whether neuroinflammation is a mere consequence of Aβ fibrillization and neurodegeneration, or it represents an early event that contributes to the AD-like pathology remains to be elucidated. Unfortunately, the investigation of the

temporal profile of the inflammatory process in AD is complicated by the impossibility of studying AD brains prior to the development and clinical diagnosis of the full-fledged pathology.

A first strategy used to tackle the problem of studying early neuroinflammation has been to use the Braak score (Braak and Braak, 1991) as an indication of disease stage in *post-mortem* material. Using this approach, several clinicopathological studies indicated that amyloid-microglia association is significantly increased in cerebral neocortex brain prior to extensive tau-related neurofibrillary pathology (Arends et al., 2000; Vehmas et al., 2003), suggesting an early involvement of inflammation in the progression of the disease. Similarly, Hoozemans and collaborators demonstrated the up-regulation of COX-2 and cell cycle-related proteins in early stage AD (Hoozemans et al., 2002; Hoozemans et al., 2004). Furthermore, oxidative stress was shown to be quantitatively greatest early in the disease progression (Nunomura et al., 2001).

More recently, an intermediate cognitive condition between normal (i.e. non-cognitive impaired) subjects and AD patients has been identified, and clinically defined as mild cognitive impairment (MCI). MCI is therefore generally considered the initial stage in the progression towards AD (Petersen et al., 1999) (Morris et al., 2001). Up-regulation of inflammatory markers has been observed in brains and CSF from MCI patients (Parachikova et al., 2007; Bruno et al., 2009b; Galimberti et al., 2006).

These indications of an on-going inflammatory process at early stages of AD/MCI have been further confirmed by imaging techniques that have allowed researchers to study the expression of inflammatory markers in patients with probable AD, or MCI. Positron emission tomography revealed that microglial activation precedes brain atrophy in AD (Cagnin et al., 2001) and is already in place at the MCI stage (Okello et al., 2009).

However, MCI is already characterized by extracellular plaque deposition and shares most of the neuropathological features with AD (Morris and Price, 2001; Haroutunian et al., 2009). Whether inflammation is an early response to the AD pathology preceding its clinical signs and plaque deposition remained to be proven.

1.6.3 The role of inflammation in AD-like amyloid pathology

The initial observations from McGeer and collaborators supported the 'inflammatory hypothesis' of AD, according to which inflammation concurs with other neurotoxic mechanisms in causing the neuronal and synaptic failure characteristic of AD (McGeer and McGeer, 2002).

In favor of this hypothesis, it is well established that neuroinflammation can induce, mediate and further exacerbate neuronal damage *in vitro* (for a review: Block et al., 2007). Activated microglia can damage healthy neurons in co-culture systems (Kaushal and Schlichter, 2008). In particular, conditioned media from Aβ activated microglia induced cell death in primary cortical neurons (Floden et al., 2005). The process of inflammation-induced neuronal damage involves the release from activated microglia of soluble factors that are recognized by neurons and can affect the neuronal fate. In particular, activated microglia is known to release a large array of cytokines and reactive oxygen species. Pro-inflammatory cytokines can be cytotoxic by themselves or exacerbate an on-going neurotoxic process (for review see: Griffin, 2006). On the other hand, NO and reactive oxygen species can directly damage neurons through oxidative stress (for a review: Brown and Bal-Price, 2003).

Inhibition of inflammatory pathways *in vivo* with non steroidal anti-inflammatory drugs (NSAIDs) has been shown to improve amyloid pathology and cognitive performance when administered early to APP Tg mice (for a review: Klegeris and McGeer, 2005). The main mechanism of action of NSAIDs is to

inhibit cyclooxygenase (COX) activity, the rate-limiting enzyme for the production of inflammatory prostaglandines (Minghetti, 2004). Two forms of the enzymes have been described: COX-1 and COX-2, and NSAIDs vary in their ability to inhibit them. The best characterized drug in this class in the AD field has been ibuprofen, a non-selective COX inhibitor, which has been shown to reduce Aβ deposition and microglial activation by different groups and in different Tg models (Morihara et al., 2005; Lim et al., 2000; Yan et al., 2003). Several authors reported that the anti-inflammatory effect was accompanied by restoration of memory and synaptic function (Kotilinek et al., 2008; McKee et al., 2008). Other non-specific COX inhibitors such as naproxen (Kotilinek et al., 2008) and indomethacin (Quinn et al., 2003; Sung et al., 2004) have also been shown to have beneficial effects. The effects have been less clear with increased COX-2 selectivity: nimesulide, a relatively selective COX-2 inhibitor, had no effect in Tg2756 (Sung et al., 2004); celecoxib, a selective COX-2 inhibitor, was reported to have no effect (Jantzen et al., 2002) or even worsen the pathology (Kukar et al., 2005). The highest efficacy of COX-1 inhibitors over COX-2 specific inhibitors is not surprising given that microglia, the cell type that are mostly involved in CNS inflammation, express mostly COX-1, while COX-2 in the CNS is a neuronal specific enzyme (Kaufmann et al., 1996).

However, several lines of evidence suggest that inflammation represents a protective mechanism of the innate immune system, aimed at reducing amyloid deposition in the CNS. Sustained chronic inflammation achieved with over-expression models has been shown to ameliorate the AD-like phenotype (Shaftel et al., 2007). Administration of lipopolysaccharide (LPS) to APP Tg mice results in reduced levels of Aβ and fewer plaques (DiCarlo et al., 2001). Furthermore, studies with Tg mice demonstrated that complement activation (Maier et al., 2008) and microglial phagocytosis (Simard et al., 2006) are essential for amyloid clearance from the brain.

In contrast with the above two views, some authors argue that the immune system does not play a role in the disease and is merely a simple bystander (Das et al., 2006). This argument has been strengthened by a recent study that showed how crossing an APP Tg mice with CD11b-HSVTK mice (in which nearly complete ablation of microglia is achieved after ganciclovir application) did not affect amyloid plaque formation and maintenance or amyloid-associated neuritic dystrophy (Grathwohl et al., 2009). In sum, the role of inflammation in plaque-related pathology remains highly debated.

1.6.4 Anti-inflammatory drugs and AD

Based on the encouraging data from APP Tg mice models showing amelioration of pathology and cognitive restoration upon NSAIDs treatment, several clinical trials were initiated to test the therapeutic effect of NSAIDs in AD (for a review see: McGeer and McGeer, 2007).

The results of such trials have been mostly disappointing. In an effort to minimize gastrointestinal side effects, COX-2 specific inhibitors have been selected. Nimesulide (Aisen et al., 2003), rofecoxib (Aisen et al., 2003; Thal et al., 2005) and celecoxib (Sainati et al., 2000) all proved to have no beneficial effects in AD patients. Two small pilot clinical trials involving non-selective COX inhibitors indomethacin and diclofenac have shown some promise (Rogers et al., 1993; Scharf et al., 1999). Even though some positive effects were detected, the trials were underpowered and the n was further reduced due to drop-out following side effects, primarily gastrointestinal.
Taken together, the available clinical evidence in prospective studies suggests that NSAIDs are not useful therapeutic tools once the AD pathology is fully established.

However, a different picture emerges from retrospective, epidemiological data such as the Rotterdam study (in, V et al., 2001) and the Baltimore longitudinal study for aging (Stewart et al., 1997). In these studies the incidence of AD and the consumption of NSAIDs (non-selective COX inhibitors, since the use of COX-2 specific agents is relatively recent) have been tracked and carefully documented in a long period of time. These retrospective studies indicated that long term use of NSAIDs such as ibuprofen or diclofenac has a protective effect against AD. Furthermore, the protective effect appeared to be proportional to the duration of usage (with highest protection measured for NSAIDs exposure longer than 2 years), and was more consistent with some agents (ibuprofen) rather than others (aspirin).

Similar, protective effects have been observed in retrospective, case-control studies, where the incidence of AD was compared among family members or non-familial cohorts. Sustained use of anti-inflammatory drugs (NSAIDs and or glucocorticoids) was associated with delayed onset and reduced risk of AD among siblings or twins (Breitner et al., 1994; Breitner et al., 1995; Yip et al., 2005). Importantly, even though arthritis appeared to be the most common cause for anti-inflammatory treatment, it did not drive the correlation. Non-familial case-control reports, which have the advantage of a larger sample size, mostly agreed with the familial studies in showing a significant (or a trend towards significant) protective effect of NSAIDs in AD (Beard et al., 1998; Wolfson et al., 2002; Lindsay et al., 2002; Vlad et al., 2008).

Taken together the clinical data strongly suggested that inflammation has a key, negative role in the pre-clinical phases of the pathology, and that its inhibition can retard or diminish the incidence of the disease. On the other hand, interfering with inflammation in late stages of the disease (i.e. in already diagnosed AD) has a detrimental effect, suggesting that inflammation plays different role in early vs. late stages of the pathology.

Despite the evidence discussed above, the field of AD currently considers neuroinflammation as a late entry in the amyloid cascade, and a mere consequence of plaque deposition. Our knowledge of the role of inflammation in early, pre-clinical stages of AD is therefore very limited. Even though some scattered data are available in the literature, no paper has focused on the characterization of an early, pre-plaque inflammatory process in APP models and the exact contribution of microglial activation in early vs. late stages of the pathology remains to be elucidated.

1.7 Objectives and Rationale

In defining the aims of this thesis we took into account that:

1. A long, pre-symptomatic phase is known to precede the onset of the clinical AD. This stage represents the ideal time window for the identification of better diagnostic markers and of early pharmacological targets. However, this latent stage still escapes current diagnosis and it can only be studied in Tg models.

2. Soluble, oligomeric forms of Aβ, rather than plaques, should be considered the main culprit of the AD-like amyloid pathology.

3. Epidemiological and imaging data suggest that inflammation plays a role in the early stages of AD. However, the extent of a pro-inflammatory process prior to plaque deposition has not been elucidated in mice models.

Based on the above, we generated and used a novel Tg mouse model over-expressing the human APP protein. We used IHC, biochemical and behavioural technique to investigate early, pre-plaque pathological events in these mice.

Furthermore, we studied the role of inflammation in pre-plaque inflammation by administering the anti-inflammatory drug minocycline.

The following working hypotheses were formulated:

- **Hypothesis 1** Prior to plaque deposition Aβ starts accumulating and oligomerizing within pyramidal neurons of cerebral cortex and hippocampus. The accumulation of toxic Aβ-oligomers should be accompanied by alterations in the behaviour, synaptic counts and biochemical markers.

- **Hypothesis 2** Inflammation is one of the earliest, oligomers-induced pathological events in the AD-like neuropathology. It should precede plaque deposition and it should be deleterious to the CNS, while its inhibition should result in amelioration of the pathology.

These hypotheses were tested by setting specific objectives, which were:

1) To characterize a novel APP mouse model of AD, with special attention to the role of oligomers in the pre-plaque phase (Chapter 2)

2) To study the occurrence and features of a pro-inflammatory response in pre-plaque Tg mice (Chapter 3)

3) To study the effect of an anti-inflammatory drug on the early, pre-plaque phase of the pathology (Chapter 4)

CHAPTER 2

Transgenic Mice as a Model of Pre-Clinical Alzheimer's Disease

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2.1 Abstract

At diagnosis, Alzheimer's disease (AD) brains are extensively burdened with plaques and tangles and display a degree of synaptic failure most likely beyond therapeutic treatment. It is therefore crucial to identify early pathological events in the progression of the disease. While it is not currently feasible to identify and study early, pre-clinical stages of AD, transgenic (Tg) models offer a valuable tool in this regard.

Here we investigated cognitive, structural and biochemical CNS alterations occurring in our newly developed McGill-Thyl-APP Tg mice (over-expressing the human amyloid precursor protein with the Swedish and Indiana mutations) prior to extracellular plaque deposition. Pre-plaque, 3-month old Tg mice already displayed cognitive deficits concomitant with reorganization of cortical cholinergic pre-synaptic terminals. Conformational specific antibodies revealed the early appearance of intracellular amyloid β (Aβ)-oligomers and fibrillar oligomers in pyramidal neurons of cerebral cortex and hippocampus. At the same age, the cortical levels of insulin degrading enzyme -a well established Aβ-peptidase, were found to be significantly down-regulated.

Our results suggest that, in the McGill-Thy1-APP Tg model, functional, structural and biochemical alterations are already present in the CNS at early, pre-plaque stages of the pathology. Accumulation of intraneuronal neurotoxic Aβ-oligomers (possibly caused by a failure in the clearance machinery) is likely to be the culprit of such early, pre-plaque pathology. Similar neuronal alterations might occur prior to clinical diagnosis in AD, during a yet undefined 'latent' stage. A better understanding of such pre-clinical AD might yield novel therapeutic targets and or diagnostic tools.

2.2 Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is characterized by progressive memory loss, extracellular amyloid plaques - composed by aggregated fibrillar amyloid-β peptide (Aβ, for a review: Selkoe, 2000a) and intracellular neurofibrillary tangles, composed by paired helical filaments of hyper-phosphorylated tau (Iqbal and Grundke-Iqbal, 2008).

According to the amyloid cascade hypothesis, the most widely accepted hypothesis for the etiology of AD, the main neurotoxic agent in the pathology is the Aβ peptide, derived by sequential cleavage of the amyloid precursor protein (APP, for reviews: Selkoe, 2000b; Hardy and Selkoe, 2002; Golde, 2005). Once generated, monomeric Aβ has a high tendency to aggregate into oligomers of different sizes, and eventually form insoluble extracellular fibrils, which are the main component of amyloid plaques seen in AD brains (Walsh et al., 1999; Harper et al., 1999).

When AD is diagnosed, the amyloid pathology is mainly extracellular, with the accumulation of a variable number of diffused and dense plaques surrounded by dystrophic neurites (Mirra et al., 1991). In both humans and transgenic (Tg) models plaque pathology is accompanied by structural and functional synaptic alterations (Bell and Cuello, 2006), in which the cholinergic system appears to be the most vulnerable (Francis et al., 1999). However, it is now widely accepted that plaque load does not correlate with cognitive status (Terry, 1997), suggesting that amyloid plaques represent a relatively inert tombstone of aggregated Aβ. On the other hand soluble, oligomeric forms of Aβ have been shown to be highly neurotoxic (Walsh and Selkoe, 2007; Klein, 2006; Glabe, 2005). Importantly, soluble levels of Aβ strongly correlate with the degree of dementia in AD patients and Tg mice (Lesne et al., 2008; McLean et al., 1999), further supporting the new concept that Aβ-oligomers play a key role in the AD neuropathology.

Despite intense efforts from the scientific community, pharmacological treatment available to AD patients is merely symptomatic and can only delay, but not arrest, the progression of the disease (Cuello, 2007). It is starting to be acknowledged that the limited therapeutic effect of current AD drugs could be due to the late clinical diagnosis of the disease, which is achieved when the neuronal function is irreversibly compromised and beyond therapeutic rescue (Becker et al., 2008). In order to maximize the possibilities of finding a cure for AD it is therefore crucial to identify events occurring at the very first (i.e. pre-clinical) stages of the pathology, preceding the formation of amyloid plaques.

An intermediate cognitive condition between normal (i.e. non-cognitive impaired) subjects and AD patients is clinically defined as mild cognitive impairment (MCI), which is therefore generally considered the initial stage in the progression towards AD (Morris et al., 2001). However, MCI is already characterized by extracellular plaque deposition and shares most of the neuropathological features with AD (Morris and Price, 2001; Haroutunian et al., 2009).

It is likely that neuropathological alterations start to take place even earlier than in MCI, in a pre-clinical, yet un-diagnosable stage of the amyloid pathology. Such an early, pre-plaque stage of the pathology cannot be easily investigated in humans, but can be modeled in Tg animals. Tg animal models of the Aβ amyloid pathology reproduce faithfully a number of key aspects of the human AD condition, notably behavioral deficits and extracellular amyloid deposits, accompanied by the accumulation of neurotoxic Aβ-oligomers, dystrophic neurites and neuroinflammation (Morrissette et al., 2009). Young, pre-plaque mice -that will invariably develop the full amyloid pathology- represent an ideal model to study early events in the progression of the amyloid pathology.

Even though pre-plaque cognitive deficits and/or LTP disruption have been documented previously by us (Echeverria et al., 2004c) and others (Billings et al., 2005) (a finding highly indicative of a neuronal dysfunction independent of plaque deposition), the extent and characteristics of such early, pre-plaque amyloid pathology in Tg mice models has not been elucidated.

In this study, we took advantage of our newly developed Tg model of AD-like pathology, coded McGill-Thy1-APP, to characterize behavioral and neuropathological events that occur in the early, pre-plaque stage of the amyloid pathology.

We demonstrate that, prior to the extracellular deposition of amyloid plaques (starting at 4 months of age), and coincidental with the first cognitive deficits, McGill-Thy1-APP mice already display alterations in the number of cholinergic presynaptic boutons in the frontal cortex. Immunolabelings with conformational specific antibodies Nu1 and OC also suggest that the early, pre-plaque pathology is accompanied by the formation and intraneuronal accumulation of neurotoxic Aβ-oligomers and fibrillar oligomers. Such early accumulation of intracellular Aβ material might be further exacerbated by the down-regulation of insulin degrading enzyme (IDE), one of best characterized Aβ peptidases, which is expressed mainly in the neuronal cytoplasm.

2.3 Materials and Methods

Expression construct. The construct used was constituted by the 5' and 3' regions of the murine thymocyte antigen promoter (*Thy-1.2*) (van der Putten H. et al., 2000), carrying the human APP_{751} (*hAPP*) cDNA bearing the Swedish double mutation (K670N, M671L) and the Indiana mutation (V717F). The cDNA contained the entire coding region and approximately 900 bp of 3' un-translated sequence (see Fig.1). Thy1.2 promoter is expressed primarily in the telencephalon, notably by selected populations of pyramidal neurons of the cerebral cortex and

hippocampus, which are regions most vulnerable to the Alzheimer's pathology. The transgene expression starts post-natally (and therefore it does not interfere with embryonic development) and continues throughout life, resulting in a progressive amyloid pathology.

Transgenic mice generation. The McGill-Thy1-APP transgenic mice were generated by the standard microinjection technique (Gordon et al., 1980) into one-cell embryos C3B6F-1 (C3HxC57Bl/6). The founders and the offspring mice were genotyped by PCR amplification of DNA from proteinase K- digested tail biopsies. The primers used were: 5'-CTGCATCCAGATTCAC-3' and 5'-AAGAGGTGGTTCGAGAGGTG-3', which generated a 110 bp band. Transgene mRNA expression in the Tg mouse cortex was confirmed by RT-PCR (Halmekyto et al., 1991) using the same primers. Transgene copy number was determined in purified genomic DNA (gDNA) from tail biopsies by Southern blotting according to standard procedures. Briefly, 10 μg of gDNA were digested with *Hind*III endonuclease, excising a fragment of about 4000 bp from the transgenic construct, and separated on a 0.9% agarose gel together with copy number controls (from 1 to 32 copies). A digoxygenin-labelled probe (Roche Molecular Biochemicals, Laval, Canada) was obtained by PCR amplification of the 5' region of the Thy1-APP construct. The primers used were: forward: ATCCCACTCGCACAGCAG (located on the linking region between the Thy sequence and the APP cDNA), reverse: GGAATCACAAAGTGGGGATG (nucleotide 336 on cDNA). Chemiluminescence detection of digoxygenin-labelled probes was performed according to the manufacturer's instructions. Throughout the study-period, animals were housed in a controlled environment (temperature 22˚C, humidity 50-60%, 12 hrs light/12 hrs dark schedules). All procedures were approved beforehand by the Animal Care Committee of McGill University, and the guidelines of the Canadian Council on Animal Care were followed.

Perfusion and tissue preparation technique. Tg and Non Tg littermates mice were deeply anesthetized with equithesin (pentobarbital-based, 2.5 ml/Kg, i.p.) and perfused through the heart with ice-cold saline solution (pH 7.4) for 1 min. The brains were then quickly removed and divided into right and left hemispheres on ice. Cortex, hippocampus and cerebellum were dissected from the left hemisphere, snap-frozen in dry ice and stored at -80˚C for biochemical analysis. The right hemisphere was fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4) for 24 h at 4˚C. The tissue was then cut into 40µm thick sections with a freezing sledge microtome (SM 2000R, Leica) at -20˚C and free-floating sections were collected in phosphate buffered saline (PBS) and processed for immunohistochemistry.

Western blotting. Western blotting was performed as previously described (Bruno et al., 2009a). Briefly, cortical samples from the left hemisphere of five Tg and five Non Tg littermates at 3 months of age were sonicated in 250 µl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 2 µg/ml of aprotinin, 2 µg/ml of leupeptin, 100 µg/ml PMSF, pH 7.4). Samples were centrifuged at 13,000 rpm for 45 min at 4˚C and protein content of the supernatants was determined using the Dc-protein assay, (BioRad, USA). 20-50 µg of total protein were loaded on a 10% acrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked for 1h at room temperature in 5% skim milk and then incubated over night at 4˚C with the primary antibody (rabbit polyclonal universal APP, working dilution 1:1000, Chemicon, Millipore, USA; mouse monoclonal anti-Aβ 6E10, working dilution 1:1000, Signet laboratories, California, USA; rabbit polyclonal anti-IDE, working dilution 1:1000, Calbiochem, USA; goat anti-NEP, working dilution 1:250). After washing in TBS-T buffer, the membranes were incubated with the proper peroxidase-conjugated secondary antibody (working dilution 1:10.000, Jackson ImmunoResearch Lab, from Medicorp, Montreal, Canada) for 1h at room temperature. βIII-tubulin (working dilution: 1:40.000, Promega, Madison, WI,

USA) or β-actin (working dilution 1:20.000, Abcam, USA) were used as loading controls. Signal intensity was quantified by densitometry (MCID4 image analysis system, Imagining Research Inc., St. Catherine's, ON). Groups were obtained simultaneously and normalized with respect to βIII-tubulin (neuronal specific) immunoreactivity. All experiments were performed in triplicate.

Human Aβ ELISA. Human Aβ40 and Aβ42 levels were measured by an *in vitro* ELISA kit (Invitrogen, from Medicorp, Montreal, Canada), according to manufacturer instructions. Briefly, five 3-month old Tg mice and two Non Tg age-matched littermates were sacrificed by decapitation, and brains were quickly dissected on ice. After separating the hemispheres, cerebral cortex, hippocampus and cerebellum were microdissected, snap frozen in liquid nitrogen and stored at -80˚C till use. Brain samples from the left hemisphere were dissolved in 10 wet weight volumes of 5M guanidine hydrochloride and incubated at room temperature gently rocking for 4h, till complete disaggregation of the tissue occurred. For the assay, samples were further diluted ten times with the provided standard diluent buffer and centrifuged at 13,000 rpm for 20 min at 4˚C. Samples and standards were run in duplicate and developed accordingly to manufacturer's instructions. To ensure reliability of the measurements, the standard curve was prepared in standard diluent buffer containing 0.5M guanidine hydrochloride. Absorbance of samples and standards was read at 450nm; Graph-Pad Prism5 curve fitting software was used to generate the standard curve and calculate Aβ concentration in the samples.

Immunohistochemistry

Bright field immunohistochemistry: free floating immunohistochemical staining was performed as previously described (Hu et al., 2003a). Mouse monoclonal primary antibodies used were: McSA1 (from MediMabs, Montreal, Canada, (Grant et al., 2000) working dilution: 1:4000), which recognizes the first 12 amino acid of the Aβ sequence; conformational specific Nu1 (from Dr Klein's

laboratory, Northwestern University (Lambert et al., 2007), working dilution 1:3000); neuronal specific NeuN (Chemicon, Temecula CA, USA; working dilution 1:20.000). Rabbit polyclonal primary antibodies used were: anti-VAChT (vesicular acetylcholine transporter, generous gift from Dr Edwards (UCSF); working dilution 1:10.000); conformational specific OC (from Dr Glabe's laboratory, University of California, (Kayed et al., 2007) working dilution 1:5000). For mouse monoclonal primary antibodies, sections were incubated with a secondary goat-anti-mouse IgG (working dilution 1:100; MP Biomedicals, Irvine California, USA) followed by a mouse anti peroxidase monoclonal antibody complex (MAP/HRP complex, MediMabs, Montreal, Canada; (Semenenko et al., 1985)). For sections processed with rabbit polyclonal primary antibodies, a biotinylated goat-anti rabbit secondary antibody was used (working dilution 1:200), followed by amplification with the avidin-biotin complex (both from Vector laboratories Inc., Burlingame, California, USA). All stainings were developed with 0.06% DAB (Sigma-Aldrich Canada, Oakville, ON) and 0.01% H_2O_2.

Digital images were acquired on an Axioplan 2 Imaging microscope (Zeiss), equipped with an AxioCam HRc digital camera (Zeiss), using Axiovision 4 Imaging program (Zeiss).

Bright-field double labeling: free-floating sections were double-labelled using the SG kit (Vector, Burlingame, California, USA) according to the manufacturer instructor. Briefly, sections were incubated with both primary antibodies according to standard procedure and sequentially developed using DAB (brown reaction) and SG (blue reaction). When two monoclonal were used (NeuN and McSA1 double labeling) the 'mouse-on-mouse' MOM blocking kit was applied according to manufacturer's instructions (Vector laboratories Inc., Burlingame, California, USA)

Thioflavin-S staining was performed as previously described (Hu et al., 2003).

Confocal double labelling: Free floating double immunofluorescence staining was performed as previously described (Majdi et al., 2007). Primary antibodies used

were: monoclonal Aβ specific McSA1 (Grant et al., 2000), working dilution 1:16000), monoclonal conformational specific Nu1 (Lambert et al., 2007; working dilution 1:300), monoclonal APP-specific 22C11 (Roche Applied Sciences, Laval, Canada, working dilution 1:500), rabbit polyclonal OC (Kayed et al., 2007; working dilution 1:500). Secondary antibodies used were: donkey anti-mouse Rhodamin conjugated (Jackson Immunoresearch, from Medicorp, Montreal, Canada; working dilution 1:200) and biotinylated goat anti-rabbit (Vector laboratories Inc., Burlingame, California, USA; working dilution 1:200) followed by treatment with Streptavidin Alexa 488 (Molecular probe, Invitrogen, Carlsbad, CA, USA). Stainings were revealed using a Zeiss LSM 510 confocal microscope equipped with argon and helium neon lasers (Zeiss Canada, Toronto, ON). A plan-apochromat 40x objective was used. Optical sections were approximately 0.6μm thick as indicated by pinhole settings. Control images (including omission of one or both primaries, and complete double labelling on Non Tg tissue) were acquired applying the same settings used for the experimental sections.

"Mouse-on-mouse" protocol for the detection of two distinct monoclonal antibodies on the same section: the protocol was applied according to manufacturer's instructions (MOM blocking kit, Vector laboratories Inc., Burlingame, California, USA). As an example, for the 22C11-McSA1 double-labeling, sections from Tg and Non Tg animals were first incubated with 22C11 and a biotinylated-anti mouse secondary antibody, followed by Streptavidin Alexa treatment. The residual sites on the 22C11 antibody un-bound to the secondary antibody were then blocked with the specific blocking agent, and the sections were further incubated with McSA1 and a Rhodamin conjugated anti-mouse secondary antibody. The efficacy of the blocking step was confirmed using several controls, including sections incubated with 22C11 and both secondary antibodies (without McSA1), and full double labeling in Non Tg littermates. A similar protocol was used for the double labeling McSA1-Nu1 and Nu1-22C11.

Quantification of immunohistochemical VAChT staining. Quantification of presynaptic cholinergic terminals in frontal cortex was performed essentially as previously described (Bell et al., 2006). Three-month old Tg (n=8) and Non Tg littermates (n=6) were used for the pre-plaque time point. Fourteen-month old Tg (n=6) and Non Tg littermates (n=4) were used for the post-plaque time point. Three free-floating sections per animals were stained according to standard protocols described above, and a total of 8 pictures (4 corresponding to lamina V and 4 corresponding to lamina VI) were captured using an Olympus BX51 microscope equipped with a 63x oil-immersion lens (for a total of 24 pictures per animal). Pictures were then up-loaded into the MCID5 Elite Image analysis system (Imagining Research Inc., St. Catherine's, ON), and the density of immunopositive punctae was quantified using a software designed for silver grain counting. A single detection threshold, obtained by trial and error and which provided the best overlap between the computer's detection and the true immunohistochemical staining, was saved and re-applied to all the pictures. Areas occupied by blood vessels or plaques (in case of old Tg mice) were excluded. M.T.F. was blind to the source of all material and, in the effort to reduce variability, was the only individual to perform the quantification.

Nu1 immunohistochemistry on unfixed tissue and Dot blot. After transcardial perfusion with ice-cold saline, the cortex from a 6 month-old Tg mouse was divided in three parts and each probed with the Nu1 antibody using a different technique. The same procedure was done in parallel on a Non Tg, age-matched littermate.

Dot blot of tissue crude homogenates. The samples were sonicated in 350 µl of lysis buffer, and centrifuged at 13,000 rpm for 1 hour at 4˚C. The supernatants were collected and re-centrifuged for 10 minutes. These final supernatants were immunodepleted by sequential incubation of one hour at 4˚C with Protein A-Sepharose, fast Flow followed by protein G-Sepharose, fast Flow (Amersham, UK). After protein content quantification, the samples were diluted to a final

concentration of 30 μg/μl, and 1 μl was spotted on a nitrocellulose membrane. The membrane was blocked with fat milk 5% 1h at room temperature, and incubated with Nu1 (1:1000) for 1h at room temperature. The signal was detected according to standard procedure described for Western blotting above.

Immunohistochemistry on unfixed tissue Immunohistochemistry of fixed cortex samples was performed as described above. The other hemi-cortex was snap frozen in liquid nitrogen-cooled isopentane, and 20 μm sections were cut with a cryostat. Fixed and unfixed sections were processed using the same batch and concentration of primary antibody (Nu1 1:3000), to ensure comparability of the results. Unfixed sections were air-dried to avoid the use of cross-linking chemicals that might artificially create oligomers.

*Morris water maze***.** Morris water maze study was performed according to standard protocols as routinely applied in our lab (Garofalo et al., 1992) adapted for mice testing. Briefly, each cohort of animals (3 months old and 10-13 months old, composed of Tg and Non Tg littermates) was trained to find the submerged platform in a 1m diameter pool of white, non-toxic colored water (temperature: 23˚C). Animals would orient themselves using distal and spatial clues available in the testing room only. Animals received 3 trials per day (10 min inter-trial period) for 5 days. During each trial animals were allowed to search for the platform for a maximum of 60s. If an animal failed to find the platform, it would be gently placed on it by the experimenter and allowed to stay there for 10s. Escape latency, path length and speed were recorded with a HVS Image tracking system (Buckingham, UK). On the sixth day the platform was removed and a probe test for memory retention was performed by allowing the mice to swim freely in the pool for 60s. Time spent in target quadrant and passes through the target platform were recorded. Blind mice or unmotivated mice (not able to reach a visible, raised platform in 60 s), and mice showing reduced speed were discarded. The final n per group was: n=8 (3 months old cohort) and n=10 (10-13 months old cohort).

*Novel Object Recognition***.** Novel object recognition was performed according to established protocols (Taglialatela et al., 2009). Briefly, 3 months old Tg and Non Tg animals were first habituated to an empty open field box (two sessions, 24 h inter-trial interval). Twenty-four hours after the last habituation session, mice were exposed to two identical, non-toxic 'familiar' objects. After a retention interval of 24 h, the animals were returned to the arena in which two objects, one familiar and one novel were located. Each session lasted 10 min, during which all the mice were allowed to freely interact with the objects and the amount of time exploring each object was recorded. Objects were randomized and counterbalanced across animals. Animals that spent <7 s exploring the objects during the 10 min test session were excluded from analysis. The number of crossings through the four quadrants was recorded as a measure of locomotor activity, while total time spent exploring both objects was recorded as a measure of curiosity and motivation. The final n per group was: n=6 Non Tg and n=13 Tg.

Data analysis. All data were analyzed using the Graph-Pad Prism 5 software. Two groups comparison was done by unpaired Student's T-test. Multiple group comparisons were analyzed by 1 or 2-way-ANOVA. Significance was set at $p<0.05$. Data are presented as mean ± S.E.M.

2.4 Results

2.4.1 Generation of McGill-Thyl-APP

We report a new Tg mouse model of the AD-like amyloid pathology, which carries the human APP751 transgene bearing the Swedish double mutation (Mullan et al., 1992) and the Indiana mutation (Murrell et al., 1991), flanking the N- and C-terminus of the Aβ domain. The transgenic strategy used was similar to the mice TgCRND8 (Chishti et al., 2001) and J20 (Mucke et al., 2000) and to the rat

transgenic models UKUR28 (Echeverria et al., 2004a) and McGill-Thy1-R-APP (Leon et al., 2010). This approach exploits the synergistic effect of the Swedish double mutation, which increases the occurrence of β-cleavage on APP (Citron et al., 1992), and the Indiana mutation, known to favor the generation of Aβ42 over Aβ40 (Suzuki et al., 1994). The transgene was cloned under the control of the murine Thy1.2 promoter (Fig. 2-1a), which yields a strong and specific expression in pyramidal neurons of the telencephalon (van der Putten H. et al., 2000). We chose the C3B6F-1strain (derived from the crossing of the light brown C3H and the dark brown C57Bl/6) as background, since it is widely used for transgenesis and it displays a higher ratio of integration of transgene (30% in comparison with the 10% achieved by using the C57Bl/6 alone).

Upon microinjections of the transgene in one-cell embryos, three putative founders were identified by PCR (Fig. 2-1b, putative founders 'd', 'f', 'h') and one showed expression of the transgene mRNA as defined by a RT-PCR assay (founder 'f', Fig. 2-1c). This line was designated McGill-Thyl-APP and was further characterized. The transgene copy number as assessed by Southern blotting was estimated to be about 20 (Fig. 2-1d).

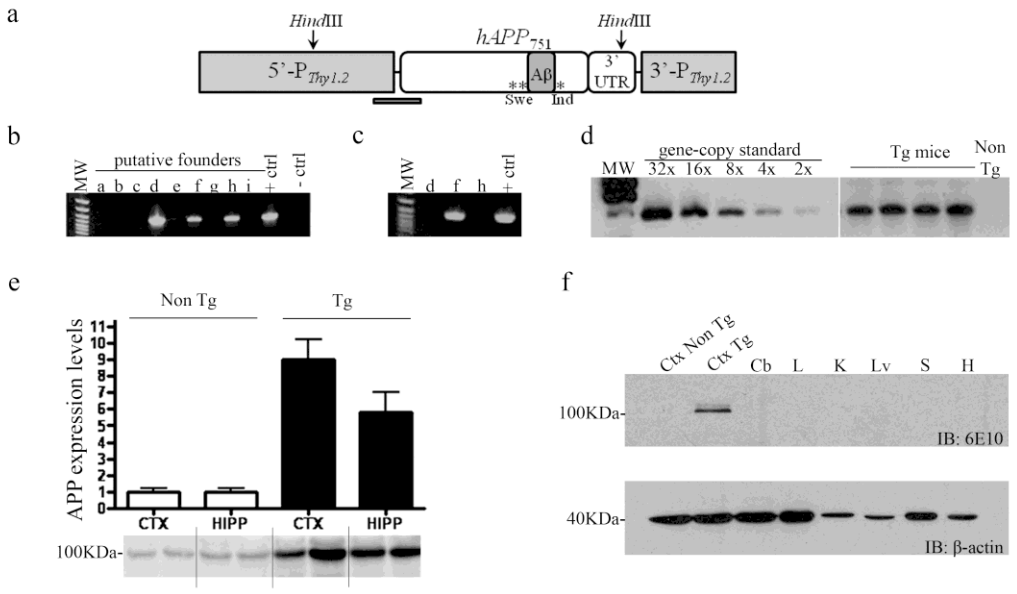

Figure 2-1. Transgene expression in McGill-Thy1-APP mice

a: Schematic of the construct used to generate the McGill-Thy1-APP mice. Note that in this construct the human APP751 sequence contains the Swedish (**) and the Indiana (*) mutations, flanking the C and N terminal of the Aβ sequence. The approximate location of the *Hind*III restriction sites used for digesting the genomic DNA is indicated by the arrows, and the approximate annealing site of the probe used in the Southern blot analysis is also shown as a grey bar.

b: PCR analysis of 9 putative founders. Three mice (d,f,h) showed to carry the transgene.

c: Reverse-transcriptase PCR revealed that only 'f' was effectively transcripting the transgene. This animal was used as the founder of the colony.

d: Representative gene copy number analysis by Southern blot: McGill-Thy1-APP mice carry on average 20 copies of the transgene. No band was detected in samples from Non Tg littermates.

e: Western blot analysis of cortical (CTX) and hippocampal (HIPP) homogenates using a universal (rodent and human) APP antibody. Note the strong over-expression of the protein (about 9 fold increase in cortex and 6 in hippocampus) in Tg animals as compared to age-matched Non Tg littermates.

f: Tissue expression profile of transgenic protein analyzed via Western blot (6E10 IB). Human APP was found only in cortex (Ctx) of Tg mice, while no expression was detectable in cerebellum (Cb) nor in peripheral organs (L: lung; K: kidney, Lv: liver; S: spleen, H: heart).

The actual over-expression of the transgene product was confirmed by Western blotting analysis with an APP-specific antibody which recognizes both human and murine APP forms (Fig. 2-1e). As expected, APP expression was found to be significantly increased in hippocampus and in cortex of McGill-Thyl-APP compared to Non Tg littermates. In particular, APP expression was 6-fold higher in the hippocampal complex and 9-fold higher in the cerebral cortex (Fig. 1e) in the Tg mice as compared to Non Tg controls as estimated by relative densitometry. There was no significant change in APP-levels in the cerebellum of Tg animals as compared to Non Tg littermates (data not shown), nor did we observe significant levels of transgenic proteins in visceral organs of Tg mice (see Fig. 2-1f).

F1 animals were then inbred and the heterozygous offspring used for further immunohistochemical, biochemical and behavioral characterization. The list of antibodies used for immunohistochemical and biochemical studies can be found in Table 2.1.

Table 2.1 List of antibodies used in the study.

Antibody name		Source	Epitope recognized	Immuno reactive species	Reference
Aβ-APP related	Universal APP	Rabbit polyclonal	Aa 99-126 on the N terminus of APP	Human, murine	Chemicon Millipore, USA
	6E10	Mouse monoclonal	Aa 1-16 on the N terminus of human Aβ	Human	Signet Laboratories, CA, USA
	McSA1	Mouse monoclonal	Aa 1-12 on the N terminus of human Aβ	Human	(Grant et al., 2000) , MediMabs, Montreal, Canada
	22C11	Mouse monoclonal	Aa 66-81 on the N terminus of APP	Human, murine	Chemicon Millipore, USA
	Nu1	Mouse monoclonal	Conformational specific, low-n Aβ-oligomers (ADDLs)	Human	(Lambert et al., 2007), Dr W. Klein, Northwestern University
	OC	Rabbit polyclonal	Conformational specific, fibrillar oligomers and fibrils	Not assessed	(Kayed et al., 2007), Dr C. Glabe, UC Irvine
Neuronal and cholinergic markers	VAChT	Rabbit polyclonal	Aa 478-530 on the C-terminus of the vesicular acetylcholine transporter	Rodent	(Gilmor et al., 1996), Dr R. Edward, UCSF
	NeuN	Mouse monoclonal	DNA-binding, neuron-specific protein NeuN	Human, murine	Chemicon, CA, USA
Aβ degrading enzymes	IDE	Rabbit polyclonal	N-terminus of human IDE	Human, murine	Calbiochem, EMD bioscience, USA
	NEP	Goat polyclonal	Extracellular domain mouse neprilysin	Murine	R&D system, MN, USA

The McGill-Thyl-APP Tg line proved to display high survival rate, as the majority of these mice live up to 2 years.

Pilot behavioral experiments demonstrated a high percentage of blind animals in the colony, most likely due to the genetic retinal degeneration characteristic of the C3H strain. We therefore decided to outbreed the Tg line with the C57Bl/6 strain, to eliminate the C3H background; no changes in the phenotype were observed following this manipulation.

2.4.2 Developmental analysis of the amyloid pathology in McGill-Thy1-APP mice

In order to investigate the time-dependent evolution of the amyloid pathology in our model, a specific mouse monoclonal antibody (McSA1, MediMabs, Montreal, Canada) recognizing the 1-12 N-terminus domain of the human Aβ peptide (Grant et al., 2000; Echeverria et al., 2004a) as used to stain brain sections of McGill-Thy1-APP mice at different time points after birth–ranging from 1 week to 10 months of age (Fig. 2-2). Controls included staining of age matched Non Tg littermates and omission of the primary antibody: no staining was observed in both conditions (data not shown).

This analysis revealed that the McGill-Thy1-APP mice accumulate intracellular McSA1-immunoreactive material as early as at 1week of age (see Fig. 2-2a-c). The reaction products appeared at this age as granular material, mainly located in neuronal soma and in the initial portion of apical dendrites of pyramidal neurons of lamina V in the cerebral cortex (Fig. 2-2c). Intracellular accumulation of McSA1-immunoreactive material was also detected in pyramidal neurons of CA1 and CA3, subiculum and hilus in the hippocampus as well as in granular neurons of the outer dentate gyrus. McSA1-immunoreactivity (IR) was also observed in the pyriform cortex and amygdala from 1 week of age. At this early stage we also observed immunoreactive fibers in the alveus and the capsula interna.

Figure 2-2. Developmental analysis of amyloid pathology in McGill-Thy1-APP mice

Representative micrographs showing the progression from pre-plaque (up to 3 months of age) to extracellular amyloid pathology (after 4th month of age) in McGill-Thy1-APP mice brain sections, as revealed by immunostaining with the McSA1 monoclonal antibody.

a-c: McGill-Thy1-APP mice present intracellular accumulation of McSA1-immunoreactive material in pyramidal neurons of hippocampus CA1 (**a**) and cerebral cortex lamina V (**a**,**b**) as early as at 1 week of age. Immunoreactive positive material appeared to accumulate in the perinuclear area and the proximal processes of the neurons (**c**).

d-f: At 1.5 months of age McGill-Thy1-APP mice show intracellular McSA1-immunoreactive material in CA1, CA2, CA3 and dentate gyrus in the hippocampus (**d**), and laminae III, V and VI in the cerebral cortex (**d**,**e**).

g-i: At 3 months of age intense McSA1-IR is detected in the pyramidal neurons throughout the hippocampal formation (**g**) and in the cerebral cortex (**g**,**h**). No amyloid plaques are detected at this age.

j-l: At 6 months of age mature, Thioflavin-S positive plaques deposit in the enthorinal cortex (ent, **j**), subiculum (s, **j**) and lamina V (**k**) of the cerebral cortex. **l**: representative pictures of a Thioflavin-S positive plaque. The same plaque was stained in two subsequent sections: the upper panel shows a double labeling for NeuN (neuronal nuclei: brown reaction) and McSA1 (Aβ-immunoreactive material: blue reaction), while the lower panel shows a Thioflavin-S reaction.

m-o: By 10 months of age the brain is heavily loaded with big, neuritic amyloid plaques (**m**,**n**). **o**: double labeling for VAChT (brown reaction) and McSA1 (blue reaction) illustrating the occurrence of cholinergic dystrophic neurites associated with a mature, dense core amyloid plaque.

Scale bar: a, d, g, j, m: 500µm; b, e, h, k, n: 50µm; c, f, i, l, o: 20µm

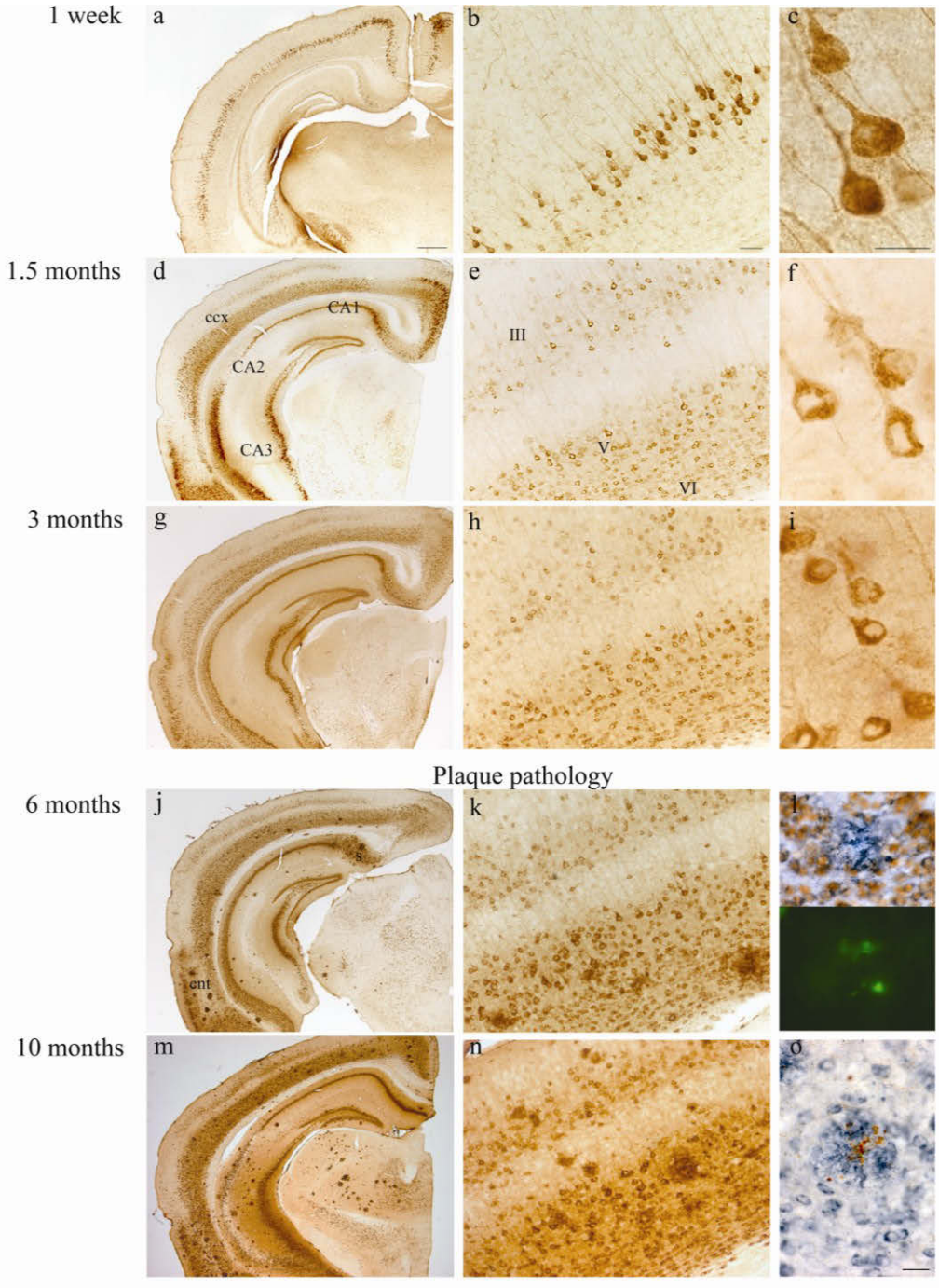

Figure 2-2. Developmental analysis of amyloid pathology in McGill-Thy1-APP mice

By 3 months of age, the intracellular McSA1 staining was widespread (see Fig. 2-2g-i): intensely immunoreactive material was detectable in pyramidal neurons of laminae III,V and VI of the neocortex, the entire hippocampus, amygdala complex and thalamus, accompanied by a marked increment in diffuse IR in the neuropil. Out of 74 animals analyzed at the age of 3 months, only 8 animals displayed occasional small plaques (20-40 μm); of these 8, only two mice presented plaque pathology in the hippocampus (i.e. 3% of total cases at this time point).

Small amyloid plaques appeared as of the fourth month of age mainly in the entorhinal cortex. The extracellular amyloid pathology was well established by 6 months of age (see Fig. 2-2 j-l). At this age, a variable number of large (50-l00 μm), extracellular deposits, positive for Thioflavin-S (i.e. mature, amyloid plaques: see Fig. 2-2l) were observed in the entorhinal cortex, subiculum and hippocampus, and in lamina V of the neo-cortex. In the following months, plaques spread into inner layers of the cerebral cortex and thalamus, and by 10 months (Fig. 2-2 m-o) the brain was heavily loaded with mature, neuritic plaques (see Fig 2-2o for an example of an amyloid plaque surrounded by dystrophic cholinergic neurites).

The epitope recognized by McSA1 is shared with the holo APP protein and APP-fragments such as soluble APP alpha (sAPPα). To establish the nature of the immunoreactive material observed intracellularly, we performed a double-labeling using McSA1 and the APP-specific 22C11 monoclonal antibody. A specific 'mouse-on-mouse' protocol for the detection of two diverse monoclonal antibodies on the same section was applied (Fig. 2-3). Confocal double-labeling in sections from Tg animals showed a distinct pattern of IR for the two antibodies: 22C11 (APP-specific, green signal) appeared diffusely distributed, while McSA1-IR (red signal) was generally associated to granule-like structures localized in the perinuclear region and in the axon. No co-localization was observed, suggesting that McSA1 does not recognize the holo-APP protein in fixed tissue sections.

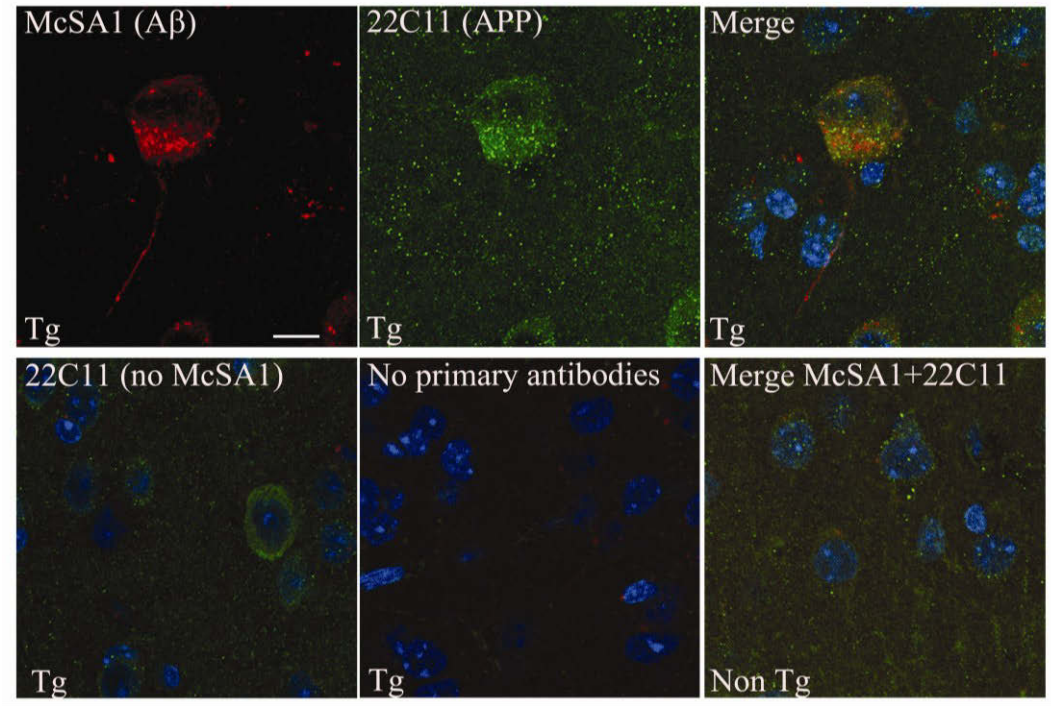

Figure 2-3 McSA1 does not recognize APP in fixed tissue

Representative micrographs of double-labeling using McSA1 and the APP-specific monoclonal antibody 22C11 by confocal microscopy immunofluorescence. The micrographs in the upper panel are the 2D projection of a Z-stack. Note that the perinuclear and axonal McSA1-IR (red signal) did not overlap with the 22C11 IR (APP, green signal). Several controls confirmed the specificity of the staining. When the same protocol was applied on Tg sections, but omitting the McSA1 primary antibody, we observed only a specific 22C11 (APP) signal. Omission of both primary antibodies completely abolished the staining. Finally, the same protocol applied on Non Tg sections rendered an immunoreaction only for 22C11 (APP).

Scale bar: 10µm

The 'mouse-on-mouse' protocol proved to be highly efficient in blocking 22C11 sites un-bound to the biotinylated secondary antibody. In fact, the immunostaining of Tg sections with 22C11 (without McSA1) followed by both secondary antibodies did not display any red signal (i.e. there was no non-specific binding of the Rhodamin anti-mouse to 22C11 sites). On the other side, confocal 22C11-McSA1 double-labeling of Non Tg mice brain sections showed only a 22C11 specific immuno-staining thus confirming the efficacy of the blocking step and the protocol applied.

The presence and levels of Aβ in the hippocampus and cortex of young, pre-plaque mice were further investigated using a well characterized commercially available ELISA kit for detection of human Aβ material. Freshly dissected brain areas from five Tg and two Non Tg mice were processed according to manufacturer's instructions to detect total Aβ40 and Aβ42 content. The results of this study are summarized in Table 2.2. The ELISA assay detected similar amounts of Aβ in the hippocampus and cortex of Tg mice (Aβ42: 52.23±27.84 ng/g wet tissue in hippocampus and 61.99± 29.66 ng/g wet tissue in cortex; Aβ40: 21.96±8.01 ng/g wet tissue in hippocampus and 22.54±7.25 ng/g wet tissue in cortex). Importantly, this analysis revealed a ratio Aβ42:Aβ40 of 2.05±0.65 and 1.68±0.56 in cortex and hippocampus, respectively, a finding indicative of a favored production of Aβ42 over Aβ40. As expected, human Aβ was not detectable in Non Tg animals, and barely measurable in cerebella of Tg animals.

Table 2.2 Human Aβ42 and Aβ40 levels in 3 month-old (pre-plaque) McGill-Thy1-APP, as measured via ELISA (mean ±S.E.M).

brain area (n=5)	Aβ42 (ng/g wet tissue)	Aβ40 (ng/g wet tissue)	Aβ42:Aβ40
cortex	61.99± 29.66	22.54±7.25	2.05±0.65
hippocampus	52.23±27.84	21.96±8.01	1.68±0.56
cerebellum	2.35±0.19	1.72±0.36	1.55±0.25

2.4.3 Cognitive deficits are detected prior to plaque deposition

We next investigated the cognitive status of McGill-Thy1-APP mice in relation to the amyloid pathology. We first used the Morris water maze (MWM) task (Fig. 2-4). Learning and memory functions were evaluated in two cohorts of transgenic animals, corresponding to the early, pre- (3 months old) and post-plaque (13 months old) phases of the amyloid pathology. Results were compared with two cohorts of age-matched, Non Tg littermates. Latency in finding the hidden platform, swim lengths, thigmotaxis and floating behavior were automatically recorded during the 6 days of testing. On the last day, a visual test was performed by raising the platform and allowing the mice to reach it. Animals displaying altered swim speed, floating or thigmotaxic behavior, or delayed latency to reach the platform during the visual test were excluded from the study. The operator was blind to the genotype of the animals throughout the duration of the testing.

Tg mice exhibited learning deficits already at 3 months of age (Fig. 2-4a, 2-way-ANOVA, effect of genotype: $p<0.000l$), when the amyloid pathology in cortex and hippocampus was restricted to intracellular accumulation of Aβ-immunoreactive material. Post-hoc analysis revealed that, at this age, the latency of Tg mice in finding the hidden platform on day 5 was significantly increased

Figure 2-4 Cognitive deficits in the McGill-Thy1-APP mice start at the pre-plaque phase of the amyloid pathology and further progress with Aβ extracellular deposition

a-d: Morris Water Maze performance of Tg and Non Tg age-matched littermates. Note that learning impairment in finding the hidden platform on day 5 was already detected in 3 month old Tg mice as compared to age-matched, Non Tg littermates (**a**), while memory was not significantly affected at this age in this task (**b**); however, a trend towards impairment is evident. Tg mice with progressive, post-plaque amyloid pathology showed more pronounced learning (**c**) and memory (**d**) impairments.

e,f: Novel Object Recognition (NOR) test on 3 month-old mice. No preference for either of the two objects was recorded during the training phase of the task (when two equal objects are presented, **e**). However, Non Tg mice showed a clear preference for the novel object during the probe test 24h later, while Tg mice failed to discriminate between the familiar and the novel object (**f**, $p<0.05$). Data are presented as time spent exploring the novel object over total time spent in exploration. The 0.5 discrimination ratio, equivalent to chance, is highlighted.

(a,c: data were analyzed by 2-way-ANOVA followed by Bonferroni post-hoc test; b,d: two group comparison was analyzed by unpaired Student's T-Test; e,f: multiple groups comparison was made using the one-way-ANOVA followed by Tukey's post-hoc test. *=$p<0.05$, **=$p<0.01$).

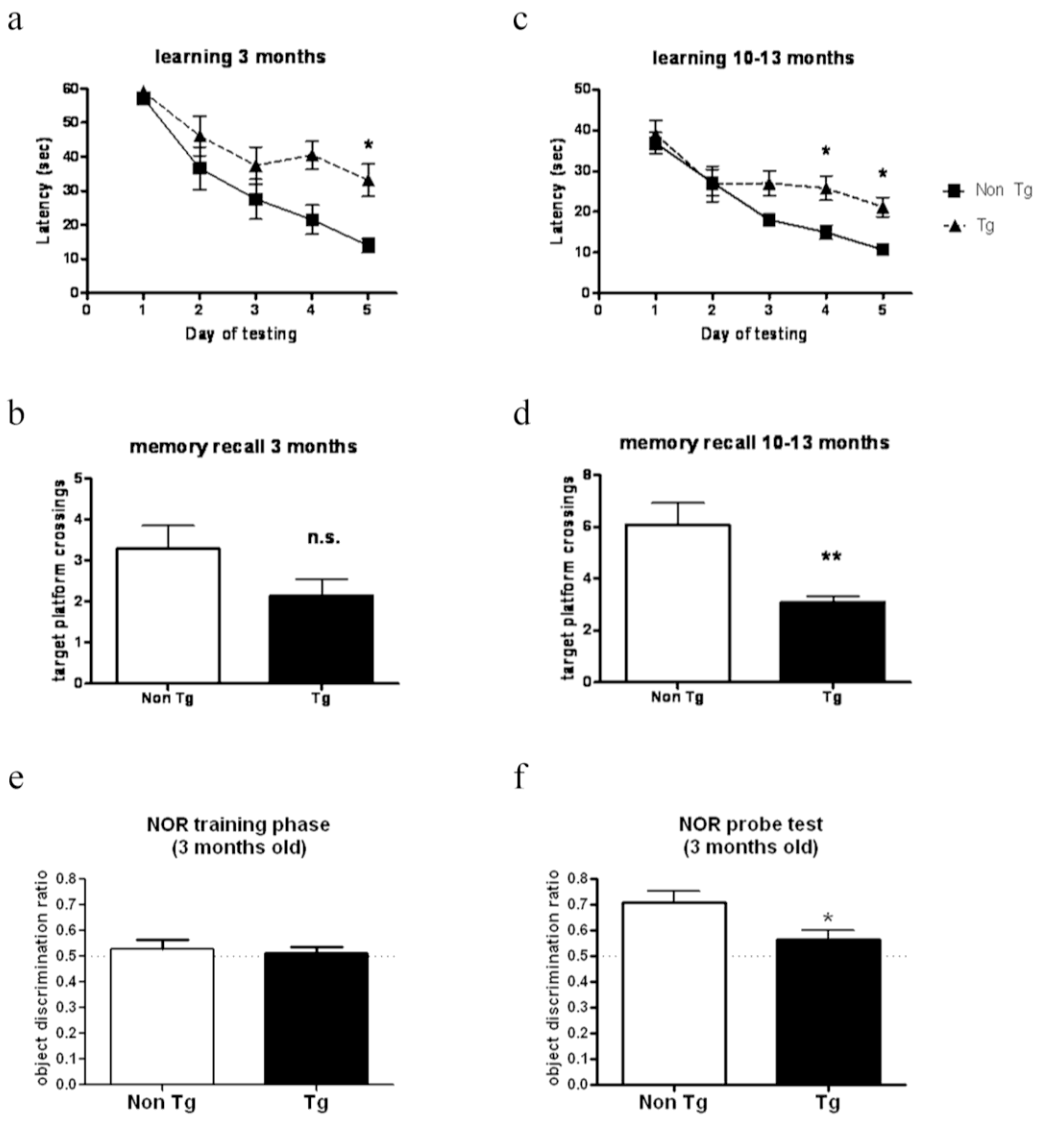

Figure 2-4 Cognitive deficits in the McGill-Thy1-APP mice start at the pre-plaque phase of the amyloid pathology and further progress with Aβ extracellular deposition

($p<0.05$, Bonferroni post-hoc test) as compared to control animals. This deficit in learning was accompanied by a trend in memory-deficit, as revealed by the decreased number of passes through the target platform during the probe test (Fig. 2-4b). By 13 months of age, Tg mice performed significantly worse than the Non Tg littermates in the learning phase of the MWM (Fig. 2-4c, 2-way-ANOVA, effect of genotype: $p<0.0001$), showing a consistent impairment since day 4 of testing ($p<0.05$, Bonferroni post-hoc test). At this late, extracellular phase of the pathology, the McGill-Thyl-APP mice also showed significant deficits in the probe test analysis (Fig. 2-4d, $p<0.01$, unpaired Student's T-test), indicating a marked memory impairment.

To confirm the occurrence of early cognitive impairments in young, pre-plaque McGill-Thy1-APP mice, we applied the Novel Object Recognition (NOR) paradigm. The time spent exploring a novel object over a familiar one was recorded as a measure of memory, and results compared with Non Tg age-matched littermates.

Both Tg and Non Tg animals did not show a preference for either of the two identical objects presented during the training phase of the test (the discrimination ratio being 0.5, i.e. equal to chance, Fig.2-4e). As expected, during the probe test performed 24 h later, Non Tg animals displayed a clear preference for the novel object ($p<0.05$, one-way-ANOVA followed by Tukey's post-hoc test). On the contrary, Tg mice failed to distinguish the novel object from the familiar one ($p>0.05$, one-way-ANOVA followed by Tukey's post-hoc test), and they displayed a significantly lower discrimination ratio than Non Tg littermates (Fig 2-4f, $p<0.05$, one-way-ANOVA followed by Tukey's post-hoc test), indicative of memory impairment. No significant differences were found between Tg and Non Tg animals in number of crossings and total time spent exploring, ruling out the possibility that the results observed were due to a lack of interest or insufficient locomotion (data not shown).

These results strongly support the notion that soluble forms of Aβ, in the absence of plaques, are sufficient to trigger AD-like neuronal dysfunction *in vivo*.

2.4.4 Cholinergic alterations are detected as early as at 3 months in McGill-Thy1-APP mice

One of the most important neuropathological landmarks of the AD brain is the development of cholinergic deficits. Down-regulation of cholinergic markers has been shown to positively correlate with cognitive impairments (Minger et al., 2000) and is accompanied by atrophy and/or loss of basal forebrain cholinergic neurons in advanced AD cases (Whitehouse et al., 1982; Pearson et al., 1983) There is substantial neurochemical and structural evidence that the cholinergic terminals are preferentially affected by the AD pathology and are the first to be compromised (Francis et al., 1999; Bell et al., 2006). Furthermore, we and others have shown that in early stages of the plaque pathology in Tg mice (Wong et al., 1999; Hu et al., 2003; Hernandez et al., 2001), and in MCI (DeKosky et al., 2002; Ikonomovic et al., 2003) cholinergic markers undergo a transitory up-regulation.

In order to investigate whether a similar dysregulation would occur at even earlier, pre-plaque stages of the amyloid pathology, we measured the density of presynaptic cholinergic boutons in the frontal cortex of young, pre-plaque mice (Fig. 2-5). Bouton density of random (plaque-free) neuropil area of old, post-plaque Tg mice, was included in the study. A specific antibody raised against vesicular acetylcholine transporter (VAChT) was used to label presynaptic cholinergic bouton sites, which appeared as distinct punctae throughout the neocortical neuropil. Bouton density (mean number of boutons per 1000 μm2) was assessed in laminae V-VI of frontal cortex via computer-assisted image analysis according to published, well established protocols (Wong et al., 1999; Bell et al., 2006; Wong et al., 1998). Representative pictures of VAChT

stainings in young and old Tg mice and age-matched littermates are shown in Fig. 2-5.

This analysis revealed that cholinergic boutons were significantly up-regulated in the frontal cortex of young, pre-plaque mice (25% up-regulation, Fig. 5c, $p=0.01$). When data from lamina V and lamina VI were analyzed separately by 2-way-ANOVA, an even stronger effect of genotype was revealed (Fig. 2-5d, $p<0.01$). Post-hoc test indicated that the up-regulation was driven by lamina VI ($p<0.001$, Bonferroni post-hoc test), while lamina V levels remained unchanged. An identical trend was observed in previous studies in pre-plaque Tg2576 mice where, at 8 months of age, no overt extracellular pathology was yet detectable (Wong et al., 1999) but a significant up-regulation of cholinergic terminals was measured in lamina VI.

As expected, aged mice (14-month old) displayed significantly lower levels of cholinergic boutons than young mice (35.20 boutons per 1000 μm2 in old mice versus 47.59 boutons per 1000 μm2 in young animals, corresponding to a 26% down-regulation in old mice, $p<0.05$). When comparing cholinergic density in old Tg mice versus old Non Tg littermates, we found a further, significant down-regulation of cholinergic terminals (23% down-regulation in Tg mice, Fig. 2-5g, $p<0.05$). In this age group, most of the differences between Tg and Non Tg mice were driven by changes in lamina V (Fig. 2-5f). These results are in line with previous studies in 8-month old double Tg (APP/PS1) mice, characterized by extensive plaque deposition (Wong et al., 1999).

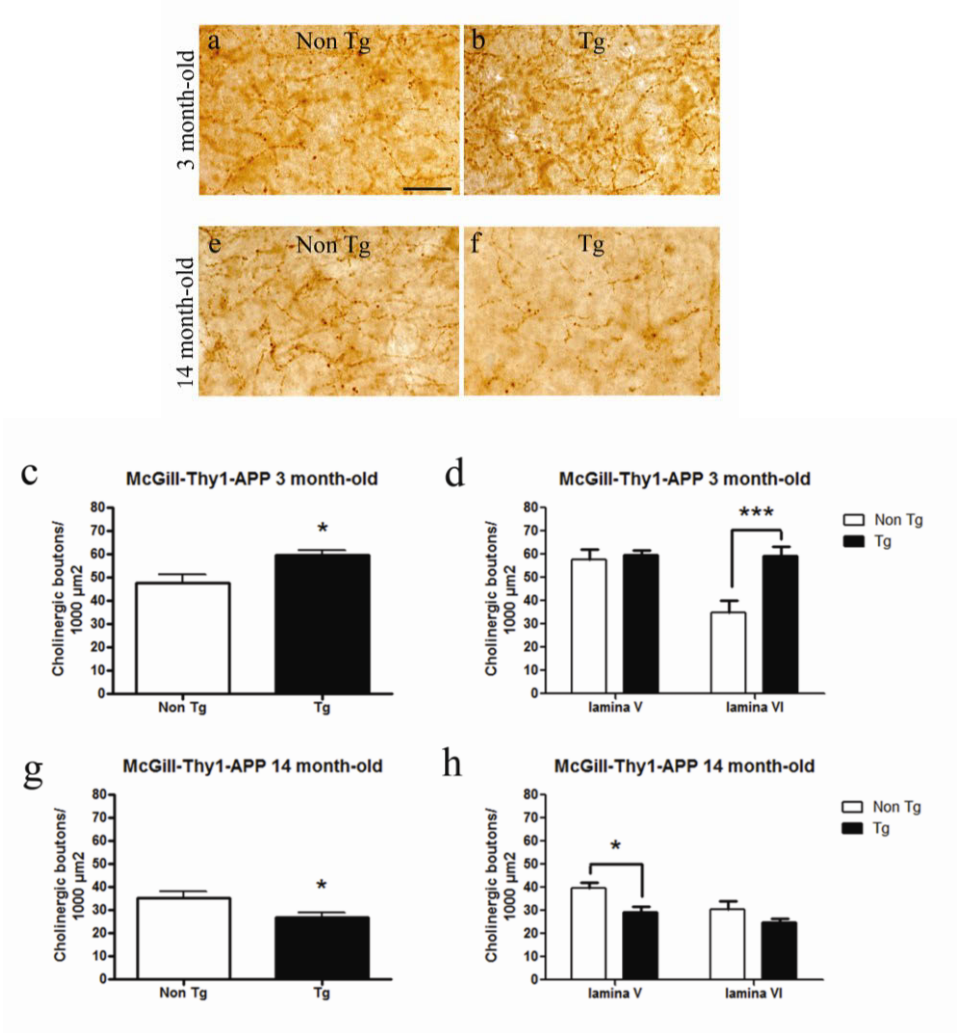

Figure 2-5 Alterations in the density of cholinergic pre-synaptic boutons in frontal cortex of young (pre-plaque) and old (plaque-burdened) Tg mice.

a-d: Representative pictures of VAChT-IR in frontal cortex of young, 3 month old Tg (**b**) and Non Tg littermate (**a**). Note the increase in immunoreactive punctae, corresponding to pre-synaptic cholinergic terminals, in the Tg mouse. The up-regulation was quantified by computer-assisted image analysis (**c**,**d**), and found to be statistically significant in lamina V and VI ($p<0.05$ Student's T-test). A further analysis revealed that the up-regulation was mainly driven by lamina VI (**d**, $p<0.001$, 2-way-ANOVA followed by Bonferroni post-hoc test).

e-h: Representative micrographs of VAChT-IR in frontal cortex of 14 month old Tg (**f**) and Non Tg littermate (**e**). Note the decreased density of cholinergic bouton in the Tg mouse compared to Non Tg littermate. The down-regulation was quantified by computer-assisted image analysis (**g**,**h**), and found to be statistically significant in lamina V and VI ($p<0.05$ Student's T-test). The down-regulation was mainly driven by lamina V (**h**, $p<0.05$, 2-way-ANOVA followed by Bonferroni post-hoc test).

(*=$p<0.05$, ***=$p<0.001$), scale bar: 20µm

Taken together, these findings confirm and further extend our previous observations in other Tg models, and support the concept that, prior to plaque deposition and concomitant with behavioral deficits, structural synaptic alterations are already detectable in the neocortex of Tg mice.

2.4.5 Intracellular Nu1-positive Aβ-oligomers appear as soon as at 1 week in the cerebral cortex, and at 1.5 month in the hippocampus of McGill-Thy1-APP mice

At the time of the early appearance of behavioral deficits and cholinergic alterations (3 months of age), the Aβ-positive material detected in the telencephalon of McGill-Thyl-APP mice appeared to be intraneuronal, suggesting a link between intracellular Aβ accumulation and neuronal dysfunction.

To further define the nature of the intracellular McSA1-immunoreactive material observed, we performed an immunohistochemical analysis applying the conformational Aβ-specific antibody Nul (Fig. 2-6). This monoclonal antibody was generated against ADDLs (Alzheimer Disease amyloid-β Diffusible Ligands), i.e. soluble, low-n Aβ-oligomers. Nu1 antibody was shown to be specific for Aβ42 over Aβ40, with preferential binding to Aβ-oligomers over monomers (Lambert et al., 2007; De Felice et al., 2007). The Nu1 monoclonal antibody was used to stain sections from Tg mice at different time points, ranging from 1 week to 10 months of age. No staining was observed in age matched Non Tg littermates brain sections or with omission of the primary antibody (data not shown). Representative micrographs of age-dependent Nu1 immunostaining on Tg mice brain sections are illustrated in Fig. 2-6.

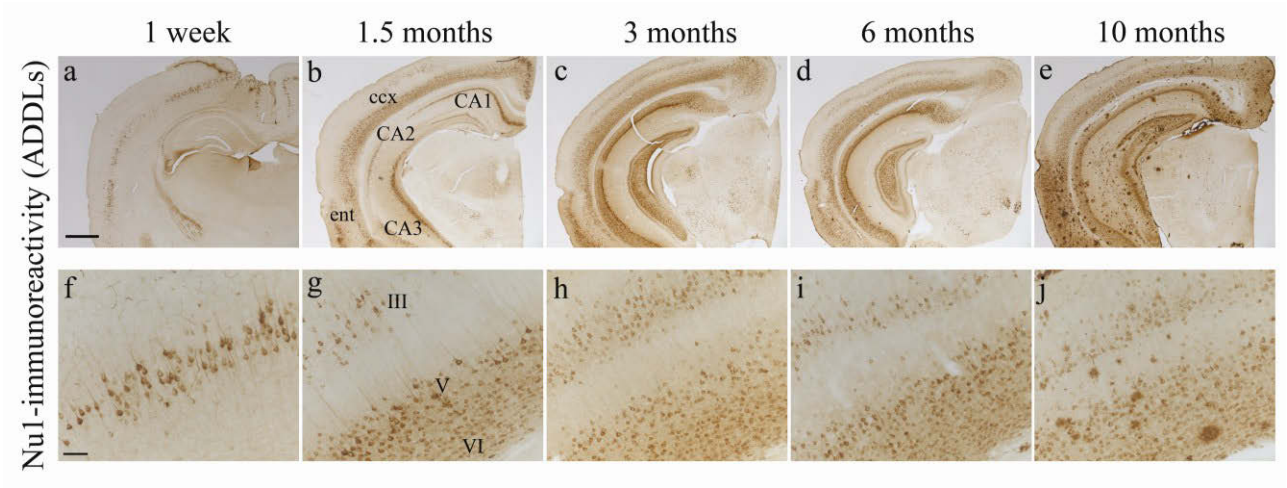

Figure 2-6 Early intracellular accumulation of Aβ low-n oligomers (ADDLs) in the telencephalon of the McGill-Thy1-APP mice, as detected with the Nu1 monoclonal antibody.

Representative micrographs of Nu1 immunostaining on Tg mice brain sections at different ages. Note that Nu1-IR was detected in pyramidal neurons of lamina V of the cerebral cortex (ccx) as early as at 1 week of age (**a**,**f**). At this early time point, no IR was observed in the hippocampal formation. At 1.5 months of age, ADDLs-positive pyramidal neurons began to appear in lamina III, hippocampus and entorhinal cortex (ent) (**b**). Strong Nu1-IR was observed throughout the cerebral cortex, entorhinal cortex, amygdala and the hippocampal formation at 3 months of age, but the ADDLs pathology was still exclusively intracellular at this time point (**c**,**h**). At later stages, when the plaque pathology is established, numerous Nu1-positive deposits of varying sizes were observed throughout the telencephalon (**d**,**e**) in addition to the intense intracellular IR associated with pyramidal neurons.

Scale bars: a-e: 500µm; f-j: 50µm

In brief, Nu1-IR was detected in pyramidal neurons of lamina V of the cerebral cortex as early as at 1 week of age (Fig. 2-6 a,f). With age, the ADDLs pathology appears to spread to lamina III, hippocampus and entorhinal cortex (b). ADDLs were exclusively found intracellularly until 3 months of age (Fig. 2-6 c,h). When the plaque pathology was established, ADDLs-IR was also observed in deposits of varying sizes throughout the telencephalon (Fig. 2-6 d,e).

To ensure that the IR observed in fixed tissue was truly due to Aβ-oligomers and not to artificial aggregates induced by cross-linking agents (such as tissue fixatives), we performed a Nu1 staining in duplicate on the same sample under either fixed and not-fixed conditions (Fig. 2-7). Similar IR was observed in both cases (Fig. 2-7c), and positive signal for Nu1 was also found on crude homogenates tested with dot blot technique (Fig. 2-7b).

To confirm that the Nu1-immunoreactive material observed was indeed Aβ and not holo-APP or soluble APP fragments, we performed a double labeling with 22C11 (Fig. 2-7d). Confocal microscopy revealed the presence of Nu1-immunoreactive vesicles-like structures in the perinuclear area and axons, which did not co-localize with the more diffuse 22C11-IR.

On the other hand, when we compared the oligomeric-specific Nu1-IR with the Aβ-specific McSA1-IR (Fig. 2-7e), we observed a large degree of co-localization.

Figure 2-7 Aβ-specificity of the Nu1 antibody

a-c: Nu-1-IR in crude homogenates and non-fixed tissue

a: Schematic representation of the tissue analysis and brain sampling.

b: Dot blot analysis of crude brain homogenates from a Non Tg and a Tg mouse. Nu1 monoclonal antibody recognized the oligomeric form of Aβ in a fresh, non-denatured tissue extract from the McGill-Thy1-APP Tg mouse.

c: Representative pictures of Nu1 immunoreactivity on brain sections from a Non Tg and the McGill-Thy1-APP Tg mouse after conventional immunohistochemistry (i.e. aldehyde fixation on 40µm thick sections), and in fresh, unfixed frozen tissue sections (20µm thick). Note that Nu1 specifically recognized intracellular Aβ-oligomers in the Tg mouse either under fixation or in unfixed tissue sections, where no fixation-induced cross linking has occurred.

d: Representative micrographs of double-labeling with Nu1 and the APP-specific monoclonal antibody 22C11 by confocal microscopy immunofluorescence. Note that the Nu1-IR (red signal) did not overlap at large with the 22C11-IR (green signal).

e: Representative micrographs of double-labeling with the McSA1 antibody and the Aβ-specific Nu1 antibody by confocal microscopy immunofluorescence. Note that the Nu1-IR (green signal) largely overlapped with the McSA1-IR (red signal).

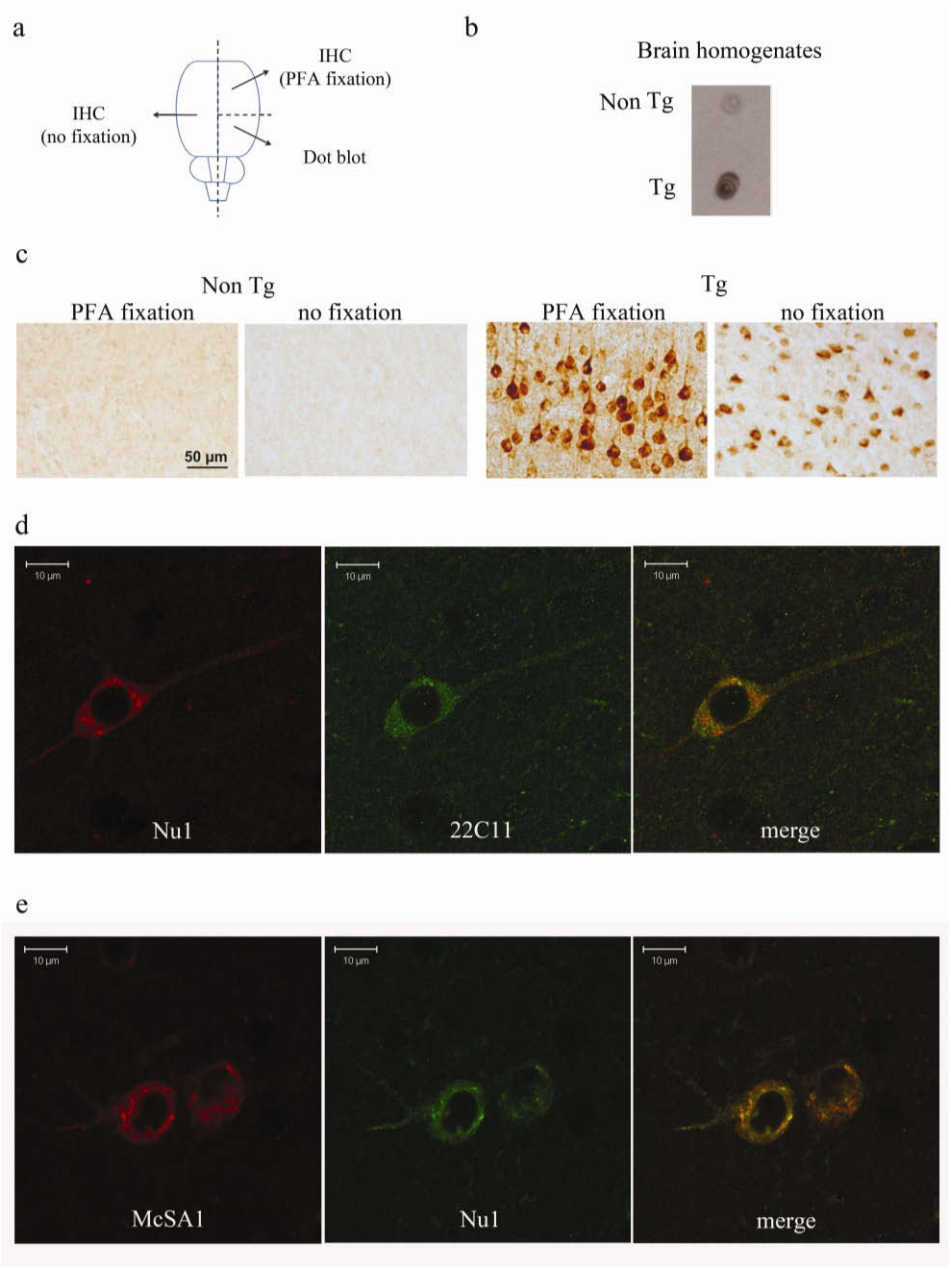

Figure 2-7. Aβ-specificity of the Nu1 antibody

These results provide further evidence that, at very early stages of the amyloid pathology (and concomitant with cognitive and synaptic alterations), Aβ starts accumulating within pyramidal neurons of the cortex and hippocampus. Importantly, Nu1-IR in young, pre-plaque animals suggested that Aβ aggregation is an early event that can begin in intracellular compartments. Intracellular Aβ-oligomers are likely to be involved in the neuronal dysfunction already detected at 3 months of age.

2.4.6 Intracellular OC-immunoreactive fibrillar oligomers appear as soon as at 1 week of age in the parietal cortex, and at 3 months in the hippocampus of McGill-Thy1-APP mice

To confirm our Nu1 findings of intracellular accumulation of Aβ-oligomers (ADDLs) and to further investigate the aggregation state of the Aβ material, we took advantage of a second well-characterized conformational specific antibody, OC, which recognizes a "generic" epitope specifically associated with amyloid fibrils, without cross-reaction with prefibrillar oligomers, random coil monomer or APP (Kayed et al., 2003; Kayed et al., 2007).

The OC polyclonal antibody was applied to Tg mice brain sections at different time-points, ranging from 1 week to 6 months of age. Controls included the staining of age-matched Non Tg littermates and omission of the primary antibody, where no staining was observed (data not shown). Representative micrographs of OC immunostaining on Tg mice brain sections at different ages are illustrated in Fig. 2-8.

Figure 2-8. Early appearance and late disappearance of intracellular fibrillar oligomers of Aβ in the telencephalon of the McGill-Thy1-APP mice, as detected with the OC polyclonal antibody

a-h: Representative micrographs of OC immunostaining of Tg mice brain sections at different ages. Note that OC-immunoreaction was detected in a few pyramidal neurons of the Tg cerebral cortex lamina V as early as at 1 week of age (**a**,**e**). At higher magnification, the OC-IR appeared to be associated with numerous, small granules in the perinuclear region and proximal processes. Parietal cortex and entorhinal cortex displayed a strong OC-IR at this time point, while weaker IR was observed in the amygdala. No OC-IR was observed in the hippocampal formation at this age. However, axonal staining was specifically observed in the alveum (*) and capsula interna (**) of 1 week-old Tg mice (**a**), when no signal was apparent in the Non Tg, age-matched littermates. At 1.5 months of age we observed an increase in the intracellular OC-IR in the cortex (**b,f**), with occasional OC-positive neurons in lamina III. At 3 months of age, the intracellular OC-IR spread to the CA1 and subiculum areas of the hippocampus (**c**), but weaker and more diffuse than the IR observed in the cortex. The specific intracellular OC-IR appeared to decrease at 3 months of age (**g**), while a strong staining was observed in fibers of the CA3 layer of the hippocampus (**c, ***) and polymorphic neurons of the hilus of the dentate gyrus (**c,****). By the 6th month of age few neurons appeared to accumulate OC-positive material (**h**). OC-immunoreactive material appeared at this age as large granules mainly in the basal portion of the perinuclear area. The intracellular OC-immunoreactive material was, at all time points, Thioflavin-S negative, thus was interpreted as fibrillar oligomers.

While amyloid plaques are normally observed at 6 months of age, numerous small, OC-positive deposits were observed throughout the telencephalon (**d**).

i-l: confocal immunofluorescence double-labeling OC-Nu1 on sections from 3-month old McGill-Thy1-APP mice. A representative micrograph of a pyramidal neuron in the lamina V of the cerebral cortex is illustrated. Note that in the Tg sample, intracellular OC-IR did not appear to colocalize with Nu1-IR: OC mainly stained granule-like structures in the basal region of the neuronal soma. Non Tg specimens did not display any non-specific staining.

Scale bars: a-d: 500µm; e-h: 10µm, i-l: 20µm

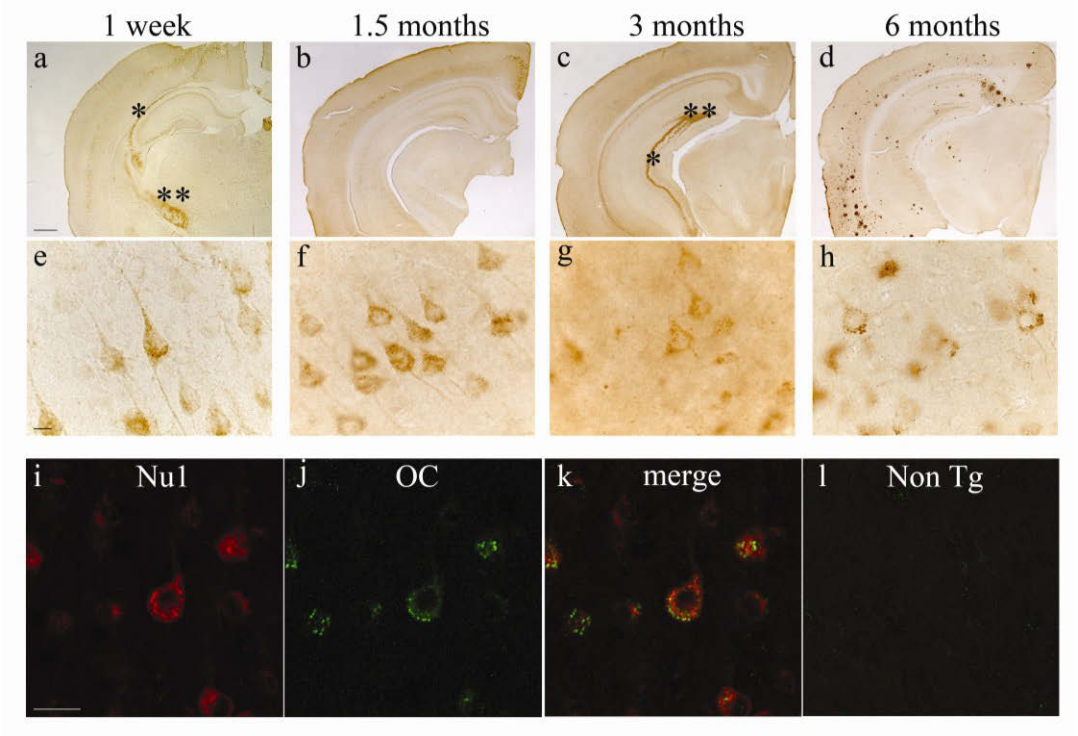

Figure 2-8. Early appearance and late disappearance of intracellular fibrillar oligomers of Aβ in the telencephalon of the McGill-Thy1-APP mice, as detected with the OC polyclonal antibody

Intracellular OC-IR was detected in Tg cerebral cortex lamina V from 1 week of age (Fig. 2-8 a,e). At this early stage we also observed specific axonal staining in the alveum (*) and capsula interna (**). With age, total OC-IR became progressively stronger and, by 3 months of age, spread to the hippocampus (Fig. 2-8c). Interestingly, the specific intracellular IR appeared to decrease after 3 months of age, and by six months of age this immunoreactive material was hardly visible within neurons. Instead, numerous small, OC-positive deposits became apparent throughout the telencephalon (Fig. 2-8d). Such early OC-immunoreactive aggregates appeared to precede the deposition of true amyloid, Thioflavin-S positive plaques.

We next performed a double labeling OC-Nu1 (Fig. 2-8i-k) in young, pre-plaque mice. This study revealed a different IR pattern of the two conformational-specific antibodies within the same pyramidal neuron in the cerebral cortex. In fact OC-immunoreactive material was mostly concentrated in granule-like structures in the basal part of the cell body, while the Nu1-immunoreactive material tended to be more abundant and associated with vesicles throughout the neuronal soma and processes. Little or no co-localization was observed via confocal fluorescent microscopy.

Taken together, these results confirmed the presence of intracellular Aβ-oligomers within pyramidal neurons of the cortex and hippocampus of young, pre-plaque Tg mice (as suggested by the Nu1 staining), further indicating that Aβ can aggregate intraneuronally *in vivo*. Interestingly, OC-immunoreactive fibrillar oligomers, were immunologically distinct from those revealed with the Nu1 monoclonal antibody, suggesting that at a given time point, oligomers corresponding to different stage of Aβ aggregation co-exist within the same neuron.

2.4.7 IDE, but not NEP, is down-regulated in the cerebral cortex of young, pre-plaque McGill-Thy1-APP mice

Our immunohistochemical observation of a progressive accumulation of intracellular Aβ-immunoreactive material, as detected with McSA1, Nu1 and OC antibodies, strongly suggested an imbalance between intracellular production and clearance of Aβ. We therefore decided to investigate the levels of insulin degrading enzyme (IDE) and neprilysin (NEP), the two main Aβ degrading enzymes (Iwata et al., 2005), which are known to be down-regulated in AD (Cook et al., 2003; Wang et al., 2005) and in late (i.e. post-plaque) stages of the amyloid pathology in Tg models (Caccamo et al., 2005; Hirata-Fukae et al., 2008).

Western blot analysis of cortical homogenates from 3-month old Tg mice (i.e. pre-plaque) was performed and the results compared to Non Tg age-matched littermates (Fig. 2-9a). IDE levels in Tg animals were found to be 2.7 fold lower than controls ($p<0.001$, unpaired Student's T-test). However, NEP levels were unchanged at this age ($p=0.1$), with a mild but not significant trend towards an increase. The lack of change in NEP levels was confirmed in another set of experiments, in which membrane-enriched fractions from four Tg and four Non Tg 3-month old mice were probed (data not shown).

Finally, we performed an immunohistochemical double labeling of McSA1 and IDE in sections from 3-month old Tg and Non Tg mice. Interestingly, we observed that IDE was abundantly expressed in neurons of laminae II and IV neurons, which tend to be less affected by the amyloid pathology. Qualitative observations via confocal microscopy suggested that McSA1-positive neurons displayed lower levels of IDE-immunoreactive material (Fig. 2-9b). Taken together, these results would indicate a link between early, pathological accumulation of Aβ-immunoreactive species and diminished intracellular Aβ clearance.

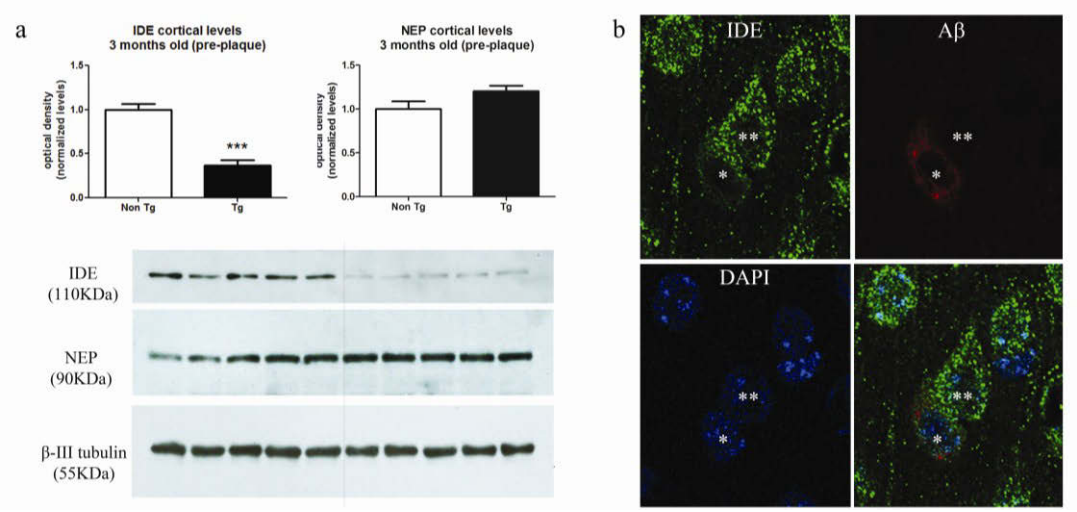

Figure 2-9. Aβ-degrading enzyme IDE, but not NEP, is markedly down-regulated as early as at 3-months of age in McGill-Thy1-APP mice

a: Cortical levels of IDE and NEP were assessed by Western blotting in young, pre-plaque Tg mice, and compared to age-matched, Non Tg littermates. IDE was found to be significantly down-regulated (36% of Non Tg, $p<0.001$) in Tg mice, while NEP levels were unchanged.

b: Representative micrograph of a double labelling McSA1 (Aβ □□and IDE by confocal microscopy. Note that Aβ-bearing neurons (*) appeared to have lower levels of IDE, compared to neighbouring, Aβ-negative neurons (**). Blue reaction corresponds to cellular nuclei stained with DAPI.

(a: data were analyzed by Student T-test. ***=$p<0.001$)

2.5 Discussion

Here we describe our newly developed Tg mouse model of AD-like amyloid pathology, named McGill-Thy1-APP, in which a single transgene encoding for a triple mutated variant of the human APP protein is expressed under the control of the Thy 1.2 murine promoter. As a result of the synergistic effect of the double Swedish and the Indiana familial AD mutations, Tg mice express on average 2 fold higher levels of Aβ42 than of Aβ40 in both cortex and hippocampus (Table 2). As described in other Tg models generated with a similar construct (Chishti et al., 2001; Mucke et al., 2000), McGill-Thy1-APP mice develop full amyloid plaque pathology and cognitive deficits in young age. In addition, McGill-Thy1-APP mice display a more precocious pathology, with the first Aβ-IR detected by one week of age (Fig. 2-2, 2-6, 2-8). As similar other transgenic models with the over-expression of a mutated form of the amyloid precursor protein, the McGill-Thy1-APP model does not recapitulate the occurrence of NFT which are observed in the human pathology. For a comparison with the most relevant Tg models of AD-like pathology see (Morrissette et al., 2009; McGowan et al., 2006; Dodart and May, 2005).

Preliminary stereological results would also indicate that in this Tg model cell death does occur at early stages of the pathology in areas rich in neurons displaying intracellular Aβ burden (Van De Berg et al., 2010). This finding is analogous to the neuronal cell death associated with the presence of intracellular Aβ burden reported by Casas et al. (Casas et al., 2004). The marked and early pathology did not affect animal survival, as we did not detect spontaneous death in the McGill-Thy1-APP colony up to 2 years and the mice were otherwise indistinguishable from Non Tg littermates. This is a remarkable advantage, considering that most Tg mice are known to have a shorter life span, a limiting factor for long-term experimental therapeutics investigations.

Our immunohistochemical analysis revealed that in the McGill-Thy1-APP Tg model the extracellular plaque phase of the pathology begins around four months of age, is well established by six months (see Fig. 2-2) and is characterized by mature (Thioflavin-S positive), neuritic plaques. Prior to this phase, McGill-Thyl-APP mice show a well defined pre-plaque stage of the pathology (up to 3 months of age), when strong McSA1-IR is detected in pyramidal neurons in the telencephalon in the absence of extracellular amyloid deposition (Fig. 2-2).

It is reasonable to believe that such McSA1-IR in the cortex and hippocampus of McGill-Thy1-APP mice corresponds to the Aβ42 and Aβ40 peptides measured by ELISA in the same brain regions. In fact, the lack of co-localization between intraneuronal McSA1- and 22C11-IR definitely ruled out cross-reactivity of this antibody with full length APP or N-terminally fragments thereof. Moreover, at the same age (i.e. in the pre-plaque phase), cortical pyramidal neurons appeared to be strongly immunoreactive to another Aβ-specific antibody (Nu1) and to OC, further indicating that in these animals the Aβ peptide is produced and accumulated intracellularly prior to plaque deposition.

The occurrence of intraneuronal Aβ peptides in pre-plaque stages of the amyloid pathology in our model is in agreement with previous evidence in a wide range of *in vitro* models and animal species (LaFerla et al., 2007; Wirths et al., 2004). This feature is highly relevant since it has been described in human AD brains (Gouras et al., 2000), even preceding the formation of paired helical filament-positive structures (Fernandez-Vizarra et al., 2004). Intracellular Aβ accumulation was also observed in Down's Syndrome (DS) individuals, who invariably develop the neuropathologic features of AD and represent a unique situation in which to study the early and sequential development of the amyloid pathology in humans (Gyure et al., 2001;Mori et al., 2002;Busciglio et al., 2002).

The mere presence of soluble, intracellular Aβ material is sufficient to negatively affect higher cognitive functions, as memory and learning deficits were detected

already at 3 months of age in McGill-Thy1-APP Tg mice (see Fig. 2-4). Similar cognitive impairment prior to plaque deposition was previously reported by our lab in a Tg rat model with a phenotype limited to intracellular Aβ pathology (Echeverria et al., 2004c) and later and more comprehensively by LaFerla and collaborators in pre-plaque stage of the triple transgenic mice (Billings et al., 2005). These findings further support the concept that neuronal dysfunction takes place much earlier than the appearance of amyloid plaques, and that the pre-plaque phase of the pathology in Tg models is already characterized by important neuropathological alterations.

Consistent with the concept that young, pre-plaque mice can be used to model the very first stages of the amyloid pathology in AD, young, 3-month old Tg mice displayed mild memory deficits in the MWM task (Fig. 2-4b), further confirmed by the NOR test (Fig. 2-4 e,f). It is most likely that a seemingly moderate cognitive impairment in humans escapes the current diagnostic tools. On the other hand a large cohort of young, pre-plaque Tg mice allows the researcher to identify mild, but significant, cognitive deficits, and further explore neurochemical and structural correlates of such behavioral alterations.

Synaptic counts in the neocortex are amongst the most reliable structural correlates of cognitive deficits. While total synaptic numbers are known to be globally down-regulated in late stages of the disease (Scheff et al., 1990), there is abundant evidence that the cholinergic system is specifically vulnerable to the amyloid pathology (Francis et al., 1999). In fact, cholinergic terminals in the cerebral cortex appear not only to be dramatically down-regulated in AD brains, but are also the first recruited to the core of amyloid plaques, becoming dystrophic (Bell et al., 2006). For this last reason, we were interested in assessing the impact of soluble forms of Aβ on cholinergic terminals in early stages of the amyloid pathology, thus prior to and in the absence of amyloid plaques. Our finding of an up-regulation of cholinergic terminals in the frontal cortex of young, pre-plaque mice is in agreement with previous studies from our group performed on early

plaque pathology of another Tg model, the Tg2576 mouse (Wong et al., 1999; Hu et al., 2003) and by Duff and collaborators in the PS/APP Tg mouse (Hernandez et al., 2001). Recently, these findings have been further supported by DeKosky and collaborators in human cases, where up-regulation of choline acetyltransferase activity was found in hippocampus and frontal cortex of subjects with MCI (DeKosky et al., 2002; Ikonomovic et al., 2003). In both Tg models and human cases, the cause of such cholinergic alteration is unknown, but it is likely due to compensatory mechanisms aimed at counteracting the initial neuronal dysfunction of the cortical pyramidal neurons.

These results not only confirm that the cholinergic is amongst the earliest systems to be affected by the incipient amyloid pathology (even prior to plaque deposition): they also indicate that in the pre-plaque stage of the amyloid pathology, neuronal dysfunction revealed by cognitive deficits corresponds to significant neurochemical structural alterations detectable in the frontal cortex.

Aβ-oligomers are known to strongly impair neuronal functionality *in vitro* (Deshpande et al., 2006), *ex vivo* (Lambert et al., 1998; Townsend et al., 2006b; Walsh et al., 2002; Cleary et al., 2005), and *in vivo* (Lacor et al., 2007; Shankar et al., 2007; Almeida et al., 2005; Bhaskar et al., 2009) and to induce striking structural synaptic alterations in primary hippocampal neurons and organotypic cultures (Tomic et al., 2009; Gong et al., 2003; Walsh et al., 2000; Takahashi et al., 2004b). In addition, the intracellular accumulation of Aβ-oligomers has been recently shown to contribute to the deregulation of the PI3K-Akt-mTOR pathway and the demise of neurons (Echeverria et al., 2005; Arvanitis et al., 2007). It is therefore possible that Aβ-oligomers play a key role in the neurodegenerative changes as well as the pre-plaque cognitive deficits and synaptic alterations observed in McGill-Thy1-APP mice. Our immunohistochemical study revealed the presence of two specific forms of soluble Aβ-oligomers: ADDLs (detected with the monoclonal Aβ specific Nu1 antibody) and fibrillar oligomers (detected with the polyclonal OC antibody) (Fig. 2-6,7,8). Importantly, these species were detected

within cortical and hippocampal neurons prior to plaque deposition but concomitant with cognitive and synaptic alterations, strongly suggesting a link between neurotoxic oligomers and structural and functional alterations in the CNS. Given that both molecular forms of the Aβ amyloid peptide are found in human cases and are elevated in AD brains (Kandel, 1991), our observations in pre-plaque Tg mice models are relevant to the human pathology, and can make an important contribution to our understanding of the progression of AD.

Our immunohistochemical study allowed us to make the following observations:

1) soluble Aβ-oligomers could be detected within cortical and hippocampal neurons as early as at 1 week of age. This is, to the best of our knowledge, the earliest occurrence of intracellular Aβ-oligomers reported in Tg animals and strongly indicates that the onset of the amyloid-like pathology takes place at much earlier stages than was previously believed.

2) both ADDLs and fibrillar oligomers could be detected within neuronal compartments prior to plaque deposition, indicating that at least a portion of the Aβ oligomerization process occurs within the neuronal compartments *in vivo* in a Tg model of AD-like pathology. This concept is in agreement with previous reports from *in vitro* cell models of human origin (Takahashi et al., 2004b) and, most importantly, with evidence from AD cases (Kokubo et al., 2005; Buxbaum et al., 1998). Given the well known neurotoxicity of such Aβ-oligomers, it is very likely that their intracellular accumulation is sufficient to negatively affect the functionality of cortical and hippocampal neurons. In line with this idea, previous *in vitro* work from our lab has shown that intracellular Aβ accumulation elicits a deregulation of the ERK/CREB/CRE signalling pathway (Oddo et al., 2006), which is classically involved in synaptic plasticity-related protein synthesis (Sarsoza et al., 2009).

3) some OC-IR was specifically observed in axons, such as in the alveus and the capsula interna at early stages of the pathology (1 week old, Fig. 2-8a), a

finding in line with reports from AD brains, where Aβ-oligomers were observed in abnormal processes and synaptic compartments (Gouras et al., 2005; D'Andrea et al., 2001). This finding is consistent with the idea that oligomeric material is axonally transported to and from the hippocampus, as previously suggested (Savage et al., 1998), and negatively impacts the connection between these two areas.

4) we observed, at least in the hippocampus, a different time-dependent occurrence of the two specific oligomeric forms of Aβ, i.e. ADDLs and fibrillar oligomers, with the ADDLs appearing at earlier time points (1.5 month, see Fig. 2-6b) and the OC punctate staining following somewhat later (3 months of age, see Fig. 8c). While the intracellular aggregation of monomeric Aβ into Aβ-oligomers has been documented in previous studies, using the triple Tg model (Selkoe, 2001a), the present work documents a further and later step of the Aβ aggregation process, from low-n oligomers into fibrillar oligomers. The later occurrence of intracellular fibrillar oligomers suggests that oligomeric, low-n material immunoreactive to Nu1 can further aggregate or undergo conformational changes leading to the formation of fibrillar oligomers within the neuronal compartment.

5) intraneuronal fibrillar oligomers disappeared after six months of age, leaving space for numerous small extracellular OC-IR deposits (Fig. 2-8). These deposits appear to precede true, Thioflavin-S positive plaques. Similarly to what we have observed in the McGill-Thy1-APP mice, Sarsoza and collaborators have recently shown that OC recognizes a subset of Thioflavin-S negative amyloid plaques in AD, DS and Tg2576 mice (Akiyama et al., 1988; Qiu et al., 1998). These early, OC positive deposits are most likely immature plaques or seeds of further aggregation. Since OC-positive fibrillar oligomers were shown to share peptide conformation with amyloid fibrils, it is possible that the aggregation of intracellular fibrillar oligomers gives rise, with a mechanism still to be elucidated, to the mature fibers that compose the amyloid plaque. One might speculate that, after a critical mass is reached within the neuronal soma, the

fibrillar material is released to the extracellular space creating a seed for a plaque, as suggested by several authors (Barnes et al., 1992).

A hypothetical evolution of the intracellular Aβ-immunoreactive material into extracellular plaques is schematically represented in Fig. 2-10.

The formation of neurotoxic low-n oligomers of Aβ, and later of fibrillar oligomers, within the neuronal compartments strongly indicates an excessive and uncontrolled accumulation of Aβ peptide. Aβ peptide was shown to have an extremely high turnover (Bernstein et al., 1999), indicating a very efficient proteolytic clearance mechanism, in physiological conditions. Amongst the numerous proteases that can potentially participate in Aβ turnover, NEP and IDE are considered the most important.

Several lines of evidence suggest that IDE is exclusively responsible for the degradation of monomeric, soluble Aβ, while NEP would be in charge of the degradation of both monomeric and fibrillar Aβ. Consistent with this, IDE occurs mainly in a soluble form in the cytoplasm and, to a smaller extent, extracellularly, while NEP occurs almost exclusively as a membrane anchored protein and acts as an ectoenzyme at the cell surface.

Our observations of reduced levels of IDE in young, pre-plaque mice is, to the best of our knowledge, the earliest report of a down-regulation of IDE levels in AD-like pathology. This finding suggests that at early stages of the disease, the degradation of the intracellular Aβ deposits is already impaired. On the other hand, NEP was found to be yet unchanged, suggesting that the extracellular clearance mechanisms are still functional at this early stage.

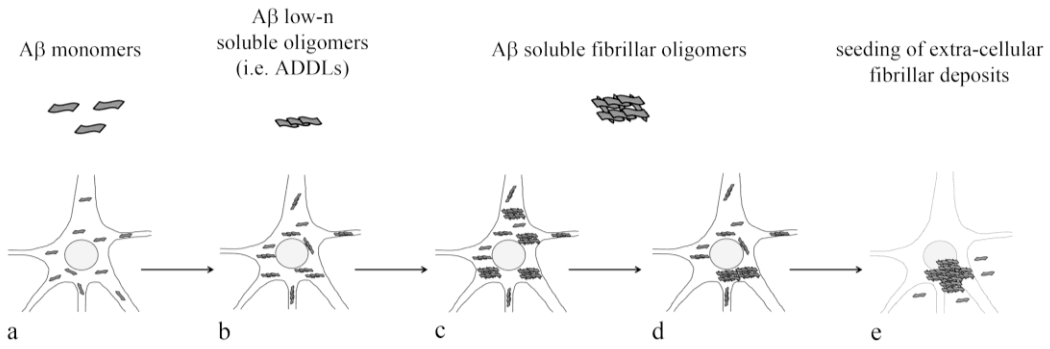

Figure 2-10. Hypothetical evolution of intracellular Aβ-immunoreactive material

The scheme illustrates a possible sequence of intracellular Aβ aggregation in early, pre-plaque stages of the AD-like amyloid pathology, based on the here reported studies. In this model, over-expression of the human APP protein with the Swedish and Indiana mutations leads to early accumulation of McSA1-immunoreactive material (likely monomeric Aβ) within perinuclear compartment of pyramidal neurons (**a**). A large portion of this Aβ-immunoreactive material appears to aggregate into low-n oligomers throughout the intracellular compartment (**b**). Only part of it is recognized by the OC antibody (**c**), suggesting that some Aβ material can further aggregate and-or undergo conformational changes characteristic of fibrillar oligomers. While intracellular low-n Aβ oligomers did not seem to decrease or change with age, OC-IR material, indicating the presence of fibrillar oligomers, appeared to decrease and move to different intracellular compartments, mainly in the basal part of the soma and closer to the plasma membrane(**d**). The nature of the latter compartments is yet unresolved, but the possibility exists that they are late endosomes on their way to extrusion. This hypothesis is supported by the late disappearance of intracellular OC-IR with the concomitant appearance of small, Thioflavin-S negative, OC-immunoreactive deposits (**e**), which might arise from the demise of intracellular Aβ-burdened neurons (depicted with dotted line). These diffuse deposits might represent seeding of mature extracellular amyloid plaques.

In agreement with previous reports, IDE appeared to be expressed predominantly by neurons. Interestingly, although we did not perform a quantitative study, our confocal analysis suggests that Aβ-positive neurons display decreased levels of IDE compared to neighboring, Aβ-negative neurons. Taken together, these results suggest that intracellular Aβ accumulation parallels and is possibly linked to a failure in the degradation machinery. It is likely that analogous changes might occur in early pre-plaque, pre-clinical stages of the AD pathology.

2.6 Conclusions

We have shown that in the McGill-Thy1-APP Tg mouse model of the AD-like amyloid pathology, a number of functional, structural and biochemical alterations of the CNS are already present prior to and independently of the deposition of extracellular amyloid plaques, but concomitant with the intracellular accumulation of Aβ-oligomers in pyramidal neurons of the cortex and hippocampus. It is highly likely that a similar pre-clinical, pre-plaque pathology might occur in AD, prior to the development of MCI and the full amyloid pathology seen later. Such early state of the pathology, which escapes the current diagnostic methods in humans but can be modeled in Tg animals, is likely characterized by intracellular Aβ-oligomers-driven synaptic dysfunction and neuronal demise. Elucidating the intracellular Aβ-mediated neurotoxic mechanisms in young, pre-plaque animal models might provide new clues for a better understanding of the incipient AD amyloid pathology, akin to the proposed ‘latent’ stage of AD. This approach might yield novel targets for early pharmacological treatment or diagnostic strategies.

2.7 Aknowledgments

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Connecting Text: Chapter 2 to 3

In Chapter 2 we took advantage of our newly generated APP Tg model of AD-like amyloid pathology, coded McGill-Thy1-APP, to characterize neuropathological events occurring at early, pre-plaque stages of the disease. These investigations allowed us to show that cognitive impairments and cholinergic alterations occur early in the progression of the disease. These pathological features were associated with intracellular Aβ-oligomers in pyramidal neurons of cerebral cortex and hippocampus, in the absence of plaque pathology. The findings presented in Chapter 2 therefore suggested that the mere presence of pathological concentration of intracellular Aβ-oligomers might be sufficient to trigger the neuronal demise characteristic of AD.

As discussed in Chapter 2, a vast body of evidence indicates that Aβ can interfere with the normal synaptic activity of the neurons and their calcium homeostasis. It is very likely that neurons burdened with intracellular Aβ will be highly impaired and-or dying. Microglial cells are ideally positioned to sense any alterations in the neuronal functionality and quickly respond to pathological events. Thus, we hypothesized that microglial cells would present an early, moderate activation state in response to neuronal pathology at pre-plaque stages of the CNS amyloidosis.

At the time when we started this research, the occurrence of an early, pre-plaque microglial response had been somehow neglected. Most studies had focused on the inflammatory response in relation to the deposition of amyloid plaques.

To test our hypothesis we used an array of immunohistochemical and biochemical techniques to characterize the status of microglial cells in young, 3 months old Tg mice, as described in Chapter 3.

The results of these investigations might shed some light on early events occurring in AD progression, which corresponds to pre-clinical stages of the disease. A better understanding of such pre-clinical phases is crucial in our search of new diagnostic tools, biomarkers and or early therapeutic agents.

CHAPTER 3

Intracellular Aβ-Oligomers and Early Inflammation in a Model of Alzheimer's Disease

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3.1 Abstract

Life-long use of NSAIDs drugs has been shown to diminish the incidence of Alzheimer's disease (AD), suggesting a key role of inflammation in early stages of the pathology. While amyloid plaque-associated inflammation has been extensively studied in human and animal models, little is known about the inflammatory process prior to plaque deposition, i.e. in preclinical stages of AD. In this study we investigated microglial and neuronal inflammatory markers in pre-plaque transgenic McGill-Thy1-APP mice. We found evidence that prior to plaque deposition classical markers of microglial activation such as MHC-II, i-NOS and CD40 are already up-regulated in hippocampus of transgenic mice. Microglial cells from transgenic mice in the pre-plaque stage displayed intermediately activated morphology and appeared to be recruited towards intracellular Aβ-oligomers burdened neurons. The inducible, neuron-specific COX-2 enzyme was found to be up-regulated and specifically expressed by neurons in close relationship with Aβ-bearing cells, at this early stage of the AD-like pathology. Our study suggests that neuroinflammation might be one of the earliest pathological responses to intracellular accumulation of Aβ-oligomers.

3.2 Introduction

Alzheimer's disease (AD) is the leading cause of dementia in the elderly, affecting more than five million people in North America (Holtzman, 2010).
It is well established that, at the clinical diagnosis stage, AD-brains display the classical pathological features of extracellular amyloid plaques (composed primarily of aggregated amyloid beta peptide, Aβ) and of intracellular and extracellular neurofibrillary tangles (composed of paired filaments of abnormally phosphorylated tau protein) (for reviews: Cuello, 2007; Selkoe, 2001b). At this stage of the AD neuropathology, an overt CNS inflammation is an invariable

component of the extracellular amyloid plaque pathology. Such inflammatory process is characterized by peri-plaque microgliosis and astrogliosis, and the presence of inflammatory and immune reaction markers (Akiyama et al., 2000; McGeer et al., 2006).

The significance and contribution of the inflammatory process has been highlighted by epidemiological, retrospective studies indicating a lower incidence of AD in populations receiving long term treatment with NSAIDs (Andersen et al., 1995; McGeer et al., 1990; Stewart et al., 1997; for a review: Klegeris and McGeer, 2005). These data would suggest that inflammation has a key, negative role in the pre-clinical phases of the pathology, and that its inhibition can retard or diminish the incidence of the disease.

In contrast, prospective trials applying NSAIDs to clinically diagnosed AD cases have failed to revert or slow down the disease, often worsening it (Aisen et al., 2003; Martin et al., 2008; Thal et al., 2005). These findings suggest that the later, plaque-associated inflammatory reaction represents a bystander or a defensive mechanism in the AD neuropathology. Taken together, the clinical evidence is consistent with the concept that inflammation plays different roles during the progression of the disease: it would contribute to and accelerate the AD neuropathology in its pre-clinical stages, while it would be neutral or even beneficial in later, clinical stages. Elucidating the nature and the significance of the early inflammatory process in the progression of the AD pathology might provide new therapeutic opportunities to arrest or delay the disease progression.

Despite the strong indication from epidemiological studies, while much effort has been put in characterizing the late, plaque-associated inflammatory response, little is known about inflammation in pre-clinical AD. The main obstacle in this regard is the impossibility to predict the conversion of non-cognitively impaired

individuals into mild cognitive impairment (MCI) or AD sufferers in the human. On the other hand, transgenic (Tg) animal models allow the investigation of events prior to the full-fledged AD-like amyloid pathology.
We therefore resolved to use young, pre-plaque Tg mice of our newly generated McGill-Thy1-APP model of AD-like amyloid pathology (Ferretti et al., 2011a) to define the earliest occurrence of a possible pro-inflammatory reaction. McGill-Thy1-APP mice will invariably develop the full amyloid pathology at 6 months of age, thus representing a suitable model to study early events in the progression of the pathology.

In these investigations we gathered biochemical and morphological evidence that, already at 3 months of age, in the absence of extracellular plaque deposition but coincidental with intracellular Aβ-oligomers and behavioral deficits, a pro-inflammatory process is taking place in the hippocampus and cortex of Tg mice. We observed that such inflammatory reaction involved both neurons and microglia and was associated with intraneuronal accumulation of neurotoxic Aβ-oligomers.

3.3 Materials and methods

Perfusion and tissue preparation technique. Tg and Non-Tg littermates mice were deeply anesthetized with Equithesin (pentobarbital-based anaesthetic, 2.5ml/Kg, i.p.) and perfused through the heart with ice-cold saline solution (pH 7.4) for 1 minute. The brains were then quickly removed and divided into right and left hemisphere on ice. Cortex, hippocampus and cerebellum were dissected from the left hemisphere, snap-frozen in dry ice and stored at -80˚C for biochemical analysis. The right hemisphere was fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4) for 24 hours at 4˚C. The tissue was then cut into 40μm thick sections with a freezing sledge microtome (SM 2000R, Leica) at -

20˚C and free-floating sections were collected in phosphate buffered saline (PBS) and processed for immunohistochemistry.

Western blotting. Hippocampal samples from the left hemisphere of 5 Tg and 5 Non-Tg littermates, 3 and 10-13 months old, were homogenized in 250µl of lysis buffer (Cell Signalling, USA) containing a complete protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). Samples were centrifuged at 13,000 rpm for 45 min at 4˚C. Following total protein content quantification (Dc-protein assay, BioRad, USA), 20-100µg of protein were separated using 10-15% SDS-polyacrylamyde gels and semi-dry transferred to nitrocellulose membrane for subsequent Western blotting. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and then incubated with the primary antibody overnight at 4˚C. Primary antibodies used were: rabbit polyclonal iNOS (1:500, Santa Cruz, USA); CD40 (1:500); COX-2 (1:2000, Cayman Chemicals, USA); mouse monoclonal: MHC-II (1:500. Abcam, USA) and βIII-tubulin (1:40.000, Promega, Madison, WI, USA). Signal intensity was quantified by densitometry (MCID5 image analysis system, USA). Groups were obtained simultaneously and normalized with respect to βIII-tubulin (neuronal specific) immunoreactivity. All experiments were performed in triplicate.

Immunohistochemistry. Bright field immunohistochemistry and double labelling: free floating immunohistochemical staining was performed as previously described (Hu et al., 2003). Mouse monoclonal primary antibodies used were: rabbit polyclonal Iba-1 (ionized calcium-binding adaptor molecule 1, DAKO, USA, working dilution 1:10.000); mouse monoclonal McSA1 (Medimabs, Montreal; Grant et al., 2000; working dilution: 1:4000), which recognizes the first 12 aminoacids of the Aβ sequence; rat monoclonal CD11b (working dilution 1:50, Chemicon, USA); rat monoclonal; MAC-2 (working dilution 1:100, from ATCC; Cedarlane, Canada); rabbit polyclonal CD68 (working dilution 1:500, from Serotec, USA); conformational specific Nu1 (Lambert et al., 2007; working

dilution 1:3000); COX-2 (Cayman, USA, working dilution 1:4000)**.** For mouse monoclonal primary antibodies, sections were incubated with a secondary goat-anti-mouse IgG (1:100; MP Biomedicals, Irvine CA, USA) followed by a mouse anti peroxidase monoclonal antibody complex [MAP/HRP complex, Medimabs, Montreal, Canada; (Semenenko et al., 1985)]. For rat monoclonal antibodies, goat-anti rat IgG was first applied to the sections (Jackson Immunoresearch, working dilution 1:100), followed by a rat anti-peroxidase monoclonal antibody complex (RAP/HRP complex Medimabs, Montreal, Canada). For sections processed with rabbit polyclonal primary antibodies, a biotinylated goat-anti rabbit secondary antibody was used, followed by amplification with the avidin-biotin complex (both from Vector laboratories Inc., Burlingame, California, USA). All single stainings were developed with 0.6% DAB (Sigma-Aldrich Canada, Oakville, ON) and 0.01% H_2O_2. For double labeling, we used the SG kit (Vector, Burlingame, California, USA) according to the manufacturer's instructions. Briefly, sections were incubated with both primary antibodies according to standard procedure and sequentially developed using DAB (brown signal) and SG (blue signal). Fluorescence double-labeling: free floating double immunofluorescence staining was performed as previously described (Majdi et al., 2007). Primary antibodies dilutions used were: CD68 1:100; McSA1 1:400; COX-2 1:100; NeuN (mouse monoclonal from Chemicon) 1:2000. Secondary antibodies used were: donkey anti-mouse and anti-rabbit Rhodamin conjugated (Jackson Immunoresearch, USA; working dilution 1:200), biotinylated goat anti-mouse and anti-rabbit (Vector laboratories Inc., Burlingame, California, USA; working dilution 1:200) followed by treatment with Streptavidin Alexa 488 (Molecular probe, Invitrogen, Eugene, Oregon, USA). Digital images were acquired on an Axioplan 2 Imaging microscope (Zeiss), equipped with an AxioCam HRc digital camera (Zeiss), using Axiovision 4 Imaging program (Zeiss). Confocal double labeling: free floating double immunofluorescence staining was performed as described above. Primary antibody dilutions used were: Nu1 1:3000, McSA1 1:16.0000, CD68 1:100, Iba-1 1:2000. Secondary antibodies used were: donkey

anti-rat Rhodamin-conjugated (Jackson Immunoresearch, USA; working dilution 1:200); donkey anti-rabbit Rhodamin-conjugated (Jackson Immunoresearch, USA; working dilution 1:200); biotinylated goat anti-mouse (Vector laboratories Inc., Burlingame, California, USA; working dilution 1:200) followed by treatment with Streptavidin Alexa 488 (Molecular probe, Invitrogen, Eugene, Oregon, USA). To avoid cross-reactivity between the goat anti-mouse antibody and the rat CD68 antibody (Nu1-CD68 double labeling), an Alexa Fluor 488 goat anti-mouse IgG highly cross-adsorbed was used (Molecular probe, Invitrogen, Eugene, Oregon, USA, working dilution 1:200). Stainings were revealed using a Zeiss LSM 510 confocal microscope equipped with argon and helium neon lasers (Zeiss Canada, Toronto, ON). A plan-apochromat 40× objective was used within the CA1 area of the hippocampus. Optical sections were approximately 0.6μm thick as indicated by pinhole settings. Using the same settings, no staining was detectable in the control sections (omission of primaries) and no Aβ-signal was observed in the complete double labeling on Non-Tg tissue.

Quantification of size and diversity of Iba-1 immunoreactive in the CA1 region cells. For this study we used 5 Non-Tg and 8 Tg mice of the 2-week old cohort; 6 Non-Tg and 6 Tg mice of the 3month-old cohort, and 5 Non-Tg and 5 Tg from the 10-13 month-old cohort. Three sections per animal were stained with Iba-1 antibody, after which the slides were coded. Eight pictures per section were taken at 63x magnification in the CA1 area of the hippocampus and imported into the MCID 4 Image Analysis Software. Cell bodies of all the cells in focus in each picture were manually outlined, and target sizes as well as number of target elements were measured in an automatic fashion. After the quantification, the slides were un-coded and the data analyzed. Cell soma size (cross-sectional area) was normalized on the Non-Tg value. The density data was expressed as the number of Iba-immunoreactive cells per field (38.206μm square). MTF was blind to experimental groups at all stages of the quantification.

Density of Iba-1 immunoreactive cells bordering CA1 pyramidal neurons: For this study we used 4 Non-Tg and 4 Tg mice of the 3-month old cohort. Three sections per animal were double-labeled with Iba-1 and either NeuN (Non-Tg mice) or McSA1 (Tg mice). After coding the slides, 4 pictures per section were taken at 40x magnification in the CA1 area and imported into the MCID 4 Image Analysis Software. A box tool was drawn in order to accommodate the CA1 pyramidal neurons and the Iba-1-immunoreactive cells immediately bordering them. The same box tool was then applied to all the pictures and the software set to quantify the brown (i.e. Iba-1 positive) elements within the box. After the quantification, the slides were un-coded and the data analyzed. The density data are expressed as number of Iba-immunoreactive cells per box (17.621μm square). Given the differences in variances, this set of data was analyzed using the Student's T-test with the Welsh correction. MTF was blind to experimental groups at all stages of the quantification.

Quantification of CD68 immunoreactive elements. For this study we used 4 Non-Tg and 5 Tg mice of the 3-month old cohort. Three sections per animal were labeled with CD68 according to the procedure described above. After coding the slides, 8 pictures per section were taken at 63x magnification in the CA1 area of the hippocampus and imported into the MCID 4 Image Analysis Software. CD68-immunoreactive granules, target size and number of target elements were measured in an automatic fashion, using a program originally designed to grain count. After the quantification, the slides were un-coded and the data analyzed. CD68 granule size was normalized on the Non-Tg value. Given the non-parametric distribution of the data, this set of data was analyzed using the Mann-Whitney test.

Quantification of Nu1 immunoreactivity. For this study we used 8 Tg mice of the 2-week old cohort and 2 Tg mice from the 3-month old cohort. One negative littermate per age group was included as control. Two sections per animal were

labeled with Nu1 according to the procedure described above. After coding the slides, 2 pictures per section were taken at 40x magnification in the CA1 area of the hippocampus and imported into the MCID 4 Image Analysis Software. Total and proportional target areas were measured in an automatic fashion. Proportional target area per picture was summed (giving the proportional target size per section), and these values averaged between the two sections, to yield the proportional target area per animal. After the quantification, the slides were un-coded and the data analyzed. The data were normalized on the average of 3-month old Tg target area value.

Data analysis. All data were analyzed using the Graph-Pad Prism 5 software. Unless differently specified, two groups comparison was done by Student's T-test.

3.4 Results

3.4.1 Neuroinflammatory features associated with amyloid plaques.

In studying the earliest occurrence of an inflammatory reaction in the McGill-Thy1-APP Tg mice, we first verified that the well-established inflammatory process does occur in the post-plaque stages of the amyloid pathology in this animal model. Immunostaining with Iba-1 (a structural marker of microglial cells (Ahmed et al., 2007) revealed fully activated, rounded microglial cells tightly surrounding the periphery of mature amyloid plaques (Fig 3-1a). Enlarged microglial cells were also observed surrounding diffuse amyloid deposits (Fig 3-1b). In addition, in the McGill-Thy1-APP Tg mice, peri-plaque microglia were clearly reactive to CD11b antibodies (a classical marker defining activated microglia (Santambrogio et al., 2001).

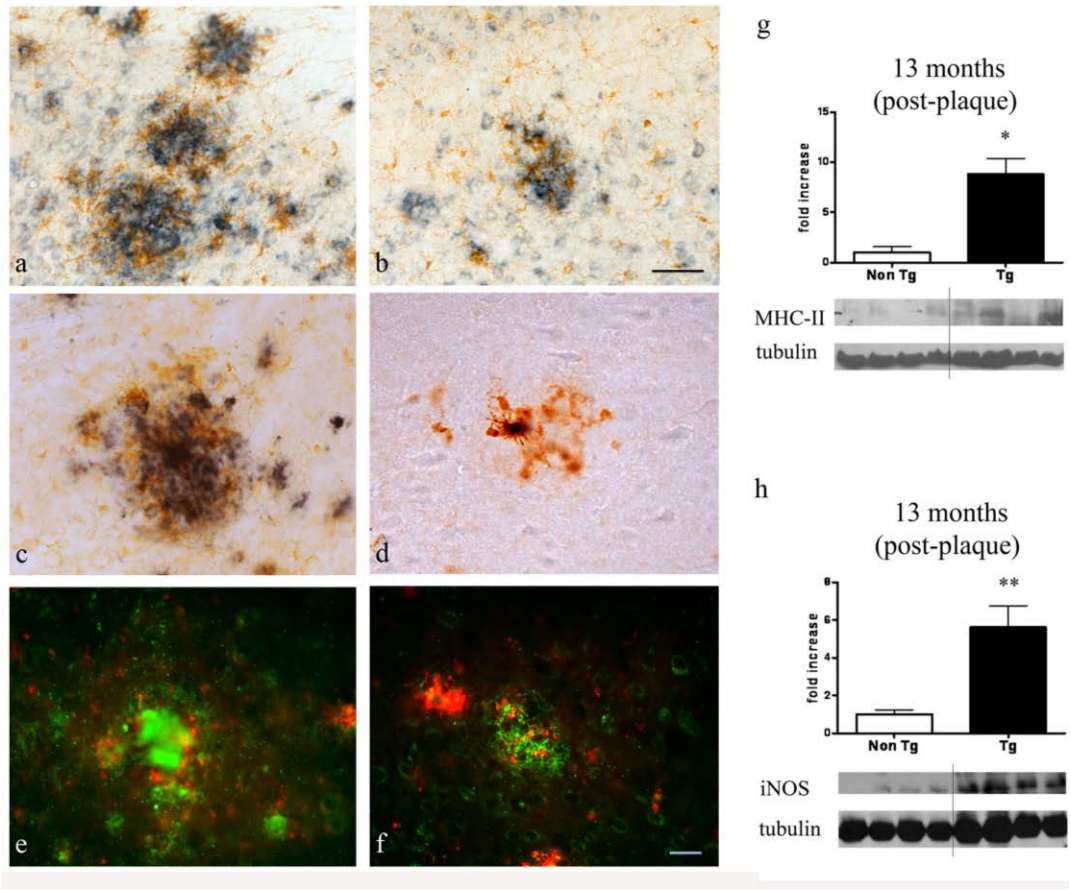


Figure 3-1 Characteristic of plaque-associated microglial activation in old McGill-Thy1-APP mice.

Left panel: Representative micrographs illustrating the presence of activated microglia surrounding dense and diffuse amyloid plaques in 13 month-old McGill-Thy1-APP Tg. Enlarged, ameboid microglial cells appeared to cluster around dense (**a**) and diffuse (**b**) amyloid plaques, as revealed by a double labeling Aβ (McSA1-IR, blue signal) and Iba-1 (brown signal). These cells expressed the typical marker of microglial activation, CD11b (**c**, brown signal), and were positive for MAC-2 (**d**), a marker of phagocytosis. The lysosomal marker CD68 (red signal) revealed enlarged microglial cells surrounding both dense (**e**) and diffuse (**f**) plaques (stained with the Aβ specific antibody McSA1, green signal).

Right panel: Representative Western blot from hippocampal homogenates of 13 month-old Tg mice compared to age-matched, Non-Tg littermates. Note the significant up-regulation of MHC-II ($p<0.05$) and iNOS ($p<0.01$), indicating the occurrence of an inflammatory process at this time point. Data were analyzed by Student's t-test. Scale bars: a,b: 50um; c-f: 20um.

Furthermore, we observed abundant expression of MAC-2, a marker of active phagocytosis (Rotshenker, 2009) around plaques (Fig 3-1d), and several MAC-2 immunoreactive (IR) cells in the corpus callosum. In line with this finding, CD68 (macrosialin, a marker indicative of lysosomal activity (da Silva and Gordon, 1999), revealed enlarged lysosomal profiles around diffuse (Fig 3-1e) and mature (Fig 3-1f) amyloid plaques.

To confirm the immunohistochemical findings, we investigated the levels of MCH-II (major histocompatibility complex –II) and iNOS (inducible nitric oxide synthase), classical markers of inflammation (Holmin and Mathiesen, 1999; Wong et al., 1996), via Western blotting. Hippocampal homogenates from 13 month-old Tg animals (corresponding to a late, post-plaque phase of the pathology) were processed and the results compared with age-matched Non-Tg littermates (Fig. 3-1). In agreement with previous observations from AD brain and from Tg models (Luth et al., 2001) this analysis revealed that both MCH-II and iNOS were significantly up-regulated in hippocampus of Tg mice as compared to Non-Tg littermates (13 months of age, Fig. 3-1g and h, $p<0.01$).

3.4.2 Microglia activation precedes plaque deposition

Having assessed the occurrence of strong microglial activation in old, plaque-burdened mice, we next investigated whether a pro-inflammatory process does occur prior to the extracellular Aβ pathology. We chose 3 month-old Tg mice, since at this time point the McGill-Thy1-APP Tg mice display cognitive deficits concomitant with the intracellular accumulation of neurotoxic Aβ-oligomers (Ferretti et al., 2011a). Levels of MHC-II, iNOS and CD40, classical markers of microglia activation (Tan et al., 1999) were investigated via Western blotting in hippocampal homogenates, and the results compared with age-matched Non-Tg littermates (Fig. 3-2).

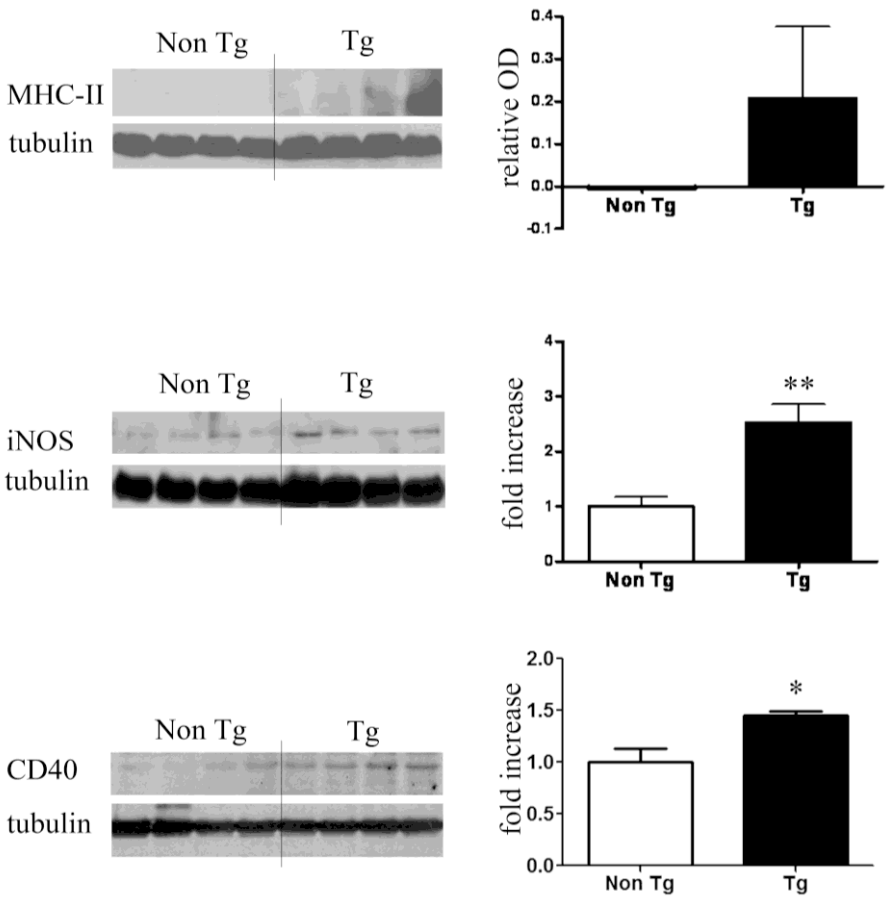


Figure 3-2 Early inflammatory response is detected in the hippocampus of the McGill Thy1-APP mice prior to plaque deposition

Representative Western blot of MHC-II, iNOS and CD40 (typical markers of microglial activation) in hippocampal homogenates from young Tg mice and age-matched littermates. Note the significant up-regulation of the three markers in Tg animals as compared to control (*, $p<0.05$, **, $p<0.01$, Student's t-test).

We found that at 3 months of age, at a stage when the phenotype is limited to intracellular Aβ accumulation, MCH-II was virtually undetectable in Non-Tg littermates, but it was detectable in Tg mice hippocampi. At this early, pre-plaque time point, CD40 and i-NOS were already up-regulated in Tg hippocampi as compared to Non-Tg littermates.

Taken together, these results suggested that early microglial activation is already in place in the hippocampus of McGill-Thyl-APP mice at a time point when no plaque deposition is apparent, and concurrently with a marked intracellular accumulation of Aβ oligomers.

3.4.3 Morphological alterations of microglia prior to plaque deposition

Progressive changes in the morphology of microglial cells, from a highly ramified (resting) phenotype to ameboid-shaped, activated phenotype (Nimmerjahn et al., 2005) are known to accompany their activation process. Therefore, we used a morphological approach to further investigate the degree of microglial activation in hippocampal CAl region of McGill-Thyl-APP mice.

We applied Iba-l to immuno-stain brain sections from 3 and 10-13 month-old Tg mice and Non-Tg age-matched littermates (Fig. 3-3). The vast majority of the microglial cells in control animals of both ages displayed a resting morphology (as exemplified in Fig. 3-3a), with a small, roundish or flattened soma and long, thin and highly ramified processes. However, in 3 month-old Tg mice, corresponding to the pre-plaque stage of the amyloid pathology, we observed intermediately activated cells, with larger cell soma and retraction and thickening of the processes (as exemplified in Fig 3-3b).

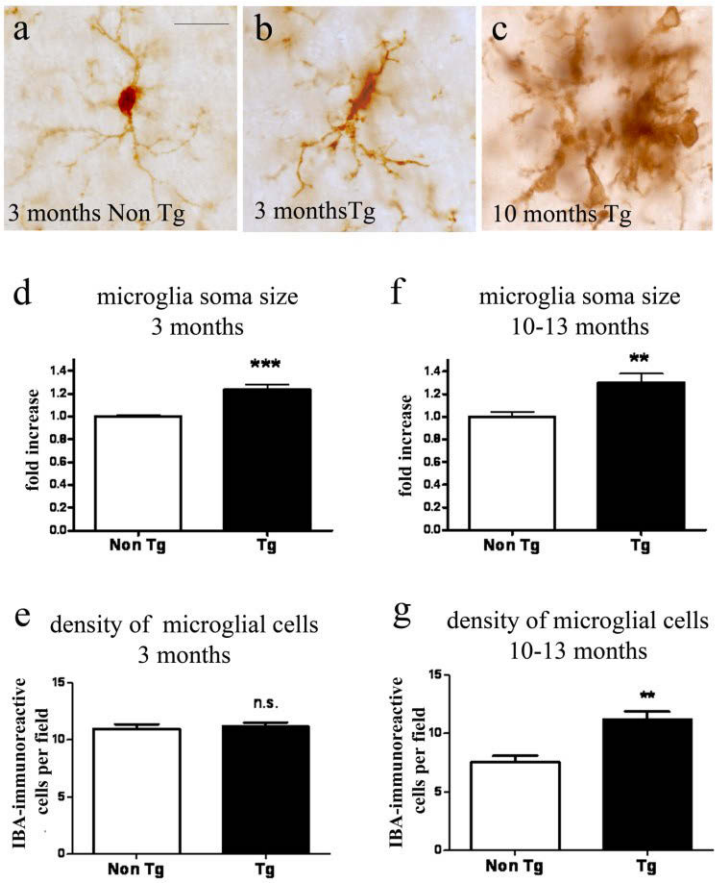


Figure 3-3. Activation of microglial cells in McGill Thy1-APP mice is already detected at early, pre-plaque stage of the pathology.

a,b,c: Representative micrographs illustrating the phenotype of a resting microglial cell in a Non-Tg, 3 month-old mouse (**a**), an intermediately activated microglial cell form a Tg 3 month-old mouse (**b**) and large, ameboid activated microglial cells around a plaque (**c**). Enlargement of the soma size and the retraction and thickening of processes in **b** are indicative of microglia activation in the Tg mice at the early, pre-plaque stage of the pathology. **d,e**: Quantification of cell soma size (**d**) and density (**e**) of Iba-1 immunoreactive cells in Tg 3 month-old mice compared to age-matched Non-Tg littermates. Note that the soma size was found to be significantly increased in Tg mice at the three months time point, while the overall density of microglial cells was unchanged at this early stage. **f,g**: activation and recruitment of microglia in 10-13 month-old, post-plaque Tg mice compared to age-matched, Non-Tg littermates. **f**: significant increase of cell soma size of Iba-1 immunoreactive cells (30%) was found in Tg mice hippocampus at 10-13 months old compared to Non-Tg littermates. A significant increase in the density of Iba-1 cells in hippocampus was also found at this age in Tg mice compared to Non-Tg littermates. (Data were analyzed by Student's-t test, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Scale bars: a-c: 20µm

Cell soma size of Iba-l-immunoreactive cells was quantified using a computer-assisted image analysis system. With this approach we found a significant 20% increase in soma size (cross-sectional area) in Tg hippocampi compared to Non-Tg littermates already at 3 months of age (Fig 3-3d, $p<0.001$), suggesting that an early microglial activation was indeed present at this age point. Despite the observed microglial activation, we did not detect an increment in the density of microglial Iba-1 positive cells this early stage (Fig 3-3e, $p<0.71$) in the overall neuropil of the CA1 region. Therefore, activation should occur within the resident microglial population.

At later stages of the pathology (10-13 months), plaque-associated microglial cells displayed an ameboid shape indicative of strong activation (Fig 3c). Computer-assisted quantification of the cell soma size of such cells revealed a 30% increase in microglial cell soma size in Tg compared to Non-Tg hippocampi (Fig. 3-3f, $p<0.01$). At this age, a significant increase in the density of microglial cells was also observed in the overall neuropil area of the CA1 in Tg mice compared to Non-Tg littermates (Fig. 3-3g, $p<0.01$), indicating a pathology-driven recruitment of new Iba-1-IR cells.

3.4.4 Morphologically activated microglia are detected in association with Aβ burdened neurons.

Extracellular application of Aβ-oligomers can induce neuroinflammation in naïve rats (Bruno et al., 2009a). Young, pre-plaque McGill-Thy1-APP mice display intracellular accumulation of Aβ-oligomers within pyramidal neurons of cortex and hippocampus, which is associated with cognitive, biochemical and structural alteration of the CNS (Ferretti et al., 2011a). Therefore, we hypothesized that the intermediate activation of microglia observed in pre-plaque Tg mice could be caused by the presence of intracellular Aβ-oligomers.

Figure 3-4. Recruitment of microglial cells in McGill-Thy1-APP mice towards Aβ burdened neurons.

a-c. Representative micrographs illustrating a double labeling for Aβ-oligomers (Nu1-immunoreaction, blue signal) and microglia (Iba-1, brown signal) in the cerebral cortex of a 3 month-old (pre-plaque) Tg mouse. Note the presence of morphologically activated microglia surrounding (arrow heads) and/or contacting with processes one or more Aβ-oligomer-bearing neurons (asterisks). On occasion the microglia attachment produces indentations in Aβ-oligomer-burdened neurons (filled, thick arrow in **c**).

d-f. Recruitment of microglial cells towards Aβ-loaded pyramidal neurons of the hippocampus CA1 region in the McGill-Thy1-APP Tg mice at 3 months of age. **e**: Non-Tg age-matched littermate tissue double labeled with Iba-1 (microglia-specific, brown signal) and NeuN- (neuron-specific, blue signal) antibodies. **f**: Tg 3 month-old tissue double labeled with Iba-1 (brown signal) and McSA1 antibodies (Aβ-immunoreactivity, blue signal). Note that in Non-Tg specimens microglial cells seldom border pyramidal neurons (**e**), while they infiltrate and surround this layer in the CA1 region of Tg mice (**f**). Computer-assisted image analysis (in **d**) revealed a significantly increased number of microglial cells surrounding CA1 in Tg animals compared to Non-Tg. Data were analyzed with the Student's t-test with the Welsh correction for unequal population variances

Scale bars: a-c: 20um, e,f: 100μm.

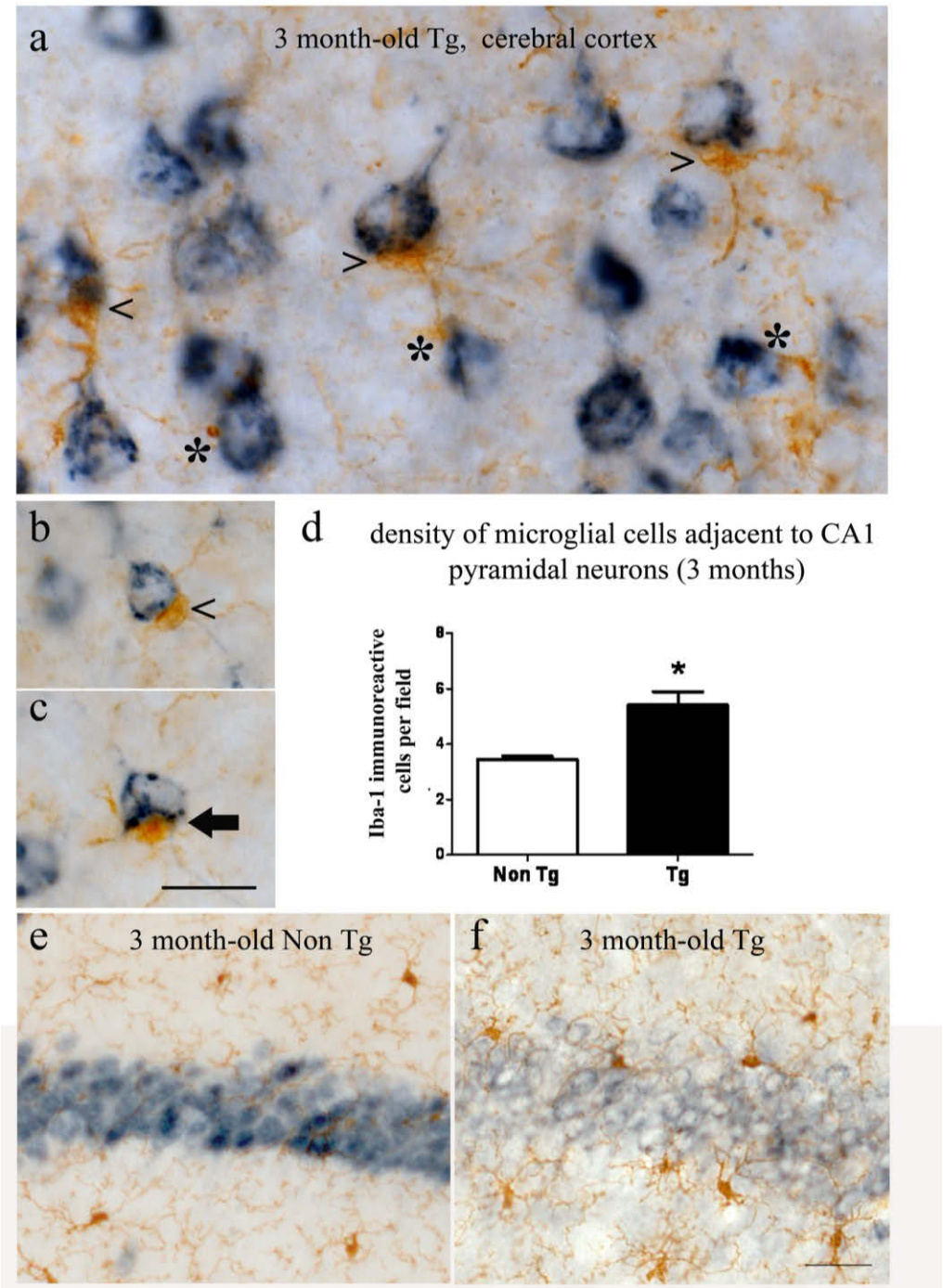


Figure 3-4. Recruitment of microglial cells in McGill-Thy1-APP mice towards Aβ burdened neurons.

To study the interaction between the intracellular accumulation of Aβ and microglia, we performed a double-labeling using Iba-1antibodies and Nu1, an oligomeric specific monoclonal antibody (Lambert et al., 2007). This approach allowed us to observe several microglial cells that appeared to be specifically recruited towards Aβ-burdened neurons, in both hippocampus and cortex of young, pre-plaque Tg mice (Fig.3-4). Iba-1 immunoreactive cells often surrounded Aβ-oligomer-burdened neurons (Fig. 3-4a-c, arrow heads), and-or contacted neighboring neurons with their processes (Fig.4a, asterisks). In some cases the microglial attachment produced an indentation in the Aβ-oligomer-burdened neurons (Fig. 3-4c, filled arrow).

A quantitative study of the microglial distribution of the hippocampus was therefore performed in the CA1 area, where the cyto-architecture facilitates the identification of potential migration of microglial cells towards Aβ-oligomer-burdened neurons (Fig. 3-4d-e). This study revealed a significant increase in the density of Iba-1 positive cells in Tg mice compared to Non-Tg littermates in the area immediately adjacent to pyramidal neurons ($p<0.05$, Fig. 3-4d). Fig. 3-4e illustrates the random distribution of microglial (Iba-IR, brown signal) cells on the CA1 region of the hippocampus of Non-Tg mice, while Fig. 4f shows the relocation of microglial cells in direct contact with pyramidal neurons in young McGill-Thy1-APP Tg mice. Since CA1 pyramidal neurons in Tg animals are overtly burdened by intracellular Aβ (Fig. 3-4e, blue signal), such rearrangement suggests an ongoing, specific migration of inflammatory cells towards intracellular-Aβ accumulating neurons.

3.4.5 Microglia from young Tg animals display enlarged CD68-immunoreactive lysosomes, but no phagocytosis of Aβ.

We then investigated whether the intermediately activated microglia observed in young, Tg mice would display a phagocytotic profile, using CD68 as a marker of microglial lysosomes.

While microglia from Non-Tg animals displayed a punctate CD68 staining uniformly distributed along the cell body, CD68 depositis in Tg animals appeared to be concentrated in larger granules associated with the cell soma (Fig.3-5). Image analysis of CD68-IR profiles confirmed a significant increase in the size in Tg animals, suggesting an increased phagocytotic activity in microglia from Tg animals prior to and independent of plaque deposition ($p<0.05$, Fig. 3-5, upper panel).

To investigate whether phagocytosis of Aβ oligomers occurred at this time point, we performed a double-fluorescent staining using CD68 and the Aβ-oligomers specific antibody Nu1 (Lambert et al., 2007). Even though we observed several examples of microglial cells closely associated with Aβ-burdened neurons (Fig 3-5, lower panel), we did not find evidence for co-localization which could indicate on-going phagocytosis.

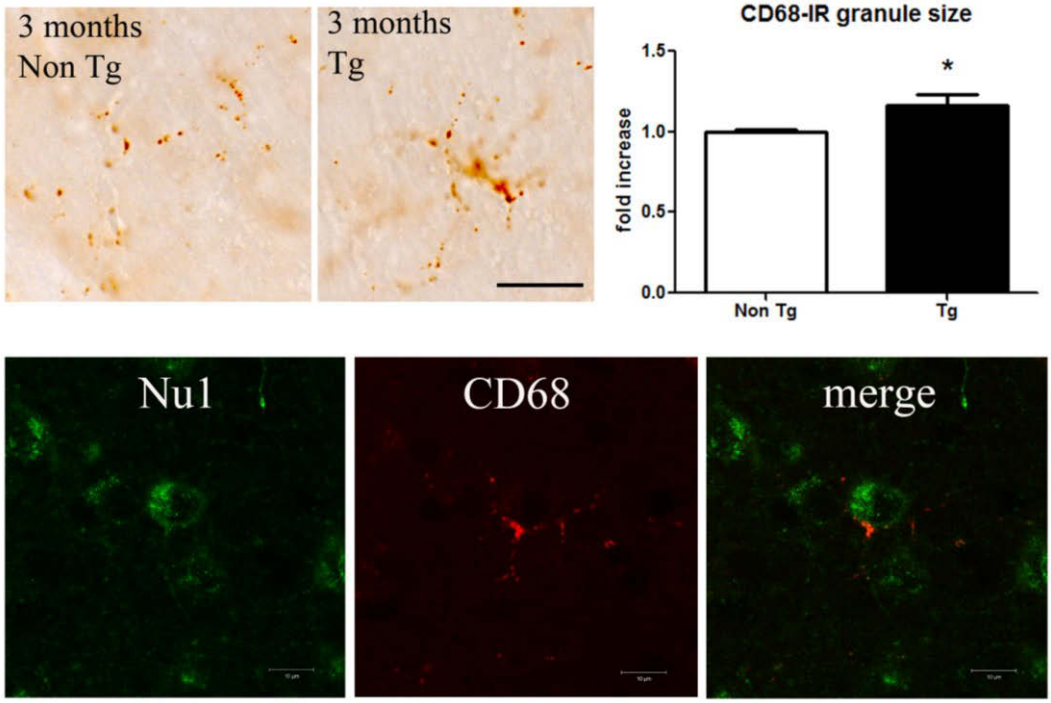


Figure 3-5. CD68 immunoreactivity reveals enlarged lysosomes in young, pre-plaque Tg mice

Upper panel: representative micrographs illustrating the immunoreactivity for the microglial lysosomal specific marker CD68 in a young, 3 month-old Non-Tg animals and in an age-matched Tg littermates. Note the presence of larger and clustered CD68 immunoreactive granules in the Tg littermate, compared to control. Computer-assisted image analysis revealed a significant 16% increase in the size of CD68 immunoreactive granules in Tg animals, compared to Non-Tg littermates (*, $p<0.05$, Mann-Whitney U test). Scale bar: 10um.

Lower panel: representative micrographs of confocal fluorescence double labeling of CD68 immunoreaction with Aβ-oligomer specific antibody Nu1 in young, pre-plaque Tg mice. The absence of co-localization might indicate ineffective phagocytosis of Aβ by microglial cells at this early stage. Scale bar: 10μm

3.4.6 Neuronal COX-2 up-regulation in areas associated with Aβ-bearing neurons

COX-2 is constitutively expressed in brain neurons (Yamagata et al., 1993). It has been shown to be up-regulated in acute conditions of brain injury (Iadecola et al., 1999), and its expression is known to be altered in AD (Pasinetti and Aisen, 1998).

Western blot analysis of hippocampal homogenates revealed that COX-2 was significantly up-regulated in Tg animals compared to Non-Tg littermates (Fig. 3-6a, $p<0.001$) as early as at 3 months of age, i.e. in the absence of visible extracellular amyloid pathology. Immunostaining showed that COX-2 was expressed in the hippocampus mainly by neurons of the dentate gyrus (Fig 3-6c) and CA2-3. Double immunofluorescence labeling for COX-2 and NeuN, a well-established marker of neurons, confirmed the neuronal origin of the COX-2 immunoreactivity (see inset in Fig. 3-6d).

The majority of COX-2 over-expressing neurons at early, pre-plaque stages of the amyloid pathology, showed low or no intracellular Aβ accumulation. However, using an immuno-fluorescent double labeling approach to reveal simultaneous COX-2 and Aβ-IR, the COX-2-overexpressing neurons were seen neighboring intracellular Aβ-burdened neurons (Fig. 3-6d-f).

At later stages of the pathology COX-2 levels appeared to return to normal values; nevertheless, a trend to an up-regulation of COX-2 was still observed (Fig. 3-6b, $p=0.07$).

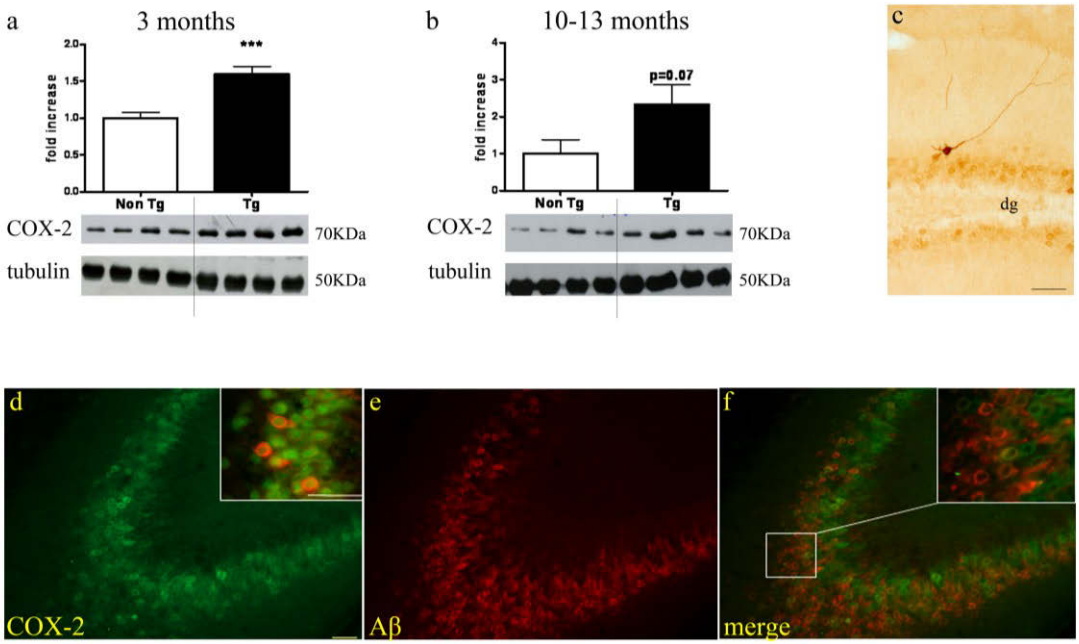


Figure 3-6. Early, pre-plaque up-regulation and topology of COX-2 in hippocampus of McGill Thy1-APP mice.

a,b: Representative Western blot analysis of hippocampal homogenates for COX-2 from young, pre-plaque (3 month-old) and old, post-plaque (10-13 month- old) mice. Note that COX-2 levels are significantly (***, $p<0.001$) up-regulated in Tg mice already at 3 months of age, compared to age-matched, Non-Tg littermates, and remain elevated at 10-13 months at levels close to significance ($p=0.07$). **c**: representative micrographs illustrating COX-2 immunoreactive neurons in the dentate gyrus (dg) of a Tg 3 month-old mouse.

d,e,f: relationship between COX-2 over-expressing neurons and Aβ-bearing neurons in CA2-3 hippocampal areas of a 3-month old Tg mouse. **d**: immunofluorescence labeling COX-2, showing specific neuronal staining (in the inset: double labeling with COX-2-red, and NeuN-green, confirmed the neuronal origin of the COX-2 immunoreactive material). **e**:Aβ immunofluorescence labeling confirming high intraneuronal accumulation of Aβ-immunoreactive material in the absence of plaque deposition at the three months time point **f**: merge. Note, in the inset, that COX-2 over-expressing neurons were found in close association with Aβ-bearing neurons, but little to no co-expression of Aβ and COX-2 was found. Scale bars: 50μm

3.5 Discussion

We provided evidence that in the McGill-Thy1-APP Tg model neuroinflammation is an early event in the progression of the AD-like CNS amyloid pathology, preceding plaque deposition but associated with the accumulation of Aβ oligomers.

In support of the above view, *in vivo* and *post-mortem* studies (Bruno et al., 2009b; Cagnin et al., 2001; Okello et al., 2009) showed an up-regulation of inflammatory markers in MCI patients, which are currently considered as the initial stage in the progression towards AD (Morris et al., 2001). However, the question of the state of the neuroinflammation in pre-clinical (pre-plaque) stages of AD cannot be answered definitely yet in humans since at the moment there are no universally accepted biomarkers signaling pre-MCI or AD condition. Even when MCI is considered as early AD (Morris et al., 2001; Petersen et al., 1999) it has to be taken into account that it is already characterized by extracellular plaque deposition and neurofibrillary tangles and shares most of the neuropathological features with AD (Morris and Price, 2001; Petersen, 2009).

On the other hand, past studies on animal models of AD have hinted at an early inflammatory process: up-regulation of inflammatory markers was reported in pre-plaque Tg2576 mice (Abbas et al., 2002), Pd/APP mice (Heneka et al., 2005a; Murphy, Jr. et al., 2000) and in the 3xTg mice (Janelsins et al., 2005).

The alterations observed in the brains of young, pre-plaque mice, suggest a classical activation state (M1) of microglial cells in early stages of the progression of the AD-like amyloid pathology. M1 macrophages are efficient phagocytotic cells, which produce high levels of reactive oxygen and nitrogen intermediates and participate in cell-mediated immunity involved in Th1 responses (Mantovani et al., 2005). The immunoreactivity pattern for CD68 (Fig 3-5) suggested active (or attempted) phagocytotic process in microglia

from pre-plaque mice, a process associated with up-regulation of iNOS (Fig 3-2) and release of nitric oxide. This will likely affect the neuronal function, as nitric oxide and reactive oxygen species can directly damage neurons through oxidative stress (Brown and Bal-Price, 2003; Dawson et al., 1991). Hallmarks of oxidative stress are in fact present in AD brains and neuronal oxidative damage has been proposed as one of the earliest pathological events in the progression of the disease (Good et al., 1996; Nunomura et al., 2001; Smith et al., 1997).

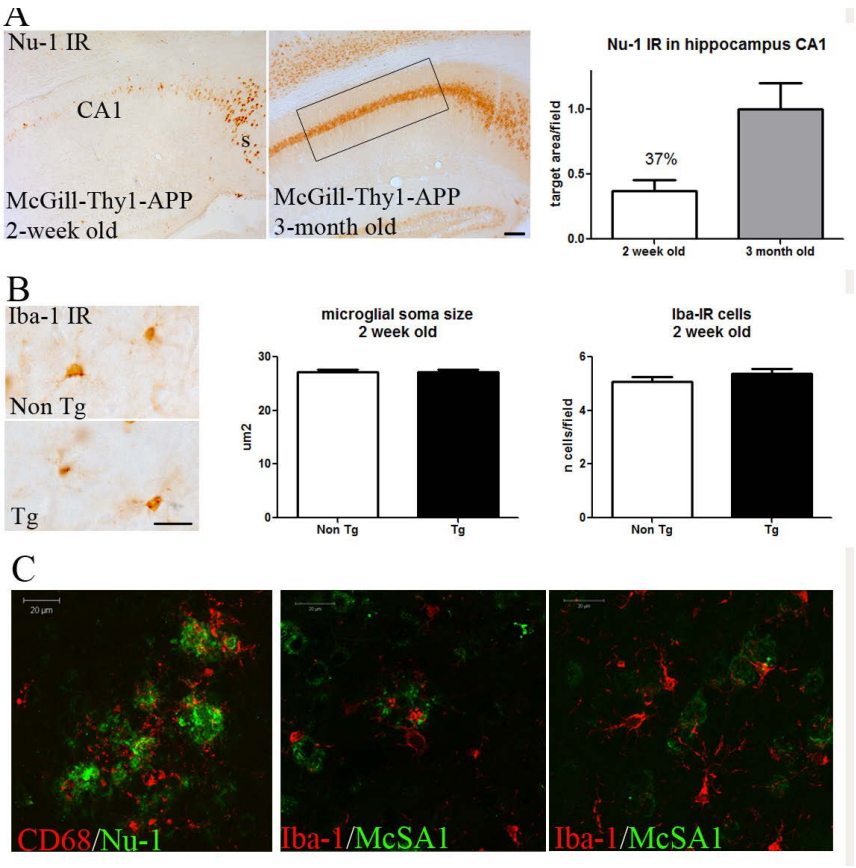
On the other hand, the up-regulation of MHC-II and CD40 (Fig 3-2) suggests an increased antigen presenting activity, with further recruitment of inflammatory cells. However, our data would indicate that an increase of Iba-1 immunoreactive microglial cells occurs only later in the progression of the disease (Fig 3-3e,g). It is therefore assumed that at early stages the inflammatory reaction is mainly manifested by the activation of resident microglial cells with no infiltration of peripheral macrophages nor proliferation of the local cells.

Interestingly, we could not detect phagocytosis of Aβ material by microglial cells in the pre-plaque phase (Fig 3-5). Additional experiments performed at later time points (12-month old) similarly showed no active Aβ phagocytosis by microglial cells (see Supplementary 1C). This negative result could be explained by the rapid degradation of the epitope in the microglial lysosomes. However, our observations are in line with evidence indicating that resident microglial cells in the adult brain are poor phagocytic cells (Reichert and Rotshenker, 1996). Adult microglial cells have been shown to be less capable of phagocytosing Aβ than embryonic microglial cells (Floden and Combs, 2006). *In vivo*, microglial phagocytosis of Aβ has been definitively shown so far only in Tg animals manipulated with irradiation (Simard et al., 2006). Even when sophisticated two-photon *in vivo* imaging was used, only close association between microglia and plaque was shown, but no actual phagocytosis. Nevertheless, several studies have reported a role of microglia in restricting amyloid plaques (for a review see:

Morgan, 2009), suggesting that additional mechanisms (i.e. enzymatic degradation) rather than active phagocytosis might be involved. Indeed, microglial cells express and secrete proteolytic enzymes that degrade Aβ, such as IDE (insulin-degrading enzyme), neprilysin, matrix metalloproteinase 9 (MMP9), and plasminogen (for a review see: Lee and Landreth, 2010).

What causes the early, pre-plaque microglial activation and recruitment towards Aβ-burdened neurons remains unclear, but it is likely to be linked to the abnormal accumulation of intracellular Aβ-oligomers. In fact, co-labeling of Nul and Iba-1 revealed morphologically activated microglial cells in close relationship with pyramidal neurons bearing oligomers of Aβ (Fig. 3-4). This investigation confirmed the intense intracellular Nu1-immunoreactivity in CA1 neurons we previously reported (Ferretti et al., 2011a). Further supporting the hypothesis that inflammatory responses are unleashed by the early accumulation of Aβ-oligomers, we have not detected any microglial response in juvenile, 2-week old Tg mice which display little to none immunoreactivity to Nu1. In fact, at this early time-point, no differences were noted in the soma size nor in the number of Iba-1 immunoreactive cells between Tg and Non Tg littermates (see Supplementary Fig. 1A,B).

One could speculate that the microglial reaction observed in young, pre-plaque McGill-Thy1-APP mice is triggered by diffusible factors from damaged or dying Aβ-burdened neurons. In fact, it is well known that unhealthy neurons can release a variety of signals to surrounding microglia (for a review see: Hanisch and Kettenmann, 2007). The intracellular accumulation of pathologic, mis-aggregated proteins is highly toxic to the neurons, and it has been shown to affect the fate of surrounding microglial cells (Yoshiyama et al., 2007). A similar recruitment of microglial cells towards neurons has been demonstrated recently using a two-photon *in vivo* imaging technology in a mouse model of AD (Fuhrmann et al., 2010). Importantly, in that report, microglia appeared to migrate specifically towards dying neurons, and was involved in their elimination.



Supplemetary Figure 1.

A. Representative micrographs of Nu-1 (Aβ-oligomers) immunoreactivity in juvenile (2-week old) and adult (3-month old) McGill-Thy1-APP mice. Note the presence of intense Nu-1 staining in the subiculum (s) at both ages, while CA1 pyramidal neurons display only a scarce immunoreactivity at 2-week of age. The square indicates the region used for quantitative analysis. Computer assisted image analysis revealed that Nu-1 immunoreactivity in juvenile mice (n=8) was in the order of 37% of that of adult mice (n=2).

B. Representative micrographs of Iba-1 immunoreactivity (microglial cells) in the CA1 region of 2-week old mice. No changes in the morphology nor in the number of microglia were observed at this time point (see centre and right panel, respectively).

C. Representative confocal micrographs of fluorescence immunolabeling for microglial markers (CD68 and Iba-1, red signal) and Aβ markers (Nu-1 and McSA1, green signal) in plaque burdened, 12-month old McGill-Thy1-APP mice. Note the absence of co-localization, suggesting a lack of active phagocytosis.
Scale bars: A= 500um, B= 20um, C= 20um

On the other hand, microglial activation and recruitment could be caused by leaking of some Aβ material itself from the neurons. The intra and extracellular pools of Aβ are assumed to be in dynamic balance (Oddo et al., 2006; Yang et al., 1999). Indeed, injections of synthetic preparation of Aβ-oligomers have been shown to elicit an inflammatory response in rat hippocampus, indicating that microglial cells respond to the presence of oligomeric forms of Aβ in the extracellular space (Bruno et al., 2009a).

The inflammatory response we observed in the hippocampal formation and cortex of McGill-Thy1-APP mice at 3 months of age, corresponding to a pre-plaque stage of the pathology, was also characterized by up-regulation of neuronal COX-2 (Fig. 3-6a). Previous studies in different Tg models showed increased levels of COX-2 in astrocytes associated with fibrillar deposits of Aβ (Matsuoka et al., 2001) or no changes (Kotilinek et al., 2008). This is therefore the first time that COX-2 is shown to be up-regulated in pre-amyloid plaque stage in animal models. As in human studies (Minghetti, 2004), we observed COX-2 immunoreactivity restricted to neurons. Although associated with Aβ-bearing neurons, COX-2-IR material at large did not correspond with Aβ-immunoreactive neurons (Fig. 3-8d-f). This finding resembles what is observed in the ischemic model, where COX-2 was shown to be up-regulated in the peri-infarct areas but not in the core of the lesion (Yokota et al., 2004). Both observations suggest that COX-2 is involved in the secondary progression of the injury; however, its role in such processes remains to be established. Even though COX-2 is known to mediate inflammation and cell damage in different experimental models (Seibert et al., 1994), several lines of evidence support the idea that COX-2 has a physiological role in synaptic function and spatial learning (Teather et al., 2002). Furthermore, COX-2 in early AD has been linked to expression of cell-cycle markers (Hoozemans et al., 2002; for a review see: Herrup and Yang, 2007). In sum, it

remains to be elucidated whether in early stages of the amyloid pathology the COX-2 over-expression is mediating the neurotoxic or neuroprotective effects.

In our experimental observations, microglial activation, COX-2 up-regulation and release of inflammatory markers were detected at a time when Tg mice show the first cognitive deficits (Ferretti et al., 2011b), leading us to speculate that Aβ-oligomer-driven inflammation plays a role in perturbing higher CNS functions. In fact, pro-inflammatory cytokines have been demonstrated to induce major depressive disorders and mild cognitive alterations in animal models and patients (for a review: Dantzer et al., 2008).

However, the role of the inflammatory response in AD is still controversial. Indeed, Rivest and collaborators have shown that recruitment of macrophages from periphery can be helpful in restricting plaque density in later stages of the pathology in the APP+PS1 model of AD (Simard et al., 2006). In agreement with this view, LPS-induced neuroinflammation in post-plaque Tg mice actually resulted in clearing of diffuse plaques and reduction of overall Aβ levels (Malm et al., 2005; Quinn et al., 2003). On the other hand, the same experimental approach proved to be detrimental in early stages of the pathology, accelerating plaque formation and increasing overall and intracellular Aβ levels (Qiao et al., 2001; Sheng et al., 2003).

Taken together these results raise the intriguing possibility that microglial activation might play a dual role, accelerating the pathology at early stages but clearing pre-existing deposits at later stages. Such complex role might be mediated by subsets of microglial populations, or by differential involvment of microglial versus circulating macrophages. Our data would support a model in which microglial cells are first primed by intracellular accumulation of Aβ, and generate a ’bad’ inflammatory reaction that is not capable of clearing the neurotoxic peptide. Only later, possibly concurring with brain parenchyma invasion by macrophages, a ’good’ inflammation takes place. This late process might help restrict the amyloid

deposits but cannot rescue the neuronal function. Such tentative model is substantiated by recent evidence from the ADAPT clinical trial, so far the only preventative antinflammatory study. Unfortunately the trial was interrupted for safety reasons (Meinert et al., 2009; Lyketsos et al., 2007). However, the post-trial analysis showed that in patients void of cognitive deficits NSAIDs can be protective against AD, while the same treatment accelerates the disease process in patients with pre-existing pathology (Lyketsos et al., 2007; Breitner, 2011).

A better understanding of the consequences of the early and late inflammatory responses in AD might provide some clues to explain the differential effects of NSAIDs as preventive or therapeutic. Such in-depth understanding would help the identification of the best therapeutic window for the initiation of preventive AD treatment aiming at delaying the onset of the disease.

3.6 Conclusions

In brief, this report highlights the occurrence of a pro-inflammatory process preceding the formation of extracellular amyloid plaques but coincidental in time with the earliest signs of detectable cognitive impairments in a Tg model. It is therefore likely that a similar pathological process takes place in humans at a stage well before MCI and AD, when plaques and tangles are already established. Elucidating the nature and the role of such early inflammatory process in the progression of the AD pathology might provide new therapeutic opportunities to arrest or delay the disease progression.

3.7 Acknowledgments

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Connecting Text: Chapter 3 to 4

The investigations presented in Chapters 2 and 3 indicated that in the McGill-Thy1-APP Tg model of AD-like amyloid pathology, the pre-plaque stage of the amyloid pathology is already characterized by significant neuronal dysfunction. In fact, we observed cognitive impairments and cholinergic alterations in Tg mice as early as at in young, 3 months of age. These neuropathological events occurred in the absence of amyloid plaques but coincidental with the accumulation of intracellular Aβ-oligomers (Chapter 2).

The pre-plaque stage of the amyloid pathology was also associated with microglial activation, as detected with morphological and biochemical methods. Interestingly, we observed a specific spatial association between activated microglial cells and intracellular Aβ-burdened neurons; furthermore, no microglial activation was observed prior to overt oligomeric pathology. Indeed, as discussed in Chapter 3, these findings were highly suggestive that the mere presence of intracellular Aβ oligomers is sufficient to unleash a microglial reaction.

However, it remained to be elucidated whether such inflammatory reaction would represent a beneficial, protective event, or rather would be a moving force towards disease progression. While several studies exist in the literature indicating that inflammation has mostly a detrimental role in post-plaque phases of the disease, very little evidence is available on its role in early, pre-plaque phases.

In an effort to clarify the role of microglial activation in early, pre-plaque stages of the disease, we studied the effect of the anti-inflammatory drug minocycline, when administered to young, pre-plaque McGill-Thy1-APP mice. In particular, we sought to elucidate the role of microglial activation in BACE activity and APP processing prior to overt plaque pathology would take place.

While further investigations will be required to assess the clinical potential of minocycline as a preventive agent, our findings underscore the importance of the glia-neuron-glia cross-talk in early AD.

CHAPTER 4

Minocycline Corrects Early, Pre-Plaque Neuroinflammation and Inhibits BACE-1 in a Transgenic Model of AD-like Amyloid Pathology

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4.1 Abstract

A growing body of evidence indicates that inflammation is one of the earliest neuropathological events in AD. Accordingly, we have recently showed the occurrence of an early pro-inflammatory reaction in the hippocampus of young, 3 month old transgenic McGill-Thy1-APP mice, in the absence of amyloid plaques but associated with intracellular accumulation of Aβ-oligomers. However, the role of such pro-inflammatory process in the progression of the pathology remained to be elucidated. To clarify this, we administered minocycline, a tetracyclic derivative with anti-inflammatory and neuroprotective properties to young, pre-plaque McGill-Thy1-APP mice for one month. The treatment ended at the age of 3 months, when the mice were still devoid of plaques. Minocycline treatment corrected the up-regulation of i-NOS and COX-2 observed in young Tg placebo mice. Furthermore, the down-regulation of inflammatory markers correlated with a reduction of APP protein levels and APP-related products. BACE-1 activity and levels were found to be up-regulated in Tg placebo mice, while minocycline treatment restored the levels to normality. The anti-inflammatory and BACE-1 effects could be partly explained by the inhibition of the NFkB pathway. Even though the inter-play between inflammation and amyloid pathology requires further investigation, our study suggests that the pharmacological modulation of neuroinflammation might represent a promising approach for preventing AD onset.

4.2 Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative condition affecting more than 35 million people world-wide (Wimo A. and Prince M., 2010). Neuropathological examination of AD brains reveals intraneuronal neurofibrillary tangles (composed by paired filaments of abnormally phosphorylated tau protein (Iqbal and Grundke-Iqbal, 2008), and massive accumulation of extracellular amyloid plaques composed by aggregated amyloid beta peptide (Aβ) (Selkoe, 2000a).

The 4 kDa Aβ peptide originates from the sequential endoproteolytic processing of a type-I membrane protein called amyloid precursor protein (APP). The initiating event for Aβ production is the cleavage of APP by the β-secretase cleaving enzyme (BACE-1), a neuronal specific aspartyl protease (Vassar et al., 1999). This event generates a soluble N-terminus exodomain (soluble APPβ) liberated into the lumen and a β-C-terminus fragment (β-CTF) bound to the membrane. Gamma-secretase cleavage of the membrane-anchored β-CTF releases Aβ peptides of different lengths, including Aβ38, 40 and 42 (Wolfe et al., 1999). In the non-amyloidogenic pathway, β-secretase cleavage is prevented by the action of a α-secretase which liberates an N-terminus exodomain (soluble APPα) and an α-C-terminus fragment (α-CTF; for a review: Selkoe, 2001b).

The deposition of Aβ42-amyloid plaques is considered a central event in the AD neuropathology and is accompanied by the formation of dystrophic neurites and the up-regulation of inflammatory mediators (such as cytokines, chemokines, iNOS, COX-1 and 2) (Akiyama et al., 2000).

The expression of several inflammatory mediators in the CNS is under the control of an inducible transcription factor called nuclear factor kappa B (NFkB) (Baeuerle and Henkel, 1994). The active form of human NFkB is a dimer

composed of the two DNA binding subunits p50 and p65. These genes are constitutively expressed in both glia and neurons and their mRNA levels are further increased in response to signals such as tumor necrosis factor alpha (TNF-α) and phorbol 12-myristate 13-acetate (PMA). Dimers are normally in an inactive form, sequestered in the cytoplasm by the NFkB inhibitor, IkB (for a review: Kaltschmidt et al., 2005). Activation by inflammatory stimuli results in phosphorylation and degradation of IkB, translocation of NFkB into the nucleus and expression of target genes including IkB as an autoregulatory loop (Verma et al., 1995). Target genes include inflammatory cytokines (e.g. interleukins IL-1 and 6, TNF-α), chemokines (e. g., MIP-1α, MCP1, RANTES), inducible enzymes (such as COX-2 and iNOS), acute phase proteins and immune receptors (Baeuerle and Henkel, 1994); in addition the BACE-1(Rossner et al., 2006) and APP genes (Grilli et al., 1995) have been shown to contain a binding site for NFkB. Increased NFkB and IkB expression has been shown in AD (Terai et al., 1996; Kaltschmidt et al., 1997; Ferrer et al., 1998; Yoshiyama et al., 2001) and elevated NFkB activity was detected in Tg models of AD (Sung et al., 2004).

While amyloid plaque-associated inflammation has been extensively studied in human and animal models, little is known on the inflammatory process prior to plaque deposition. This is an issue of great potential interest, since new insights on the earliest stages of the AD amyloid pathology are pivotal to the discovery of predictive biomarkers and new therapeutic targets. Strong evidence in the literature indeed indicates that inflammation could be one of such early events in the progression of AD. Epidemiological data demonstrated that life-long use of non steroidal anti-inflammatory drugs (NSAIDs) protects against AD, suggesting the existence of a latent pre-clinical inflammatory process which would facilitate the disease progression (for a review: McGeer and McGeer, 2007). Inflammation could in fact be detected in mild cognitive impairment patients (Okello et al., 2009; Parachikova et al., 2007; Bruno et al., 2009b), which represent the prodromal stage of AD (for a review see: Ferretti and Cuello, 2011).

Along the same lines, we have described the occurrence of an early, pre-plaque inflammatory response in our newly generated McGill-Thy1-APP mouse transgenic (Tg) model of AD-like amyloid pathology which would mimic the pre-morbid AD. In this Tg model plaques deposition begins around 4-5 months of age (Ferretti et al., 2011b); however, up-regulation of inflammatory markers and activation and mobilization of microglia could be detected as early as at 3 months of age. Such pro-inflammatory reaction appeared to be supported by the resident microglia population, and was associated with the intracellular accumulation of Aβ-immunoreactive oligomeric species (Ferretti et al., 2011a).

Taken together, these findings support the notion that a pro-inflammatory process should take place at very early stages of the AD pathology. However, the role of such early neuroinflammation in the progression of the AD-like pathology remains to be elucidated.

To define the role of the early, pro-inflammatory events observed at pre-plaque stages of the AD-like amyloid pathology, we tested the therapeutic effect of minocycline, a tetracyclic derivative with anti-inflammatory properties in young, pre-plaque McGill-Thy1-APP Tg mice. The treatment lasted one month and ended when the mice were 3 month old, thus prior to the appearance of the first plaques. This strategy allowed us to specifically investigate the role of inflammation in early, pre-plaque stages of the amyloid pathology, which likely correspond to pre-clinical stages in the human.

We gathered biochemical and morphological evidence indicating that the early, pre-plaque neuroinflammation can be blocked by minocycline treatment. The reduction of inflammatory markers was accompanied by reduced activity of BACE-1 and correction of the NFkB pathway.

4.3 Materials and Methods

Animals and treatment. For this studies we used our in-house APP Tg mouse model of AD-like amyloid pathology coded McGill-Thy1-APP (Ferretti et al., 2011b). These mice carry the human APP transgene with the Swedish and the Indiana mutations under the control of the murine Thy1.2 promoter. A total of 14 Tg mice (n=7 placebo and n=7 minocycline) were utilized. For controls, 15 Non Tg, age-matched littermates were included (n=8 placebo and n=7 minocycline). The animals were housed in groups of up to four in individually ventilated cages under standard conditions (22 °C, 12 h light-dark cycle) receiving food and water *ad libitum*. All procedures were approved by the Animal Care Committee of McGill University and followed the guidelines of the Canadian Council on Animal Care. Minocycline hydrochloride was purchased from Sigma (M9511) and a fresh solution of 5 mg/ml (10 mM) was prepared in filtered PBS and stored at -80°C for one week. Since the pH of minocycline hydrochloride is acidic (4), we corrected it to neutrality by adding NaOH (as described by (Cornet et al., 2004). The animals were injected intraperitoneally (i.p.) with 200 ul of solution (i.e. 1 mg, which, for an average mouse weighing 20g, corresponds to 50 mg/Kg), alternating the injection side, every day for one month. Placebo animals received an equal volume of filtered, pH 7.4 PBS. The mice were 2 month old when they started the treatment, and were sacrificed at 3 months of age. At the end of the treatment signs of peritoneal irritation were observed in the mice receiving minocycline.

Perfusion and tissue preparation technique. Tg and Non Tg littermate mice were deeply anesthetized with equithesin (pentobarbital-based, 2.5ml/Kg, i.p.) and perfused through the heart with ice-cold saline solution (pH 7.4) for 1 minute. The brains were then quickly removed and divided into right and left hemisphere on ice. Cortex, hippocampus and cerebellum were dissected from the left hemisphere, snap-frozen in dry ice and stored at -80˚C for biochemical analysis. Cortical

samples were used for the determination of inflammatory marker levels and APP related products, while hippocampi were used for BACE-1 activity assay and BACE, NFkB and IkB quantification. The right hemisphere was fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4) for 24 hours at 4˚C. The tissue was then cut into 40um thick sections with a freezing sledge microtome (SM 2000R, Leica, Wetzlar, Germany) and free-floating sections were collected in phosphate buffered saline (PBS) and processed for immunohistochemistry.

Western blotting

Inflammatory markers. Cortical samples from the left hemisphere were homogenized in 250µl of lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 0.1% deoxycholic acid, 2 µg/mL of aprotinin, 2 µg/mL of leupeptin, 100 µg/mL phenylmethanesulfonyl fluoride, pH 7.4). The samples were centrifuged at 13,000 rpm for 45 min at 4˚C. Following total protein content quantification (Dc-protein assay, Bio-Rad, Hercules, CA, USA), 100ug of protein were separated using 10% SDS-polyacrilamyde gels and semi-dry transferred to nitrocellulose membranes for subsequent Western blotting. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and then incubated with the primary antibody overnight at 4˚C. Primary antibodies used were: rabbit polyclonal anti iNOS and IL-1β (both 1:500, Santa Cruz Biotechnology Inc., CA, USA); COX-2 (1:2000, Cayman Chemicals, Ann Arbor, MI, USA); and mouse monoclonal anti βIII-tubulin (1:40,000, Promega, Madison, WI, USA). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson (Jackson Immunoresearch Laboratories, West Grove, PA, USA). HRP signal was revealed with a chemiluminescence assay (ECL, GE Healthcare, Amersham, UK) on films. Signal intensity was quantified by densitometry (MCID4 image analysis system, Imaging Research Inc., St. Catherine's, ON, Canada). The levels for each marker were normalized

with respect to βIII-tubulin (neuronal specific) immunoreactivity. All experiments were performed in triplicate.

Tris-Tricine Western blotting for APP and APP related products Total proteins (100-250ug) from the cortical homogenates (prepared as indicated above) were run in pre-cast commercially available 10-20% Tris-Tricine gels (Criterion, Bio-Rad laboratories, Hercules, CA, USA). The proteins were semi-dry transferred on nitrocellulose (for 6E10 detection) or PVDF (for pab27576) for 2h at 12V. The membranes were boiled for 5 min in PBS, then blocked for 2h with milk 10% and incubated with the specific antibody O/N at 4˚C. Antibodies used were: monoclonal mouse antibodies: 6E10 (directed against the residues 1-16 of human Aβ, from Signet, provided by Covance, Princeton, NJ, USA; 1:1000), neuron specific βIII tubulin (Promega, Madison, WI, USA; 1:40,000); rabbit polyclonal pab27576, directed against the C-terminus of APP (generous gift from Dr Multhaup, Freie University, Berlin, 1:250). The quantification was performed as described above; for CTF, the duplet was quantified. For the calibration curve, a recombinant CTF (C100 protein purified recombinantly from E.coli) was used (generous gift from Dr Multhaup, Freie University, Berlin). The C100 had a C-terminal Hexa-His tag and a N-terminal start-methionine. The calibration curve appeared to be linear (r^2=0.99) in the range between 7 and 0.43ng per lane.

BACE-1, NFkB and IkB The hippocampi were briefly sonicated in 100ul of Cell Extraction Buffer (provided with the BACE activity kit, see below), incubated on ice for 15 mins and centrifuged at 10.000rpm for 5 min at 4˚C. Protein content was quantified as described above and 100ug of total protein were separated using 10% SDS-polyacrylamyde gels and transferred to PVDF membrane for subsequent Western blotting. Membranes were blocked with 5% non-fat milk in TBS-T and then incubated with the primary antibody overnight at 4˚C. Primary antibodies used were rabbit polyclonal BACE-1 (PA1-757, Meridian Road Rockford, IL, USA; 1:250); NFkB (p65, 1:1000) and IkB (1:2000, both from Santa Cruz Biotechnology Inc., CA, USA), and actin (Abcam, Cambridge, MA,

USA; 1:20,000). Band quantification was performed as described above. All experiments were performed in triplicate.

Immunohistochemistry and quantification of Iba-1 immunoreactive cells

Bright field immunohistochemistry. Free floating immunohistochemical staining was performed as previously described (Hu et al., 2003) (Côté et al., 1993) using the rabbit polyclonal primary antibody Iba-1 (ionized calcium-binding adaptor molecule 1, Wako Chemicals USA, Inc., Richmond, VA, 1:10,000). For the detection, a biotinylated goat-anti rabbit secondary antibody was applied followed by amplification with the avidin-biotin complex (ABC elite kit, both from Vector laboratories Inc., Burlingame, California, USA). All stainings were developed with 0.06% DAB (Sigma-Aldrich Canada, Oakville, ON) and 0.01% H_2O_2.

Quantification of Iba-1-immunoreactive (ir) cell soma size and density The staining and the quantification were performed according to published protocols (Ferretti et al., 2011a) . All the stainings were performed simultaneously. Three sections per animal were chosen in the area corresponding to bregma -2.9 (Franklin and Paxinos, 1997), stained with Iba-1 and mounted on gelatinated slides, after which the slides were coded. Digital images were acquired on an Axioplan 2 Imaging microscope (Zeiss, Toronto, ON, Canada), equipped with an AxioCam HRc digital camera (Zeiss), using AxioVision 4 Imaging program (Zeiss). The micrographs were taken with a 63x Zeiss plan-Apochromat oil immersion objective in the CA1 area of the hippocampus (8 micrographs per section, 3 sections per animal for a total of 24 micrographs per animal,) and 4 micrographs were taken in the lateral posterior thalamic nucleus as control (total of 12 micrographs per animal). The images were imported into the MCID 5 Image Analysis Software (Imaging Research Inc., St. Catherine's, ON, Canada) as TIFF files and transformed (with the 'target accent' function) to allow optimal detection by the program. Cell bodies of all the cells in the focal plane of each micrograph were manually out-lined by a blinded observer, and target size, number of target elements; intensity and form factor were measured in an automatic fashion.

After quantification, the slides were un-coded and the data analyzed. The density data are expressed as number of Iba1-ir cells per field (38.206μm square). MTF was blind on the nature of the material at all stages of the quantification.

ELISA for human Aβ. Human Aβ40 and Aβ42 levels were quantified from cortical homogenates using a commercially available ELISA kit (Invitrogen, Carlsbad, CA, USA, distributed by Medicorp, Montreal, Canada). Each sample was mixed in an equal volume of Guanidine-HCl (to a final concentration of 5M Guanidine) and incubated for 3h at RT. The resulting samples were further diluted 1:10 in the provided dilution buffer (to a final concentration of 0.5M Guanidine) and tested in duplicate. The amount of Aβ was extrapolated from a calibration curve of synthetic human Aβ 40 and 42 using the curve fitting function of Graph-Pad Prism 5 software (La Jolla, CA, USA). The calibration curve was prepared according to the manufacturer's instructions in the presence of 0.5M Guanidine to ensure comparability with experimental samples. The data were normalized on total ug of protein content per each sample. Controls included: omission of samples (background), chromogenic substrate alone (blank) and Non Tg samples. No signal was detectable in Non Tg samples (data not shown).

BACE-1 activity. We used two commercially available kits for the measurement of BACE-1 enzymatic activity from biological sample (from R&D, Minneapolis, MN and Abcam, Cambridge, MA, USA). The assay was conducted according to manufacturer's instructions. Briefly, hippocampal samples (see above for preparation details) were diluted to a final concentration of 2.5ug/50ul. 2.5ug of total protein from the resultant samples were loaded into a black 96-well microplate and the fluorogenic substrate was added in the dark. The substrate is conjugated to the EDANS and DABCYL reporter molecules. Cleavage of the peptide by β-secretase separates EDANS and DABCYL allowing for the release of a fluorescent signal. The reaction was incubated at 37˚C for one hour in the dark, and the signal was measured using a Fluostar Optima (from BMG Labtech GmbH, Ortenberg, Germany) with a 355nm excitation and 520nm emission

wavelengths. Each sample was run in duplicate; the assay was repeated twice and results from the two experiments were pooled after normalization on Non Tg Placebo values. Negative controls included: omission of the fluorogenic peptide (background fluorescence of the tissue), omission of the sample (blank) and addition of BACE-1 inhibitor to the samples (provided by the kit). 2.5ug of recombinant human peptide (rhBACE-1, R&D, Minneapolis, MN, USA) was run as a positive control.

Post natal day 7 samples for BACE-1 Western blot. Two pups from postnatal day 7 were euthanized and the brains carefully removed; one brain was immediately homogenized as described above for regular Western blotting, while the cortex of the second brain was used to establish a mixed glial culture according to standard protocols with minor modifications (Kaushal and Schlichter, 2008). Briefly, the cells were disaggregated using papain (Worthington, Vassar Ave., Lakewood, NJ, USA) and mild mechanical stress with a polished Pasteur pipette. The cells were then re-suspended in DMEM containing 10% FBS, and Penicillin-Streptomycin and cultured in a 10cm petri dish. The medium was changed every 3-4 days, and after 2 weeks the cells were collected by scraping on ice, sonicated and processed for Western blotting.

Data analysis. All data were analyzed using the Graph-Pad Prism 5 software (La Jolla, CA, USA). Multiple groups comparison was done by 1-Way-ANOVA followed by Tukey post-hoc test. Kruskall-Wallis was used for not-normally distributed data. When necessary, the interaction between genotype and effect of the drug was studied using 2-Way-ANOVA. Correlation studies were done using the Spearman's test for not normal distributed data. Significance was set at $p<0.05$. All data are presented as mean ± S.E.M.

4.4 Results

4.4.1 Minocycline corrects neuroinflammation in pre-plaque McGill-Thy1-APP Tg mice

Young, pre-plaque 2 month old Tg mice (n=7) received 50 mg/Kg/day of minocycline via daily intraperitoneal (ip) injection for a month. Control groups included vehicle treated Tg mice (Tg Placebo, n=7), vehicle treated Non Tg, age-matched littermates (Non Tg Placebo, n=8) and Non Tg, age-matched littermates treated with minocycline (Non Tg Mino, n=8). After three weeks of the treatment, one Non Tg mouse treated with minocycline had to be euthanized for a swollen intestine and two Tg mice in the minocycline group died. No mouse died in the placebo groups. At the end of the treatment the animals (n=8 Non Tg Placebo, n=7 Non Tg Mino and Tg Placebo; n=5 Tg Mino) were sacrificed by transcardial perfusion and the brains processed for biochemical and immunohistochemical analysis.

We first assessed the ability of minocycline to reduce the CNS pro-inflammatory process, by measuring levels of iNOS, COX-2 and IL-1β via Western blotting (Fig 4-1A-C). Confirming and further expanding our previous sets of experiments (Ferretti et al., 2011a), we found that iNOS and COX-2 were significantly up-regulated in cortical homogenates from Tg mice compared to Non Tg littermates (Fig.4-1A,B, $p<0.05$). IL-1β showed a trend towards up-regulation which did not reach significance (Fig 4-1C). Minocycline treatment was able to inhibit neuroinflammation, as the levels of iNOS, IL-1β and COX-2 of Tg mice treated were significantly different from Tg Placebo ($p<0.01$) but not significantly different from Non Tg controls.

Figure 4-1. Minocycline corrects neuroinflammation in young, pre-plaque Tg mice.

A-C Representative Western blots for iNOS, COX-2, IL-1β (typical markers of microglial and neuronal activation) in control, Non Tg Placebo mice (Non Tg), Tg Placebo mice (Tg Pl) and Tg mice treated with minocycline (Tg Mino). Note the significant up-regulation of iNOS and COX-2 in Tg Placebo compared to Non Tg Placebo. Minocycline restored iNOS and COX-2 to levels similar to those of Non Tg Placebo, and significantly reduced the levels of IL-1β (*=$p<0.05$, **=$p<0.01$, 1-way-ANOVA with Tukey post-hoc test). See main text for the values of Non Tg treated with minocycline.

D Schematic illustrating the sampling of images in the CA1 area of the hippocampus and from the lateral posterior thalamic nucleus as utilized for the morphological study in E-G.

E Representative micrographs illustrating the ir of Iba-1 in microglial cells in the hippocampus of the Non Tg Placebo (Non Tg), Tg Placebo (Tg Pl) and Tg treated with minocycline (Tg Mino) mice. Note the altered morphology of the microglial cells in Tg Placebo compared to Non Tg placebo: enlargement of the soma size and polarization and thickening of the processing are indicative of microglial activation in Tg placebo. Minocycline treatment resulted in correction of microglial soma size (note the small, roundish morphology), with some residual thickening of processes and increase in spines.

F Representative micrographs illustrating the ir of Iba-1 in microglial cells in the lateral posterior thalamic nucleus of the Non Tg Placebo, Tg Placebo and Tg treated with minocycline. The cells were notably smaller and less ramified than in the hippocampus, but no differences in the morphology could be observed between experimental groups. Scale bar for E and F: 20um.

G,**H** Quantification of cell soma size and density of microglial cells from hippocampus (**G**) and thalamus (**H**) in Non Tg placebo, Tg Placebo and Tg treated with minocycline. Note that minocycline treatment (Tg Mino) resulted in significant reduction of microglial cells soma size compared to Tg placebo (Tg Pl) in hippocampus (**G**). No significant differences were observed in the soma size of cells in the thalamus (**H**). No significant changes in microglial cell density were observed across experimental groups in any area. (*=$p<0.05$, 1-way-ANOVA followed by Tukey post-hoc test).

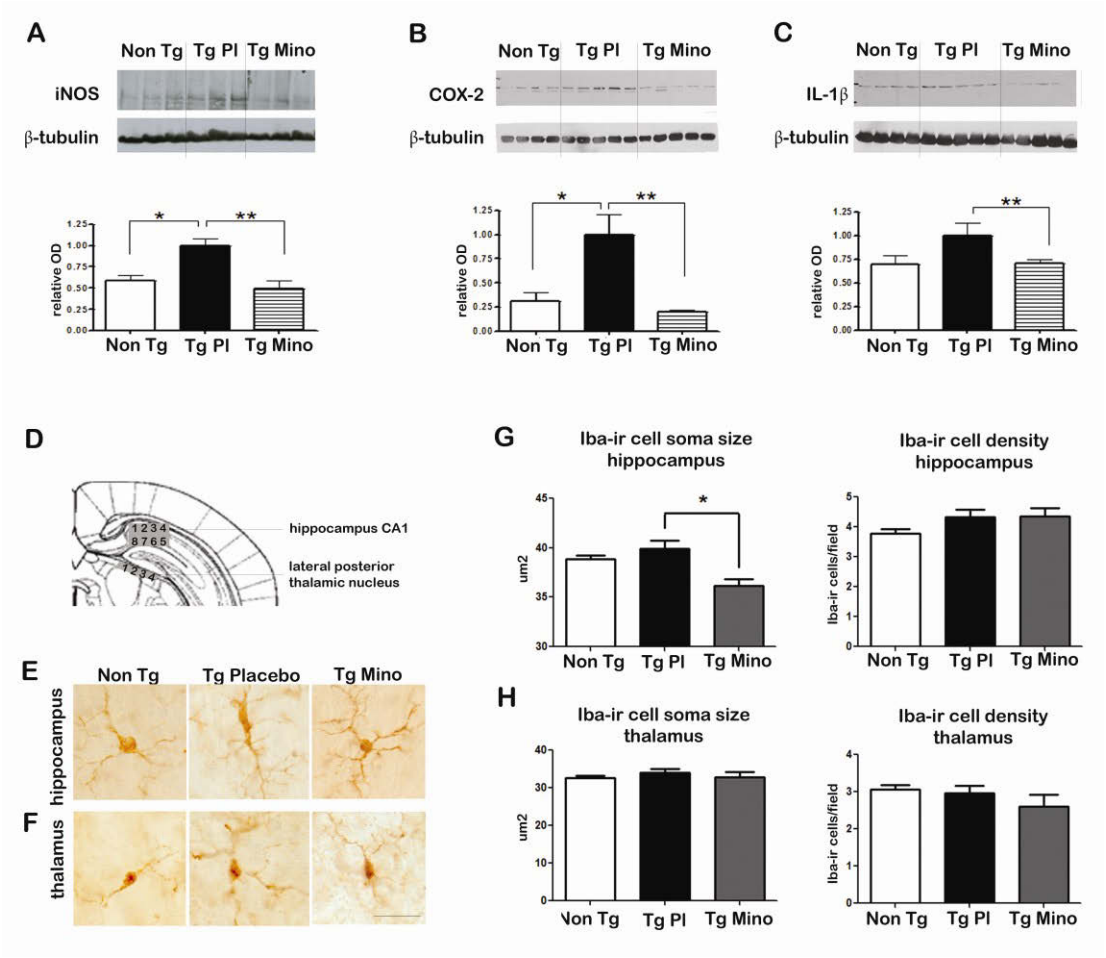

Figure 4-1. Minocycline corrects neuroinflammation in young, pre-plaque Tg mice.

Minocycline treatment did not significantly alter the levels of these markers in Non Tg animals. The following fold-increases compared to Non Tg Placebo were observed in Non Tg Mino: iNOS 1.49±0.19; IL-1β 0.99±0.04; COX-2 1.31±0.15. None of these reached significance ($p=0.08$, 0.95 and 0.31, respectively, Student's t-test), even though a trend was observed for the iNOS levels.

To confirm the biochemical data, we used a morphological approach to study the activation state of microglial cells. Iba-1, a structural marker for microglia (Ahmed et al., 2007), was used to stain brain sections from Tg and Non Tg animals. As expected, most microglial cells in Non Tg placebo mice displayed a resting morphology, with a small soma size and symmetrical, fine arborization of the processes (Fig. 4-1E). As previously reported, we observed an altered morphology of the microglial cells from the hippocampus of Tg Placebo animals. The cells displayed an enlarged soma size, with notable polarization and thickening of the processes, all indicative of microglial activation. In contrast, microglial cells from minocycline-treated Tg animals displayed a small, roundish soma, similar to Non Tg placebo. Interestingly, we noticed an increase in the complexity of microglia ramification following minocycline treatment in Tg animals: the number of processes emanating from the cells appeared to be elevated and they were often thick and decorated by spines. Microglial soma size and density was measured with the assistance of the MCID 5 Image Analysis System, according to published protocols (Ferretti et al., 2011a). Reinforcing our biochemical results, we observed a significant down-regulation of microglial cells size in the hippocampus of Tg animals treated with minocycline (Fig. 4-1G, $p<0.05$) indicative of a reduced activation state. No differences in the density of microglial cells were observed, suggesting that the effect of minocycline was not mediated by a reduction in microglial proliferation or infiltration of peripheral monocytes.

No significant differences were found between the microglial soma size in Non Tg Placebo (38.86 $\mu m^2 \pm 0.37$) and Non Tg Mino (39.64 $\mu m^2 \pm 1.16$).

The same analysis was performed in the lateral posterior thalamic nucleus in the same sections (Fig. 4-1F) as a control area which is spared by the amyloid pathology at this age (Ferretti et al., 2011b). In agreement with previous reports (Lawson et al., 1990) we noticed that microglial cells in this area were smaller, less ramified and less dense than in the hippocampus. The analysis of microglial cells soma size and density in the thalamus revealed no differences between groups (Fig 1H). This result indicated that the effect of minocycline was specific to the hippocampus, an area burdened with intracellular Aβ-ir material.

4.4.2 Minocycline treatment affects APP metabolism

Next, we investigated the effects of the anti-inflammatory treatment on APP metabolism and Aβ levels (Fig 4-2). For these studies we used cortical homogenates from Tg Placebo and Tg animals treated with minocycline.

We first applied the commercially available monoclonal antibody 6E10 which recognizes the residues 1-16 of human Aβ. This Western blot analysis revealed several ir bands in Tg Placebo animals between 10 kDa and 50 kDa. These bands did not appear in the Non Tg homogenates, and are likely to be oligomeric forms of Aβ. Minocycline treatment resulted in the clearance of most of the 6E10-ir bands; in particular, a strong (-70%), significant down-regulation of a 12 kDa band (Fig 4-2A, B) was observed. We noticed that the levels of the 12 kDa band strongly correlated with the levels of iNOS across samples (Fig. 4-2C, $r=0.81$, $p=0.004$, Spearman's correlation analysis): where placebo Tg mice displayed the highest values for both 12 kDa-6E10 and iNOS, while all but one Tg mice treated with minocycline displayed reduced levels of both markers.

Figure 4-2. Minocycline effects on APP metabolism.

Cortical brain homogenates from Tg animals treated with vehicle (Tg Pl) or Minocycline (Tg Mino) were subjected to Western blotting and ELISA to determine the levels of Aβ species, full length APP (flAPP) and CTF.

A. Representative WB of cortical (ctx) homogenates from Tg animals treated with vehicle or with minocycline, using 6E10 antibody. Note the strong down-regulation of the ca 12 kDa-ir band (asterisk) which likely represents a mixture of β-CTF of APP and Aβ trimers.

B. Densitometric analysis of the 12 kDa band detected with 6E10 (normalized on neuronal-specific β-tubulin). Minocycline treated animals showed significantly lower levels compared with placebo (**, $p<0.01$, Student's t-test).

C. Correlation analysis of the levels of the 12 kDa band ir to 6E10 and the levels of iNOS per each sample. The correlation was found to be highly significant (Tg Pl: black dots; Tg Mino: grey dots; $r=0.81$; $p=0.004$, Spearman's correlation).

D. Representative WB of ctx homogenates from placebo and minocycline treated animals using the pab27576 antibody, specific to the C terminus of APP. The pab27576 detects two prominent bands in the blots: a high molecular weight band (ca 100 kDa), correspondent to full length APP (flAPP) and a faster band (ca 12 kDa) corresponded to the CTF fragments. A calibration curve using known amounts of recombinant C100 (rCTF) was run in the same gel for quantification purposes (rCTF ran slightly slower due to the presence of a C-terminal hexa-His tag).

E. Quantification of flAPP and CTF levels in ctx homogenates from Tg Placebo and Tg treated animals. FlAPP relative optical density (OD) values were normalized on neuronal-specific β-tubulin. Note the significant down-regulation of flAPP in Tg animals treated with minocycline compared with Tg Placebo. CTF absolute levels were extrapolated from the calibration curve of rCTF. There was a not-significant trend towards reduction following minocycline treatment. Normalization of the relative OD of CTF over the relative OD of flAPP confirmed a non-significant trend towards a reduction (ratio CTF/flAPP). All data were analyzed with Student's t-test (**, $p<0.01$).

F. Quantification of human Aβ levels in cortical homogenates from Tg Placebo and Tg Mino using ELISA. The treatment reduced both Aβ40 and Aβ42, but the effect did not reach significance ($p=0.09$ and 0.08, respectively, Student's t-test; see main text for exact values).

G. The ratios between Aβ (pg/mg tot protein) and CTF (ng/mg tot protein) were calculated per each sample and compared across groups. We observed 24.29±6.43 pg of Aβ42 and 15.14±3.08 pg of Aβ40 per each ng of CTF. Minocycline treatment resulted in a reduction of the ratio (which did not reach significance), suggesting that less Aβ was produced per each CTF molecule after the treatment with minocycline.

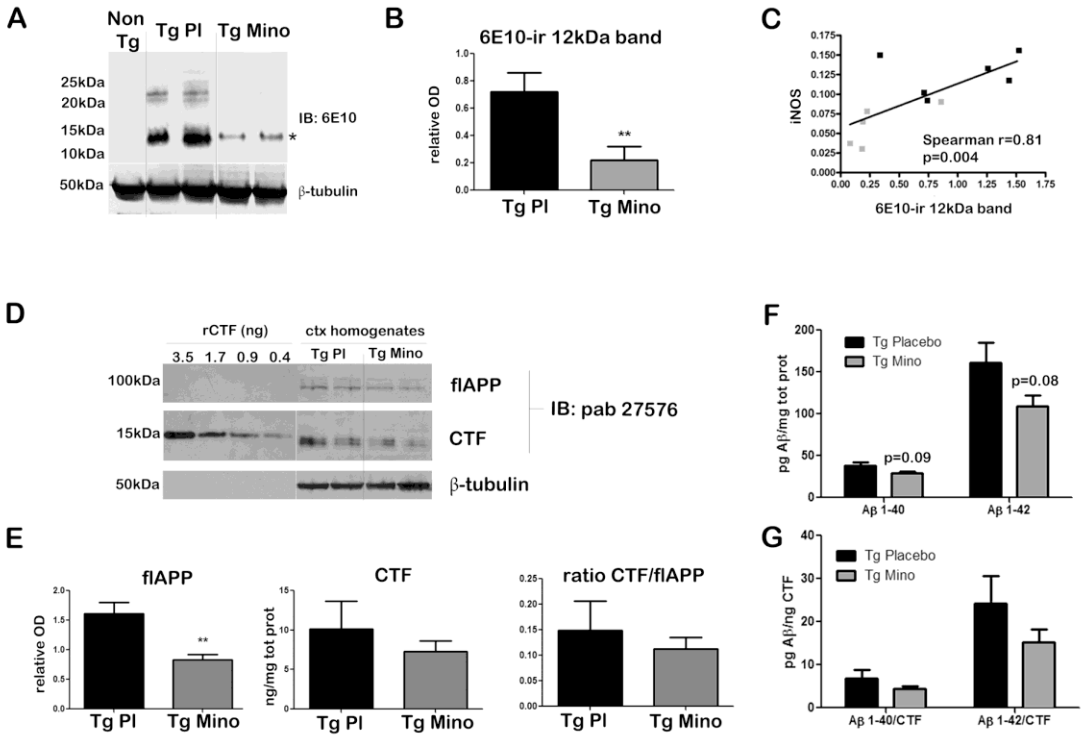

Figure 4-2. Minocycline effects on APP metabolism.

These results would suggest a link between APP metabolism and inflammation.

The 12 kDa band could not be definitively identified: in fact, 6E10 is directed against the N-terminus residues of Aβ and thus can recognize both β-CTF fragments of APP (that migrate around 12 kDa) and Aβ species (such as Aβ-trimers). To elucidate the nature of the 12 kDa band we therefore performed additional analysis on the homogenates that enabled us to specifically quantify absolute values of CTF fragments and human Aβ.

To detect CTF fragments without cross-reactivity with Aβ we used a specific antibody directed against the C terminus of the APP holoprotein called pab27576 (generous gift from Dr Multhaup, Freie University, Berlin). This serum recognized full length APP (flAPP, which appeared at 100 kDa) and CTFs (which migrated around 12 kDa). In order to quantify absolute levels of CTF in the sample, in the same gels we included a calibration curve of recombinant CTF (rCTF) (Fig 4-2D). Following this analysis, we quantified the levels of flAPP and CTF fragments (2E).

We observed a significant down-regulation of flAPP relative levels (Fig 4-2E, $p<0.01$) following minocycline treatment. The down-regulation of flAPP was confirmed using other APP-specific antibodies such as 6E10 and 22C11 (data not shown).

Extrapolation of the levels of CTF in the brain homogenates from the calibration curve revealed that Tg placebo animals had 10.13 ± 3.50 ng of CTF per mg of total protein. Following minocycline treatment, CTF fragments (both relative and absolute levels) appeared reduced (7.27 ± 1.34 ng/mg) but not reaching significance. Importantly, the reduction in CTF following minocycline treatment was not completely explained by the reduction in APP levels.

In fact, when we normalized the CTF levels on flAPP levels, the trend towards a reduction was still present (Fig 4-2E, ratio CTF/flAPP). These results suggested that the 12 kDa band recognized by 6E10 was not exclusively constituted by CTF, as the values detected by the two antibodies were not perfectly correspondent. We therefore sought to quantify Aβ levels in the same samples.

To obtain a direct quantitative measurement of the Aβ levels we performed an ELISA analysis of soluble human Aβ40 and Aβ42. The study revealed the presence of 160.9 ± 24.37 pg/mg tot protein of Aβ42 and 38.28 ± 4.25 pg/mg tot protein of Aβ40 in Tg placebo animals. Following minocycline treatment we measured 109.0 ± 13.43 pg/mg tot protein of Aβ42 and 28.76 ± 2.42 pg/mg of Aβ40. Though a strong trend was observed towards reduction (p=0.08 and 0.09, respectively), this did not reach significance (Fig. 4-2 F).

We then calculated the ratio between Aβ (pg/mg total protein) and CTF (ng/mg total protein) in each sample: this analysis revealed that in the Tg placebo animals there were 24.29±6.43pg of Aβ42 and 6.76±2.15pg of Aβ40 per each ng of CTF. A trend towards a decrease in animals treated with minocycline was found (15.14±3.08pg of Aβ42 and 4.36±0.58pg of Aβ40 per each ng of CTF), indicating fewer molecules of Aβ per molecule of CTF following minocycline treatment (Fig. 4-2G).

4.4.3 Minocycline inhibits BACE-1 activity in young, pre-plaque Tg mice

The strong reduction of the 12 kDa band immunoreactive with 6E10 and the altered CTF/flAPP ratio observed following minocycline treatment suggested that the β cleavage of APP could be affected by the anti-inflammatory drug.

Therefore, we proceeded to measure BACE-1 activity in the hippocampal samples, using a well characterized fluorometric assay (Fig 4-3A,B).

Figure 4-3. Minocycline inhibits BACE-1 in young, pre-plaque Tg mice.

A. BACE-1 activity was quantified from hippocampal homogenates using a well characterized fluorometric assay. Fluorescent units (FU) data were normalized on Non Tg Placebo values. Note the significant up-regulation of BACE-1 activity in Tg Placebo compared to Non Tg Placebo (***, $p<0.001$), which was corrected following minocycline treatment (*, $p<0.05$ compared to Tg Placebo). The data were analyzed with the 1-way-ANOVA followed by Tukey's post-hoc test.

B Specificity of the BACE-1 fluorometric assay: note how the fluorescent units (FU) detected from brain homogenates (of a Non Tg Placebo animal) are 7 times higher than the background (bg). The signal was completely abolished by co-incubation with a specific BACE inhibitor (BACEi), and was in the range of activity of a recombinant human BACE protein (rhBACE, 2.5ug).

C. Specificity of the polyclonal BACE-1 antibody used for Western blotting: the specific band (close to 75 kDa) appeared in brain extracts from post natal day 7 (pnd 7) and from adult cortex (ctx), while it was hardly detectable in cerebellum (cereb) sample and was not detectable in a glial preparation from pups.

D. Representative Western blots of BACE-1, NFkB and IkB from the same hippocampal samples used for the enzymatic assay (ns=non-specific band recognized by the polyclonal antibody directed against NFkB).

E. Quantification data of BACE-1, NFkB and IkB from hippocampal homogenates. Band intensity was quantified via densitometry and the values were normalized on the neuronal specific- β-tubulin content. Note the significant up-regulation of BACE-1 levels in Tg Placebo, which was corrected by the minocycline treatment ($p<0.05$). The levels of NFkB and BACE-1 in each sample strongly correlated ($p<0.001$, Spearman's correlation analysis). Given the not-normal distribution of the data, the values were analyzed with Kruskal Wallis test and Spearman's correlation analysis.

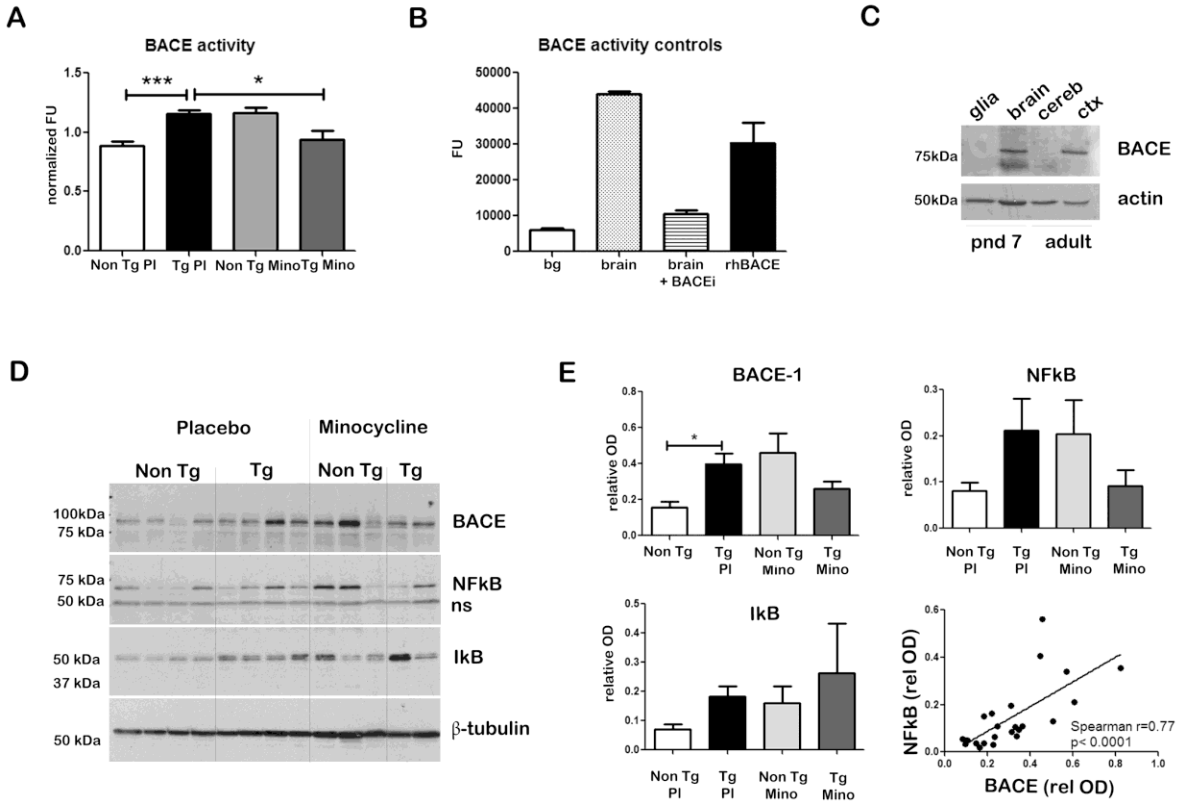

Figure 4-3. Minocycline inhibits BACE-1 in young, pre-plaque Tg mice.

This kit allowed a specific and robust detection of BACE-1 from brain homogenates: the fluorescent signal detected in brain homogenates was 7 times higher that background, and was reduced to background levels by co-incubation with the kit BACE inhibitor (Fig 4-3B).

With this approach we found that Tg animals displayed significantly increased BACE-1 activity compared to Non Tg ($p<0.001$). Supporting our biochemical findings on APP processing, we found that minocycline was able to reduce BACE-1 activity in Tg animals, and blunted it to levels that were similar to Non Tg ($p<0.05$ Tg placebo vs. Tg Mino). On the other hand, Non Tg animals receiving minocycline displayed a strong up-regulation of BACE-1 activity; 2-way-ANOVA revealed that there was a strong interaction between the genotype and the treatment ($p<0.001$), suggesting that minocycline exerted differential effects according to the genotype.

To assess whether the effect observed was due to a pure enzymatic inhibition or to reduction in the protein content we used Western blotting to quantify BACE-1 levels in the same samples (Fig 4-3D). Even though this assay appeared to be less sensitive than the enzymatic activity assay, we found a significant up-regulation of BACE-1 protein levels in Tg animals compared to Non Tg (Fig. 4-3E, $p<0.05$). Minocycline treated animals had intermediate levels of BACE-1, which were not significantly different from the Tg placebo nor from the Non Tg levels. Confirming our activity data, Non Tg treated with minocycline displayed elevated levels of BACE-1 (interaction between genotype and treatment: $p<0.01$, 2-way-ANOVA). The band observed was most likely specific, as it did not appear in glial cultures from mice pups and was barely detectable in cerebellum (4-3C).

Taken together, these results demonstrated that Tg animals, even prior to plaque deposition, showed increased levels and activity of BACE-1. Such up-

regulation is likely to be related to an inflammatory process, as minocycline could significantly reduce BACE-1 activity and corrected BACE-1 levels.

Since BACE-1 and many inflammatory mediators such as iNOS, COX-2 and IL-1β are known to be under the transcriptional control of NFkB (Baeuerle and Henkel, 1994) (Kaltschmidt et al., 2005), we measured the levels of NFkB and its inhibitor IkB in the same samples (Fig.4-3D). We observed elevated levels of NFkB and IkB in the Tg Placebo animals compared with Non Tg Placebo, consistent with pro-inflammatory condition. Treatment with minocycline in Tg animals resulted in lower levels of NFkB and higher levels of IkB. Minocycline-treated Non Tg showed increased NFkB and IkB. None of these changes reached significance, but we found a very strong correlation between BACE-1 levels and NFkB levels (Fig. 4-1E, Spearman's correlation analysis, $p<0.001$). These findings suggested that BACE-1 levels might be under the direct or indirect control of NFkB.

4.5 Discussion

Early microglial activation and its inhibition with minocycline. In this study we confirmed and extended our past work in young, pre-plaque Tg mice on the occurrence of a pro-inflammatory process independent of plaque deposition in AD-like amyloid pathology. The occurrence of an early, pre-plaque neuroinflammatory response in animal models of AD has been hinted by other studies (Abbas et al., 2002; Heneka et al., 2005a; Murphy, Jr. et al., 2000; Janelsins et al., 2005); however, the features and pathological role of this process still need to be elucidated.

In agreement with our previous observations (Ferretti et al., 2011a) here we gathered biochemical (fig 1A-C) and morphological evidence (Fig 4-1D-H) both indicating the presence of a microglial activation in the CNS of pre-plaque mice.

In contrast with our previous study, in this cohort we observed a slight increase in the density of microglial cells in CA1 area of the hippocampus, in the absence of a significant increase in microglial soma size. The cohort used in this study might therefore represent a slightly more advanced stage of the pathology than the one previously observed, in which proliferation of microglial and-or invasion of monocytes had been already initiated.

Microglial activation might be amongst the earliest events in AD and a better understanding of its mechanism could yield to the identification of potential diagnostic biomarkers as well as new pharmacological targets for AD prevention (Hoozemans et al., 2006). In the present studies we therefore aimed at exploring the nature of the interaction between activated glia and neurons in the earliest stages of the AD pathology.

To investigate the pathological participation of such pro-inflammatory process in the AD-like amyloid pathology we chose to administer the tetracyclic derivative minocycline. In addition to its antimicrobial activities, the drug easily crosses the blood brain barrier and has been shown to be beneficial in several CNS neuropathological conditions and in neurodegeneration (for a review: Griffin et al., 2010). Minocycline appears to exert its action through a plethora of mechanisms including inhibition of key inflammatory enzymes (such as iNOS, MMP9 and 5LO), blocking caspase-dependent and independent apoptosis and demonstrating anti-oxidant effects (for a review: Jordan et al., 2007).

As expected, minocycline was indeed effective in reducing inflammation, as COX-2, iNOS and IL-1β levels were all found to be down-regulated in Tg animals treated with minocycline compared to placebo (Fig 4-1A-C). Following minocycline treatment, microglial soma size of hippocampal cells appeared to be significantly reduced (Fig 4-1E,G), along with an increase in the complexity of microglial arborization (as illustrated in the representative micrograph in E). Both biochemical and morphological findings suggested decreased pro-inflammatory

activity. Interestingly, this effect was specific to the hippocampus, a region burdened with intracellular Aβ-oligomers (Ferretti et al., 2011b). In fact, microglial cells of the thalamus, an area largely devoid of Aβ material at this early age, appeared to be not affected by the treatment (Fig 4-1F,H). This result indicates that minocycline specifically interfered with a pathological inflammatory process dependent on intracellular Aβ accumulation.

Minocycline effects on the amyloid pathology. Having assessed the ability of minocycline to inhibit the pre-plaque inflammatory process, we set ourselves to study the consequences of such anti-inflammatory treatment on the intracellular, pre-plaque phase of the amyloid pathology. This is an issue of high relevance as pre-plaque or even early plaque stages of the amyloid pathology in Tg models likely mimic pre-clinical stage of AD (Ashe and Zahs, 2010).

Previous reports have studied the effect of minocycline on the full-blown amyloid pathology in APP Tg mice (Choi et al., 2007; Fan et al., 2007), where this drug appeared to improve the neuropathology and behavioural deficits. The only preventative treatment present in the literature showed a beneficial effect of minocycline in pre-plaque APP23 Tg mice, but the animals were sacrificed after the onset of plaque pathology (Seabrook et al., 2006). Our study differed from the above mentioned and cannot be compared to them, in that the treatment was started and finished when the animals were devoid of plaques. Therefore, instead of plaque number (the main end-point reported in previous studies) we focused our investigation on the cerebral levels of APP, APP related products and soluble Aβ following minocycline treatment. Soluble levels of Aβ are particularly important indicators of the disease state, as their amounts were shown to correlate with the degree of dementia in patients (McLean et al., 1999). On the other hand, it is well established that the amyloid burden does not correlate with the severity of the disease (Terry et al., 1991; Gomez-Isla et al., 1997).

In this regard we noticed that, at this early time point, inhibition of inflammation correlated with the down-regulation of APP and APP-ir products (Fig. 4-2).

The most significant effect we observed was the reduction of a 12 kDa band recognized with the monoclonal antibody 6E10. This band co-migrates with trimers of synthetic Aβ (MTF, unpublished observation) and is considered by some authors as oligomeric Aβ (McLaurin et al., 2006; Lesne et al., 2006). However, the epitope recognized by 6E10 is shared by the C-Terminus fragments of the amyloidogenic pathway (β-CTF) and 6E10 is often used to detect β CTF from cell lysates and homogenates (Sastre, 2010). The fact that CTFs migrate around 12kDa, as Aβ trimers do, complicated the interpretation of the band. To clarify the nature of this material we sought to specifically quantify CTF fragments and human Aβ in the same samples. This analysis also allowed us to determine the relative abundance of the two species in each brain.

Western blots using a specific antibody directed against the C terminus of full length APP (the pab 27576) revealed a reduction in the CTF content, which did not reach significance (Fig 4-2D,E). Even though the band recognized by pab27576 perfectly overlapped with the band seen with 6E10, the results did not fully match our analysis with 6E10. It is possible that the discrepancy is due to different specificity of the antibodies. Alternatively, some CTF material from the non-amyloidogenic pathway (αCTF which can be detected by pab 27576 but not by 6E10) might have affected our quantification. On the other hand, we considered the possibility that the 12 kDa band is constituted mostly of Aβ species (trimers) and performed a highly sensitive ELISA assay for human Aβ40 and Aβ42. Again, our analysis of soluble Aβ material did reveal some degree of reduction following minocycline treatment, but the high variability resulted in no statistical significance (Fig. 4-2F). Hence, the pattern observed with 6E10 was not fully reproduced by neither that of CTF nor Aβ.

While further investigations are needed to clarify this point, we favor the hypothesis that the 12 kDa band represents a mixture of β-CTF and Aβ-oligomers;

in this view, minocycline treatment resulted in the reduction of both species, which together reached significance.

The simultaneous presence of Aβ and APP related products in early, pre-plaque stages of the disease in Tg models of AD is a highly controversial issue (Wirths et al., 2001; Rosario et al., 2006; Philipson et al., 2009; Aho et al., 2010; Winton et al., 2011). In particular, their relative abundance and their specific contribution to the neuropathology have not been clarified. We therefore took advantage of the data set presented here to explore the relative abundance of soluble Aβ and CTF, and the effect of an anti-inflammatory treatment on their ratio. To do so we compared the absolute levels of CTF (extrapolated from a recombinant CTF calibration curve) and Aβ (measured via ELISA) from each sample. In Tg Placebo animals we observed an average of 6.76pg Aβ40/ng of CTF and 24pg of Aβ42/ng of CTF. In other words (calculating the ratio between pg of CTF and pg of Aβ in each sample), this indicated an average of 231 pg of CTF per each pg of Aβ40 and 56 pg of CTF per each pg of Aβ42 in the placebo. In terms of molar ratio, it appears that McGill-Thy1-APP mice harbor about 84 molecules of CTF per each molecule of Aβ 40 and about 24 molecules of CTF per each mole of Aβ 42. It is therefore very likely that, while the species co-exist, β-CTF fragments represent the vast majority of the material seen with 6E10 (as suggested by McAlpine et al., 2009).

Interestingly, minocycline treatment appeared to reduce the ratio Aβ /CTF, suggesting that fewer molecules of Aβ were generated per each molecule of CTF. Taken together, our results indicate that minocycline affected APP metabolism at multiple levels: reducing APP (Fig 4-2D,E), inhibiting β cleavage (Fig 4-2A and E) and possibly affecting gamma secretase and or Aβ clearance (as suggested by the altered ratio of CTF with Aβ levels).

Minocycline effects on BACE-1.To further elucidate the effect of minocycline on APP processing we have studied the levels and activity of BACE-1, the most important β -site cleaving enzyme in the brain (for a review: Vassar, 2004).

Our analysis revealed that BACE-1 levels and activity were up-regulated in the McGill-Thy1-APP Tg model, in agreement with reports from sporadic AD (Fukumoto et al., 2002; Yang et al., 2003; Li et al., 2004; Harada et al., 2006) and Tg models (Heneka et al., 2005a; Zhao et al., 2007; O'Connor et al., 2008). As described in young V717V Tg mice (Heneka et al., 2005a), BACE-1 levels and activity were up-regulated in McGill-Thy1-APP mice prior to plaque deposition. Therefore, deregulation of APP processing might be an early event in the progression of the AD-like amyloid pathology.

Since the anti-inflammatory treatment with minocycline was able to correct BACE-1 up-regulation in pre-plaque Tg mice, it is very likely that the early, pro-inflammatory process is responsible for the deregulation of BACE-1. This view is in line with the body of evidence indicating that neuroinflammation has a pivotal role in regulating BACE-1. In fact, several recent studies indicated that BACE-1 behaves as a stress-response protein and its levels are increased by cytokines (Sastre et al., 2003), oxidative stress (Tamagno et al., 2002), ischemia (Wen et al., 2004), hypoxia (Webster et al., 2002) and energy inhibition (Velliquette et al., 2005).

Whether BACE-1 levels are regulated at the transcriptional or translational level is still a matter of debate, as different experimental paradigms led to different set of results (Heneka et al., 2005a; Zhao et al., 2007; O'connor et al., 2008). We could not measure the mRNA levels of BACE due to the incompatibility of this analysis with other biochemical tests. However, we measured the levels of NFkB, a key transcription factor which is known to regulate the expression of several inflammatory markers as well as BACE-1 (Kaltschmidt et al., 2005; Rossner et al., 2006).

Consistent with the notion of a pro-inflammatory state in these brains, we found up-regulated levels of NFkB and its inhibitor, IkB in Tg animals compared to Non Tg placebo (though this change did not reach significance due to the high variability in the groups). Interestingly, NFkB levels were reduced following minocycline treatment, and strongly correlated with BACE-1 levels in each sample. This result suggests that BACE-1 levels are tightly linked to NFkB levels *in vivo*. Supporting this hypothesis, Mullan and collaborators have recently shown that the NFkB inhibitor celastrol is capable of inhibiting BACE and reducing amyloidogenic pathway in a mouse Tg model of AD (Paris et al., 2010). Similarly, reduction in BACE-1 and Aβ levels was found in Tg mice acutely treated with the NSAID ibuprofen (Heneka et al., 2005b); this drug is endowed with multiple COX- independent mechanisms of action, including inhibition of NFkB signaling (Tegeder et al., 2001), PPAR-gamma activation (Sastre et al., 2003) and gamma secretase modulation (Weggen et al., 2001). It is very likely that, like ibuprofen, minocycline exerts its beneficial effects via multiple mechanisms of action.

Based on these results, one could speculate that the inflammation-induced hyperactivity of NFkB is responsible for the increased transcription of BACE-1 in Tg animals. This might represent a possible mechanism of the glia-to-neuron or neuron-to-glia communication in early AD, whereby the activation state of microglia can instruct the processing of APP in neurons. Alternatively, the reduction of inflammatory markers and the reduction in BACE-1 (levels and activity) following minocycline treatment might be parallel, unrelated events sharing the same up-stream events (i.e. inhibition of NFkB in glia and neurons).

Minocycline adverse effects. It is important to note that the application of 50 mg/Kg/day of minocycline i.p. resulted in some toxicity: 1 over 8 mice (12.5%) in the Non Tg group and 2 out of 7 (28%) mice in the Tg group died, while the remaining mice showed signs of liver toxicity and peritoneal irritation. These

adverse effects rendered the animals incompatible with the behavioural testing for learning and memory, such the Morris water maze task. Liver toxicity (Bocker et al., 1991) and peritoneal inflammation (Fagan et al., 2004) are well known side effects of i.p. administration of minocycline which are seldom referred to in numerous experimental published studies. We cannot exclude the possibility that the peripheral toxicity and consequent inflammation could have a role in the CNS effects observed in the treated mice. The occurrence of some inflammation in the Non Tg mino group is supported by the rise in BACE-1 activity and levels and NFkB in this group. Inflammatory markers such as COX-2, iNOS and IL-1β were measured in these animals, and they were found to be not significantly different from Placebo; however, iNOS levels were slightly up-regulated ($p=0.08$). Interestingly, in the Tg mice, where an inflammatory process was already starting to appear, the treatment with minocycline did not induce further CNS inflammation, but rather blunted the inflammation already present in the brain.

One has to conclude that the treatment had opposite effects on Non Tg and Tg animals in terms of modulation of inflammatory markers: it stimulated NFkB signaling in animals that were devoid of inflammation at the beginning of the treatment, but helped resolving an already on-going inflammatory process in the Tg animals. In both Non Tg and Tg animals we did not notice an increase in the density of microglial cells following treatment, ruling out the possibility that the differential effects could be due to infiltration of peripheral monocytes. There are examples in the literature of differential modulation of inflammatory pathways in control versus activated cells: NFkB activation, for instance, appeared to mediate opposite effects on BACE transcription according in the presence or in the absence of stimulants, such as Aβ (Bourne et al., 2007).

While dose and administration route of the drug need to be optimized to avoid adverse effects, our results overall indicate that the use of minocycline can be beneficial in pre-plaque stages of AD-like amyloid pathology.

4.6 Conclusions

This report demonstrated that early, pre-plaque inflammatory process occurring at early stages of the AD-like amyloid pathology can be modulated pharmacologically by the application of minocycline. The down-regulation of inflammatory markers was accompanied by the reduction of APP levels and correction of BACE-1 hyper-activity. Our results would indicate that inflammation has a pivotal role in early stages of the disease instructing APP metabolism. Interfering with inflammation could be a useful therapeutic approach in early, pre-plaque stages of the AD-like amyloid pathology.

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CHAPTER 5

General Discussion

The main objective of this thesis was to elucidate early pathological events which precede plaque deposition in the progression of the AD pathology. We felt that, while the plaque-associated pathology had been extensively studied in humans and animal models, there was a significant lack of knowledge on factors involved in the first stages of the disease. We strongly believed that a better understanding of such early events will help us in identifying early pharmacological targets for AD prevention and-or diagnosis.

Taking advantage of our in-house Tg models of the AD-like amyloid pathology, we have shown that:

1. Pre-plaque phases of the AD-like amyloid pathology already display behavioral deficits and cholinergic alterations (Chapter 2).

2. Aβ aggregation into oligomers is an early event that precedes plaque formation and occurs intraneuronally (Chapter 2,3).

3. Microglial activation can be detected in pre-plaque phases of the AD-like amyloid pathology and is associated with the occurrence of neurons burdened with pathological accumulation of intracellular Aβ-oligomers (Chapter 3,4)

4. Pharmacological reduction of inflammation with minocycline in early, pre-plaque stages can alter the APP synthesis and metabolism (Chapter 4)

The use of Tg animals was based on the assumption that young animals displaying pre-plaque pathology would represent a model of early, pre-clinical stages of AD. In the present chapter we will discuss this assumption and the relevance of our findings in relation to current body of knowledge.

5.1 Tg mice models of AD

5.1.1 Advantages and limitations

The cloning of APP in 1987 (Kang et al., 1987) made it possible to generate Tg animals over-expressing mutated, familial AD-causing forms of APP (for a review: Philipson et al., 2010).

Early Tg models over-expressing APP with FAD mutations successfully proved that APP processing is sufficient to provoke the deposition of plaques and that such plaque-deposition is accompanied by cognitive impairments (Games et al., 1995; Hsiao et al., 1996). These seminal reports represented a convincing experimental proof of concept supporting the amyloid cascade hypothesis (see Introduction).

In the following years, several Tg models were developed by different groups. In general, a more aggressive pathology (earlier onset and higher amyloid burden) could be achieved by using very efficient neuronal promoters (such as Thy1.2, Chishti et al., 2001) and combining APP mutations with PS1 mutations (a strategy first used by Holcomb et al., 1998) up to five mutations (5x FAD Tg model, Oakley et al., 2006).

It is noteworthy that, while all aforementioned APP models successfully reproduced the amyloid plaque pathology (amyloid plaques, dystrophic neurites, loss of cholinergic terminals in cerebral cortex and hippocampus, cognitive deficits), they failed to exhibit convincing NFT. As murine Aβ is not prone to develop plaques, it seems that murine tau is not prone to develop NFT. This is thought to be due to the different isoform expression between human and mice (Goedert et al., 1989). Alternatively, it is conceivable that rodent brains are lacking of factors required to unleash the formation of pair helical filaments in an

environment of pathological Aβ accumulation. Therefore, at the moment, the only models of tauopathy available over-express human tau gene with mutations linked to FTD alone (Lewis et al., 2000; Gotz, 2001) or in combination with APP and PS mutations (Oddo et al., 2003). In this regard, generating Tg rat models (as the McGill-Thy1-R-APP; Leon et al., 2010) might represent a better strategy, given the higher degree of similarity between rat and human tau. Rats in fact possess all the human isoforms, albeit if not in the same proportion (Hanes et al., 2009).

Probably due to lack of NFT pathology, none of the Tg models currently available, even in the more aggressive combinations, develop a dramatic neurodegeneration as seen in AD. It is therefore accepted that Tg mice are incomplete models of AD, but rather models of AD-like amyloid pathology. Despite the apparent limitations, the APP Tg animal models have been largely used in the drug discovery process.

The failure of many clinical trials based on Tg animal studies (Zahs and Ashe, 2010) has generated some skepticism around the validity of Tg models. However, Tg animals offer an invaluable opportunity for basic science and for drug development, once its limitations are recognized and accepted.

Tg models over-expressing APP and or PS are in fact, as mentioned above, not models of AD, but they are models of AD-like amyloid pathology. They are not homologous models (in that the cause of the pathology is not the same in human and mice). They are rather isomorphic models that faithfully mimic the symptoms of the amyloid pathology. In fact, they reproduce the pathophysiology (amyloid plaques, dystrophic neurites, cholinergic loss) and symptomatology (cognitive dysfunction, emotional components) of the disease. Further to it, they respond to drug treatment such as ChEI (for a review: Gotz and Ittner, 2008).

These models are therefore extremely useful tools in the first stages of the drug discovery process, such as in the basic research phase and in target ID confirmation/discovery.

On the other hand, the use of Tg models over-expressing APP and or PS in pre-clinical efficacy studies has to be carefully analyzed and interpreted. In particular, when translating the results from Tg animal work into the clinic, one should considered at least the following points:

1. Effect on tau pathology: the candidate drug should be tested at least on two different models, one of which displaying tauopathy. This approach would allow assessing the interaction between the compound and the NFT pathology.

2. Effect on neurodegeneration: the pathology displayed by Tg mice most likely represents the equivalent of an mild, early AD in human, before overt neurodegeneration has occurred. Therefore, drugs that prove to be beneficial in animal models are more likely to be candidates for preventative studies in human, rather than therapeutic (Ashe and Zahs, 2010).

5.1.2 Young Tg mice as a model of pre-clinical AD

It is becoming increasingly clear that even old Tg mice, with extensive plaque pathology but lacking tau and neuronal loss, represent a relatively early stage of the pathology compared to a human brain. Furthermore, a human AD brain has carried the pathology for decades, at the moment of diagnosis, while mice only live up to two years. In that regard, as it has been recently highlighted by Ashe and collaborators, Tg mice are most likely models of early AD/MCI (Ashe and Zahs, 2010). If aged Tg mice represent early AD/MCI, it is quite natural to accept that young, pre-plaque Tg mice likely model what happens in the human brain decades before the appearance of the first plaque.

This, though, remains an assumption, based on the current impossibility to study such early, pre-plaque events in humans. In fact, in *post-mortem* human

brain material, it is still impossible to predict the progression from NCI to MCI and AD. However, a number of recent studies are starting to indicate that a long pre-symptomatic phase of the pathology does occur in AD.

Several reports have been recently published with the results of long lasting longitudinal studies. While long and expensive, these studies allow correlating *in vivo* parameters (such as MRI brain mass, PET scanning for amyloid, CSF measurements of Aβ/tau) with the eventual AD diagnosis. It is becoming increasingly clear that AD patients present a number of neurological alterations (Sperling et al., 2010) years before the diagnosis. This clearly indicates that the AD pathology starts much earlier than what has been initially thought.

To investigate the pre-symptomatic phase of AD, other groups have taken advantage of the existence of familial AD cases. This condition represents the closest situation to that observed in Tg mice, in which the pathology is driven by genetic factors resulting in over-production of Aβ. Interestingly, and consistent with results with Tg models, cognitive alterations start to appear years before the fully-developed pathology in familial AD (Costa-Baena et al., 2011; Parra et al., 2010; Quiroz et al., 2010).

Finally, young DS patients who display a progressive over-production of Aβ material have been used to study early events in the AD pathology. Importantly, young AD individuals showed accumulation of intracellular Aβ (Mori et al., 2002) and oxidative stress (Nunomura et al., 2000) prior to plaque deposition. The presence of pathological accumulation of Aβ immunoreactive material in such cases would support the notion of an evolution of the Aβ amyloid pathology from intra- to extracellular, as found by us and others in Tg models.

In conclusion, recent evidence supports the idea that AD is characterized by a long, a-symptomatic phase whose features closely resemble the early pathology seen in pre-plaque Tg mice. The development of new imaging techniques and

further studies on familial AD cases should define as to which extent our assumptions regarding the staging of the AD amyloid pathology are correct.

5.2 Intracellular Aβ

5.2.1 Technical considerations

In Chapter 2, using immunohistochemical and biochemical techniques, we showed that Aβ accumulation within pyramidal neurons appears to be one of the earliest events in the progression of the pathology in the McGill-Thy1-APP Tg mice. Even though the concept of the intracellular generation of Aβ is not a matter of debate, there are indeed some technical limitations and possible caveats in our study.

The unequivocal detection of Aβ via IHC is complicated by the fact that most antibodies used (such as 6E10 and McSA1) are directed against the N terminus of Aβ, and can easily cross-react with the holo APP protein and APP-related products. To convince ourselves that the intraneuronal staining observed was actually Aβ material, we performed a number of control experiments and used a panel of antibodies.

We believe that at least part of the material we have observed is *bona fide* Aβ for the following reasons:

1. We dissociated APP-IR from that of McSA1. As illustrated in Fig.2-3, Chapter 2, we have detected a differential pattern of immunoreactivities where the 22C11- IR (APP) appeared diffusely distributed, while that of McSA1-IR (Aβ) was largely associated to granule-like structures in the perinuclear region and in the axon. This pattern has also been described by others (Rosario et al., 2006). Little to no subcellular colocalization was

observed, suggesting that McSA1 does not recognize the holo-APP protein in 4% paraformaldehyde fixed free-floating tissue sections.

2. In a separate study, we used pre-absorption as a control for the specificity of the immunoreaction. In this procedure, the antibody is incubated with a protein (or peptide) of interest prior to use in IHC; if the protein (or peptide) specifically reacts with the antibody, this will prevent the binding of the antibody to the protein in the tissue, resulting in no staining. Pre-absorption of McSA1 with synthetic Aβ42 in fact completely abolished the intracellular immunostaining. On the other hand, the same molar concentration of synthetic, soluble sAPPPα did not show any effects, indicating that McSA1 does not significantly react with this APP-related product (supplementary figure in Leon et al., 2010). Full-length APP could not be used as a control since this large protein is not commercially available.

3. Such intracellular material was also reactive to the Nu1 antibody (Lambert et al., 2007), a well characterized monoclonal antibody for the identification of Aβ-oligomers (ADDLs). Again, in our hands, in the McGill-Thy1-APP Tg mice dual immunostaining revealed a differential intracellular staining pattern for Nu1 and APP (Fig. 2-7, Chapter 2).

4. The intracellular material at 3 month of age appeared to be IR also with OC, a fibrillar specific antibody (Kayed et al., 2007). Unless assuming that APP can aggregate and form oligomers, one has to conclude that this material is oligomeric Aβ.

Based on these controls we strongly believe that the intracellular IR observed is largely not APP nor sAPPα, as proposed by some authors for the so-called triple Tg mice (Winton et al., 2011). However, we cannot exclude the possibility that it

is partly coincidental with βCFT (McAlpine et al., 2009). In support of the Aβ theory, other authors have reported specific intracellular Aβ staining (Rosario et al., 2006; Lord et al., 2006; Wirths et al., 2001). The nature of the intracellular material has been elegantly confirmed in recent studies applying ELISA on microdissected neurons (Aoki et al., 2008; Hashimoto et al., 2010).

Furthermore, our biochemical studies (using ELISA and WB) confirmed the presence of Aβ before plaque deposition (Ferretti et al., 2011b). The limitation of this technique is that, since we used gross homogenates, we could not discriminate between intracellular, extracellular and membrane bound material. Preliminary studies showed that the vast majority of the Aβ and β-CTF material is membrane bound (Dr Canneva, unpublished).

Our investigations revealed 61.99± 29.66ng/g wet tissue of Aβ42 and 22.54±7.25 ng/g wet tissue of Aβ40 in cortex of pre-plaque Tg mice. Further to it, preliminary studies suggest that there are about 20 molecules of β-CTF for each molecule of Aβ (Chapter 4). These results would indicate that most of the material present at this age is β-CTF. More investigations will be required to confirm the relative abundance of the two species and their relative contribution in the earliest pathological stages.

Finally, it has to be taken into consideration that our studies were conducted in Tg animal that express high (9x) levels of APP. It is possible that the mechanism of APP processing and Aβ trafficking are altered by the over-expression system. Some authors argue that high levels of intracellular Aβ (i-Aβ) are an artifact of over-expression, which do not apply to the human condition (Winton et al., 2011). However, we observed similar i-Aβ also in the Tg rat model, which carries only one copy of the transgene. Furthermore, in sporadic AD cases, quantification of Aβ42 revealed a concentration equal to 200 nM in microdissected pyramidal neurons (Hashimoto et al., 2010). While the absolute amounts might vary between

animals and humans, the occurrence of i-Aβ is therefore quite likely in early stages of the AD pathology.

5.2.2 Relevance

The occurrence of i-Aβ coincided with a number of pathological, biochemical and behavioral alterations, suggesting a causal role of Aβ (Chapter 2,3). Clearly, the results shown here are suggestive but not sufficient to claim that Aβ is the only cause of all the alterations observed. Further manipulations, such as inhibiting Aβ production either affecting the γ-secretase activity or via immunization, would be necessary to make this point. However, there is strong evidence in the literature indicating that i-Aβ can be neurotoxic.

Some of the first evidence in this direction came from the McGill laboratory of Leblanc: injection of human Aβ42 in primary neurons proved to be neurotoxic, while human Aβ40 appeared to be inert (Zhang et al., 2002).

It has been argued that the levels of i-Aβ injected in a cell or measured in an over-expressing system are not comparable with the AD situation. However, recent quantification of Aβ42 in pyramidal neurons microdissected from human brains demonstrated a concentration equal to 200 nM in sporadic AD cases and 90 nM in controls (Hashimoto et al., 2010). These results indicate that the localized concentration of Aβ in neurons can reach toxic levels.

Furthermore, even if Aβ turns out to be a minor component of the intracellular pool, this would not rule out its pathophysiological relevance. In fact, the sub-cellular localization of even small amounts of Aβ in crucial intracellular organelles (such as mitochondria), nucleus or synaptic terminals can result in powerful toxic effect. Thus, there is evidence that Aβ is specifically associated with the mitochondrial membrane (Hansson Petersen et al., 2008). In such subcellular location Aβ could induce the well known mitochondrial dysfunction

described in AD (Caspersen et al., 2005; Lustbader et al., 2004) as well as in cellular expression models (Grant et al., 1999).

Interestingly, Dr Multhaup's group recently gathered convincing evidence that Aβ can also traffic to the nucleus where it can act as a transcription factor, modulating the expression of target genes (Barucker et al., 2011).

Furthermore, the occurrence of i-Aβ in synaptic terminals in AD has been studied by Gouras and collaborators (Takahashi et al., 2004a). In particular, it was noted that the presence of Aβ was accompanied by dystrophic appearance, suggesting a specific toxic effect of Aβ in this cellular compartment.

Besides the sub-cellular localization, another factor to take into consideration when studying intracellular Aβ is the aggregation status in which the peptide is found. A vast body of evidence suggests that Aβ is not toxic *per se*, but rather it acquires toxicity as it undergoes structural re-organization and oligomeric conformations (Lorenzo and Yankner, 1994; Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 2002). Hence, even low levels of Aβ-oligomers might be highly toxic. The exact localization of Aβ aggregation is therefore a crucial point that needs to be elucidated.

It is largely accepted in the Alzheimer's field that Aβ aggregation takes place extracellularly. However, it is conceptually hard to understand how the Aβ material which is released in the extracellular space at low, picomolar concentrations can aggregate and ultimately form plaques.

Our results strongly indicate that several steps of Aβ aggregation might occur intracellularly (Chapter 2). Even the formation of fibrillar oligomers (OC positive) appears to start within the soma of pyramidal neurons. However, this transient localization is soon followed by extracellular release and formation of amyloid plaques. The high concentration of Aβ material in the acidic environment of the intracellular vesicles would facilitate Aβ fibrillogenesis.

The pathological intracellular accumulation of Aβ-oligomers is likely a negative neuronal event and one of high potential therapeutic relevance. A better understanding of the mechanism leading to such accumulation and its resulting toxicity is crucial for the search of agents capable of preventing, at early stages, the further evolution of the AD pathology.

5.2.3 Aβ modifications

The studies presented here focused on the quantitative and qualitative characterization of the two most common Aβ species generated during APP processing, Aβ40 and Aβ42. However, more species can be generated by the action of γ-secretase, each of them can undergo further modifications, and most of them could have toxic effects.

It is well established that γ-secretase is an imprecise enzyme that can cleave APP at different sites generating a range of Aβ peptides with variable C termini and length (Wiltfang et al., 2002). Shorter Aβ species (including Aβ 1-37, 1-38, 1-39) have been described, and are considered not toxic. The production of these species is considered independent from Aβ40 and Aβ42 by some authors (Czirr et al., 2008). However, there is evidence in the literature of compounds that can modulate the gamma cleavage (so called 'γ-secretase modulators'). These drugs in fact reduce Aβ42 and increase Aβ38, suggesting that the two species are in dynamic balance. Interestingly, some NSAIDs have been shown to modulate γ-secretase activity (Weggen et al., 2001).
A thorough analysis of shorter Aβ species in the Tg mouse and rat is in progress.

Aβ N terminal contains serine (8) and tyrosine (10) residues that can be subjected to a number of modifications. N-terminal isomerization, racemization, and oxidation are spontaneous modifications of long lived proteins. The Aβ N-terminus is in fact subject to a variety of truncations and post-translational

modifications which have been involved in pathogenesis and oligomers formation (Geddes et al., 1999). N-truncated forms have been identified in the AD brain (Masters et al., 1985; Mori et al., 1992). In DS, this species were reported well before the appearance of plaques (Teller et al., 1996). In particular, N-terminally truncated Aβ starting with pyroglutamate (AβpE3) appears to be the predominant form in plaques (Saido et al., 1995). Tg mice expressing pE3 in neurons exhibit neuronal loss and neurological alteration, confirming the neurotoxicity *in vivo* (Wirths et al., 2009). Inhibiting glutamyl cyclase (the enzyme responsible for N truncated formation) in APP Tg mice resulted in reductions in Aβ, diminished plaque formation and gliosis, as well as improved cognitive performance (Schilling et al., 2008). Importantly, Wirths and collaborators demonstrated the presence of intra- and extracellular AβpE3 oligomers in human SAD (Wirths et al., 2010).

Besides truncation, phosphorylation and nitrosylation can also take place at the N terminal of Aβ.

Serine 8 phosphorylation of extracellular Aβ has been shown to occur in AD brain (Kumar et al., 2011). Importantly, this modification occurs by the action of an ecto-protein kinase at the plasma membrane and facilitates oligomeric formation. Oligomers of phosphorylated Aβ can be detected in Tg mice at very early stages, suggesting that this is a possible early event in the progression of the disease (Kumar et al., 2011).

Extensive oxidative stress and up-regulation of iNOS are well known features of AD brains (Honda et al., 2004). Nitric oxide generation from neurons and glia can induce localized oxidative stress and protein and lipids modifications. Interestingly, oxidation of Aβ at tyrosine 10 has been demonstrated in a subset of core plaques in AD and DS and is suggested to be an early event (Head et al., 2001). Nitration at the same residue has also been demonstrated; it appeared to

increase Aβ aggregation and mediate most of its neurotoxic effects (Kummer et al., 2011).

Further investigations will be required to assess whether the i-Aβ detected in young Tg mice presents any of the above modifications, and how these can be linked to inflammation.

5.3 Pre-plaque inflammation

Our studies in Chapter 3 led us to identify a previously un-noticed microglia activation that specifically accompanies intracellular Aβ burdened neurons. The relevance and limitations of this finding are discussed below. Furthermore, we offer an overview of additional inflammatory players (astrocytes and oxidative stress) that were not part of our investigations but surely play a pivotal role in early stages of AD-like amyloid pathology.

5.3.1 Evidence for early inflammation in AD and other conditions

The relevance of inflammation in early stages of AD has been highlighted by recent biochemical and imaging studies in MCI, which is considered the prodromal phase to AD. Higher levels of key inflammatory markers have been observed in MCI brains and blood compared to controls (Parachikova et al., 2007; Bruno et al., 2009b; Roberts et al., 2009). Interestingly, a specific protein expression pattern (blood signature) is associated with AD and MCI. Overall, MCI blood shows signs of dysregulation of hematopoiesis, immune responses, apoptosis and neuronal support (Britschgi and Wyss-Coray, 2009). These data were confirmed by recent *in vivo* imaging results, indicating an ongoing activation of microglia in MCI patients (Okello et al., 2009; Edison et al., 2008).

The investigation of these markers in pre-clinical stages in humans is complicated by the fact that it is presently impossible to predict who will convert from NCI to MCI and AD. The ability of NSAIDs to protect against AD remains the most convincing, yet indirect evidence, for a role of inflammation in early stages of AD (McGeer and McGeer, 2007).

However, it is interesting to know that in other neurodegenerative conditions with a well defined progression, the occurrence of inflammation in early (pre-clinical) stages is starting to be reported. For instance, Huntington's disease (HD) is a progressive genetic neurodegenerative disease characterized by abnormal motor movements, cognitive decline and early death. It is an autosomal dominant disease where a long asymptomatic phase precedes the age of onset (between 35 and 45 years of age). Interestingly, strong up-regulation of inflammatory cytokines has been documented in pre-manifest HD mutation carriers many years before the onset of motor abnormalities (Bjorkqvist et al., 2008). Transmissible spongiform encephalopathies, or prion diseases, are infectious neurodegenerative diseases characterized by the aggregation of misfolded prion protein (PrP). Mutated PrP causes severe motor dysfunction and eventually death in mice, but this occur several months after its inoculation (Chandeler, 1961). In pre-symptomatic animals, some synaptic changes have been shown to precede the onset of widespread degeneration and are accompanied by microglial activation (Cunningham et al., 2003; Boche et al., 2006). Similarly, a recent imaging study showed that astrogliosis precede neuronal death (Tamguney et al., 2009) in animal models of the disease.

It appears therefore likely that early, synaptic alterations are sufficient to trigger a microglial response. The features and the role of such activation in neurodegeneration remain to be elucidated.

5.3.2 Systemic inflammation and AD

The relevance of inflammation in the progression of AD is also hinted by several clinical and animal studies indicating that systemic inflammation can precipitate the symptoms of dementia.

It is well accepted that systemic inflammation, caused by infection, surgery or injury can induce episodes of delirium in elderly and demented patients (Lemstra et al., 2008; Lerner et al., 1997; van Munster et al., 2008; for a review see: Van Gool et al., 2010).

In a prospective study it was shown that the degree of decline over 6 months was 10 fold higher in AD patients who experienced, over the 6 month, one or more episodes of systemic inflammation (Holmes et al., 2009). Similarly, studies in animal models of neurodegeneration indicate that systemic inflammation can exacerbate the local brain inflammation (Cunningham et al., 2005; Sly et al., 2001). In some cases, such as in the ME7 model of prion disease and in models of Parkinson's disease, this resulted in exacerbation of behavioral impairment and neuronal death (Cunningham et al., 2005; Pott Godoy et al., 2008). In AD models, exacerbation of CNS inflammation via systemic inflammation results in an acute increase in Aβ levels (Sly et al., 2001), followed by a significant decrease and clearance of plaques (Quinn et al., 2003). However, it is not known whether this effect is accompanied by effect on the cognitive status.

The observation that systemic inflammation can precipitate the pathology of AD patients is only one of many indications that the peripheral immune system and the brain significantly affect each other (Solomon, 1987). On one side, induction of systemic inflammation in animals induces a number of long-lasting alterations called 'sickness behavior', which mimic depression symptoms (Dantzer et al., 2008). This is of extreme relevance to the AD field, since depression is an early feature of MCI; accumulating evidence suggests that

depression correlates with inflammatory markers (Leonard, 2007). Conversely, environmental enrichment can improve the functionality of the immune system and reverse cognitive deficits in AD models (Nichol et al., 2008). It is therefore possible that depression, AD and delirium represent a continuum, and that the transition can be modulated by the peripheral immune system (Van Gool et al., 2010).

Why systemic inflammation affects more markedly the CNS in a context of neurodegeneration is not clear. The most convincing hypothesis put forward so far is the "microglia priming hypothesis". According to this hypothesis, a given pre-existing pathology (such as Wallerian degeneration, synaptic failure or frank neuronal death) induces a low grade inflammatory status where microglial cells are primed. Upon a secondary influence, such as infection and injury, microglia can be stimulated to an aggressive state of inflammation and release neurotoxic factors (for a review: Perry et al., 2010).

It is therefore crucial to study and characterize the status of the priming of the microglial cells in AD. While most studies have been conducted in plaque stages, our investigations open the possibility that microglia priming occurs much earlier, in pre-clinical stages of the disease. Our results on a pre-inflammatory status in pre-plaque AD would predict that systemic inflammation, even in pre-clinical AD individuals, might control the onset of the disease.

5.3.3 Genome wide association studies

Another line of evidence for the involvement of the immune system in AD comes from genetic investigations. The occurrence of mutations or polymorphism of immunoregulatory genes in AD has been widely studied. Two approaches have

been used: candidate gene versus genome-wide approaches (for a review: Bertram et al., 2010).

Candidate gene studies investigated the association of specific genes (chosen on the base of functional hypotheses, mostly Aβ-centered) with the incidence of the disease. The only strong finding that emerged from candidate gene studies was the APOE correlation, whereby carriers of the APOE4 allele have 4-fold increased risk as compared to non-carriers (Strittmatter et al., 1993; Poirier et al., 1993, discussed in the Introduction section of this thesis). However, for most of the nearly 700 candidate AD genes investigated over the past 30 years, the outcomes have been inconsistent.

The opposite approach consists in the screening of vast arrays of genes, in a hypothesis-free manner. The advent of microarray technology has made this high-throughput research feasible, and several hundreds of thousands of single-nucleotide polymorphisms (SNPs) can now be assessed in one experiment. Genome wide association studies (GWAS) in AD have yielded reproducible and consistent results.

Interestingly, association signals related to pathways involved with cholesterol metabolism and the immune response are significantly over-represented in these studies (Jones et al., 2010). Consistent genome-wide significant association signals were in fact reported for CD33, clusterin, and CR1. CD33 is an immunoglobulin-like lectin that is involved in cell-cell interactions and adaptive and innate immune systems (Crocker et al., 2007).Clusterin is related to the regulation of brain cholesterol and lipid metabolism, and the inhibition of neuronal apoptosis/potentiation of neuroprotection (Nuutinen et al., 2009). CR1 is the main receptor of the complement C3b protein, a key inflammatory protein activated in AD (Khera and Das, 2009).

5.3.4 Inflammaging and the age dependent hypothesis of AD

Besides the genetic risks factors discussed above, the only confirmed risk factor for sporadic AD is aging.

Inflammation plays an important role in normal aging, and age-related changes within the innate immune system have been implicated in the development of age-related neurodegenerative disorders (Blasko et al., 2004b).

While in the past aging was solely associated with immunodepression (Linton and Dorshkind, 2004), it is now well established that normal aging is associated with a complex immune system deregulation. Such deregulation includes a low grade, but chronic, activation of the innate system, which likely compensates the exhaustion of the capacity of the adaptive system response (Giunta et al., 2008). This phenomenon has been called 'inflammaging' (Franceschi et al., 2007).

Low grade chronic inflammation is considered a reliable marker of high risk morbidity and mortality (Bruunsgaard et al., 2003; Schmaltz et al., 2005) and is linked to cognitive impairment (Weaver et al., 2002). It is therefore very likely that such low grade inflammation, in certain circumstances, could favour the development of AD.

Importantly, not all aged individual will display cognitive decline, and even fewer will develop AD. Many centenarians live a long life spared from most of the neurodegenerative disease. Interestingly, genetic and biochemical data emerging from the study of such individuals indicate that, while a general low grade inflammation is present, this is balanced by the over-expression of anti-inflammatory cytokine transforming growth factor beta (TGF-β), the natural anti-inflammatory cortisol, the reduction in coagulation factors; the up-regulation of IL-15 (which contributes to the maintenance of memory T cells) and changes in lipid profile. Furthermore, centenarians display specific gene polymorphism all related to reduced immune response (Franceschi et al., 2007; Giunta et al., 2008).

These changes are consistent with the idea that the body of centenarians compensated the classical, low grade inflammatory status with the concomitant development of strong and effective anti-inflammatory responses.

On the other hand, it is likely that in individuals who fail to mount such anti-inflammatory response, neurodegeneration arises. In line with this idea, NCI patients with AD-like brains (so-called "high pathology controls") demonstrated dramatically lower levels of pro-inflammatory markers than AD patients (Lue et al., 1996).

On the whole, the above mentioned evidence of chronic inflammation in normal and successful aging indirectly point out the importance of inflammation for the onset of age-related diseases such as AD, and strongly suggests that inflammation might be a key player in the onset of AD.

5.4 Pre-plaque inflammation: additional features

5.4.1 The many faces of microglial activation: M1 and M2

It is generally accepted that microglial activation is accompanied by morphological changes and the up-regulation of membrane and cytoplasmatic markers (Perry et al., 2010). In our studies, microglial activation was therefore investigated by means of classical biochemical and morphological methods. Biochemically, we relied on the expression of well-known markers of microglial activation: iNOS, CD40, IL-1β. Morphologically, we defined as ‘activated’ a microglial cell that would present retraction and thickening of the processes, and hypertrophic soma size. However, it has to be noted that microglial activation is an extremely complex phenomenon.

First of all, microglia are never ‘resting’, but rather are always ‘active’. Elegant *in vivo* imaging studies (Nimmerjahn et al., 2005; Davalos et al., 2005) revealed that microglial cells are never quiescent, but constantly scan the microenvironment. Importantly, they appear to be actively involved in a number of functions (including remodeling and stripping of dendritic spines), in the absence of any tissue damage (Tremblay and Majewska, 2011). Thus, the term ‘resting’ is considered largely imprecise, and should be substituted by ‘surveilling’ microglia.

Secondary, the microglial phenotype is extremely plastic and versatile, as other members of the bigger family of monocyte-macrophage lineage. Therefore microglial function cannot be exclusively extrapolated from their morphology (Perry et al., 2010). In fact, even when faced with tissue disturbances, the nature of the microglial response varies enormously in terms of expression of inflammatory markers, morphological changes and phagocytic capability. Furthermore, heterogeneous microglial profiles can be found in the same setting.

In an attempt to put order in this topic Mantovani and collaborators recently proposed a dual classification of activated macrophages; based on this, macrophages can either take on a “classically activated” phenotype (also called M1) or an “alternatively activated” phenotype (also called M2) (Mantovani et al., 2004; Mantovani et al., 2002). The terminology is derived from the dichotomy known for T helper lymphocytes (Th): Th1 cells (which promote inflammation and evoke cell-mediated immunity) and Th2 cells (that produce anti-inflammatory interleukins and activate strong antibody responses (Romagnani, 2000). The idea behind this classification is that, when presented with a potential threat, macrophages are capable to recognize the nature of the encountered challenge and act accordingly.

In case of minor disturbances (e.g., neuronal stress) microglia may secrete anti-inflammatory cytokines and supportive growth factors. This profile corresponds to the 'alternatively activated' microglia or M2, and it is generally considered benign. It can be modeled *in vitro* by application of the inhibitory cytokines IL-4, IL-10, IL-13 or glucocorticoids, and it is characterized by high levels of IL-10 and low of IL-12 and inflammatory cytokines. YM-1 and Arginase-1 are also markers of M2. Down-regulation of inflammatory cytokines is achieved through inhibition of NFkB and stat-1 pathways. M2 macrophages elicit type II response in leukocytes, resulting in immunoregulation and tissue remodeling.

In presence of a serious threat (such as a pathogen invasion), macrophages can release toxic factors to kill the pathogen and recruit help by releasing pro-inflammatory cytokines: these macrophages cells are called M1. A stereotypical M1 response can be elicited *in vitro* by activating macrophages with IFNgamma (alone or in combination with additional inflammatory agents such as LPS or Aβ). This response is characterized by antigen presenting activity, elevated IL-12 and 23, and up-regulation of iNOS and reactive oxygen species.

M1 and M2 are the extremes of a continuum. Further sub-classifications have been proposed in the M2 category, to include intermediate states that correspond to different functions of 'wound healing' or 'immune regulators' (Mosser and Edwards, 2008). 'Wound healing' M2 macrophages respond to IL-4, promoting Arginase activity and YM1 expression, both contributing to the production of the extracellular matrix. On the other hand, glucocorticoids released in conditions of stress can inhibit the pro-inflammatory activity of macrophages and convert them into anti-inflammatory cells. These 'immune regulators' M2 macrophages are not involved in tissue remodeling but secrete large amount of IL-10, thus inhibiting the immune response.

In sum, it is becoming increasingly clear that macrophages are a heterogeneous population with distinct functions and there is not as yet a specific marker recognizing a specific population. Furthermore, the phenotype is not fixed, and any given macrophage can switch from one to the other following stress or therapeutic treatment. The switch from M1 to M2 states appear to be crucial involved in a number of pathological conditions, including cancer and diabetes (Mosser and Edwards, 2008).

In the CNS, it has been shown that microglial cells can differentiate along a similar spectrum of phenotypes (Michelucci et al., 2009; Colton et al., 2006). In fact, the expression profile of microglia in response to typical M1 and M2 stimulants is remarkably similar to macrophage pattern with low phagocytotic ability, *in vitro*.

As seen for macrophages, microglial phenotype is not fixed. In models of *in vivo* neuronal injury microglial response is staged. For example, Wallerian degeneration induces a transient up-regulation of classical inflammatory markers (Palin et al., 2008). However, the synthesis of some of these inflammatory mediators is restricted in time, and the microglial phenotype is quickly reverted into an anti-inflammatory phenotype (Palin et al., 2008; Perry et al., 2010).

In a brain affected by a chronic neurodegenerative disease, it is very likely that at any given time some microglia will be acutely responding to a local apoptotic or synaptic degeneration event with an M1 phenotype, while most of them will be found in a M2 state. This might explain why the inflammatory profile observed in prion disease appears to be an alternative activation (Perry et al., 2002b). As discussed above, such M2 microglia cells are primed, and they can be further converted into M1 upon a secondary stress (such as LPS). Thus, the switch M1-M2-M1 is critical in neurodegeneration and it might be responsible for the progression of the disease.

5.4.2 M1 and M2 in AD

In vitro, fibrillar Aβ and even more so oligomeric Aβ are capable of inducing an M1 profile, with up-regulation of iNOS, IL-1β, COX-2 and IL-6 and PPARγ. Both preparations reduce phagocytotic activity (Michelucci et al., 2009). These data supported the idea that brains burdened with Aβ would present mostly a M1 phenotype. However, the results from animal studies and from the clinical samples have been controversial.

Colton and colleagues reported a hybrid activation state in the APPswe models, where a significant up-regulation of M2 markers (such as Arginase1 and YM1) was concomitant with up-regulation of TNFα, typical M1 marker (Colton et al., 2006). In the APP-PS1 model, Jimenez showed that microglial cells switch from a mostly M1 profile (at early plaque stages) to M2 profile with a more advanced stage of the pathology (Jimenez et al., 2008). However, the authors noted that the profile of alternative microglia surrounding plaque was not 'pure' M2, as, for instance, the cells did not express Arginase1.

In the McGill-Thy1-APP model we investigated enzymes (iNOS), cytokines (IL-1β) and membrane receptors (CD40, MHC-II) involved in antigen presenting functions, which are mostly involved in M1 function. This led us to speculate that microglial cells in this model prior to plaque deposition are mostly M1, even though they appear unable to phagocytose Aβ. A more extensive investigation of the mRNA and protein levels of both M1 and M2 cytokines is in progress.

Preliminary data (Appendix 1) suggested that both M1 and M2 signaling co-exist at this early stage. Our pilot experiments suggest a mostly M1 profile, with up-regulation of M1 cytokines IL-12 p40 and p70 (implicated in the natural killer activation) and MIP1b (Th1 chemokine for lymphocytes and monocytes) and down-regulation of eotaxin (Th2 activator). However, at the same age, we noticed some up-regulation of basic FGF and M-CSF; M-CSF elevation has been

confirmed with ELISA (p=0.06). FGF is involved in tissue remodeling and is released by M2 activated macrophages (Mantovani et al., 2002). M-CSF is a strong hematopoietic factor that induces monocytes to differentiate into macrophages (Geissmann et al., 2010) of the M2 phenotype (Verreck et al., 2004). It is therefore very likely that M1 and M2 factors are coexisting in the conditions under investigation. One possible explanation is that a subset of microglial cells are involved in the transient response to an acute event (such as apoptosis or axonal degeneration) by up-regulating M1 factors, while others have already converted into an anti-inflammatory M2 phenotype. More studies are required to confirm this hypothesis.

At later stages, we found a significant up-regulation of M1 cytokines MIG, M-CSF and MIP1b and we observed a down-regulation of IL-10 (anti-inflammatory). However, the anti-inflammatory cytokines LIF (possibly neuroprotective: Blesch et al., 1999) and IL-3 (involved in hematopoiesis) were found to be significantly up-regulated.

In AD brains, while a vast literature supports the up-regulation of M1 classical markers (Akiyama et al., 2000), there is also evidence for elevated levels of M2 cytokines (such as TGF-β; Wyss-Coray et al., 1997) and M2 enzymes (such as Arginase1; Colton et al., 2006).

The available results from *in vitro*, *in vivo* and AD brain samples, although conflicting, taken together would suggest that during AD progression microglial cells change their activation state as a function of the disease stage. Also, the evidence would indicate that the innate immune cells in AD exhibit a hybrid activation state that includes characteristics of classical (M1) and alternative (M2) activation. To better elucidate the role of microglial activation in AD it will be therefore necessary to have a deeper understanding of the balance between M1 and M2 and their reciprocal temporal and spatial evolution.

5.4.3 Adaptive response in AD

Activation of microglia (either M1 or M2) is tightly linked to the activity and recruitment of lymphocytes. The involvement of microglial activation in the AD pathology might lie in the activation (or lack thereof) of lymphocytes. While extensive data exist on the occurrence of microglial activation in AD, the state of the adaptive response is much less clear.

CD4+ and CD8+ T lymphocytes have been found in the brain parenchyma of AD patients (Rogers et al., 1988), but no B cells. The level of T-cells in the AD brain is slightly higher than controls, but low compared to other neurodegenerative diseases such as MS (for review see: Lafaille, 1998). Of course, it remains to be determined whether brain penetration of T-cells is involved in the etiopathogenesis of AD, or it is simply an epiphenomenon.

Interestingly, a different picture emerges from the blood of AD patients. Here, a general decrease in T and B cells was observed, while the number of natural killer (NK) cells was not affected (Richartz-Salzburger et al., 2007; Speciale et al., 2007). Furthermore, hypo-responsiveness of suppressor as well as helper T-cells was described in AD (Skias et al., 1985; Singh et al., 1986; Richartz-Salzburger et al., 2007).

The hypofunctionality of T and B cells might explain why specific antibody titers for Aβ are strongly decreased in patients with AD (Du et al., 2001) and might be involved in the onset of the disease.

This view was strongly supported by the finding that passive or active immunization effectively clears Aβ plaques and restores cognitive function in animal models (for a review: Nitsch and Hock, 2008). It is currently thought that reduction of Aβ plaques following immunization protocols is due to microglia-mediated phagocytosis and clearance of Aβ.

In sum, a defective functionality of the adaptive arm of the immune response is likely to be involved in the etiology of AD. The importance of the interaction between the adaptive and the innate response was highlighted by immunotherapy studies, showing that plaque clearance is mediated by microglia. Hence, the cross-talk between the adaptive and the innate systems in the early stages of AD is an important issue that remains to be elucidated.

5.4.4 Astrocytes

As briefly mentioned in the Introduction, astrocytes are intimately associated with the neurons and can quickly detect neuronal alterations. It is therefore to be expected that the pathological intracellular accumulation of Aβ will affect the phenotype of astrocytes.

Astrogliosis has been reported in AD and in Tg model of AD-like amyloid pathology, whenever plaque appear (Verkhratsky et al., 2010). However, there are indications that astrogliosis might be an early neuropathological event. Activation of ERK in astroglia has been detected in frontal cortex of patients with mild AD (Webster et al., 2006). This possibility is supported by the finding that astrogliosis is an early feature of DS brains (Griffin et al., 1989). In particular, S100 (a molecule involved in cell cycle and proliferation of astrocytes (Selinfreund et al., 1991) has been found to be elevated in DS as early as in the fetus. Furthermore, recent imaging studies demonstrated that astrogliosis can be detected in pre-symptomatic phases of mice models of Prion disease (Tamguney et al., 2009).

Under stress conditions, astrocytes can express a variety of inflammatory markers, including some (like iNOS and IL-1β) that are up-regulated in young Tg McGill-Thy1-APP mice. It is therefore possible that part of the pro-inflammatory reaction detected in pre-plaque stages of the disease is also supported by astrocytes. While a thorough investigation of astrogliosis was beyond the scope

of this work, preliminary results from the group indicate that GFAP levels were unchanged in young pre-plaque mice, and started to rise only at the onset of plaque pathology (Dr Do-Carmo, personal communication) as well as in our rat transgenic line (JSW, Gratz, preliminary data). However, astrocytic activation can occur also in the absence of overt GFAP up-regulation (Rodriguez et al., 2009).

A careful analysis of astrocytic morphology and the expression of markers that have been implicated in AD (such as S100) might elucidate the role of astrocytes in early stages of AD.

5.4.5 Oxidative stress

Signs of oxidative stress (lipids, proteins and DNA) are readily detected in AD brains (Smith et al., 2000; Perry et al., 2002a). Interestingly, there is evidence supporting the concept that oxidative stress is an early event in the progression of the disease in AD (Nunomura et al., 2001; Pratico et al., 2002) and in Tg models (Pratico et al., 2001). Our results indicating increased expression of iNOS in pre-plaque Tg mice would suggest that high levels of NO and oxidative stress accompany early stages of Aβ accumulation. While this is still an assumption, if confirmed it would be of high relevance in our understanding of early mechanism involved in AD.

Oxidation of key proteins can in fact lead to loss of function and might be responsible for some of the neuropathological signs of AD. A clear example is the peroxynitration of proNGF that our group detected in AD (Bruno et al., 2009a). The vast majority of the proNGF protein found in AD cases was peroxynitrated, and we demonstrated that peroxynitrated NGF lacks of trophic ability.

Furthermore, a mounting body of evidence indicates that oxidative stress can direct the APP metabolism toward the amyloidogenic pathway (increasing BACE levels and activity; Tamagno et al., 2002) and induce Aβ aggregation (Head et al., 2001; Kummer et al., 2011).

5.5 Pre-plaque inflammation: causes and implications

5.5.1 Causes

What causes the early, pre-plaque inflammation detected in young McGill-Thy1-APP mice is still to be elucidated. Since the only difference between Tg and Non Tg animals is the presence of the APP transgene, microglial activation has to derive either directly or indirectly form the presence of APP and-or APP related products.

We proposed that intraneuronal Aβ-oligomers induce microglial activation based on the following points:

- Aβ-oligomers have been shown to elicit microglial activation *in vitro* (Maezawa et al., 2011) and *in vivo* (Bruno et al., 2009a);

- activated microglia closely associated with iAβ –oligomers burdened neurons, and

- no microglial activation was detected at 2 weeks of age when Aβ-oligomers are not present (Chapter 3)

However, other candidates definitely exist and will be briefly reviewed here.

APP-related fragments. Several APP-related fragments have been shown to interact with microglia *in vitro*; these findings however have not been unequivocally demonstrated *in vivo*. Soluble APP fragments are likely candidates to activate neighboring microglial cells. APPα and APPβ have been shown to activate microglia *in vitro*, via p38 and JNK signaling (Barger and Harmon, 1997; Bodles and Barger, 2005). In particular sAPPα can induce the release of glutamate and iNOS. There is evidence that also CTF can exert neurotoxic effects

accompanied by a strong inflammatory reaction involving both astrocytes and microglia. CTF *in vitro* can elicit the expression of TNFα, IL-1β, iNOS and MMP9 through MAPKs- and NFkB-dependent mechanism (Chang and Suh, 2005). How an intracellular, vesicle-associated peptide can interact with glial cells is presently harder to conceptualize; however, endocytotic dysfunction might result in the over-expression of this fragment at the membrane, where it can be sensed by surrounding glial cells.

Non -APP factors. Besides the release of APP and APP-related products, it is quite possible that microglia are activated by the release of other soluble factors secondary to a pathological Aβ-APP intracellular accumulation and the ensuing neurodegeneration. Injured neurons secrete a variety of factors that actively induce microglial activation ('on signal'). On the other hand, the decreased release of neuronal modulatory factors ('off signals') can also instruct a pro-inflammatory response (Biber et al., 2007).

Damage-associated molecular pattern molecules (DAMPs) (Bianchi, 2007) are released from injured or degenerating cells. This group of molecules includes heat shock proteins, histones, oxidized lipids, DNA, ATP, and potentially many others. Microglia express a variety of specific receptors (PRR, RAGE, purinergic receptors) to such molecules, and have been shown to respond to their release *in vitro* and *in vivo* (Davalos et al., 2005). Since AD is associated with widespread mitochondrial abnormalities (Hirai et al., 2001) it is very likely that i-Aβ burdened neurons are releasing ATP, oxidized lipids and other agents; all of them can act as 'on signals'.

On the other hand, neuronal activity and neurotransmitters levels can affect the microglial state. Similar to neurons, glial cells express a number of neurotransmitter receptors that enable them to respond to signals of neuronal origin (Hanisch and Kettenmann, 2007). In particular, glutamate release in known to activate microglia (Farber and Kettenmann, 2005). Uncontrolled calcium entry and resulting excitotoxicity has been involved in AD (Demuro et al., 2010, see

also the Introduction section of this thesis). The resulting glutamate release could therefore activate microglia.

While glutamate activates microglia, all other neurotransmitters have been shown to act as 'off signals' (Biber et al., 2007). Both ACh and NA are known to down-regulate microglia (Ulloa, 2005; Heneka et al., 2010; Carnevale et al., 2007). Since ACh and NA deficits are well characterized in AD, a local decrease in the levels of these neurotransmitters might therefore mediate microglial activation in early stages of AD. It remains to be elucidated whether these alterations occur at early, pre-plaques stages of the disease.

5.5.2 Other implications of early inflammation: cell cycle events

As mentioned in the Introduction and above, tissue remodeling and proliferation are part of the normal phase of resolution of inflammation. Far from being temporally and physically separated events, the programs responsible for tissue damage and regeneration are started about the same time and coexist in the same environment. Evidence from several fields points in the direction that the immune system can regulate proliferation and growth of organs (Filbin, 2006; Viebahn and Yeoh, 2008). In cancer, it appears as if activation of immune cells can favor growth (Coussens and Werb, 2002; Mantovani et al., 2008). It is therefore quite clear that the immune system can cross-talk with cell-cycle in mitotic cells.

The interaction between an inflammatory environment and post-mitotic cells like neurons is harder to predict. It has been shown that microglial activation favors neurogenesis. This effect seems to be related to a M2 type of activation, while M1 factors mostly appeared to inhibit neurogenesis (Ziv et al., 2006). In adult neurons, Aβ-activated microglia can induce cell cycle re-entry, suggesting a link between inflammation and cell cycle events (Wu et al., 2000).

Cell cycle re-entry in neurons is a potentially harmful event (Herrup and Yang, 2007). It has been proposed that the machinery responsible for cell cycle has been converted to other functions in neurons, in particular related to synaptic plasticity (Arendt, 2003). Thus, cell cycle re-entry can interfere with the proper functionality of neurons and could be responsible for a number of synaptic alterations. It has been demonstrated that neurons entering cell cycle events eventually die (Herrup and Busser, 1995).

Interestingly, aberrant cell cycle re-entry is associated with and precedes neurodegeneration in a number of conditions, including AD (Herrup, 2010; Hoozemans et al., 2006; Arendt, 2003). AD brains showed up-regulated levels of a variety of molecules critically involved in the activation and progression of the cell cycle, including cyclins, cyclin-dependent kinases (CDK) and their inhibitors, and COX-2 (Arendt, 2003).

In line with the idea that AD shows signs of aberrant proliferation, it was early noted that AD neurons show features of developing neurons in their morphology (noted by Fischer, as referred by Goedert, 2009), and in their protein expression profile. AD dystrophic neurites in fact present misplacement and aberrant phosphorylation of tau characteristic of the developing CNS (Goedert et al., 1993; McKee et al., 1989) and re-expression of fetal forms of tubulin (Geddes et al., 1990).

What causes the neurons in AD to attempt a cell cycle re-entry is not clear; however a recent study has shown that a preventive treatment with NSAIDs can block such a process in a Tg model (Varvel et al., 2009). These results would suggest that microglia activation is an up-stream event leading to cell cycle re-entry in models of AD-like amyloid pathology.

In this context, it is of relevance to note that we consistently found up-regulated levels of neuronal COX-2 (an enzyme that has been involved in cell

cycle events) in early, pre-plaque stages of the pathology in the McGill-Thy1-APP mice. Interestingly, the neurons immunoreactive for COX-2 are not accumulating Aβ, suggesting that COX-2 expression is induced in a paracrine fashion. As discussed in Chapter 3, the cause of such up-regulation remains to be elucidated; however, one possible interpretation could be that COX-2-over-expressing neurons are attempting to re-enter the cell cycle.

One possible implication of the occurrence of the early, pre-plaque microglia activation could therefore be the induction of an aberrant proliferative state in neighboring neurons. Such aberrant proliferative process would negatively affect the neuronal function, and contribute to early cognitive impairments. The occurrence of an aberrant proliferative process in the brains of pre-plaque Tg mice and early stages of the disease is supported by the following observations:

1. Cholinergic (Chapter 2) and glutamatergic (Bell et al., 2006) pre-synaptic boutons were found to be up-regulated in early stages of the disease, suggesting an augmented synaptic plasticity.

2. MMP9, a key enzyme involved in tissue remodeling and wound repair, is up-regulated in early stages of the disease (Bruno et al., 2009a,b).

3. We measured elevated levels of NFkB and its inhibitor, IkB, in pre-plaque Tg mice (Chapter 4). NFkB is known to be a positive regulator of cell growth in non-neuronal cell types (Guttridge et al., 1999) and up-regulation of the NFkB pathway accompanies abnormal proliferative events, such as tumorigenesis (Aggarwal, 2004). In neurons, NFkB has been found to be anti-apoptotic (Yu et al., 1999) and pro-survival (Bhakar et al., 2002).

Interestingly, the treatment with the anti-inflammatory drug minocycline was able to correct all of the above alterations in young, pre-plaque McGill-Thy1-APP mice. Following minocycline treatment we observed:

1. Normalization of the cortical density of cholinergic pre-synaptic boutons (Appendix 2)
2. Reduced levels of COX-2 (Chapter 4)
3. Reduced levels and activity of MMP9 (Bruno et al., 2009a)
4. Reduced levels of NFkB (Chapter 4)
5. Amelioration of cognitive impairments (Bruno et al., 2009a and Appendix 3)

These findings, taken together, support the idea that inflammation might be an upstream event responsible for a number of functional alterations in early stages of AD neuropathology, including aberrant cell cycle re-entry.

5.5.3 Glia-neurons cross talk: inflammation and APP processing

In our studies we attempted to establish the role of inflammation in the progression of the disease by applying an anti-inflammatory drug minocycline to young, pre-plaque mice. Our results indicate that, at least in early stages of the disease, dampening inflammation can result in a reduced APP expression and BACE1 cleavage (Chapter 4). Given the experimental setup, we could not discriminate whether the effect we saw was specifically mediated by a reduction of glial activation, or it was due to an effect on the neuronal metabolism.

However, a growing body of evidence would support the concept that activated glia can instruct neurons to produce APP and Aβ. Thus, Sastre and collaborators have convincingly shown that inflammatory cytokines can induce BACE-1 expression and that anti-inflammatory drugs can inhibit this process via

activation of PPARγ (Sastre et al., 2003). This pathway was shown to be relevant *in vitro* and *in vivo* (Sastre et al., 2006; Heneka et al., 2005b).

Perhaps the most intriguing piece if evidence of the link between glial cells and APP processing comes from the HIV field. Here, a series of neuroinflammatory responses resulting in neurologic dysfunctions have been described in the CNS of a significant number of individuals. The neuropathological syndrome is collectively termed HIV-associated encephalitis and is accompanied by dementia (Anthony and Bell, 2008). To date, the mechanisms leading to dementia in AIDS patients are not fully understood; however, it is thought that activated macrophage, microglia, and astrocytes play a critical role (for a review: Gartner, 2000). In fact, HIV associated dementia correlates most closely with neuroinflammation rather than directly with viral load or HIV encephalitis (Glass et al., 1995).

Interestingly, inflammation in HIV is invariably accompanied by increased APP levels (An et al., 1997) and intense intracellular accumulation of Aβ-IR products (Achim et al., 2009). While a direct link has not been established unequivocally, it is generally thought that inflammation leads to up-regulation of APP and its cleavage products. This could, in turn, be largely responsible for the neurological deficits observed in HIV.

5.5.4 Glia-neurons cross talk: inflammation and tau

While our studies allowed us to explore the role of inflammation in early stages of AD amyloid pathology, we did not assess the involvement of inflammation in tau pathology. The reason for this is that the McGill-Thy1-APP does not develop a full tau pathology and is not a suitable model for such an investigation. However, there is evidence in the literature supporting the notion that inflammation is also tightly linked to tau pathology.

Intracellular aggregation of tau is accompanied by microglial activation that precedes tangle formation in the P301S Tg model of tauopathy (Yoshiyama et al., 2007). Indeed, systemic inflammation induced with LPS and viral infection resulted in exacerbated tau pathology (Kitazawa et al., 2005; Lee et al., 2010; Sy et al., 2011). The mechanisms by which activated microglia cells can affect the phosphorylation of tau in healthy neurons have been recently revealed in an elegant *in vitro* study, and they appear to involve IL-1 receptor and p38 (Bhaskar et al., 2010). These results suggest that inflammation could be mediating the well known spreading of tau pathology observed in AD and AD models.

Accordingly, anti-inflammatory treatment appeared to be mostly beneficial in the 3xTg (McKee et al., 2008) and in models of tauopathy, where it successfully dumped inflammation and reduced phosphorylation of tau (Garwood et al., 2010). However, treatment with the drug minocycline appeared to have more complex effects in the 3xTg, where it reduced neuroinflammation, but did not resolve the tau pathology (Parachikova et al., 2010).

In sum, early pre-plaque inflammation might be responsible for at least part of the hyper-phosphorylation of tau; however, further studies are required to elucidate the cross-talk between inflammation and tau, especially in the presence of Aβ.

Conclusions: Inflammation as a Therapeutic Target in AD

In Chapter 4 we gathered evidence for an overall positive effect of anti-inflammatory treatment in early stages of the pathology. Epidemiological data indicating a protection against AD in NSAIDs chronic users would support this idea (McGeer and McGeer, 2007). However, clinical studies applying NSAIDs in AD failed to prove any beneficial effects (Aisen et al., 2003). Similar failure of anti-inflammatory treatment with minocycline has been observed in ALS (Gordon et al., 2007). Here, the anti-inflammatory treatment appeared to be beneficial in animal models, while worsened the pathology in humans.

It is starting to be acknowledged that animal models mimic early, pre-symptomatic phases of the disease. It is therefore likely that for any anti-inflammatory treatment to be effective, it has to be administered in a preventive fashion. Recent evidence from ALS models would support this notion (Keller et al., 2011). Thus, when in ALS models the treatment with minocycline was initiated after the disease onset, it altered glial responses and exaggerated neuroinflammation were observed (Keller et al., 2011).

A recent preventive study (AD anti-inflammatory prevention trial, ADAPT) was designed to test the hypothesis that sustained use of naproxen or celecoxib would prevent AD in a healthy elderly population. While halted after 3 years due to safety concerns, extended results of the trial have been recently published, with the follow-up observations in the ensuing 18-24 months. Cumulatively, the results of this trial indicate the existence of a therapeutic window in which naproxen can reduce the incidence of AD. In fact, asymptomatic individuals administered with naproxen were protected against AD. However, naproxen accelerated AD onset in individuals that were dementia-free, but slightly cognitively impaired at the beginning of the treatment (Leoutsakos et al., 2011; Breitner et al., 2011).

Taken together, the available evidence indicates that inflammation is a potential therapeutic target for the very first stages of the disease. On the other hand, its inhibition thereafter can be detrimental.

Our preliminary results in Tg mice would agree with this view (Appendix 3). In fact, we observed that minocycline treatment restored memory function when administered to 1-2 month old mice, but worsened the cognitive performance when administered later than 2 months of age. It is therefore likely that the pre-plaque inflammation is not a homogeneous phenomenon, but is also staged; the features of the different stages of such process need to be further elucidated.

In conclusion, our work indicates that inflammation is a crucial player in the very first stages of the amyloid pathology. For it to be used as a target for disease prevention, it will be necessary to find reliable predictive biomarkers. A better understanding of the mechanisms of the early, pre-plaque pathology should yield new effective cues for diagnostic tools.

Appendix 1. Preliminary investigation of cytokine levels in McGill-Thy1-APP Tg mice

The levels of cytokines and growth factors were assessed in the hippocampi from 3 month-old (A) and 13 month-old (B) Tg animals using a LUMINEX platform (Bio-Plex Pro mouse cytokine 9-Plex and 23-Plex, Biorad, Hercules CA), following the manufacturer's instructions. The results were compared with Non Tg, age-matched littermates.

A. Four Non Tg and four Tg samples from young, 3 month old animals, were used. For the pilot experiment, the four samples were pooled (yielding to one pooled sample per group), thus no statistical analysis is presented. The up-regulation of M-CSF in Tg animals was confirmed using an ELISA kit (Mouse M-CSF immunoassay, R&D systems, Minneapolis, MN). The data are presented as mean ± SEM and were analyzed with the Student's T-test.

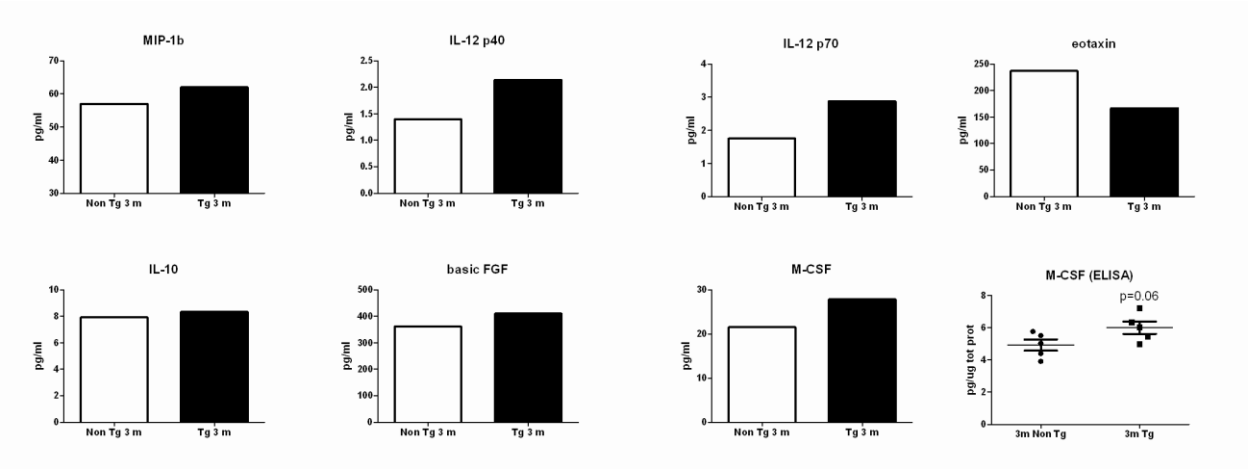
B. Five Non Tg and five Tg samples from old, 14 month old animals, were used. Note the significant up-regulation of several M1 markers (such as M-CSF, MIG and MIP1b). Some anti-inflammatory markers were also up-regulated (LIF, IL-13). The anti-inflammatory cytokine IL-10 was, on the contrary, down-regulated. The data are presented as mean ± SEM; they were analyzed with the Student's T-test with the Welsh's correction for unequal variances when necessary.

**=$p<0.01$.

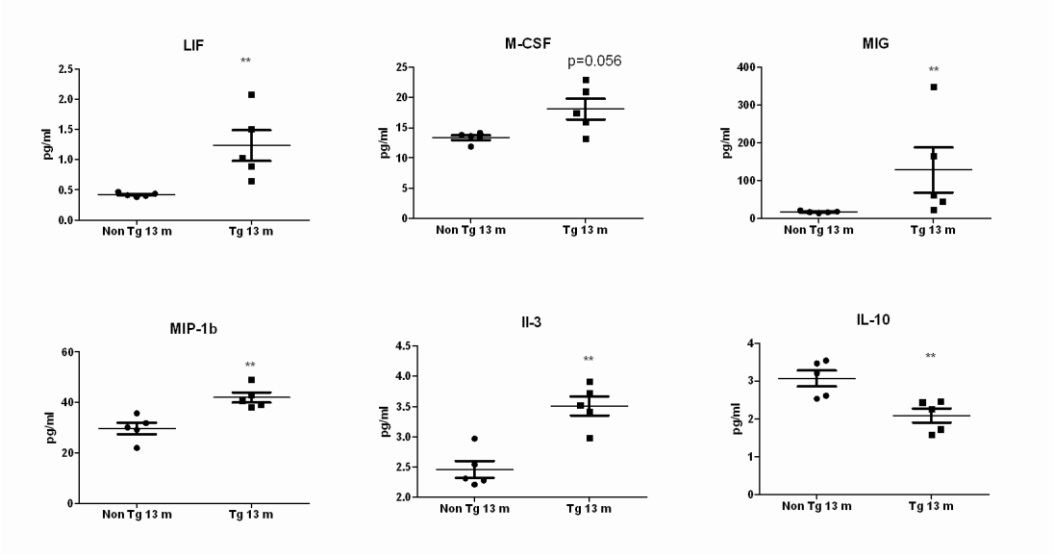
APPENDIX 1

Preliminary investigation of cytokine levels in McGill-Thy1-APP Tg mice

A.

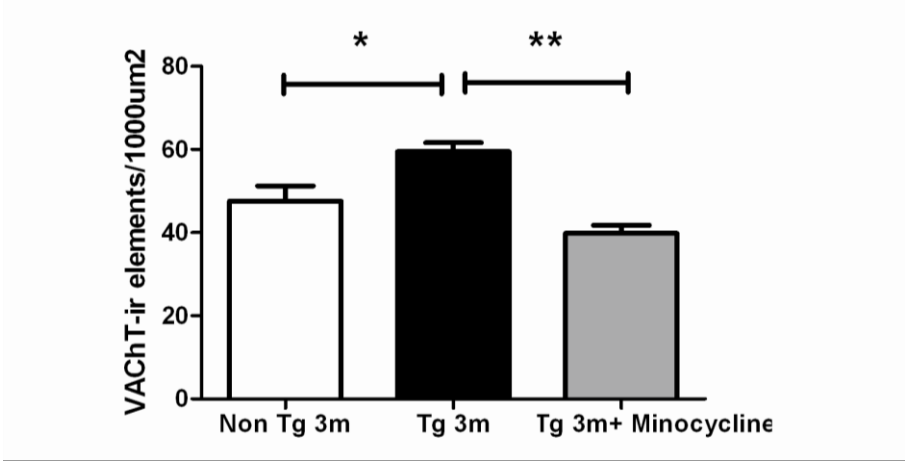


B.



APPENDIX 2

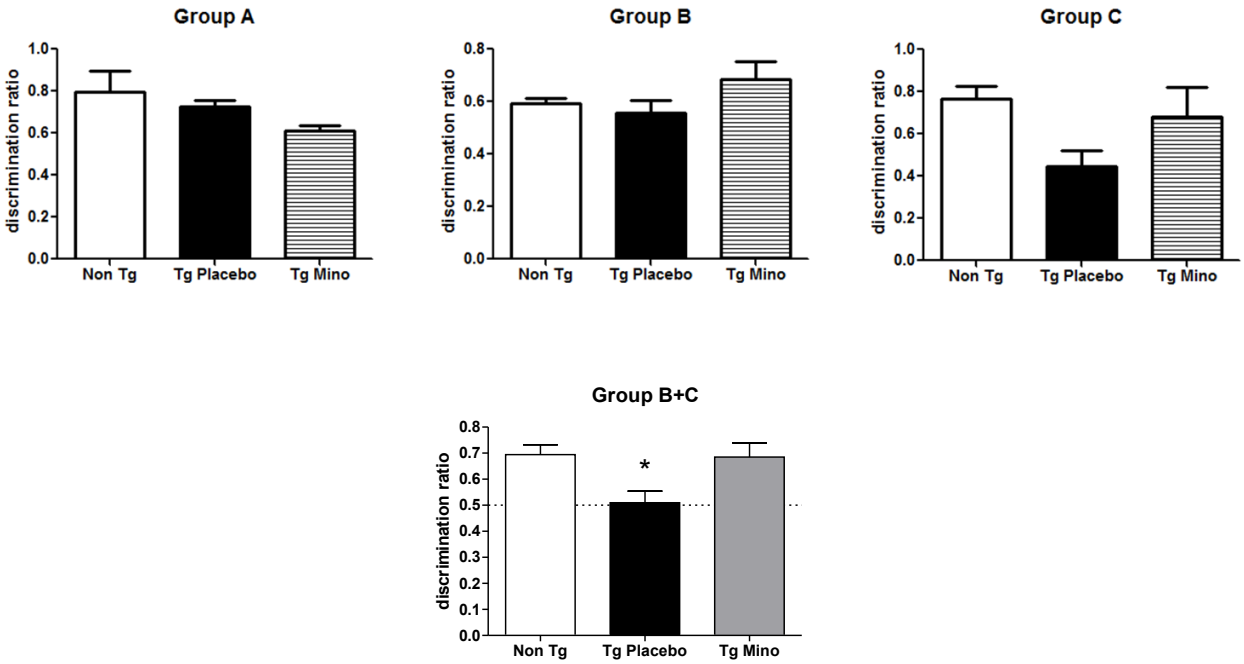
Minocycline corrects the up-regulation of cholinergic boutons in young, pre-plaque Tg mice



The number of VAChT-IR elements was measured in lamina V-VI of cerebral cortex from young, 3 month old Non Tg animals (n=6), Tg animals (n=8) and Tg animals treated with minocycline 50mg/Kg/day (n=10). For details on the treatment see Bruno et al, 2009a. The quantification was performed according to published protocols (Ferretti et al., 2011a). Note that minocycline treatment was able to revert the paradoxical up-regulation of cholinergic boutons in young, pre-plaque Tg mice. Data are shown as mean ± SEM, and were analyzed with 1-way ANOVA with Tukey's post-hoc. (*= $p<0.05$, **= $p<0.01$).

APPENDIX 3

Differential effects of minocycline treatment on mice cognitive function according to disease stage in McGill-Thy1-APP Tg mice



McGill-Thy1-APP mice were treated with either vehicle or minocycline 50mg/Kg/day via food pellets (Harlan Laboratories, Indianapolis, IN) for 2 months. The animals were divided into 3 groups according to their age: **Group A** started the treatment at later than 2 months of age, **Group B** mice were exactly 2 month of age when they entered the experiment, while **Group C** were 1/1.5 month old when they started the treatment. The effect of minocycline on the cognitive status was assessed by using the Novel Object Recognition task, according to published protocols (Ferretti et al, 2011a). Surprisingly, the treatment appeared to worsen the behavioral performance in older animals (Group A), while it appeared to be beneficial in younger animals (Groups B and C). Pooling the data from B and C revealed a significant effect of minocycline (1-way ANOVA with Tukey post-hoc test, *=$p<0.05$; Ferretti et al., in preparation). Data are presented as mean ± SEM, and are shown as time spent exploring the novel object over total time spent exploring.

Thesis References

Abbas N, Bednar I, Mix E, Marie S, Paterson D, Ljungberg A, Morris C, Winblad B, Nordberg A, Zhu J (2002) Up-regulation of the inflammatory cytokines IFN-gamma and IL-12 and down-regulation of IL-4 in cerebral cortex regions of APP(SWE) transgenic mice. J Neuroimmunol 126:50-57.

Abbott NJ, Ronnback L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7:41-53.

Achim CL, Adame A, Dumaop W, Everall IP, Masliah E (2009) Increased accumulation of intraneuronal amyloid beta in HIV-infected patients. J Neuroimmune Pharmacol 4:190-199.

Aggarwal BB (2004) Nuclear factor-kappaB: the enemy within. Cancer Cell 6:203-208.

Ahmed Z, Shaw G, Sharma VP, Yang C, McGowan E, Dickson DW (2007) Actin-binding proteins coronin-1a and IBA-1 are effective microglial markers for immunohistochemistry. J Histochem Cytochem 55:687-700.

Aho L, Pikkarainen M, Hiltunen M, Leinonen V, Alafuzoff I (2010) Immunohistochemical visualization of amyloid-beta protein precursor and amyloid-beta in extra- and intracellular compartments in the human brain. J Alzheimers Dis 20:1015-1028.

Aisen PS, Schafer KA, Grundman M, Pfeiffer E, Sano M, Davis KL, Farlow MR, Jin S, Thomas RG, Thal LJ (2003) Effects of rofecoxib or naproxen vs placebo on Alzheimer disease progression: a randomized controlled trial. JAMA 289:2819-2826.

Akiyama H, et al. (2000) Inflammation and Alzheimer's disease. Neurobiol Aging 21:383-421.

Akiyama H, Shii K, Yokono K, Yonezawa K, Sato S, Watanabe K, Baba S (1988) Cellular localization of insulin-degrading enzyme in rat liver using monoclonal antibodies specific for this enzyme. Biochem Biophys Res Commun 155:914-922.

Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC, Snyder PJ, Carrillo MC, Thies B, Phelps CH (2011) The diagnosis of mild cognitive impairment due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:270-279.

Albert MS, Moss MB, Tanzi R, Jones K (2001) Preclinical prediction of AD using neuropsychological tests. J Int Neuropsychol Soc 7:631-639.

Almeida CG, Tampellini D, Takahashi RH, Greengard P, Lin MT, Snyder EM, Gouras GK (2005) Beta-amyloid accumulation in APP mutant neurons reduces PSD-95 and GluR1 in synapses. Neurobiol Dis 20:187-198.

Alzheimer A (1907) Über eine eigenartige Erkrankung der Hirnrinde. Allgemeine Zeitschrift für Psychiatrie und Psychisch-Gerichtliche Medizine 64:146-148.

An SF, Giometto B, Groves M, Miller RF, Beckett AA, Gray F, Tavolato B, Scaravilli F (1997) Axonal damage revealed by accumulation of beta-APP in HIV-positive individuals without AIDS. J Neuropathol Exp Neurol 56:1262-1268.

Andersen K, Launer LJ, Ott A, Hoes AW, Breteler MM, Hofman A (1995) Do nonsteroidal anti-inflammatory drugs decrease the risk for Alzheimer's disease? The Rotterdam Study. Neurology 45:1441-1445.

Andersson PB, Perry VH, Gordon S (1991) The kinetics and morphological characteristics of the macrophage-microglial response to kainic acid-induced neuronal degeneration. Neuroscience 42:201-214.

Ando Y, Brannstrom T, Uchida K, Nyhlin N, Nasman B, Suhr O, Yamashita T, Olsson T, El SM, Uchino M, Ando M (1998) Histochemical detection of 4-hydroxynonenal protein in Alzheimer amyloid. J Neurol Sci 156:172-176.
Anthony IC, Bell JE (2008) The Neuropathology of HIV/AIDS. Int Rev Psychiatry 20:15-24.

Aoki M, Volkmann I, Tjernberg LO, Winblad B, Bogdanovic N (2008) Amyloid beta-peptide levels in laser capture microdissected cornu ammonis 1 pyramidal neurons of Alzheimer's brain. Neuroreport 19:1085-1089.

Arends YM, Duyckaerts C, Rozemuller JM, Eikelenboom P, Hauw JJ (2000) Microglia, amyloid and dementia in alzheimer disease. A correlative study. Neurobiol Aging 21:39-47.

Arendt T (2003) Synaptic plasticity and cell cycle activation in neurons are alternative effector pathways: the 'Dr. Jekyll and Mr. Hyde concept' of Alzheimer's disease or the yin and yang of neuroplasticity. Prog Neurobiol 71:83-248.

Arispe N, Rojas E, Pollard HB (1993) Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. Proc Natl Acad Sci U S A 90:567-571.

Arvanitis DN, Ducatenzeiler A, Ou JN, Grodstein E, Andrews SD, Tendulkar SR, Ribeiro-da-Silva A, Szyf M, Cuello AC (2007) High intracellular concentrations of amyloid-beta block nuclear translocation of phosphorylated CREB. J Neurochem 103:216-228.

Ashe KH, Zahs KR (2010) Probing the biology of Alzheimer's disease in mice. Neuron 66:631-645.

Atwood CS, Moir RD, Huang X, Scarpa RC, Bacarra NM, Romano DM, Hartshorn MA, Tanzi RE, Bush AI (1998) Dramatic aggregation of Alzheimer abeta by Cu(II) is induced by conditions representing physiological acidosis. J Biol Chem 273:12817-12826.

Baeuerle PA, Henkel T (1994) Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 12:141-179.

Bailey TL, Rivara CB, Rocher AB, Hof PR (2004) The nature and effects of cortical microvascular pathology in aging and Alzheimer's disease. Neurol Res 26:573-578.

Barger SW, Harmon AD (1997) Microglial activation by Alzheimer amyloid precursor protein and modulation by apolipoprotein E. Nature 388:878-881.

Barnes K, Turner AJ, Kenny AJ (1992) Membrane localization of endopeptidase-24.11 and peptidyl dipeptidase A (angiotensin converting enzyme) in the pig brain: a study using subcellular fractionation and electron microscopic immunocytochemistry. J Neurochem 58:2088-2096.

Bartus RT, Dean RL, III, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. Science 217:408-414.

Barucker C, Harmeier A, Weiske J, Albring KF, Fauler B, Lurz R, Huber O, Multhaup G (2011) A novel function of Aβ in gene regulation as an alternative pathway of toxicity. 10th AD/PD Conference, Barcelona, Spain.

Beard CM, Waring SC, O'Brien PC, Kurland LT, Kokmen E (1998) Nonsteroidal anti-inflammatory drug use and Alzheimer's disease: a case-control study in Rochester, Minnesota, 1980 through 1984. Mayo Clin Proc 73:951-955.

Beatty WW, Salmon DP, Butters N, Heindel WC, Granholm EL (1988) Retrograde amnesia in patients with Alzheimer's disease or Huntington's disease. Neurobiol Aging 9:181-186.

Bechmann I, Galea I, Perry VH (2007) What is the blood-brain barrier (not)? Trends Immunol 28:5-11.

Becker RE, Greig NH, Giacobini E (2008) Why do so many drugs for Alzheimer's disease fail in development? Time for new methods and new practices? J Alzheimers Dis 15:303-325.

Bell KF, Cuello AC (2006) Altered synaptic function in Alzheimer's disease. Eur J Pharmacol 545:11-21.

Bell KF, Ducatenzeiler A, Ribeiro-da-Silva A, Duff K, Bennett DA, Cuello AC (2006) The amyloid pathology progresses in a neurotransmitter-specific manner. Neurobiol Aging 27:1644-1657.

Bernstein HG, Ansorge S, Riederer P, Reiser M, Frolich L, Bogerts B (1999) Insulin-degrading enzyme in the Alzheimer's disease brain: prominent localization in neurons and senile plaques. Neurosci Lett 263:161-164.

Bero AW, Yan P, Roh JH, Cirrito JR, Stewart FR, Raichle ME, Lee JM, Holtzman DM (2011) Neuronal activity regulates the regional vulnerability to amyloid-beta deposition. Nat Neurosci.14:750–756.

Bertram L, Lill CM, Tanzi RE (2010) The genetics of Alzheimer disease: back to the future. Neuron 68:270-281.

Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA (2002) Constitutive nuclear factor-kappa B activity is required for central neuron survival. J Neurosci 22:8466-8475.

Bhaskar K, Konerth M, Kokiko-Cochran ON, Cardona A, Ransohoff RM, Lamb BT (2010) Regulation of tau pathology by the microglial fractalkine receptor. Neuron 68:19-31.

Bhaskar K, Miller M, Chludzinski A, Herrup K, Zagorski M, Lamb BT (2009) The PI3K-Akt-mTOR pathway regulates Abeta oligomer induced neuronal cell cycle events. Mol Neurodegener 4:14.

Bianchi ME (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 81:1-5.
Biber K, Neumann H, Inoue K, Boddeke HW (2007) Neuronal 'On' and 'Off' signals control microglia. Trends Neurosci 30:596-602.

Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM (2005) Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 45:675-688.

Bjorkqvist M, et al. (2008) A novel pathogenic pathway of immune activation detectable before clinical onset in Huntington's disease. J Exp Med 205:1869-1877.

Bjurstom H, Wang J, Ericsson I, Bengtsson M, Liu Y, Kumar-Mendu S, Issazadeh-Navikas S, Birnir B (2008) GABA, a natural immunomodulator of T lymphocytes. J Neuroimmunol 205:44-50.

Blasko I, Beer R, Bigl M, Apelt J, Franz G, Rudzki D, Ransmayr G, Kampfl A, Schliebs R (2004a) Experimental traumatic brain injury in rats stimulates the expression, production and activity of Alzheimer's disease beta-secretase (BACE-1). J Neural Transm 111:523-536.

Blasko I, Stampfer-Kountchev M, Robatscher P, Veerhuis R, Eikelenboom P, Grubeck-Loebenstein B (2004b) How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes. Aging Cell 3:169-176.

Blesch A, Uy HS, Grill RJ, Cheng JG, Patterson PH, Tuszynski MH (1999) Leukemia inhibitory factor augments neurotrophin expression and corticospinal axon growth after adult CNS injury. J Neurosci 19:3556-3566.

Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci 8:57-69.

Boche D, Cunningham C, Docagne F, Scott H, Perry VH (2006) TGFbeta1 regulates the inflammatory response during chronic neurodegeneration. Neurobiol Dis 22:638-650.

Bocker R, Estler CJ, Ludewig-Sandig D (1991) Evaluation of the hepatotoxic potential of minocycline. Antimicrob Agents Chemother 35:1434-1436.

Bodles AM, Barger SW (2005) Secreted beta-amyloid precursor protein activates microglia via JNK and p38-MAPK. Neurobiol Aging 26:9-16.

Bourne KZ, Ferrari DC, Lange-Dohna C, Rossner S, Wood TG, Perez-Polo JR (2007) Differential regulation of BACE1 promoter activity by nuclear factor-kappaB in neurons and glia upon exposure to beta-amyloid peptides. J Neurosci Res 85:1194-1204.

Bowen DM, Smith CB, White P, Davison AN (1976) Neurotransmitter related enzymes and indices of hypoxia in senile dementia and other abiotrophies. Brain 99:459-496.

Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol (Berl) 82:239-259.

Breitner JC, Baker LD, Montine TJ, Meinert CL, Lyketsos CG, Ashe KH, Brandt J, Craft S, Evans DE, Green RC, Ismail MS, Martin BK, Mullan MJ, Sabbagh M, Tariot PN (2011) Extended results of the Alzheimer's disease anti-inflammatory prevention trial. Alzheimers Dement 7:402-411.
Breitner JC, Gau BA, Welsh KA, Plassman BL, McDonald WM, Helms MJ, Anthony JC (1994) Inverse association of anti-inflammatory treatments and Alzheimer's disease: initial results of a co-twin control study. Neurology 44:227-232.

Breitner JC, Welsh KA, Helms MJ, Gaskell PC, Gau BA, Roses AD, Pericak-Vance MA, Saunders AM (1995) Delayed onset of Alzheimer's disease with nonsteroidal anti-inflammatory and histamine H2 blocking drugs. Neurobiol Aging 16:523-530.

Britschgi M, Wyss-Coray T (2009) Blood protein signature for the early diagnosis of Alzheimer disease. Arch Neurol 66:161-165.

Brody DL, Magnoni S, Schwetye KE, Spinner ML, Esparza TJ, Stocchetti N, Zipfel GJ, Holtzman DM (2008) Amyloid-beta dynamics correlate with neurological status in the injured human brain. Science 321:1221-1224.

Brown GC, Bal-Price A (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. Mol Neurobiol 27:325-355.

Bruno MA, Leon WC, Fragoso G, Mushynski WE, Almazan G, Cuello AC (2009a) Amyloid beta-induced nerve growth factor dysmetabolism in Alzheimer disease. J Neuropathol Exp Neurol 68:857-869.

Bruno MA, Mufson EJ, Wuu J, Cuello AC (2009b) Increased matrix metalloproteinase 9 activity in mild cognitive impairment. J Neuropathol Exp Neurol 68:1309-1318.

Bruunsgaard H, ndersen-Ranberg K, Hjelmborg JB, Pedersen BK, Jeune B (2003) Elevated levels of tumor necrosis factor alpha and mortality in centenarians. Am J Med 115:278-283.

Busciglio J, Pelsman A, Wong C, Pigino G, Yuan M, Mori H, Yankner BA (2002) Altered metabolism of the amyloid beta precursor protein is associated with mitochondrial dysfunction in Down's syndrome. Neuron 33:677-688.

Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22:183-192.

Buxbaum JD, Thinakaran G, Koliatsos V, O'Callahan J, Slunt HH, Price DL, Sisodia SS (1998) Alzheimer amyloid protein precursor in the rat hippocampus: transport and processing through the perforant path. J Neurosci 18:9629-9637.

Caccamo A, Maldonado MA, Bokov AF, Majumder S, Oddo S (2010) CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 107:22687-22692.

Caccamo A, Oddo S, Sugarman MC, Akbari Y, LaFerla FM (2005) Age- and region-dependent alterations in Abeta-degrading enzymes: implications for Abeta-induced disorders. Neurobiol Aging 26:645-654.

Cagnin A, Brooks DJ, Kennedy AM, Gunn RN, Myers R, Turkheimer FE, Jones T, Banati RB (2001) In-vivo measurement of activated microglia in dementia. Lancet 358:461-467.

Carey RM, Balcz BA, Lopez-Coviella I, Slack BE (2005) Inhibition of dynamin-dependent endocytosis increases shedding of the amyloid precursor protein ectodomain and reduces generation of amyloid beta protein. BMC Cell Biol 6:30.

Carnevale D, De SR, Minghetti L (2007) Microglia-neuron interaction in inflammatory and degenerative diseases: role of cholinergic and noradrenergic systems. CNS Neurol Disord Drug Targets 6:388-397.

Casas C, Sergeant N, Itier JM, Blanchard V, Wirths O, van der KN, Vingtdeux V, van de SE, Ret G, Canton T, Drobecq H, Clark A, Bonici B, Delacourte A, Benavides J, Schmitz C, Tremp G, Bayer TA, Benoit P, Pradier L (2004) Massive CA1/2 neuronal loss with intraneuronal and N-terminal truncated Abeta42 accumulation in a novel Alzheimer transgenic model. Am J Pathol 165:1289-1300.

Caspersen C, Wang N, Yao J, Sosunov A, Chen X, Lustbader JW, Xu HW, Stern D, McKhann G, Yan SD (2005) Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. FASEB J 19:2040-2041.

Caughey B, Lansbury PT (2003) Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. Annu Rev Neurosci 26:267-298.

Cerpa W, Farias GG, Godoy JA, Fuenzalida M, Bonansco C, Inestrosa NC (2010) Wnt-5a occludes Abeta oligomer-induced depression of glutamatergic transmission in hippocampal neurons. Mol Neurodegener 5:3.

Chandeler RL (1961) Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet 1:1378-1379.
Chang KA, Suh YH (2005) Pathophysiological roles of amyloidogenic carboxy-terminal fragments of the beta-amyloid precursor protein in Alzheimer's disease. J Pharmacol Sci 97:461-471.

Chishti MA, et al. (2001) Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695. J Biol Chem 276:21562-21570.

Choi Y, Kim HS, Shin KY, Kim EM, Kim M, Kim HS, Park CH, Jeong YH, Yoo J, Lee JP, Chang KA, Kim S, Suh YH (2007) Minocycline attenuates neuronal cell death and improves cognitive impairment in Alzheimer's disease models. Neuropsychopharmacology 32:2393-2404.

Cirrito JR, Deane R, Fagan AM, Spinner ML, Parsadanian M, Finn MB, Jiang H, Prior JL, Sagare A, Bales KR, Paul SM, Zlokovic BV, Piwnica-Worms D, Holtzman DM (2005) P-glycoprotein deficiency at the blood-brain barrier increases amyloid-beta deposition in an Alzheimer disease mouse model. J Clin Invest 115:3285-3290.

Cirrito JR, Kang JE, Lee J, Stewart FR, Verges DK, Silverio LM, Bu G, Mennerick S, Holtzman DM (2008) Endocytosis is required for synaptic activity-dependent release of amyloid-beta *in vivo*. Neuron 58:42-51.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360:672-674.

Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, Ashe KH (2005) Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci 8:79-84.

Coffey PJ, Perry VH, Rawlins JN (1990) An investigation into the early stages of the inflammatory response following ibotenic acid-induced neuronal degeneration. Neuroscience 35:121-132.

Colton CA, Mott RT, Sharpe H, Xu Q, Van Nostrand WE, Vitek MP (2006) Expression profiles for macrophage alternative activation genes in AD and in mouse models of AD. J Neuroinflammation 3:27.

Combs CK, Karlo JC, Kao SC, Landreth GE (2001) beta-Amyloid stimulation of microglia and monocytes results in TNFalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. J Neurosci 21:1179-1188.

Cook DG, Leverenz JB, McMillan PJ, Kulstad JJ, Ericksen S, Roth RA, Schellenberg GD, Jin LW, Kovacina KS, Craft S (2003) Reduced hippocampal insulin-degrading enzyme in late-onset Alzheimer's disease is associated with the apolipoprotein E-epsilon4 allele. Am J Pathol 162:313-319.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921-923.

Cornet S, Spinnewyn B, Delaflotte S, Charnet C, Roubert V, Favre C, Hider H, Chabrier PE, Auguet M (2004) Lack of evidence of direct mitochondrial involvement in the neuroprotective effect of minocycline. Eur J Pharmacol 505:111-119.

Costa-Baena N, Sepulveda-Falla D, Lopera-Gomez CM, Jaramillo-Elorza MC, Moreno S, guirre-Acevedo DC, Saldarriaga A, Lopera F (2011) Pre-dementia clinical stages in presenilin 1 E280A familial early-onset Alzheimer's disease: a retrospective cohort study. Lancet Neurol 10:213-220.

Côté S, Ribeiro-da-Silva A, Cuello AC (1993) Current protocols for light microscopy immunocytochemistry. In: Immunohistochemistry II (Cuello AC, ed), pp 147-168. Chichester: John Wiley & Sons.

Coulson EJ, Paliga K, Beyreuther K, Masters CL (2000) What the evolution of the amyloid protein precursor supergene family tells us about its function. Neurochem Int 36:175-184.

Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420:860-867.

Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. Nat Rev Immunol 7:255-266.

Cuello AC (2007) Overview of the Alzheimer's Disease Pathology and Potential Therapeutic Targets. In: Pharmacological Mechanisms in Alzheimer's Therapeutics (Cuello AC, ed), New York: Springer.

Cunningham C, Deacon R, Wells H, Boche D, Waters S, Diniz CP, Scott H, Rawlins JN, Perry VH (2003) Synaptic changes characterize early behavioural signs in the ME7 model of murine prion disease. Eur J Neurosci 17:2147-2155.

Cunningham C, Wilcockson DC, Campion S, Lunnon K, Perry VH (2005) Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. J Neurosci 25:9275-9284.

Czirr E, Cottrell BA, Leuchtenberger S, Kukar T, Ladd TB, Esselmann H, Paul S, Schubenel R, Torpey JW, Pietrzik CU, Golde TE, Wiltfang J, Baumann K, Koo EH, Weggen S (2008) Independent generation of Abeta42 and Abeta38 peptide species by gamma-secretase. J Biol Chem 283:17049-17054.

D'Andrea MR, Nagele RG, Wang HY, Peterson PA, Lee DH (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38:120-134.

da Silva RP, Gordon S (1999) Phagocytosis stimulates alternative glycosylation of macrosialin (mouse CD68), a macrophage-specific endosomal protein. Biochem J 338 (Pt 3):687-694.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 9:46-56.

Das P, Smithson LA, Price RW, Holloway VM, Levites Y, Chakrabarty P, Golde TE (2006) Interleukin-1 receptor 1 knockout has no effect on amyloid deposition in Tg2576 mice and does not alter efficacy following Abeta immunotherapy. J Neuroinflammation 3:17.

Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB (2005) ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci 8:752-758.

Davies P, Maloney AJF (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 2 (8000):1403.

Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci U S A 88:6368-6371.

De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WL (2007) Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. J Biol Chem 282:11590-11601.

De Felice FG, Vieira MN, Bomfim TR, Decker H, Velasco PT, Lambert MP, Viola KL, Zhao WQ, Ferreira ST, Klein WL (2009) Protection of synapses

against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Abeta oligomers. Proc Natl Acad Sci U S A 106:1971-1976.

De Simone R, Ajmone-Cat MA, Carnevale D, Minghetti L (2005) Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures. J Neuroinflammation 2:4.

Deane R, et al. (2003) RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. Nat Med 9:907-913.

Decker H, Jurgensen S, Adrover MF, Brito-Moreira J, Bomfim TR, Klein WL, Epstein AL, De Felice FG, Jerusalinsky D, Ferreira ST (2010) N-methyl-D-aspartate receptors are required for synaptic targeting of Alzheimer's toxic amyloid-beta peptide oligomers. J Neurochem 115:1520-1529.

DeKosky ST, Ikonomovic MD, Styren SD, Beckett L, Wisniewski S, Bennett DA, Cochran EJ, Kordower JH, Mufson EJ (2002) Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment. Ann Neurol 51:145-155.

Demuro A, Parker I, Stutzmann GE (2010) Calcium signaling and amyloid toxicity in Alzheimer disease. J Biol Chem 285:12463-12468.

Deshpande A, Mina E, Glabe C, Busciglio J (2006) Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. J Neurosci 26:6011-6018.

DiCarlo G, Wilcock D, Henderson D, Gordon M, Morgan D (2001) Intrahippocampal LPS injections reduce Abeta load in APP+PS1 transgenic mice. Neurobiol Aging 22:1007-1012.

Dineley KT, Westerman M, Bui D, Bell K, Ashe KH, Sweatt JD (2001) Beta-amyloid activates the mitogen-activated protein kinase cascade via hippocampal alpha7 nicotinic acetylcholine receptors: *In vitro* and in vivo mechanisms related to Alzheimer's disease. J Neurosci 21:4125-4133.

Dodart JC, May P (2005) Overview on rodent models of Alzheimer's disease. Curr Protoc Neurosci Chapter 9:Unit 9.22.

Donahue JE, Flaherty SL, Johanson CE, Duncan JA, III, Silverberg GD, Miller MC, Tavares R, Yang W, Wu Q, Sabo E, Hovanesian V, Stopa EG (2006) RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. Acta Neuropathol 112:405-415.

Doody RS, Massman P, Mahurin R, Law S (1995) Positive and negative neuropsychiatric features in Alzheimer's disease. J Neuropsychiatry Clin Neurosci 7:54-60.

Dougherty JJ, Wu J, Nichols RA (2003) Beta-amyloid regulation of presynaptic nicotinic receptors in rat hippocampus and neocortex. J Neurosci 23:6740-6747.

Drachman DA, Leavitt J (1974) Human memory and the cholinergic system: A relationship to aging? Arch Neurol 30:113-121.

Du Y, Dodel R, Hampel H, Buerger K, Lin S, Eastwood B, Bales K, Gao F, Moeller HJ, Oertel W, Farlow M, Paul S (2001) Reduced levels of amyloid beta-peptide antibody in Alzheimer disease. Neurology 57:801-805.

Echeverria V, Ducatenzeiler A, Alhonen L, Janne J, Grant SM, Wandosell F, Muro A, Baralle F, Li H, Duff K, Szyf M, Cuello AC (2004a) Rat transgenic models with a phenotype of intracellular Abeta accumulation in hippocampus and cortex. J Alzheimers Dis 6:209-219.

Echeverria V, Ducatenzeiler A, Chen CH, Cuello AC (2005) Endogenous beta-amyloid peptide synthesis modulates cAMP response element-regulated gene expression in PC12 cells. Neuroscience 135:1193-1202.

Echeverria V, Ducatenzeiler A, Chen CH, Cuello AC (2004b) Endogenous Aß peptide synthesis modulates CRE-regulated gene expression in PC12 cells. J Biol Chem Submitted.

Echeverria V, Ducatenzeiler A, Dowd E, Janne J, Grant SM, Szyf M, Wandosell F, Avila J, Grimm H, Dunnett SB, Hartmann T, Alhonen L, Cuello AC (2004c) Altered mitogen-activated protein kinase signaling, tau hyperphosphorylation and mild spatial learning dysfunction in transgenic rats expressing the beta-amyloid peptide intracellularly in hippocampal and cortical neurons. Neuroscience 129:583-592.

Eckman EA, Eckman CB (2005) Abeta-degrading enzymes: Modulators of Alzheimer's disease pathogenesis and targets for therapeutic intervention. Biochemical Society Symposium 33:1101-1105.

Edison P, Archer HA, Gerhard A, Hinz R, Pavese N, Turkheimer FE, Hammers A, Tai YF, Fox N, Kennedy A, Rossor M, Brooks DJ (2008) Microglia, amyloid, and cognition in Alzheimer's disease: An [11C](R)PK11195-PET and [11C]PIB-PET study. Neurobiol Dis 32:412-419.

Eikelenboom P, Stam FC (1982) Immunoglobulins and complement factors in senile plaques. An immunoperoxidase study. Acta Neuropathol 57:239-242.

Eikelenboom P, Veerhuis R, Exel EV, Hoozemans JJ, Rozemuller AJ, Van Gool WA (2011) The Early Involvement of the Innate Immunity in the Pathogenesis of Late-OnsetAlzheimer's Disease: Neuropathological, Epidemiological and Genetic Evidence. Curr Alzheimer Res 8:142-150.

Eikelenboom P, Veerhuis R, Scheper W, Rozemuller AJ, Van Gool WA, Hoozemans JJ (2006) The significance of neuroinflammation in understanding Alzheimer's disease. J Neural Transm 113:1685-1695.

Etienne P, Robitaille Y, Wood P, Gauthier S, Nair NP, Quirion R (1986) Nucleus basalis neuronal loss, neuritic plaques and choline acetyltransferase activity in advanced Alzheimer's disease. Neuroscience 19:1279-1291.

Fagan SC, Edwards DJ, Borlongan CV, Xu L, Arora A, Feuerstein G, Hess DC (2004) Optimal delivery of minocycline to the brain: implication for human studies of acute neuroprotection. Exp Neurol 186:248-251.

Fan R, Xu F, Previti ML, Davis J, Grande AM, Robinson JK, Van Nostrand WE (2007) Minocycline reduces microglial activation and improves behavioral deficits in a transgenic model of cerebral microvascular amyloid. J Neurosci 27:3057-3063.

Farber K, Kettenmann H (2005) Physiology of microglial cells. Brain Res Brain Res Rev 48:133-143.

Farkas E, De Jong GI, de Vos RA, Jansen Steur EN, Luiten PG (2000) Pathological features of cerebral cortical capillaries are doubled in Alzheimer's disease and Parkinson's disease. Acta Neuropathol 100:395-402.

Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proc Natl Acad Sci U S A 100:4162-4167.

Fernandez-Vizarra P, Fernandez AP, Castro-Blanco S, Serrano J, Bentura ML, Martinez-Murillo R, Martinez A, Rodrigo J (2004) Intra- and extracellular Abeta and PHF in clinically evaluated cases of Alzheimer's disease. Histol Histopathol 19:823-844.

Ferrer I, Marti E, Lopez E, Tortosa A (1998) NF-kB immunoreactivity is observed in association with beta A4 diffuse plaques in patients with Alzheimer's disease. Neuropathol Appl Neurobiol 24:271-277.

Ferretti MT, Bruno MA, Ducatenzeiler A, Klein WL, Cuello AC (2011a) Intracellular Abeta-oligomers and early inflammation in a model of Alzheimer's disease. Neurobiol Aging, March 15, e-pub ahead of printing.

Ferretti MT, Cuello AC (2011) Does a pro-inflammatory process precede Alzheimer's disease and mild cognitive impairment? Curr Alzheimer Res 8:164-174.

Ferretti MT, Partridge V, Leon WC, Canneva F, Allard S, Arvanitis DN, Vercauteren F, Houle D, Ducatenzeiler A, Klein WL, Glabe CG, Szyf M, Cuello AC (2011b) Transgenic mice as a model of pre-clinical Alzheimer's disease. Curr Alzheimer Res 8:4-23.

Fiala M, Lin J, Ringman J, Kermani-Arab V, Tsao G, Patel A, Lossinsky AS, Graves MC, Gustavson A, Sayre J, Sofroni E, Suarez T, Chiappelli F, Bernard G (2005) Ineffective phagocytosis of amyloid-beta by macrophages of Alzheimer's disease patients. J Alzheimers Dis 7:221-232.

Filbin MT (2006) How inflammation promotes regeneration. Nat Neurosci 9:715-717.

Finder VH, Vodopivec I, Nitsch RM, Glockshuber R (2010) The recombinant amyloid-beta peptide Abeta1-42 aggregates faster and is more neurotoxic than synthetic Abeta1-42. J Mol Biol 396:9-18.

Floden AM, Combs CK (2006) Beta-amyloid stimulates murine postnatal and adult microglia cultures in a unique manner. J Neurosci 26:4644-4648.

Floden AM, Li S, Combs CK (2005) Beta-amyloid-stimulated microglia induce neuron death via synergistic stimulation of tumor necrosis factor alpha and NMDA receptors. J Neurosci 25:2566-2575.

Forstl H, Kurz A (1999) Clinical features of Alzheimer's disease. Eur Arch Psychiatry Clin Neurosci 249:288-290.

Frackowiak J, Zoltowska A, Wisniewski HM (1994) Non-fibrillar beta-amyloid protein is associated with smooth muscle cells of vessel walls in Alzheimer disease. J Neuropathol Exp Neurol 53:637-645.

Franceschi C, Capri M, Monti D, Giunta S, Olivieri F, Sevini F, Panourgia MP, Invidia L, Celani L, Scurti M, Cevenini E, Castellani GC, Salvioli S (2007)

Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev 128:92-105.

Francis PT, Palmer AM, Snape M, Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry 66:137-147.

Franklin KBJ, Paxinos G (1997) The Mouse Brain in Stereotaxic Coordinates. San Diego: Academic Press.

Fuhrmann M, Bittner T, Jung CK, Burgold S, Page RM, Mitteregger G, Haass C, LaFerla FM, Kretzschmar H, Herms J (2010) Microglial Cx3cr1 knockout prevents neuron loss in a mouse model of Alzheimer's disease. Nat Neurosci 13:411-413.

Fujio H, Takagaki Y, Ha YM, Doi EM, Soebandrio A, Sakato N (1985) Native and non-native conformation-specific antibodies directed to the loop region of hen egg-white lysozyme. J Biochem 98:949-962.

Fukumoto H, Cheung BS, Hyman BT, Irizarry MC (2002) Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch Neurol 59:1381-1389.

Funato H, Enya M, Yoshimura M, Morishima-Kawashima M, Ihara Y (1999) Presence of sodium dodecyl sulfate-stable amyloid beta-protein dimers in the hippocampus CA1 not exhibiting neurofibrillary tangle formation. Am J Pathol 155:23-28.

Galea I, Bechmann I, Perry VH (2007) What is immune privilege (not)? Trends Immunol 28:12-18.

Galimberti D, Schoonenboom N, Scheltens P, Fenoglio C, Bouwman F, Venturelli E, Guidi I, Blankenstein MA, Bresolin N, Scarpini E (2006) Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease. Arch Neurol 63:538-543.

Games D, et al. (1995) Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature 373:523-527.

Garofalo L, Elliott PJ, Cuello AC (1992) Behavioral response of rats with cortical lesions to cholinomimetics. Physiol Behav 52:971-977.

Gartner S (2000) HIV infection and dementia. Science 287:602-604.
Garwood CJ, Cooper JD, Hanger DP, Noble W (2010) Anti-inflammatory impact of minocycline in a mouse model of tauopathy. Front Psychiatry 1:136.

Gasque P, Dean YD, McGreal EP, VanBeek J, Morgan BP (2000) Complement components of the innate immune system in health and disease in the CNS. Immunopharmacology 49:171-186.

Geddes JW, Tekirian TL, Mattson MP (1999) N-terminus truncated beta-amyloid peptides and C-terminus truncated secreted forms of amyloid precursor protein: distinct roles in the pathogenesis of Alzheimer's disease. Neurobiol Aging 20:75-79.

Geddes JW, Wong J, Choi BH, Kim RC, Cotman CW, Miller FD (1990) Increased expression of the embryonic form of a developmentally regulated mRNA in Alzheimer's disease. Neurosci Lett 109:54-61.

Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K (2010) Development of monocytes, macrophages, and dendritic cells. Science 327:656-661.

Giacobini E (2004) Cholinesterase inhibitors: new roles and therapeutic alternatives. Pharmacol Res 50:433-440.

Gilmor ML, Nash NR, Roghani A, Edwards RH, Yi H, Hersch SM, Levey AI (1996) Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. J Neurosci 16:2179-2190.

Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M (2010) Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science 330:841-845.

Giuffrida ML, Caraci F, Pignataro B, Cataldo S, De BP, Bruno V, Molinaro G, Pappalardo G, Messina A, Palmigiano A, Garozzo D, Nicoletti F, Rizzarelli E, Copani A (2009) Beta-amyloid monomers are neuroprotective. J Neurosci 29:10582-10587.

Giunta B, Fernandez F, Nikolic WV, Obregon D, Rrapo E, Town T, Tan J (2008) Inflammaging as a prodrome to Alzheimer's disease. J Neuroinflammation 5:51.
Glabe CC (2005) Amyloid accumulation and pathogensis of Alzheimer's disease: significance of monomeric, oligomeric and fibrillar Abeta. Subcell Biochem 38:167-177.

Glabe CG (2008) Structural classification of toxic amyloid oligomers. J Biol Chem 283:29639-29643.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH (2010) Mechanisms underlying inflammation in neurodegeneration. Cell 140:918-934.

Glass JD, Fedor H, Wesselingh SL, McArthur JC (1995) Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia. Ann Neurol 38:755-762.

Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120:885-890.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, . (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349:704-706.

Goedert M (2009) Oskar Fischer and the study of dementia. Brain 132:1102-1111.

Goedert M, Hasegawa M, Jakes R, Lawler S, Cuenda A, Cohen P (1997) Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. FEBS Lett 409:57-62.

Goedert M, Jakes R, Crowther RA, Six J, Lubke U, Vandermeeren M, Cras P, Trojanowski JQ, Lee VM (1993) The abnormal phosphorylation of tau protein at Ser-202 in Alzheimer disease recapitulates phosphorylation during development. Proc Natl Acad Sci U S A 90:5066-5070.

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519-526.

Golde TE (2005) The Abeta hypothesis: leading us to rationally-designed therapeutic strategies for the treatment or prevention of Alzheimer disease. Brain Pathol 15:84-87.

Gomez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC, Parisi JE, Hyman BT (1997) Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. Ann Neurol 41:17-24.

Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA, Klein WL (2003) Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss. Proc Natl Acad Sci U S A 100:10417-10422.

Good PF, Werner P, Hsu A, Olanow CW, Perl DP (1996) Evidence of neuronal oxidative damage in Alzheimer's disease. Am J Pathol 149:21-28.

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A 77:7380-7384.

Gordon PH, Moore DH, Miller RG, Florence JM, Verheijde JL, Doorish C, Hilton JF, Spitalny GM, MacArthur RB, Mitsumoto H, Neville HE, Boylan K, Mozaffar T, Belsh JM, Ravits J, Bedlack RS, Graves MC, McCluskey LF, Barohn RJ, Tandan R (2007) Efficacy of minocycline in patients with amyotrophic lateral sclerosis: a phase III randomised trial. Lancet Neurol 6:1045-1053.

Gordon S (2002) Pattern recognition receptors: doubling up for the innate immune response. Cell 111:927-930.

Gotz J (2001) Tau and transgenic animal models. Brain Res Brain Res Rev 35:266-286.

Gotz J, Ittner LM (2008) Animal models of Alzheimer's disease and frontotemporal dementia. Nat Rev Neurosci 9:532-544.

Gouras GK, Almeida CG, Takahashi RH (2005) Intraneuronal Abeta accumulation and origin of plaques in Alzheimer's disease. Neurobiol Aging 26:1235-1244.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000) Intraneuronal Abeta42 accumulation in human brain. Am J Pathol 156:15-20.

Grant SM, Ducatenzeiler A, Szyf M, Cuello AC (2000) Abeta immunoreactive material is present in several intracellular compartments in transfected, neuronally differentiated, P19 cells expressing the human amyloid beta-protein precursor. J Alzheimers Dis 2:207-222.

Grant SM, Shankar SL, Chalmers-Redman RME, Tatton WG, Szyf M, Cuello AC (1999) Mitochondrial abnormalities in neuroectodermal cells stably expressing human amyloid precursor protein ($hAPP_{751}$). Neuroreport 10:41-46.

Grathwohl SA, Kalin RE, Bolmont T, Prokop S, Winkelmann G, Kaeser SA, Odenthal J, Radde R, Eldh T, Gandy S, Aguzzi A, Staufenbiel M, Mathews PM, Wolburg H, Heppner FL, Jucker M (2009) Formation and maintenance of Alzheimer's disease beta-amyloid plaques in the absence of microglia. Nat Neurosci 12:1361-1363.

Gravina SA, Ho L, Eckman CB, Long KE, Otvos L, Jr., Younkin LH, Suzuki N, Younkin SG (1995) Amyloid beta protein (A beta) in Alzheimer's disease brain.

Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43). J Biol Chem 270:7013-7016.

Grbovic OM, Mathews PM, Jiang Y, Schmidt SD, Dinakar R, Summers-Terio NB, Ceresa BP, Nixon RA, Cataldo AM (2003) Rab5-stimulated up-regulation of the endocytic pathway increases intracellular beta-cleaved amyloid precursor protein carboxyl-terminal fragment levels and Abeta production. J Biol Chem 278:31261-31268.

Greenfield JP, Tsai J, Gouras GK, Hai B, Thinakaran G, Checler F, Sisodia SS, Greengard P, Xu HX (1999) Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer beta-amyloid peptides. Proc Natl Acad Sci USA 96:742-747.

Griffin MO, Fricovsky E, Ceballos G, Villarreal F (2010) Tetracyclines: a pleitropic family of compounds with promising therapeutic properties. Review of the literature. Am J Physiol Cell Physiol 299:C539-C548.

Griffin WS (2006) Inflammation and neurodegenerative diseases. Am J Clin Nutr 83:470S-474S.

Griffin WS, Stanley LC, Ling C, White L, MacLeod V, Perrot LJ, White CL, III, Araoz C (1989) Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. Proc Natl Acad Sci U S A 86:7611-7615.

Grilli M, Ribola M, Alberici A, Valerio A, Memo M, Spano P (1995) Identification and characterization of a kappa B/Rel binding site in the regulatory region of the amyloid precursor protein gene. J Biol Chem 270:26774-26777.

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI (1986) Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U S A 83:4913-4917.

Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS, Jr. (1999) NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 19:5785-5799.

Gyure KA, Durham R, Stewart WF, Smialek JE, Troncoso JC (2001) Intraneuronal abeta-amyloid precedes development of amyloid plaques in Down syndrome. Arch Pathol Lab Med 125:489-492.

Haass C, Schlossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DB (1992) Amyloid b-peptide is produced by cultured cells during normal metabolism. Nature 359:322-325.

Haffner SM (2003) Insulin resistance, inflammation, and the prediabetic state. Am J Cardiol 92:18J-26J.

Halmekyto M, Hyttinen JM, Sinervirta R, Utriainen M, Myohanen S, Voipio HM, Wahlfors J, Syrjanen S, Syrjanen K, Alhonen L, . (1991) Transgenic mice aberrantly expressing human ornithine decarboxylase gene. J Biol Chem 266:19746-19751.

Hanes J, Zilka N, Bartkova M, Caletkova M, Dobrota D, Novak M (2009) Rat tau proteome consists of six tau isoforms: implication for animal models of human tauopathies. J Neurochem 108:1167-1176.

Hanisch UK, Kettenmann H (2007) Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci 10:1387-1394.

Hansson Petersen CA, Alikhani N, Behbahani H, Wiehager B, Pavlov PF, Alafuzoff I, Leinonen V, Ito A, Winblad B, Glaser E, Ankarcrona M (2008) The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. Proc Natl Acad Sci U S A 105:13145-13150.

Harada H, Tamaoka A, Ishii K, Shoji S, Kametaka S, Kametani F, Saito Y, Murayama S (2006) Beta-site APP cleaving enzyme 1 (BACE1) is increased in remaining neurons in Alzheimer's disease brains. Neurosci Res 54:24-29.
Hardy J (2007) Does Abeta 42 have a function related to blood homeostasis? Neurochem Res 32:833-835.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353-356.
Hardy JA, Higgins GA (1992) Alzheimer's disease: The amyloid cascade hypothesis. Science 256:184-185.

Haroutunian V, Hoffman LB, Beeri MS (2009) Is there a neuropathology difference between mild cognitive impairment and dementia? Dialogues Clin Neurosci 11:171-179.

Harper JD, Wong SS, Lieber CM, Lansbury PT, Jr. (1999) Assembly of A beta amyloid protofibrils: an in vitro model for a possible early event in Alzheimer's disease. Biochemistry 38:8972-8980.

Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J Neurosci 19:8876-8884.

Hashimoto M, Bogdanovic N, Volkmann I, Aoki M, Winblad B, Tjernberg LO (2010) Analysis of microdissected human neurons by a sensitive ELISA reveals a correlation between elevated intracellular concentrations of Abeta42 and Alzheimer's disease neuropathology. Acta Neuropathol 119:543-554.

Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D (2006) The P2Y12 receptor regulates microglial activation by extracellular nucleotides. Nat Neurosci 9:1512-1519.

Head E, Garzon-Rodriguez W, Johnson JK, Lott IT, Cotman CW, Glabe C (2001) Oxidation of Abeta and plaque biogenesis in Alzheimer's disease and Down syndrome. Neurobiol Dis 8:792-806.

Heneka MT, Nadrigny F, Regen T, Martinez-Hernandez A, Dumitrescu-Ozimek L, Terwel D, Jardanhazi-Kurutz D, Walter J, Kirchhoff F, Hanisch UK, Kummer MP (2010) Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proc Natl Acad Sci U S A 107:6058-6063.

Heneka MT, Sastre M, Dumitrescu-Ozimek L, Dewachter I, Walter J, Klockgether T, van LF (2005a) Focal glial activation coincides with increased BACE1 activation and precedes amyloid plaque deposition in APP[V717I] transgenic mice. J Neuroinflammation 2:22.

Heneka MT, Sastre M, Dumitrescu-Ozimek L, Hanke A, Dewachter I, Kuiperi C, O'Banion K, Klockgether T, van LF, Landreth GE (2005b) Acute treatment with the PPARgamma agonist pioglitazone and ibuprofen reduces glial inflammation and Abeta1-42 levels in APPV717I transgenic mice. Brain 128:1442-1453.

Heneka MT, Wiesinger H, Dumitrescu-Ozimek L, Riederer P, Feinstein DL, Klockgether T (2001) Neuronal and glial coexpression of argininosuccinate synthetase and inducible nitric oxide synthase in Alzheimer disease. J Neuropathol Exp Neurol 60:906-916.

Hernandez D, Sugaya K, Qu T, McGowan E, Duff K, McKinney M (2001) Survival and plasticity of basal forebrain cholinergic systems in mice transgenic for presenilin-1 and amyloid precursor protein mutant genes. Neuroreport 12:1377-1384.

Herrup K (2010) The involvement of cell cycle events in the pathogenesis of Alzheimer's disease. Alzheimers Res Ther 2:13.

Herrup K, Busser JC (1995) The induction of multiple cell cycle events precedes target-related neuronal death. Development 121:2385-2395.

Herrup K, Yang Y (2007) Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? Nat Rev Neurosci 8:368-378.

Hirai K, Aliev G, Nunomura A, Fujioka H, Russell RL, Atwood CS, Johnson AB, Kress Y, Vinters HV, Tabaton M, Shimohama S, Cash AD, Siedlak SL, Harris PL, Jones PK, Petersen RB, Perry G, Smith MA (2001) Mitochondrial abnormalities in Alzheimer's disease. J Neurosci 21:3017-3023.

Hirata-Fukae C, Li HF, Hoe HS, Gray AJ, Minami SS, Hamada K, Niikura T, Hua F, Tsukagoshi-Nagai H, Horikoshi-Sakuraba Y, Mughal M, Rebeck GW, LaFerla FM, Mattson MP, Iwata N, Saido TC, Klein WL, Duff KE, Aisen PS, Matsuoka Y (2008) Females exhibit more extensive amyloid, but not tau, pathology in an Alzheimer transgenic model. Brain Res 1216:92-103.

Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, Saad I, Mueller R, Morgan D, Sanders S, Zehr C, O'Campo K, Hardy J, Prada CM, Eckman C, Younkin S, Hsiao K, Duff K (1998) Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat Med 4:97-100.

Holmes C, Boche D, Wilkinson D, Yadegarfar G, Hopkins V, Bayer A, Jones RW, Bullock R, Love S, Neal JW, Zotova E, Nicoll JA (2008) Long-term effects of Abeta42 immunisation in Alzheimer's disease: follow-up of a randomised, placebo-controlled phase I trial. Lancet 372:216-223.

Holmes C, Cunningham C, Zotova E, Woolford J, Dean C, Kerr S, Culliford D, Perry VH (2009) Systemic inflammation and disease progression in Alzheimer disease. Neurology 73:768-774.

Holmin S, Mathiesen T (1999) Long-term intracerebral inflammatory response after experimental focal brain injury in rat. Neuroreport 10:1889-1891.

Holtzman JL (2010) Are we prepared to deal with the Alzheimer's disease pandemic? Clin Pharmacol Ther 88:563-565.

Honda K, Casadesus G, Petersen RB, Perry G, Smith MA (2004) Oxidative stress and redox-active iron in Alzheimer's disease. Ann N Y Acad Sci 1012:179-182.

Hoozemans JJ, Bruckner MK, Rozemuller AJ, Veerhuis R, Eikelenboom P, Arendt T (2002) Cyclin D1 and cyclin E are co-localized with cyclo-oxygenase 2 (COX-2) in pyramidal neurons in Alzheimer disease temporal cortex. J Neuropathol Exp Neurol 61:678-688.

Hoozemans JJ, Veerhuis R, Rozemuller AJ, Arendt T, Eikelenboom P (2004) Neuronal COX-2 expression and phosphorylation of pRb precede p38 MAPK

activation and neurofibrillary changes in AD temporal cortex. Neurobiol Dis 15:492-499.

Hoozemans JJ, Veerhuis R, Rozemuller JM, Eikelenboom P (2006) Neuroinflammation and regeneration in the early stages of Alzheimer's disease pathology. Int J Dev Neurosci 24:157-165.

Hoshi M, Sato M, Matsumoto S, Noguchi A, Yasutake K, Yoshida N, Sato K (2003) Spherical aggregates of beta-amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase I/glycogen synthase kinase-3beta. Proc Natl Acad Sci U S A 100:6370-6375.

Houades V, Koulakoff A, Ezan P, Seif I, Giaume C (2008) Gap junction-mediated astrocytic networks in the mouse barrel cortex. J Neurosci 28:5207-5217.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274:99-102.

Hu L, Wong TP, Cote SL, Bell KF, Cuello AC (2003) The impact of Abeta-plaques on cortical cholinergic and non-cholinergic presynaptic boutons in alzheimer's disease-like transgenic mice. Neuroscience 121:421-432.

Hyman BT, Trojanowski JQ (1997) Consensus recommendations for the postmortem diagnosis of Alzheimer disease from the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease. J Neuropathol Exp Neurol 56:1095-1097.

Iadecola C, Forster C, Nogawa S, Clark HB, Ross ME (1999) Cyclooxygenase-2 immunoreactivity in the human brain following cerebral ischemia. Acta Neuropathol 98:9-14.

Ikonomovic MD, Mufson EJ, Wuu J, Cochran EJ, Bennett DA, DeKosky ST (2003) Cholinergic plasticity in hippocampus of individuals with mild cognitive impairment: correlation with Alzheimer's neuropathology. J Alzheimers Dis 5:39-48.

In' t Veld, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, Breteler MM, Stricker BH (2001) Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. N Engl J Med 345:1515-1521.

Inestrosa N, De Ferrari GV, Garrido JL, Alvarez A, Olivares GH, Barria MI, Bronfman M, Chacon MA (2002) Wnt signaling involvement in beta-amyloid-dependent neurodegeneration. Neurochem Int 41:341-344.

Iqbal K, Alonso AC, Chen S, Chohan MO, El-Akkad E, Gong CX, Khatoon S, Li B, Liu F, Rahman A, Tanimukai H, Grundke-Iqbal I (2005) Tau pathology in Alzheimer disease and other tauopathies. Biochim Biophys Acta 1739:198-210.

Iqbal K, Grundke-Iqbal I (2008) Alzheimer neurofibrillary degeneration: significance, etiopathogenesis, therapeutics and prevention. J Cell Mol Med 12:38-55.

Ishii T, Haga S (1984) Immuno-electron-microscopic localization of complements in amyloid fibrils of senile plaques. Acta Neuropathol 63:296-300.

Iwata N, Higuchi M, Saido TC (2005) Metabolism of amyloid-beta peptide and Alzheimer's disease. Pharmacol Ther 108:129-148.

Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saido TC (2001) Metabolic regulation of brain Abeta by neprilysin. Science 292:1550-1552.

Jack CR, Jr., Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ (2010) Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet Neurol 9:119-128.

Janelsins MC, Mastrangelo MA, Oddo S, LaFerla FM, Federoff HJ, Bowers WJ (2005) Early correlation of microglial activation with enhanced tumor necrosis factor-alpha and monocyte chemoattractant protein-1 expression specifically within the entorhinal cortex of triple transgenic Alzheimer's disease mice. J Neuroinflammation 2:23.

Jantzen PT, Connor KE, DiCarlo G, Wenk GL, Wallace JL, Rojiani AM, Coppola D, Morgan D, Gordon MN (2002) Microglial activation and beta -amyloid deposit reduction caused by a nitric oxide-releasing nonsteroidal anti-inflammatory drug in amyloid precursor protein plus presenilin-1 transgenic mice. J Neurosci 22:2246-2254.

Jarrett JT, Berger EP, Lansbury PT, Jr. (1993) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32:4693-4697.

Jeynes B, Provias J (2008) Evidence for altered LRP/RAGE expression in Alzheimer lesion pathogenesis. Curr Alzheimer Res 5:432-437.

Jiang Q, Lee CY, Mandrekar S, Wilkinson B, Cramer P, Zelcer N, Mann K, Lamb B, Willson TM, Collins JL, Richardson JC, Smith JD, Comery TA, Riddell D,

Holtzman DM, Tontonoz P, Landreth GE (2008) ApoE promotes the proteolytic degradation of Abeta. Neuron 58:681-693.

Jimenez S, Baglietto-Vargas D, Caballero C, Moreno-Gonzalez I, Torres M, Sanchez-Varo R, Ruano D, Vizuete M, Gutierrez A, Vitorica J (2008) Inflammatory response in the hippocampus of PS1M146L/APP751SL mouse model of Alzheimer's disease: age-dependent switch in the microglial phenotype from alternative to classic. J Neurosci 28:11650-11661.

Jin M, Shepardson N, Yang T, Chen G, Walsh D, Selkoe DJ (2011) Soluble amyloid {beta}-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration. Proc Natl Acad Sci U S A 108:5819-5824.

Jones L, et al. (2010) Genetic evidence implicates the immune system and cholesterol metabolism in the aetiology of Alzheimer's disease. PLoS One 5:e13950.

Jordan J, Fernandez-Gomez FJ, Ramos M, Ikuta I, Aguirre N, Galindo MF (2007) Minocycline and cytoprotection: shedding new light on a shadowy controversy. Curr Drug Deliv 4:225-231.

Kaether C, Lammich S, Edbauer D, Ertl M, Rietdorf J, Capell A, Steiner H, Haass C (2002) Presenilin-1 affects trafficking and processing of betaAPP and is targeted in a complex with nicastrin to the plasma membrane. J Cell Biol 158:551-561.

Kaltschmidt B, Uherek M, Volk B, Baeuerle PA, Kaltschmidt C (1997) Transcription factor NF-kappaB is activated in primary neurons by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. Proc Natl Acad Sci U S A 94:2642-2647.

Kaltschmidt B, Widera D, Kaltschmidt C (2005) Signaling via NF-kappaB in the nervous system. Biochim Biophys Acta 1745:287-299.

Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R (2003) APP processing and synaptic function. Neuron 37:925-937.

Kandel ER (1991) Cellular mechanisms of learning and the biological basis of individuality. In: Principles of neural science (Kandel ER, Schwartz JH, Jessell TM, eds), pp 1009-1032. Englewood Cliffs, NJ: Prentice Hall.

Kang J, Lemaire H-G, Unterbeck A, Salbaum JM, Masters CL, Grzeschik K-H, Multhaup G, Beyreuther K, Muller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell surface receptor. Nature 325:733-736.

Kang JE, Lim MM, Bateman RJ, Lee JJ, Smyth LP, Cirrito JR, Fujiki N, Nishino S, Holtzman DM (2009) Amyloid-beta dynamics are regulated by orexin and the sleep-wake cycle. Science 326:1005-1007.

Kaufmann WE, Worley PF, Pegg J, Bremer M, Isakson P (1996) COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proc Natl Acad Sci U S A 93:2317-2321.

Kaushal V, Schlichter LC (2008) Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. J Neurosci 28:2221-2230.

Kayed R, Head E, Sarsoza F, Saing T, Cotman CW, Necula M, Margol L, Wu J, Breydo L, Thompson JL, Rasool S, Gurlo T, Butler P, Glabe CG (2007) Fibril specific, conformation dependent antibodies recognize a generic epitope common to amyloid fibrils and fibrillar oligomers that is absent in prefibrillar oligomers. Mol Neurodegener 2:18.

Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 300:486-489.

Keller AF, Gravel M, Kriz J (2011) Treatment with minocycline after disease onset alters astrocyte reactivity and increases microgliosis in SOD1 mutant mice. Exp Neurol 228:69-79.

Kettenmann H, Verkhratsky A (2008) Neuroglia: the 150 years after. Trends Neurosci 31:653-659.

Khera R, Das N (2009) Complement Receptor 1: disease associations and therapeutic implications. Mol Immunol 46:761-772.

Kim J, Basak JM, Holtzman DM (2009) The role of apolipoprotein E in Alzheimer's disease. Neuron 63:287-303.

Kitazawa M, Oddo S, Yamasaki TR, Green KN, LaFerla FM (2005) Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. J Neurosci 25:8843-8853.

Klegeris A, McGeer PL (2005) Non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-inflammatory agents in the treatment of neurodegenerative disease. Curr Alzheimer Res 2:355-365.

Klegeris A, Walker DG, McGeer PL (1997) Interaction of Alzheimer beta-amyloid peptide with the human monocytic cell line THP-1 results in a protein

kinase C-dependent secretion of tumor necrosis factor-alpha. Brain Res 747:114-121.

Klein WL (2006) Synaptic targeting by A beta oligomers (ADDLS) as a basis for memory loss in early Alzheimer's disease. Alzheimers Dement 2:43-55.

Koistinaho M, Lin S, Wu X, Esterman M, Koger D, Hanson J, Higgs R, Liu F, Malkani S, Bales KR, Paul SM (2004) Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-beta peptides. Nat Med 10:719-726.

Kokubo H, Kayed R, Glabe CG, Yamaguchi H (2005) Soluble Abeta oligomers ultrastructurally localize to cell processes and might be related to synaptic dysfunction in Alzheimer's disease brain. Brain Res 1031:222-228.

Koo EH (2002) The beta-amyloid precursor protein (APP) and Alzheimer's disease: does the tail wag the dog? Traffic 3:763-770.

Koo EH, Squazzo SL (1994) Evidence that production and release of amyloid b-protein involves the endocytic pathway. J Biol Chem 269:17386-17389.

Kotilinek LA, Westerman MA, Wang Q, Panizzon K, Lim GP, Simonyi A, Lesne S, Falinska A, Younkin LH, Younkin SG, Rowan M, Cleary J, Wallis RA, Sun GY, Cole G, Frautschy S, Anwyl R, Ashe KH (2008) Cyclooxygenase-2 inhibition improves amyloid-beta-mediated suppression of memory and synaptic plasticity. Brain 131:651-664.

Kukar T, Murphy MP, Eriksen JL, Sagi SA, Weggen S, Smith TE, Ladd T, Khan MA, Kache R, Beard J, Dodson M, Merit S, Ozols VV, Anastasiadis PZ, Das P, Fauq A, Koo EH, Golde TE (2005) Diverse compounds mimic Alzheimer disease-causing mutations by augmenting Abeta42 production. Nat Med 11:545-550.

Kumar S, Rezaei-Ghaleh N, Terwel D, Thal DR, Richard M, Hoch M, Mc Donald JM, Wullner U, Glebov K, Heneka MT, Walsh DM, Zweckstetter M, Walter J (2011) Extracellular phosphorylation of the amyloid beta-peptide promotes formation of toxic aggregates during the pathogenesis of Alzheimer's disease. EMBO J 30:2255-2265.

Kummer MP, Hermes M, Hammerschmidt T, Kumar S, Terwel D, Walter J, Pape HC, Konig S, Rober S, Klockgether T, Heneka MT (2011) Nitration of amyloid beta at tyrosine 10 promotes aggregation and plaque formation. 10th AD/PD Conference, Barcelona, Spain.

Kuo YM, Emmerling MR, Vigo-Pelfrey C, Kasunic TC, Kirkpatrick JB, Murdoch GH, Ball MJ, Roher AE (1996) Water-soluble Abeta (N-40, N-42) oligomers in normal and Alzheimer disease brains. J Biol Chem 271:4077-4081.

Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Bigio EH, Finch CE, Krafft GA, Klein WL (2004) Synaptic targeting by Alzheimer's-related amyloid beta oligomers. J Neurosci 24:10191-10200.

Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL (2007) Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. J Neurosci 27:796-807.

Lafaille JJ (1998) The role of helper T cell subsets in autoimmune diseases. Cytokine Growth Factor Rev 9:139-151.

LaFerla FM, Green KN, Oddo S (2007) Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci 8:499-509.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 95:6448-6453.

Lambert MP, Velasco PT, Chang L, Viola KL, Fernandez S, Lacor PN, Khuon D, Gong Y, Bigio EH, Shaw P, De Felice FG, Krafft GA, Klein WL (2007) Monoclonal antibodies that target pathological assemblies of Abeta. J Neurochem 100:23-35.

Lambert MP, Viola KL, Chromy BA, Chang L, Morgan TE, Yu J, Venton DL, Krafft GA, Finch CE, Klein WL (2001) Vaccination with soluble Abeta oligomers generates toxicity-neutralizing antibodies. J Neurochem 79:595-605.

Lassmann H (1983) Comparative neuropathology of chronic experimental allergic encephalomyelitis and multiple sclerosis. Schriftenr Neurol 25:1-135.

Lawrence T, Willoughby DA, Gilroy DW (2002) Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2:787-795.

Lawson LJ, Perry VH, Dri P, Gordon S (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39:151-170.

Lee CY, Landreth GE (2010) The role of microglia in amyloid clearance from the AD brain. J Neural Transm 117:949-960.

Lee DC, Rizer J, Selenica ML, Reid P, Kraft C, Johnson A, Blair L, Gordon MN, Dickey CA, Morgan D (2010) LPS- induced inflammation exacerbates phospho-tau pathology in rTg4510 mice. J Neuroinflammation 7:56.

Lee MK, Slunt HH, Martin LJ, Thinakaran G, Kim G, Gandy SE, Seeger M, Koo E, Price DL, Sisodia SS (1996) Expression of presenilin 1 and 2 (PS1 and PS2) in human and murine tissues. J Neurosci 16:7513-7525.

Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, Frosch MP, Selkoe DJ (2003) Enhanced proteolysis of beta-amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. Neuron 40:1087-1093.

Lemstra AW, Kalisvaart KJ, Vreeswijk R, Van Gool WA, Eikelenboom P (2008) Pre-operative inflammatory markers and the risk of postoperative delirium in elderly patients. Int J Geriatr Psychiatry 23:943-948.

Leon WC, Canneva F, Partridge V, Allard S, Ferretti MT, Dewilde A, Vercauteren F, Atifeh R, Ducatenzeiler A, Klein W, Szyf M, Alhonen L, Cuello AC (2010) A Novel Transgenic Rat Model with a Full Alzheimer's-Like Amyloid Pathology Displays Pre-Plaque Intracellular Amyloid-beta-Associated Cognitive Impairment. J Alzheimers Dis. 20:113-26.

Leonard BE (2007) Inflammation, depression and dementia: are they connected? Neurochem Res 32:1749-1756.

Leoutsakos JM, Muthen BO, Breitner JC, Lyketsos CG (2011) Effects of non-steroidal anti-inflammatory drug treatments on cognitive decline vary by phase of pre-clinical Alzheimer disease: findings from the randomized controlled Alzheimer's Disease Anti-inflammatory Prevention Trial. Int J Geriatr Psychiatry. E-pub ahead of print.

Lerner AJ, Hedera P, Koss E, Stuckey J, Friedland RP (1997) Delirium in Alzheimer disease. Alzheimer Dis Assoc Disord 11:16-20.

Lesne S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, Gallagher M, Ashe KH (2006) A specific amyloid-beta protein assembly in the brain impairs memory. Nature 440:352-357.

Lesne S, Kotilinek L, Ashe KH (2008) Plaque-bearing mice with reduced levels of oligomeric amyloid-beta assemblies have intact memory function. Neuroscience 151:745-749.

Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van SM, Gwinn-Hardy K, Paul MM, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin WL, Yen SH, Dickson DW, Davies P, Hutton M (2000) Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. Nat Genet 25:402-405.

Li R, Lindholm K, Yang LB, Yue X, Citron M, Yan R, Beach T, Sue L, Sabbagh M, Cai H, Wong P, Price D, Shen Y (2004) Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. Proc Natl Acad Sci U S A 101:3632-3637.

Li S, Hong S, Shepardson NE, Walsh DM, Shankar GM, Selkoe D (2009) Soluble oligomers of amyloid Beta protein facilitate hippocampal long-term depression by disrupting neuronal glutamate uptake. Neuron 62:788-801.

Li S, Jin M, Koeglsperger T, Shepardson NE, Shankar GM, Selkoe DJ (2011) Soluble A{beta} Oligomers Inhibit Long-Term Potentiation through a Mechanism Involving Excessive Activation of Extrasynaptic NR2B-Containing NMDA Receptors. J Neurosci 31:6627-6638.

Lilja AM, Porras O, Storelli E, Nordberg A, Marutle A (2011) Functional interactions of fibrillar and oligomeric amyloid-beta with alpha7 nicotinic receptors in Alzheimer's disease. J Alzheimers Dis 23:335-347.

Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J Neurosci 20:5709-5714.

Lindsay J, Laurin D, Verreault R, Hebert R, Helliwell B, Hill GB, McDowell I (2002) Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. Am J Epidemiol 156:445-453.

Linton PJ, Dorshkind K (2004) Age-related changes in lymphocyte development and function. Nat Immunol 5:133-139.

Litman GW, Rast JP, Fugmann SD (2010) The origins of vertebrate adaptive immunity. Nat Rev Immunol 10:543-553.

Liu Y, Teige I, Birnir B, Issazadeh-Navikas S (2006) Neuron-mediated generation of regulatory T cells from encephalitogenic T cells suppresses EAE. Nat Med 12:518-525.

Lomakin A, Teplow DB, Kirschner DA, Benedek GB (1997) Kinetic theory of fibrillogenesis of amyloid beta-protein. Proc Natl Acad Sci U S A 94:7942-7947.

Lord A, Kalimo H, Eckman C, Zhang XQ, Lannfelt L, Nilsson LN (2006) The Arctic Alzheimer mutation facilitates early intraneuronal Abeta aggregation and senile plaque formation in transgenic mice. Neurobiol Aging 27:67-77.

Lorenzo A, Yankner BA (1994) Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. Proc Natl Acad Sci U S A 91:12243-12247.

Lue LF, Brachova L, Civin WH, Rogers J (1996) Inflammation, A beta deposition, and neurofibrillary tangle formation as correlates of Alzheimer's disease neurodegeneration. J Neuropathol Exp Neurol 55:1083-1088.

Lustbader JW, et al. (2004) ABAD directly links Abeta to mitochondrial toxicity in Alzheimer's disease. Science 304:448-452.

Luth HJ, Holzer M, Gartner U, Staufenbiel M, Arendt T (2001) Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology. Brain Res 913:57-67.

Luxembourg B, Schmitt J, Humpich M, Glowatzki M, Dressler D, Seifried E, Lindhoff-Last E (2009) Cardiovascular risk factors in idiopathic compared to risk-associated venous thromboembolism: A focus on fibrinogen, factor VIII, and high-sensitivity C-reactive protein (hs-CRP). Thromb Haemost 102:668-675.

Lyketsos CG, Breitner JC, Green RC, Martin BK, Meinert C, Piantadosi S, Sabbagh M (2007) Naproxen and celecoxib do not prevent AD in early results from a randomized controlled trial. Neurology 68:1800-1808.

Ma QL, Harris-White ME, Ubeda OJ, Simmons M, Beech W, Lim GP, Teter B, Frautschy SA, Cole GM (2007) Evidence of Abeta- and transgene-dependent defects in ERK-CREB signaling in Alzheimer's models. J Neurochem 103:1594-1607.

Ma QL, Yang F, Rosario ER, Ubeda OJ, Beech W, Gant DJ, Chen PP, Hudspeth B, Chen C, Zhao Y, Vinters HV, Frautschy SA, Cole GM (2009) Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: suppression by omega-3 fatty acids and curcumin. J Neurosci 29:9078-9089.

Maezawa I, Zimin PI, Wulff H, Jin LW (2011) Amyloid-beta protein oligomer at low nanomolar concentrations activates microglia and induces microglial neurotoxicity. J Biol Chem 286:3693-3706.

Maier M, Peng Y, Jiang L, Seabrook TJ, Carroll MC, Lemere CA (2008) Complement C3 deficiency leads to accelerated amyloid beta plaque deposition and neurodegeneration and modulation of the microglia/macrophage phenotype in amyloid precursor protein transgenic mice. J Neurosci 28:6333-6341.

Majdi M, Ribeiro-da-Silva A, Cuello AC (2007) Cognitive impairment and transmitter-specific pre- and postsynaptic changes in the rat cerebral cortex during ageing. Eur J Neurosci 26:3583-3596.

Malm TM, Koistinaho M, Parepalo M, Vatanen T, Ooka A, Karlsson S, Koistinaho J (2005) Bone-marrow-derived cells contribute to the recruitment of microglial cells in response to beta-amyloid deposition in APP/PS1 double transgenic Alzheimer mice. Neurobiol Dis 18:134-142.

Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. Nature 454:436-444.

Mantovani A, Sica A, Locati M (2005) Macrophage polarization comes of age. Immunity 23:344-346.

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25:677-686.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23:549-555.

Mariani E, Monastero R, Mecocci P (2007) Mild cognitive impairment: a systematic review. J Alzheimers Dis 12:23-35.

Martin BK, Szekely C, Brandt J, Piantadosi S, Breitner JC, Craft S, Evans D, Green R, Mullan M (2008) Cognitive function over time in the Alzheimer's Disease Anti-inflammatory Prevention Trial (ADAPT): results of a randomized, controlled trial of naproxen and celecoxib. Arch Neurol 65:896-905.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. Proc Natl Acad Sci U S A 82:4245-4249.

Matsuoka Y, Picciano M, Malester B, LaFrancois J, Zehr C, Daeschner JM, Olschowka JA, Fonseca MI, O'Banion MK, Tenner AJ, Lemere CA, Duff K (2001) Inflammatory responses to amyloidosis in a transgenic mouse model of Alzheimer's disease. Am J Pathol 158:1345-1354.

Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, Bateman RJ (2010) Decreased clearance of CNS beta-amyloid in Alzheimer's disease. Science 330:1774.

Mayeux R (2010) Clinical practice. Early Alzheimer's disease. N Engl J Med 362:2194-2201.

McAlpine FE, Lee JK, Harms AS, Ruhn KA, Blurton-Jones M, Hong J, Das P, Golde TE, LaFerla FM, Oddo S, Blesch A, Tansey MG (2009) Inhibition of soluble TNF signaling in a mouse model of Alzheimer's disease prevents pre-plaque amyloid-associated neuropathology. Neurobiol Dis 34:163-177.

McGeer PL, Itagaki S, Tago H, McGeer EG (1987) Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci Lett 79:195-200.

McGeer PL, McGeer E, Rogers J, Sibley J (1990) Anti-inflammatory drugs and Alzheimer disease. Lancet 335:1037.

McGeer PL, McGeer EG (2007) NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol Aging 28:639-647.

McGeer PL, McGeer EG (2002) Local neuroinflammation and the progression of Alzheimer's disease. J Neurovirol 8:529-538.

McGeer PL, Rogers J, McGeer EG (2006) Inflammation, anti-inflammatory agents and Alzheimer disease: the last 12 years. J Alzheimers Dis 9:271-276.

McGowan E, Eriksen J, Hutton M (2006) A decade of modeling Alzheimer's disease in transgenic mice. Trends Genet 22:281-289.

McKee AC, Carreras I, Hossain L, Ryu H, Klein WL, Oddo S, LaFerla FM, Jenkins BG, Kowall NW, Dedeoglu A (2008) Ibuprofen reduces Abeta, hyperphosphorylated tau and memory deficits in Alzheimer mice. Brain Res 1207:225-236.

McKee AC, Kowall NW, Kosik KS (1989) Microtubular reorganization and dendritic growth response in Alzheimer's disease. Ann Neurol 26:652-659.

McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH (2011) The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:263-269.

McLaurin J, Kierstead ME, Brown ME, Hawkes CA, Lambermon MH, Phinney AL, Darabie AA, Cousins JE, French JE, Lan MF, Chen F, Wong SS, Mount HT, Fraser PE, Westaway D, St George-Hyslop P (2006) Cyclohexanehexol inhibitors of Abeta aggregation prevent and reverse Alzheimer phenotype in a mouse model. Nat Med 12:801-808.

McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL (1999) Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol 46:860-866.

Meda L, Cassatella MA, Szendrei GI, Otvos L, Jr., Baron P, Villalba M, Ferrari D, Rossi F (1995) Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature 374:647-650.

Medawar PB (1948) Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 29:58-69.

Meinert CL, McCaffrey LD, Breitner JC (2009) Alzheimer's Disease Anti-inflammatory Prevention Trial: design, methods, and baseline results. Alzheimers Dement 5:93-104.

Michelucci A, Heurtaux T, Grandbarbe L, Morga E, Heuschling P (2009) Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyloid-beta. J Neuroimmunol 210:3-12.

Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG, Love S (2008) Abeta-degrading enzymes in Alzheimer's disease. Brain Pathol 18:240-252.

Minger SL, Esiri MM, McDonald B, Keene J, Carter J, Hope T, Francis PT (2000) Cholinergic deficits contribute to behavioral disturbance in patients with dementia. Neurology 55:1460-1467.

Minghetti L (2004) Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. J Neuropathol Exp Neurol 63:901-910.

Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van BG, Berg L (1991) The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41:479-486.

Morgan D (2009) The role of microglia in antibody-mediated clearance of amyloid-beta from the brain. CNS Neurol Disord Drug Targets 8:7-15.

Morgan D, Gordon MN, Tan J, Wilcock D, Rojiani AM (2005) Dynamic complexity of the microglial activation response in transgenic models of amyloid deposition: implications for Alzheimer therapeutics. J Neuropathol Exp Neurol 64:743-753.

Mori C, Spooner ET, Wisniewsk KE, Wisniewski TM, Yamaguch H, Saido TC, Tolan DR, Selkoe DJ, Lemere CA (2002) Intraneuronal Abeta42 accumulation in Down syndrome brain. Amyloid 9:88-102.

Mori H, Takio K, Ogawara M, Selkoe DJ (1992) Mass spectrometry of purified amyloid beta protein in Alzheimer's disease. J Biol Chem 267:17082-17086.

Morihara T, Teter B, Yang F, Lim GP, Boudinot S, Boudinot FD, Frautschy SA, Cole GM (2005) Ibuprofen suppresses interleukin-1beta induction of pro-amyloidogenic alpha1-antichymotrypsin to ameliorate beta-amyloid (Abeta) pathology in Alzheimer's models. Neuropsychopharmacology 30:1111-1120.

Morris JC, Price AL (2001) Pathologic correlates of nondemented aging, mild cognitive impairment, and early-stage Alzheimer's disease. J Mol Neurosci 17:101-118.

Morris JC, Roe CM, Grant EA, Head D, Storandt M, Goate AM, Fagan AM, Holtzman DM, Mintun MA (2009) Pittsburgh compound B imaging and prediction of progression from cognitive normality to symptomatic Alzheimer disease. Arch Neurol 66:1469-1475.

Morris JC, Storandt M, Miller JP, McKeel DW, Price JL, Rubin EH, Berg L (2001) Mild cognitive impairment represents early-stage Alzheimer disease. Arch Neurol 58:397-405.

Morrissette DA, Parachikova A, Green KN, LaFerla FM (2009) Relevance of transgenic mouse models to human Alzheimer disease. J Biol Chem 284:6033-6037.

Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958-969.

Mucke L, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L (2000) High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J Neurosci 20:4050-4058.

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1:345-347.

Murphy GM, Jr., Zhao F, Yang L, Cordell B (2000) Expression of macrophage colony-stimulating factor receptor is increased in the AbetaPP(V717F) transgenic mouse model of Alzheimer's disease. Am J Pathol 157:895-904.

Murrell J, Farlow M, Ghetti B, Benson MD (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. Science 254:97-99.

Nathan C (2002) Points of control in inflammation. Nature 420:846-852.

Nichol KE, Poon WW, Parachikova AI, Cribbs DH, Glabe CG, Cotman CW (2008) Exercise alters the immune profile in Tg2576 Alzheimer mice toward a response coincident with improved cognitive performance and decreased amyloid. J Neuroinflammation 5:13.

Nimmerjahn A, Kirchhoff F, Helmchen F (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308:1314-1318.

Nitsch RM, Hock C (2008) Targeting beta-amyloid pathology in Alzheimer's disease with Abeta immunotherapy. Neurotherapeutics 5:415-420.

Nordberg A, Winblad B (1986) Reduced numbers of [^{3}H]nicotine and [^{3}H]acetylcholine binding sites in frontal cortex of Alzheimer brains. Neurosci Lett 72:115-121.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA (2001) Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 60:759-767.

Nunomura A, Perry G, Pappolla MA, Friedland RP, Hirai K, Chiba S, Smith MA (2000) Neuronal oxidative stress precedes amyloid-beta deposition in Down syndrome. J Neuropathol Exp Neurol 59:1011-1017.

Nuutinen T, Suuronen T, Kauppinen A, Salminen A (2009) Clusterin: a forgotten player in Alzheimer's disease. Brain Res Rev 61:89-104.

O'Connor T, Sadleir KR, Maus E, Velliquette RA, Zhao J, Cole SL, Eimer WA, Hitt B, Bembinster LA, Lammich S, Lichtenthaler SF, Hebert SS, De SB, Haass C, Bennett DA, Vassar R (2008) Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. Neuron 60:988-1009.

O'Hare E, Weldon DT, Mantyh PW, Ghilardi JR, Finke MP, Kuskowski MA, Maggio JE, Shephard RA, Cleary J (1999) Delayed behavioral effects following intrahippocampal injection of aggregated A beta (1-42). Brain Res 815:1-10.

Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, Van EL, Berry R, Vassar R (2006) Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. J Neurosci 26:10129-10140.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Abeta and synaptic dysfunction. Neuron 39:409-421.

Oddo S, Caccamo A, Tran L, Lambert MP, Glabe CG, Klein WL, LaFerla FM (2006) Temporal profile of amyloid-beta (Abeta) oligomerization in an in vivo model of Alzheimer disease. A link between Abeta and tau pathology. J Biol Chem 281:1599-1604.

Okello A, Edison P, Archer HA, Turkheimer FE, Kennedy J, Bullock R, Walker Z, Kennedy A, Fox N, Rossor M, Brooks DJ (2009) Microglial activation and amyloid deposition in mild cognitive impairment: a PET study. Neurology 72:56-62.

Ono K, Condron MM, Teplow DB (2009) Structure-neurotoxicity relationships of amyloid beta-protein oligomers. Proc Natl Acad Sci U S A 106:14745-14750.

Otvos L, Jr., Szendrei GI, Lee VM, Mantsch HH (1993) Human and rodent Alzheimer beta-amyloid peptides acquire distinct conformations in membrane-mimicking solvents. Eur J Biochem 211:249-257.

Palin K, Cunningham C, Forse P, Perry VH, Platt N (2008) Systemic inflammation switches the inflammatory cytokine profile in CNS Wallerian degeneration. Neurobiol Dis 30:19-29.

Panickar KS, Norenberg MD (2005) Astrocytes in cerebral ischemic injury: morphological and general considerations. Glia 50:287-298.

Parachikova A, Agadjanyan MG, Cribbs DH, Blurton-Jones M, Perreau V, Rogers J, Beach TG, Cotman CW (2007) Inflammatory changes parallel the early stages of Alzheimer disease. Neurobiol Aging 28:1821-1833.

Parachikova A, Vasilevko V, Cribbs DH, LaFerla FM, Green KN (2010) Reductions in amyloid-beta-derived neuroinflammation, with minocycline, restore cognition but do not significantly affect tau hyperphosphorylation. J Alzheimers Dis 21:527-542.

Pardridge WM (2005) Molecular biology of the blood-brain barrier. Mol Biotechnol 30:57-70.

Paris D, Ganey NJ, Laporte V, Patel NS, Beaulieu-Abdelahad D, Bachmeier C, March A, it-Ghezala G, Mullan MJ (2010) Reduction of beta-amyloid pathology by celastrol in a transgenic mouse model of Alzheimer's disease. J Neuroinflammation 7:17.

Parra MA, Abrahams S, Logie RH, Mendez LG, Lopera F, Della SS (2010) Visual short-term memory binding deficits in familial Alzheimer's disease. Brain 133:2702-2713.

Pasinetti GM, Aisen PS (1998) Cyclooxygenase-2 expression is increased in frontal cortex of Alzheimer's disease brain. Neuroscience 87:319-324.

Pearson RC, Sofroniew MV, Cuello AC, Powell TP, Eckenstein F, Esiri MM, Wilcock GK (1983) Persistence of cholinergic neurons in the basal nucleus in a brain with senile dementia of the Alzheimer's type demonstrated by immunohistochemical staining for choline acetyltransferase. Brain Res 289:375-379.

Pei JJ, Braak H, An WL, Winblad B, Cowburn RF, Iqbal K, Grundke-Iqbal I (2002) Up-regulation of mitogen-activated protein kinases ERK1/2 and MEK1/2 is associated with the progression of neurofibrillary degeneration in Alzheimer's disease. Brain Res Mol Brain Res 109:45-55.

Perrin RJ, Fagan AM, Holtzman DM (2009) Multimodal techniques for diagnosis and prognosis of Alzheimer's disease. Nature 461:916-922.

Perry G, Cash AD, Smith MA (2002a) Alzheimer Disease and Oxidative Stress. J Biomed Biotechnol 2:120-123.

Perry VH, Cunningham C, Boche D (2002b) Atypical inflammation in the central nervous system in prion disease. Curr Opin Neurol 15:349-354.

Perry VH, Nicoll JA, Holmes C (2010) Microglia in neurodegenerative disease. Nat Rev Neurol 6:193-201.

Petersen RC (2006) Conversion. Neurology 67:S12-S13.

Petersen RC (2009) Early diagnosis of Alzheimer's disease: is MCI too late? Curr Alzheimer Res 6:324-330.

Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E (1999) Mild cognitive impairment: clinical characterization and outcome. Arch Neurol 56:303-308.

Pfeifer M, Boncristiano S, Bondolfi L, Stalder A, Deller T, Staufenbiel M, Mathews PM, Jucker M (2002) Cerebral hemorrhage after passive anti-Abeta immunotherapy. Science 298:1379.

Philipson O, Lannfelt L, Nilsson LN (2009) Genetic and pharmacological evidence of intraneuronal Abeta accumulation in APP transgenic mice. FEBS Lett 583:3021-3026.

Philipson O, Lord A, Gumucio A, O'Callaghan P, Lannfelt L, Nilsson LN (2010) Animal models of amyloid-beta-related pathologies in Alzheimer's disease. FEBS J 277:1389-1409.

Pike CJ, Walencewicz AJ, Glabe CG, Cotman CW (1991) In vitro aging of beta-amyloid protein causes peptide aggregation and neurotoxicity. Brain Res 563:311-314.

Podlisny MB, Ostaszewski BL, Squazzo SL, Koo EH, Rydell RE, Teplow DB, Selkoe DJ (1995) Aggregation of secreted amyloid beta-protein into sodium dodecyl sulfate-stable oligomers in cell culture. J Biol Chem 270:9564-9570.

Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S (1993) Apolipoprotein E polymorphism and Alzheimer's disease. Lancet 342:697-699.

Pott Godoy MC, Tarelli R, Ferrari CC, Sarchi MI, Pitossi FJ (2008) Central and systemic IL-1 exacerbates neurodegeneration and motor symptoms in a model of Parkinson's disease. Brain 131:1880-1894.

Prasher VP, Farrer MJ, Kessling AM, Fisher EM, West RJ, Barber PC, Butler AC (1998) Molecular mapping of Alzheimer-type dementia in Down's syndrome. Ann Neurol 43:380-383.

Pratico D, Clark CM, Liun F, Rokach J, Lee VY, Trojanowski JQ (2002) Increase of brain oxidative stress in mild cognitive impairment: a possible predictor of Alzheimer disease. Arch Neurol 59:972-976.

Pratico D, Uryu K, Leight S, Trojanoswki JQ, Lee VM (2001) Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. J Neurosci 21:4183-4187.

Puzzo D, Privitera L, Leznik E, Fa M, Staniszewski A, Palmeri A, Arancio O (2008) Picomolar amyloid-beta positively modulates synaptic plasticity and memory in hippocampus. J Neurosci 28:14537-14545.

Qiao X, Cummins DJ, Paul SM (2001) Neuroinflammation-induced acceleration of amyloid deposition in the APPV717F transgenic mouse. Eur J Neurosci 14:474-482.

Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, Rosner MR, Safavi A, Hersh LB, Selkoe DJ (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation. J Biol Chem 273:32730-32738.

Quinn J, Montine T, Morrow J, Woodward WR, Kulhanek D, Eckenstein F (2003) Inflammation and cerebral amyloidosis are disconnected in an animal model of Alzheimer's disease. J Neuroimmunol 137:32-41.

Quiroz YT, Budson AE, Celone K, Ruiz A, Newmark R, Castrillon G, Lopera F, Stern CE (2010) Hippocampal hyperactivation in presymptomatic familial Alzheimer's disease. Ann Neurol 68:865-875.

Ransohoff RM, Kivisakk P, Kidd G (2003) Three or more routes for leukocyte migration into the central nervous system. Nat Rev Immunol 3:569-581.

Reichert F, Rotshenker S (1996) Deficient activation of microglia during optic nerve degeneration. J Neuroimmunol 70:153-161.

Reid LM, Maclullich AM (2006) Subjective memory complaints and cognitive impairment in older people. Dement Geriatr Cogn Disord 22:471-485.

Richartz-Salzburger E, Batra A, Stransky E, Laske C, Kohler N, Bartels M, Buchkremer G, Schott K (2007) Altered lymphocyte distribution in Alzheimer's disease. J Psychiatr Res 41:174-178.

Robert P, Ferris S, Gauthier S, Ihl R, Winblad B, Tennigkeit F (2010) Review of Alzheimer's disease scales: is there a need for a new multi-domain scale for therapy evaluation in medical practice? Alzheimers Res Ther 2:24.

Roberts GW, Gentleman SM, Lynch A, Graham DI (1991) bA4 amyloid protein deposition in brain after head trauma. Lancet 338:1422-1423.

Roberts RO, Geda YE, Knopman DS, Boeve BF, Christianson TJ, Pankratz VS, Kullo IJ, Tangalos EG, Ivnik RJ, Petersen RC (2009) Association of C-reactive protein with mild cognitive impairment. Alzheimers Dement 5:398-405.

Rodriguez JJ, Olabarria M, Chvatal A, Verkhratsky A (2009) Astroglia in dementia and Alzheimer's disease. Cell Death Differ 16:378-385.

Rogers J, Cooper NR, Webster S, Schultz J, McGeer PL, Styren SD, Civin WH, Brachova L, Bradt B, Ward P, . (1992) Complement activation by beta-amyloid in Alzheimer disease. Proc Natl Acad Sci U S A 89:10016-10020.

Rogers J, Kirby LC, Hempelman SR, Berry DL, McGeer PL, Kaszniak AW, Zalinski J, Cofield M, Mansukhani L, Willson P, . (1993) Clinical trial of indomethacin in Alzheimer's disease. Neurology 43:1609-1611.

Rogers J, Luber-Narod J, Civin WH (1988) Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. Neurobiol Aging 9:339-349.

Rogers J, Strohmeyer R, Kovelowski CJ, Li R (2002) Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. Glia 40:260-269.

Roher AE, Baudry J, Chaney MO, Kuo YM, Stine WB, Emmerling MR (2000) Oligomerizaiton and fibril asssembly of the mayloid-beta protein. Biochim Biophys Acta 1502:31-43.

Romagnani S (2000) T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol 85:9-18.

Rosario ER, Carroll JC, Oddo S, LaFerla FM, Pike CJ (2006) Androgens regulate the development of neuropathology in a triple transgenic mouse model of Alzheimer's disease. J Neurosci 26:13384-13389.

Rossner S, Sastre M, Bourne K, Lichtenthaler SF (2006) Transcriptional and translational regulation of BACE1 expression--implications for Alzheimer's disease. Prog Neurobiol 79:95-111.

Rotshenker S (2009) The role of Galectin-3/MAC-2 in the activation of the innate-immune function of phagocytosis in microglia in injury and disease. J Mol Neurosci 39:99-103.

Roychaudhuri R, Yang M, Hoshi MM, Teplow DB (2009) Amyloid beta-protein assembly and Alzheimer disease. J Biol Chem 284:4749-4753.

Sachs DH, Schechter AN, Eastlake A, Anfinsen CB (1972) An immunologic approach to the conformational equilibria of polypeptides. Proc Natl Acad Sci U S A 69:3790-3794.

Saido TC, Iwatsubo T, Mann DM, Shimada H, Ihara Y, Kawashima S (1995) Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. Neuron 14:457-466.

Sainati SM, Ingram DM, Talwalker S, and Geis G (2000) Results of a double-blind, randomized, placebo-controlled study of celecoxib in the treatment of progression of Alzheimer's disease, in 6th International Stockholm/Springfield Symposium on Advances in Alzheimer Therapy; April 5–8; Stockholm, Sweden. Abstract Book, p 180.

Santambrogio L, Belyanskaya SL, Fischer FR, Cipriani B, Brosnan CF, Ricciardi-Castagnoli P, Stern LJ, Strominger JL, Riese R (2001) Developmental plasticity of CNS microglia. Proc Natl Acad Sci U S A 98:6295-6300.

Sarsoza F, Saing T, Kayed R, Dahlin R, Dick M, Broadwater-Hollifield C, Mobley S, Lott I, Doran E, Gillen D, nderson-Bergman C, Cribbs DH, Glabe C, Head E (2009) A fibril-specific, conformation-dependent antibody recognizes a subset of Abeta plaques in Alzheimer disease, Down syndrome and Tg2576 transgenic mouse brain. Acta Neuropathol 118:505-517.

Sastre M (2010) Troubleshooting methods for APP processing in vitro. J Pharmacol Toxicol Methods 61:86-91.

Sastre M, Dewachter I, Landreth GE, Willson TM, Klockgether T, van LF, Heneka MT (2003) Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. J Neurosci 23:9796-9804.

Sastre M, Dewachter I, Rossner S, Bogdanovic N, Rosen E, Borghgraef P, Evert BO, Dumitrescu-Ozimek L, Thal DR, Landreth G, Walter J, Klockgether T, van LF, Heneka MT (2006) Nonsteroidal anti-inflammatory drugs repress beta-secretase gene promoter activity by the activation of PPARgamma. Proc Natl Acad Sci U S A 103:443-448.

Savage MJ, Trusko SP, Howland DS, Pinsker LR, Mistretta S, Reaume AG, Greenberg BD, Siman R, Scott RW (1998) Turnover of amyloid beta-protein in

mouse brain and acute reduction of its level by phorbol ester. J Neurosci 18:1743-1752.

Scharf S, Mander A, Ugoni A, Vajda F, Christophidis N (1999) A double-blind, placebo-controlled trial of diclofenac/misoprostol in Alzheimer's disease. Neurology 53:197-201.

Scheff SW, DeKosky ST, Price DA (1990) Quantitative assessment of cortical synaptic density in Alzheimer's disease. Neurobiol Aging 11:29-37.

Scheuermann S, Hambsch B, Hesse L, Stumm J, Schmidt C, Beher D, Bayer TA, Beyreuther K, Multhaup G (2001) Homodimerization of amyloid precursor protein and its implication in the amyloidogenic pathway of Alzheimer's disease. J Biol Chem 276:33923-33929.

Schilling S, Zeitschel U, Hoffmann T, Heiser U, Francke M, Kehlen A, Holzer M, Hutter-Paier B, Prokesch M, Windisch M, Jagla W, Schlenzig D, Lindner C, Rudolph T, Reuter G, Cynis H, Montag D, Demuth HU, Rossner S (2008) Glutaminyl cyclase inhibition attenuates pyroglutamate Abeta and Alzheimer's disease-like pathology. Nat Med 14:1106-1111.

Schmaltz HN, Fried LP, Xue QL, Walston J, Leng SX, Semba RD (2005) Chronic cytomegalovirus infection and inflammation are associated with prevalent frailty in community-dwelling older women. J Am Geriatr Soc 53:747-754.

Schneider A, Rajendran L, Honsho M, Gralle M, Donnert G, Wouters F, Hell SW, Simons M (2008) Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. J Neurosci 28:2874-2882.

Schweber MS (1989) Alzheimer's disease and Down syndrome. Prog Clin Biol Res 317:247-267.

Seabrook TJ, Jiang L, Maier M, Lemere CA (2006) Minocycline affects microglia activation, Abeta deposition, and behavior in APP-tg mice. Glia 53:776-782.

Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W, Lee L, Isakson P (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. Proc Natl Acad Sci U S A 91:12013-12017.

Selinfreund RH, Barger SW, Pledger WJ, Van Eldik LJ (1991) Neurotrophic protein S100 beta stimulates glial cell proliferation. Proc Natl Acad Sci U S A 88:3554-3558.

Selkoe DJ (2001a) Clearing the brain's amyloid cobwebs. Neuron 32:177-180.

Selkoe DJ (2000a) The genetics and molecular pathology of Alzheimer's disease: roles of amyloid and the presenilins. Neurol Clin 18:903-922.

Selkoe DJ (2002) Deciphering the genesis and fate of amyloid beta-protein yields novel therapies for Alzheimer disease. J Clin Invest 110:1375-1381.

Selkoe DJ (2001b) Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 81:741-766.

Selkoe DJ (1994) Amyloid beta-protein precursor: new clues to the genesis of Alzheimer's disease. Curr Opin Neurobiol 4:708-716.

Selkoe DJ (2000b) Toward a comprehensive theory for Alzheimer's disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid beta-protein. Ann N Y Acad Sci 924:17-25.

Semenenko FM, Bramwell S, Sidebottom E, Cuello AC (1985) Development of a mouse antiperoxidase secreting hybridoma for use in the production of a mouse PAP complex for immunocytochemistry and as a parent cell line in the development of hybrid hybridomas. Histochemistry 83:405-408.

Serhan CN, Chiang N, Van Dyke TE (2008) Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 8:349-361.

Seubert P, Vigo-Pelfrey C, Esch F, Lee M, Dovey H, Davis D, Sinha S, Schlossmacher M, Whaley J, Swindlehurst C, . (1992) Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. Nature 359:325-327.
Shaftel SS, Kyrkanides S, Olschowka JA, Miller JN, Johnson RE, O'Banion MK (2007) Sustained hippocampal IL-1 beta overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. J Clin Invest 117:1595-1604.

Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL (2007) Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J Neurosci 27:2866-2875.

Shankar GM, Leissring MA, Adame A, Sun X, Spooner E, Masliah E, Selkoe DJ, Lemere CA, Walsh DM (2009) Biochemical and immunohistochemical analysis of an Alzheimer's disease mouse model reveals the presence of multiple cerebral Abeta assembly forms throughout life. Neurobiol Dis 36:293-302.

Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ (2008) Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med 14:837-842.

Sheng JG, Bora SH, Xu G, Borchelt DR, Price DL, Koliatsos VE (2003) Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid beta peptide in APPswe transgenic mice. Neurobiol Dis 14:133-145.

Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, Holtzman DM, Miller CA, Strickland DK, Ghiso J, Zlokovic BV (2000) Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. J Clin Invest 106:1489-1499.

Shivers BD, Hilbich C, Multhaup G, Salbaum M, Beyreuther K, Seeburg PH (1988) Alzheimer's disease amyloidogenic glycoprotein: expression pattern in rat brain suggests a role in cell contact. EMBO J 7:1365-1370.

Simard AR, Soulet D, Gowing G, Julien JP, Rivest S (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. Neuron 49:489-502.

Simons M, De Strooper B, Multhaup G, Tienari PJ, Dotti CG, Beyreuther K (1996) Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. J Neurosci 16:899-908.

Singh TJ, Haque N, Grundke-Iqbal I, Iqbal K (1995) Rapid Alzheimer-like phosphorylation of tau by the synergistic actions of non-proline-dependent protein kinases and GSK-3. FEBS Lett 358:267-272.

Singh VK, Fudenberg HH, Brown FR, III (1986) Immunologic dysfunction: simultaneous study of Alzheimer's and older Down's patients. Mech Ageing Dev 37:257-264.

Skias D, Bania M, Reder AT, Luchins D, Antel JP (1985) Senile dementia of Alzheimer's type (SDAT): reduced T8+-cell-mediated suppressor activity. Neurology 35:1635-1638.

Sly LM, Krzesicki RF, Brashler JR, Buhl AE, McKinley DD, Carter DB, Chin JE (2001) Endogenous brain cytokine mRNA and inflammatory responses to lipopolysaccharide are elevated in the Tg2576 transgenic mouse model of Alzheimer's disease. Brain Res Bull 56:581-588.

Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci 17:2653-2657.

Smith MA, Rottkamp CA, Nunomura A, Raina AK, Perry G (2000) Oxidative stress in Alzheimer's disease. Biochim Biophys Acta 1502:139-144.

Snyder EM, Nong Y, Almeida CG, Paul S, Moran T, Choi EY, Nairn AC, Salter MW, Lombroso PJ, Gouras GK, Greengard P (2005) Regulation of NMDA receptor trafficking by amyloid-beta. Nat Neurosci 8:1051-1058.

Sokolov Y, Kozak JA, Kayed R, Chanturiya A, Glabe C, Hall JE (2006) Soluble amyloid oligomers increase bilayer conductance by altering dielectric structure. J Gen Physiol 128:637-647.

Solomon GF (1987) Psychoneuroimmunology: interactions between central nervous system and immune system. J Neurosci Res 18:1-9.

Soscia SJ, Kirby JE, Washicosky KJ, Tucker SM, Ingelsson M, Hyman B, Burton MA, Goldstein LE, Duong S, Tanzi RE, Moir RD (2010) The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. PLoS One 5:e9505.

Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P (2007) Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. Neurobiol Aging 28:1163-1169.

Sperling RA, Dickerson BC, Pihlajamaki M, Vannini P, LaViolette PS, Vitolo OV, Hedden T, Becker JA, Rentz DM, Selkoe DJ, Johnson KA (2010) Functional alterations in memory networks in early Alzheimer's disease. Neuromolecular Med 12:27-43.

Sperling RA, LaViolette PS, O'Keefe K, O'Brien J, Rentz DM, Pihlajamaki M, Marshall G, Hyman BT, Selkoe DJ, Hedden T, Buckner RL, Becker JA, Johnson KA (2009) Amyloid deposition is associated with impaired default network function in older persons without dementia. Neuron 63:178-188.

Stewart WF, Kawas C, Corrada M, Metter EJ (1997) Risk of Alzheimer's disease and duration of NSAID use. Neurology 48:626-632.

Stine WB, Jr., Dahlgren KN, Krafft GA, LaDu MJ (2003) In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. J Biol Chem 278:11612-11622.

Streit WJ, Graeber MB (1993) Heterogeneity of microglial and perivascular cell populations: insights gained from the facial nucleus paradigm. Glia 7:68-74.

Strittmatter WJ, Weisgraber KH, Huang DY, Dong LM, Salvesen GS, Pericak-Vance M, Schmechel D, Saunders AM, Goldgaber D, Roses AD (1993) Binding of human apolipoprotein E to synthetic amyloid beta peptide: isoform-specific effects and implications for late-onset Alzheimer disease. Proc Natl Acad Sci USA 90:8098-8102.

Stutzmann GE (2007) The pathogenesis of Alzheimers disease is it a lifelong "calciumopathy"? Neuroscientist 13:546-559.

Sudoh S, Frosch MP, Wolf BA (2002) Differential effects of proteases involved in intracellular degradation of amyloid beta-protein between detergent-soluble and -insoluble pools in CHO-695 cells. Biochemistry 41:1091-1099.

Sung S, Yang H, Uryu K, Lee EB, Zhao L, Shineman D, Trojanowski JQ, Lee VM, Pratico D (2004) Modulation of nuclear factor-kappa B activity by indomethacin influences A beta levels but not A beta precursor protein metabolism in a model of Alzheimer's disease. Am J Pathol 165:2197-2206.

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr., Eckman C, Golde TE, Younkin SG (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264:1336-1340.

Sy M, Kitazawa M, Medeiros R, Whitman L, Cheng D, Lane TE, LaFerla FM (2011) Inflammation induced by infection potentiates tau pathological features in transgenic mice. Am J Pathol 178:2811-2822.

Taglialatela G, Hogan D, Zhang WR, Dineley KT (2009) Intermediate- and long-term recognition memory deficits in Tg2576 mice are reversed with acute calcineurin inhibition. Behav Brain Res 200:95-99.

Takahashi RH, Almeida CG, Kearney PF, Yu F, Lin MT, Milner TA, Gouras GK (2004a) Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. J Neurosci 24:3592-3599.

Takahashi RH, Almeida CG, Kearney PF, Yu F, Lin MT, Milner TA, Gouras GK (2004b) Oligomerization of Alzheimer's beta-amyloid within processes and synapses of cultured neurons and brain. J Neurosci 24:3592-3599.

Tamagno E, Bardini P, Obbili A, Vitali A, Borghi R, Zaccheo D, Pronzato MA, Danni O, Smith MA, Perry G, Tabaton M (2002) Oxidative stress increases expression and activity of BACE in NT2 neurons. Neurobiol Dis 10:279-288.

Tamguney G, Francis KP, Giles K, Lemus A, DeArmond SJ, Prusiner SB (2009) Measuring prions by bioluminescence imaging. Proc Natl Acad Sci U S A 106:15002-15006.

Tan J, Town T, Paris D, Mori T, Suo Z, Crawford F, Mattson MP, Flavell RA, Mullan M (1999) Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation. Science 286:2352-2355.

Tanzi RE, Gusella JF, Watkins PC, Bruns GA, St George-Hyslop P, Van Keuren ML, Patterson D, Pagan S, Kurnit DM, Neve RL (1987) Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science 235:880-884.

Teather LA, Packard MG, Bazan NG (2002) Post-training cyclooxygenase-2 (COX-2) inhibition impairs memory consolidation. Learn Mem 9:41-47.

Tegeder I, Pfeilschifter J, Geisslinger G (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 15:2057-2072.

Teller JK, Russo C, DeBusk LM, Angelini G, Zaccheo D, gna-Bricarelli F, Scartezzini P, Bertolini S, Mann DM, Tabaton M, Gambetti P (1996) Presence of soluble amyloid beta-peptide precedes amyloid plaque formation in Down's syndrome. Nat Med 2:93-95.

Teplow DB (1998) Structural and kinetic features of amyloid beta-protein fibrillogenesis. Amyloid 5:121-142.

Terai K, Matsuo A, McGeer PL (1996) Enhancement of immunoreactivity for NF-kappa B in the hippocampal formation and cerebral cortex of Alzheimer's disease. Brain Res 735:159-168.

Terai K, Walker DG, McGeer EG, McGeer PL (1997) Neurons express proteins of the classical complement pathway in Alzheimer disease. Brain Res 769:385-390.

Terry RD (1997) The pathology of Alzheimer's disease: numbers count. Ann Neurol 41:7.

Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Ann Neurol 30:572-580.

Thal LJ, Ferris SH, Kirby L, Block GA, Lines CR, Yuen E, Assaid C, Nessly ML, Norman BA, Baranak CC, Reines SA (2005) A randomized, double-blind, study of rofecoxib in patients with mild cognitive impairment. Neuropsychopharmacology 30:1204-1215.

Thinakaran G, Koo EH (2008) Amyloid precursor protein trafficking, processing, and function. J Biol Chem 283:29615-29619.

Tomic JL, Pensalfini A, Head E, Glabe CG (2009) Soluble fibrillar oligomer levels are elevated in Alzheimer's disease brain and correlate with cognitive dysfunction. Neurobiol Dis 35:352-358.

Townsend M, Cleary JP, Mehta T, Hofmeister J, Lesne S, O'Hare E, Walsh DM, Selkoe DJ (2006a) Orally available compound prevents deficits in memory caused by the Alzheimer amyloid-beta oligomers. Ann Neurol 60:668-676.

Townsend M, Shankar GM, Mehta T, Walsh DM, Selkoe DJ (2006b) Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. J Physiol 572:477-492.

Tremblay ME, Majewska AK (2011) A role for microglia in synaptic plasticity? Commun Integr Biol 4:220-222.

Ulloa L (2005) The vagus nerve and the nicotinic anti-inflammatory pathway. Nat Rev Drug Discov 4:673-684.

Van De Berg WD, Partridge V, De Wilde A, Ducatenzeiler A, Cuello AC (2010) Intracellular amyloid-beta accumulation causes neuron loss in the hippocampus of Thy1-APP transgenic mice. 7th Froum of European Neuroscience (FENS), Amsterdam, The Netherlands.

van der Putten H., Wiederhold KH, Probst A, Barbieri S, Mistl C, Danner S, Kauffmann S, Hofele K, Spooren WP, Ruegg MA, Lin S, Caroni P, Sommer B, Tolnay M, Bilbe G (2000) Neuropathology in mice expressing human alpha-synuclein. J Neurosci 20:6021-6029.

Van Gool WA, van de BD, Eikelenboom P (2010) Systemic infection and delirium: when cytokines and acetylcholine collide. Lancet 375:773-775.

van Munster BC, Korevaar JC, Zwinderman AH, Levi M, Wiersinga WJ, de Rooij SE (2008) Time-course of cytokines during delirium in elderly patients with hip fractures. J Am Geriatr Soc 56:1704-1709.

Van Nostrand WE, Schmaier AH, Farrow JS, Cunningham DD (1990) Protease nexin-II (amyloid beta-protein precursor): a platelet alpha-granule protein. Science 248:745-748.

Varvel NH, Bhaskar K, Kounnas MZ, Wagner SL, Yang Y, Lamb BT, Herrup K (2009) NSAIDs prevent, but do not reverse, neuronal cell cycle reentry in a mouse model of Alzheimer disease. J Clin Invest 119:3692-3702.

Vassar R (2004) BACE1: the beta-secretase enzyme in Alzheimer's disease. J Mol Neurosci 23:105-114.

Vassar R, et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735-741.

Vehmas AK, Kawas CH, Stewart WF, Troncoso JC (2003) Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. Neurobiol Aging 24:321-331.

Vekrellis K, Ye Z, Qiu WQ, Walsh D, Hartley D, Chesneau V, Rosner MR, Selkoe DJ (2000) Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. J Neurosci 20:1657-1665.

Velliquette RA, O'Connor T, Vassar R (2005) Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. J Neurosci 25:10874-10883.

Verkhratsky A, Olabarria M, Noristani HN, Yeh CY, Rodriguez JJ (2010) Astrocytes in Alzheimer's disease. Neurotherapeutics 7:399-412.

Verma IM, Stevenson JK, Schwarz EM, Van AD, Miyamoto S (1995) Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev 9:2723-2735.

Verreck FA, de BT, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R, Ottenhoff TH (2004) Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. Proc Natl Acad Sci U S A 101:4560-4565.

Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, Nukina N, Wong PC, Xu H, Thinakaran G (2004) Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem 279:44945-44954.

Viebahn CS, Yeoh GC (2008) What fires prometheus? The link between inflammation and regeneration following chronic liver injury. Int J Biochem Cell Biol 40:855-873.

Vlad SC, Miller DR, Kowall NW, Felson DT (2008) Protective effects of NSAIDs on the development of Alzheimer disease. Neurology 70:1672-1677.

Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29:3974-3980.

Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB (1999) Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 274:25945-25952.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416:535-539.

Walsh DM, Selkoe DJ (2007) A beta oligomers - a decade of discovery. J Neurochem 101:1172-1184.

Walsh DM, Tseng BP, Rydel RE, Podlisny MB, Selkoe DJ (2000) The oligomerization of amyloid beta-protein begins intracellularly in cells derived from human brain. Biochemistry 39:10831-10839.

Walsh JS, Welch HG, Larson EB (1990) Survival of outpatients with Alzheimer-type dementia. Ann Intern Med 113:429-434.

Walter L, Franklin A, Witting A, Wade C, Xie Y, Kunos G, Mackie K, Stella N (2003) Nonpsychotropic cannabinoid receptors regulate microglial cell migration. J Neurosci 23:1398-1405.

Wang DS, Lipton RB, Katz MJ, Davies P, Buschke H, Kuslansky G, Verghese J, Younkin SG, Eckman C, Dickson DW (2005) Decreased neprilysin immunoreactivity in Alzheimer disease, but not in pathological aging. J Neuropathol Exp Neurol 64:378-385.

Wang HW, Pasternak JF, Kuo H, Ristic H, Lambert MP, Chromy B, Viola KL, Klein WL, Stine WB, Krafft GA, Trommer BL (2002) Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. Brain Res 924:133-140.

Wang HY, Lee DH, D'Andrea MR, Peterson PA, Shank RP, Reitz AB (2000) beta-Amyloid(1-42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. J Biol Chem 275:5626-5632.

Weaver JD, Huang MH, Albert M, Harris T, Rowe JW, Seeman TE (2002) Interleukin-6 and risk of cognitive decline: MacArthur studies of successful aging. Neurology 59:371-378.

Webster B, Hansen L, Adame A, Crews L, Torrance M, Thal L, Masliah E (2006) Astroglial activation of extracellular-regulated kinase in early stages of Alzheimer disease. J Neuropathol Exp Neurol 65:142-151.

Webster NJ, Green KN, Peers C, Vaughan PF (2002) Altered processing of amyloid precursor protein in the human neuroblastoma SH-SY5Y by chronic hypoxia. J Neurochem 83:1262-1271.

Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, Findlay KA, Smith TE, Murphy MP, Bulter T, Kang DE, Marquez-Sterling N, Golde TE, Koo EH (2001) A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. Nature 414:212-216.

Wells J, Vietje BP, Wells DG, Paradee J (1992) Isomorphic activation of astrocytes in the somatosensory thalamus. Glia 5:154-160.

Wen Y, Onyewuchi O, Yang S, Liu R, Simpkins JW (2004a) Increased beta-secretase activity and expression in rats following transient cerebral ischemia. Brain Res 1009:1-8.

Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. Physiol Rev 83:835-870.

Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, DeLong MR (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215:1237-1239.

Whitson JS, Glabe CG, Shintani E, Abcar A, Cotman CW (1990) Beta-amyloid protein promotes neuritic branching in hippocampal cultures. Neurosci Lett 110:319-324.

Whitson JS, Selkoe DJ, Cotman CW (1989) Amyloid beta protein enhances the survival of hippocampal neurons in vitro. Science 243:1488-1490.

Widner H, Brundin P, Bjorklund A, Moller E (1988) Immunological aspects of neural grafting in the mammalian central nervous system. Prog Brain Res 78:303-307.

Wiltfang J, Esselmann H, Bibl M, Smirnov A, Otto M, Paul S, Schmidt B, Klafki HW, Maler M, Dyrks T, Bienert M, Beyermann M, Ruther E, Kornhuber J (2002) Highly conserved and disease-specific patterns of carboxyterminally truncated Abeta peptides 1-37/38/39 in addition to 1-40/42 in Alzheimer's disease and in patients with chronic neuroinflammation. J Neurochem 81:481-496.

Wimo A., Prince M. (2010) World Alzheimer Report 2010.

Winton MJ, Lee EB, Sun E, Wong MM, Leight S, Zhang B, Trojanowski JQ, Lee VM (2011) Intraneuronal APP, Not Free A{beta} Peptides in 3xTg-AD Mice: Implications for Tau versus A{beta}-Mediated Alzheimer Neurodegeneration. J Neurosci 31:7691-7699.

Wirths O, Breyhan H, Cynis H, Schilling S, Demuth HU, Bayer TA (2009) Intraneuronal pyroglutamate-Abeta 3-42 triggers neurodegeneration and lethal neurological deficits in a transgenic mouse model. Acta Neuropathol 118:487-496.

Wirths O, Erck C, Martens H, Harmeier A, Geumann C, Jawhar S, Kumar S, Multhaup G, Walter J, Ingelsson M, german-Gunnarsson M, Kalimo H, Huitinga I, Lannfelt L, Bayer TA (2010) Identification of low molecular weight pyroglutamate A{beta} oligomers in Alzheimer disease: a novel tool for therapy and diagnosis. J Biol Chem 285:41517-41524.

Wirths O, Multhaup G, Bayer TA (2004) A modified beta-amyloid hypothesis: intraneuronal accumulation of the beta-amyloid peptide--the first step of a fatal cascade. J Neurochem 91:513-520.

Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Pradier L, Beyreuther K, Bayer TA (2001) Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice. Neurosci Lett 306:116-120.

Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398:513-517.

Wolfson C, Perrault A, Moride Y, Esdaile JM, Abenhaim L, Momoli F (2002) A case-control analysis of nonsteroidal anti-inflammatory drugs and Alzheimer's disease: are they protective? Neuroepidemiology 21:81-86.

Wong ML, Rettori V, al-Shekhlee A, Bongiorno PB, Canteros G, McCann SM, Gold PW, Licinio J (1996) Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. Nat Med 2:581-584.

Wong TP, Campbell PM, Ribeiro-da-Silva A, Cuello AC (1998) Synaptic numbers across cortical laminae and cognitive performance of the rat during ageing. Neuroscience 84:403-412.

Wong TP, Debeir T, Duff K, Cuello AC (1999) Reorganization of cholinergic terminals in the cerebral cortex and hippocampus in transgenic mice carrying mutated presenilin-1 and amyloid precursor protein transgenes. J Neurosci 19:2706-2716.

Wu Q, Combs C, Cannady SB, Geldmacher DS, Herrup K (2000) Beta-amyloid activated microglia induce cell cycling and cell death in cultured cortical neurons. Neurobiol Aging 21:797-806.

Wyss-Coray T, Masliah E, Mallory M, McConlogue L, Johnson-Wood K, Lin C, Mucke L (1997) Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. Nature 389:603-606.

Xia P, Chen HS, Zhang D, Lipton SA (2010) Memantine preferentially blocks extrasynaptic over synaptic NMDA receptor currents in hippocampal autapses. J Neurosci 30:11246-11250.

Xu H, Sweeney D, Wang R, Thinakaran G, Lo AC, Sisodia SS, Greengard P, Gandy S (1997) Generation of Alzheimer b-amyloid protein in the trans-Golgi network in the apparent absence of vesicle formation. Proc Natl Acad Sci U S A 94:3748-3752.

Yamada T, Sasaki H, Furuya H, Miyata T, Goto I, Sakaki Y (1987) Complementary DNA for the mouse homolog of the human amyloid beta protein precursor. Biochem Biophys Res Commun 149:665-671.

Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, Worley PF (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. Neuron 11:371-386.

Yamamoto-Sasaki M, Ozawa H, Saito T, Rosler M, Riederer P (1999) Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type. Brain Res 824:300-303.

Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, Citron M, Landreth G (2003) Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. J Neurosci 23:7504-7509.

Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM (1996) RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 382:685-691.

Yang AJ, Chandswangbhuvana D, Shu T, Henschen A, Glabe CG (1999) Intracellular accumulation of insoluble, newly synthesized abetan-42 in amyloid precursor protein-transfected cells that have been treated with Abeta1-42. J Biol Chem 274:20650-20656.

Yang LB, Lindholm K, Yan R, Citron M, Xia W, Yang XL, Beach T, Sue L, Wong P, Price D, Li R, Shen Y (2003) Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. Nat Med 9:3-4.

Yankner BA, Duffy LK, Kirschner DA (1990) Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. Science 250:279-282.

Yip AG, Green RC, Huyck M, Cupples LA, Farrer LA (2005) Nonsteroidal anti-inflammatory drug use and Alzheimer's disease risk: the MIRAGE Study. BMC Geriatr 5:2.

Yokota C, Kaji T, Kuge Y, Inoue H, Tamaki N, Minematsu K (2004) Temporal and topographic profiles of cyclooxygenase-2 expression during 24 h of focal brain ishemia in rats. Neurosci Lett 357:219-222.

Yoshiyama Y, Arai K, Hattori T (2001) Enhanced expression of I-kappaB with neurofibrillary pathology in Alzheimer's disease. Neuroreport 12:2641-2645.

Yoshiyama Y, Higuchi M, Zhang B, Huang SM, Iwata N, Saido TC, Maeda J, Suhara T, Trojanowski JQ, Lee VM (2007) Synapse loss and microglial activation precede tangles in a P301S tauopathy mouse model. Neuron 53:337-351.

Yu Z, Zhou D, Bruce-Keller AJ, Kindy MS, Mattson MP (1999) Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. J Neurosci 19:8856-8865.

Zahs KR, Ashe KH (2010) 'Too much good news' - are Alzheimer mouse models trying to tell us how to prevent, not cure, Alzheimer's disease? Trends Neurosci 33:381-389.

Zempel H, Thies E, Mandelkow E, Mandelkow EM (2010) Abeta oligomers cause localized Ca(2+) elevation, missorting of endogenous Tau into dendrites, Tau phosphorylation, and destruction of microtubules and spines. J Neurosci 30:11938-11950.

Zhang Y, McLaughlin R, Goodyer C, LeBlanc A (2002) Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons. J Cell Biol 156:519-529.

Zhao J, Fu Y, Yasvoina M, Shao P, Hitt B, O'Connor T, Logan S, Maus E, Citron M, Berry R, Binder L, Vassar R (2007) Beta-site amyloid precursor protein cleaving enzyme 1 levels become elevated in neurons around amyloid plaques: implications for Alzheimer's disease pathogenesis. J Neurosci 27:3639-3649.

Zhao WQ, De Felice FG, Fernandez S, Chen H, Lambert MP, Quon MJ, Krafft GA, Klein WL (2008) Amyloid beta oligomers induce impairment of neuronal insulin receptors. FASEB J 22:246-260.

Ziv Y, Ron N, Butovsky O, Landa G, Sudai E, Greenberg N, Cohen H, Kipnis J, Schwartz M (2006) Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. Nat Neurosci 9:268-275.

Zlokovic BV (2005) Neurovascular mechanisms of Alzheimer's neurodegeneration. Trends Neurosci 28:202-208.

Zlokovic BV, Martel CL, Matsubara E, McComb JG, Zheng G, McCluskey RT, Frangione B, Ghiso J (1996) Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. Proc Natl Acad Sci U S A 93:4229-4234.