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Influence of mouse genetic background on hAPP transgene-induced brain amyloidosis and inflammatory response to beta-amyloid protein.

Running title: *Inflammatory response to beta-amyloid in brain amyloidosis*

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Abstract

Influence of mouse genetic background on hAPP transgene-induced brain amyloidosis and inflammatory response to β-amyloid protein. Ph.D. Thesis Abstract. 2003. Sherri Dudal. Division of Experimental Medicine, Department of Medicine. McGill University.

Brain inflammation is a hallmark of Alzheimer Disease (AD) neuropathology. This involves microglial activation which has been well characterized around fibrillar plaques in humans and in transgenic mouse models. Plaques are initiated by the accumulation of $A\beta_{1-42}$ (beta-amyloid) which is a highly aggregating, neurotoxic protein derived from alternative processing of APP (amyloid precursor protein). Genetic factors involved in influencing the intensity of an inflammatory response have been shown clearly in other pathologies but not in AD. Therefore, an *in vitro* model of microglial activation by $A\beta_{1-42}$, was developed using mouse strains having a low (A/J) or high (C57BL/6) inflammatory response. Interferon-γ-primed C57BL/6 microglia underwent morphological changes from resting (ramified) morphology to activated (ameboid) morphology, increases in nitric oxide (NO) production, and very high levels of tumor necrosis factor α (TNF-α) when stimulated with $A\beta_{1-42}$. On the other hand, A/J microglia underwent few morphological changes, had increased levels of NO and had minimal increases in TNF-α levels when submitted to the same treatment. Thus, microglial activation to $A\beta_{1-42}$ was influenced by the magnitude in inflammatory response which may be determined by genetic factors. The timing of an inflammatory response in the process of Aβ deposition is still under debate. Therefore, to define the

kinetics of the inflammatory response in brain amyloidogenesis, TgCRND8 mice which overexpress hAPP and aggressively develop amyloidosis, were used to characterize the evolution of diffuse and fibrillar plaque formation and gliosis. Histopathological analysis revealed diffuse plaques as early as 12 weeks of age. Fibrillar (senile) plaques and microgliosis were seen at 13 weeks of age whereas astrocytic clustering began at 14-15 weeks of age. Microglial activation was found to be correlated strongly to Aβ deposition and fibrillar plaque formation. Therefore, the early appearance of the inflammatory response to Aβ deposition in brain amyloidosis suggests that microglia contribute to Aβ deposition or clearance. To determine whether the variations in inflammatory response to Aβ occur *in vivo*, TgCRND8 mice were backcrossed onto mice with low (A/J) or high (C57BL/6) inflammatory responses. These mice did not differ in their hAPP mRNA expression, Aβ load , and ratio of $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$. However, immunohistochemical analysis showed more Aβ deposition, plaque burden, and lower survival in CRND8.B6 mice compared to CRND8.AJ mice. Hence, genetic factors, possibly related to inflammatory response, can influence the rate of brain amyloidosis. This delineates the need for individual therapy to regulate the inflammatory response in AD patients.

Résumé

Analyse du déterminisme génétique de l'amyloidose cérébrale et de la réponse inflammatoire à la protéine β-amyloid chez la souris porteuse du transgène hAPP. Résumé de Ph.D. 2003. Sherri Dudal. Division de Médecine Expérimentale, Département de Médecine. Université McGill.

L'inflammation du cerveau est un facteur majeur dans la neuropathologie de la maladie d'Alzheimer (AD). L'activation des microglies est bien caractérisée autour des plaques séniles humaines et aussi dans les modèles transgéniques. Les plaques sont formées par l'accumulation de $A\beta_{1-42}$ (amyloid-beta), une protéine neurotoxique qui agrège fortement, provenant d'APP (protéine précurseur d'amyloid). Des facteurs génétiques qui gèrent l'intensité de la réponse inflammatoire ont été décrits dans plusieurs infections mais pas dans AD. Un modèle *in vitro* de l'activation des microglies a été développé chez deux lignées de souris consanguines dont une réponse inflammatoire faible (A/J) et l'autre ayant une réponse inflammatoire forte (C57BL/6). Ce modèle a été utilisé suuite pour étudier leurs réponse imflammatoire suite à l'activation des microglies par la β-amyloid. Les microglies C57BL/6 ont subi des changements morphologiques suite à l'exposition à $A\beta_{1-42}$ non-activées (ramifiées) à activées (ameboide) et une augmentation importante de production d'oxyde nitrique (NO) et du facteur alpha de nécrose de tumeur (TNF-α). Par contre, les microglies A/J stimulées à l'$A\beta_{1-42}$ ont montré peu de difference de morphologie, quoiqu'elles pésentaient une forte augmentation dans les niveaux d'oxyde nitrique (NO), et une modeste augmentation de TNF-α. Ces résultats suggèrent que des facteurs génétiques de l'hôte

influencent la réponse inflammatoire des microglies induite par la β-amyloid. Pour étudier l'effet des processus inflammatoires et de déposition de β-amyloid, des souris TgCRND8 surexprimant hAPP et développant une amyloidose aggressive, ont été utilisées pour le caractérisation de la formation des plaques diffuses et fibrillares, et la gliose. Le premier événement observé a été la déposition de Aβ à 12 semaines d'âge, suivi par la formation des plaques fibrillaires (positive pour ThioS) et microgliose à 13 semaines d'âge. Finalement, des foci composés d'astrocytes ont été détecté à 14-15 semaines d'âge. L'activation des microglies est bien correlée à la déposition de Aβ (r^2=0.799) et à la formation des plaques fibrillaires (r^2=0.854). Par conséquent, la réponse inflammatoire est détectée tôt dans le processus de déposition de l'Aβ ce que pourrait jouer un rôle dans la déposition ou l'élimination de Aβ. Ces résultats ont été verifiés *in vivo* en utilisant des souris TgCRND8 qui ont été rétro-croisées avec des lignées de souris ayant une réponse inflammatoire faible (A/J) ou forte (C57BL/6) afin de déterminer l'impact de la réponse inflammatoire sur la pathologie associé à la maladie d'Alzheimer (AD). Les deux lignées de souris présentent des niveaux d'expression d'ARN messager de hAPP, de protéines Aβ, et des ratios $A\beta_{1-42}/A\beta_{1-40}$ semblables. Toutfois l'analyse immunohistochimique a démontré une déposition de Aβ, de plaque plus importante ainsi qu'un taux de mortalité accru chez les souris ayant une réponse inflammatoire forte comparativement à celles ayant une réponse inflammatoire faible. Ainsi, des facteurs génétiques influencant la réponse inflammatoire pourraient être des cibles thérapeutiques importantes car l'inflammation agit précocement dans la neuropathologie de AD.

Dedication

To my ever-supporting husband,

Yves Dudal

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I would like to thank my supervisor, Dr. Francine Gervais, her guidance and showing me how to be an independent, critical-thinking researcher. Even though she took a leaping career change that demanded her to take on a wider scope of activities, she continued to support my graduate studies and provided me with a unique opportunity of seeing both the academic and industrial sides of research. I am also thankful to Dr. Céline Morissette that provided helpful scientific discussion and played an important role in the progression of my project. She also counselled me on management and taught me about the ever- changing world of industrial priorities. My thesis supervisory committee (Drs. Jean-Pierre Julien, Trevor Owens, Albert Descoteaux, and Nicole Bernard) provided good scientific discussion and creative ideas towards my project. Julie Paquette has given me continuous moral support, has a 'let's attack' attitude to setting up intracranial injections in mice, and has laughed at my B.C. expressions. Giovanna Sebastiani, my molecular biology expert, arrived and added to the positive atmosphere of the lab. Patrick Tremblay has meticulously reviewed my manuscripts and given me important insight on how to write an exciting and concise paper. Pascale Krzywkowski for showing me the finer points of immunohistochemistry and teaching me to be a highly critical analyst. Diane Lacombe who has patiently explained how to do proper statistical analysis and has thoroughly reviewed my papers. The *in vitro* lab at Neurochem Inc. for their support and helpful advice during the microglial experiments and the *in vivo* lab for patiently breeding my cannibalistic mice to provide me with timed newborn pups. The biochemistry group, for making sure that my Aβ preps were just right and for Bob

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Contributions of Authors

In Chapter 2, the experimental design and the experiments including the set-up of isolation, characterization, stimulation, and secretory factor analysis of primary microglia, were my original contributions. Dr. Morissette provided discussion and co-supervised the project. Dr. Skamene supported this work financially with studentship funding and reviewed the manuscript. Dr. Tremblay reviewed the manuscript. Dr. Gervais contributed to the initial experimental design and supervised the project.

In Chapter 3, this project was initiated by Julie Paquette, Francine Gervais, and myself. The work on this manuscript was co-first authored with Dr. Krzywkowski. As this study involved immunohistochemical analysis of two different brain areas, the experiments were performed in parallel on separate brain areas and then cross-analyzed. The experimental design, immunohistochemical staining, processing, imaging, and analysis as well as marker set-up was equally shared. Dr. Morissette co-supervised the project. Julie Paquette supervised breeding and perfusion/fixation protocols. Dr. Tremblay reviewed the manuscript. Dr. Gervais supervised the project and provided student financial support.

In Chapter 4, the experimental design and all experiments including Aβ ELISAs, immunohistochemistry (set-up, staining, processing, imaging, and analysis) were my original contribution. Dr. Sebastiani provided the hAPP mRNA expression and mRNase protection results and was responsible for hAPP and Hc genotyping. Dr. Sebastiani and Julie Paquette contributed to the breeding set-up which was carefully supervised by Julie Paquette. Dr. Skamene supported this work financially with studentship funding and reviewed the manuscript. Dr. Tremblay reviewed the

manuscript. Dr. Westaway contributed TgCRND8 breeders to start the mouse colony and allowed their manipulation onto different mouse backgrounds. Dr. Gervais supervised the project and provided student financial support.

Abbreviations

A2M	α-2 macroglobulin
Ab	Antibody
Aβ	beta-Amyloid
ACT	α1-antichymotrypsin
AD	Alzheimer's disease
ADDL	Aβ-derived diffusible ligand
Apo	Apolipoprotein
APP	Amyloid precursor protein
BACE	β-site APP-cleaving enzyme
C	Complement factor
CAA	Cerebral amyloid angiopathy
CD	Circular dichroism
CTF	C-terminal fragment
DS	Down's syndrome
ER	Endoplasmic reticulum
FAD	Familial AD
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-monocyte colony-stimulating factor
hAPP	human amyloid precursor protein
IFN-γ	Interferon-gamma
IL	Interleukin
Int. sig.	Internalization signal

KPI	Kunitz protease inhibitor
LDL	Low-density lipoprotein
LMW	Low molecular weight
LRP	LDL-receptor-related protein
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NEP	Neural endopeptidase
NFT	Neurofibrillary tangles
NO	Nitric oxide
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PF	Protofibril
PHF	Paired helical filaments
PrP	Prion promoter
PS	Presenilin
R	Receptor
SP	Signal peptide
Tg	Transgenic
TGF-β	Transforming growth factor beta
ThioS	Thioflavin S
TM	Transmembrane
TNF-α	Tumor necrosis factor-alpha
VLDL	Very low-density lipoprotein

Statement of originality

The following work is considered to constitute original scholarship and an advancement of knowledge in the domain of Alzheimer's Disease and neuroinflammation.

1. Demonstration of the *in vitro* differences in microglial activation by $A\beta_{1-42}$ in mouse strains with a low or high inflammatory response to stimuli.
2. Activation of microglia is not dependant on the aggregation state or concentration of Aβ.
3. Correlation of inflammation, Aβ deposition, and fibrillar plaque formation through the complete and quantitative immunohistochemical analysis of an hAPP transgenic mouse model.
4. Proof of an early role for inflammation in the process of Aβ deposition.
5. Microglia and astrocytes react to both fibrillar and diffuse plaque deposits.
6. Determination of genetic background and inflammatory response effects on brain Aβ amyloidosis and survival by backcrossing hAPP transgenic mice onto different inbred mouse backgrounds and measuring hAPP mRNA, Aβ protein, and immunohistochemical levels of plaques and inflammatory foci.

Introduction

Brain inflammation in AD. Such a small phrase that incorporates two large domains of research with so many questions yet to be answered. It used to be believed that the brain was 'immune' to infectious agents. In fact, the brain has its own immune system that is similar to the peripheral system, yet different. Microglia (the brain macrophages) and astrocytes are the main immune cells in the brain. Microglia are involved in immune surveillance and innate immunity which use astrocytes to communicate and amplify inflammatory responses. Neurons have not yet been called immune cells, but their role in immune mediation is beginning to be evident. These brain immune cells have been shown to respond to beta-amyloid which is believed to be the major culprit in the neuropathology of AD. The process of its deposition and plaque formation is referred to as amyloidosis. The role of beta-amyloid in the brain, its anabolism, and its catabolism have not been fully discerned.

Through immunohistochemical analysis, microglia and astrocytes have been seen to surround senile plaques in human brains. Patients undergoing long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDS), showed an increase in the number of individuals with conserved cognitive function over non-treated individuals [1-3]. This suggested that dampening brain inflammation delayed the development of AD. Furthermore, patients receiving anti-inflammatory therapy exhibited a 65% reduction in the number of activated microglia associated with senile plaques [4]. Thus, it became obvious that there is a link between inflammation and Alzheimer's disease.

Since then, a wider selection of markers for immunohistochemical studies increased the knowledge of the brain immune system and allowed for a better characterization of the inflammatory status in the brains of patients with AD. The immune system and more precisely, inflammation, has become accepted as contributing factors to neuropathogenesis in AD. However, the time at which the inflammatory response occurs during pathogenesis, whether this is early or late in Aβ deposition, is still under debate. Microglia have been found around senile plaques and are rarely reported around diffuse plaques which are considered by many, to be the predecessors of senile plaques. Furthermore, it still remains to be determined whether inflammation is purely detrimental to the host or whether it has a positive role in the pathogenesis through the clearance of Aβ deposits by microglia. Finally, the neuropathological decline is known to vary from patient to patient suggesting that factors may be responsible for either precipitating or slowing down the neurodegenerative process. Such aspects affecting the course of the disease may be influenced by the magnitude of the inflammatory response and its effect on the neurodegenerative process. From here, the research objectives became clear:

1. Study the role of microglial activation by Aβ and its resulting inflammatory response.
2. Determine the kinetics of the appearance of inflammation during Aβ amyloidogenesis
3. Determine whether genetic backgrounds having different magnitudes of inflammatory response, leads to phenotypic variations in the brain amyloidosis.

An *in vitro* model of microglial activation by Aβ was introduced to determine whether there were differences in the activation profiles of microglia from different strains of mice known to respond to infection with a low (A/J strain) or a high (C57BL/6 strain) inflammatory response. This would demonstrate if microglia obtained from mice of different genetic backgrounds, respond differently to Aβ.

The second objective concentrated on studying the kinetics of the appearance of an inflammatory response during the Aβ deposition process and determining whether inflammation and Aβ deposition correlated well with each other. An early-onset transgenic mouse model of AD, the TgCRND8 mouse model, was used to characterize Aβ deposition, fibrillar plaque formation (fibrillar Aβ deposits), and gliosis (glial activation) in a time-dependent fashion. If inflammation occurs primarily in older mice once fibrillar (senile) plaque formation is widespread, this would indicate that inflammation does not contribute to Aβ deposition but is mostly a consequence of it. On the other hand, the early presence of inflammation during brain amyloidogenesis would support that inflammation plays a much more dynamic role in Aβ deposition whether it be in promoting deposition or in favouring its clearance and degradation.

The third objective was to determine whether an inflammatory response can impact AD-linked pathogenesis. Two observations suggest that inflammation plays an early role in Aβ pathogenesis:

- role of long term NSAIDS treatment in slowing the development of AD
- presence of cytokines and other inflammatory mediators in the brains of AD patients which could play a role in neuronal cell loss

If microglial activation is different between the low and high responder backgrounds and inflammation plays an early role in Aβ deposition, inflammatory response could have a major impact on neuropathology in AD through its influence on brain amyloidosis. Thus, mice which differ in their magnitude of inflammatory response to Aβ during brain amyloidogenesis, should have measurable phenotypic differences. Therefore, TgCRND8 mice on low (A/J) or high (C57BL/6) inflammatory response backgrounds, were used to observe the phenotypic variations between these two sets of mice. If a correlation exists between the magnitude of the inflammatory response and the development of the disease, it would be most interesting to determine whether, in humans, an individual's genetically-determined inflammatory response to Aβ could influence the entailing AD-linked neuropathology.

CHAPTER 1: Literature Review

Alzheimer's disease (AD) is the common cause of progressive cognitive decline in aged humans and is characterized by senile plaques, neurofibrillary tangles, and neuronal loss in highly selective regions of the forebrain [5, 6]. It is the most common cause of senile dementia and affects more than 10% of the population over the age of 65 years and 50% of the population over 80 [7].

Neuropathologic Features of AD

There are two main neuropathologic features of AD: neurofibrillary tangles (NFT's) and neuritic plaques which are spread diffusely through the cerebral cortex and hippocampus [8]. These areas of the brain are important for cognition, learning, and memory. Tangles are situated inside the neuron and consist of bundles of filaments in cell bodies, axons, and dendrites [9]. Plaques are located outside the neuron and consist of spherical structures with a central core of amyloid protein surrounded by distended abnormal neuronal dendrites and small axons. Both tangles and plaques are believed to be the major causes of cognitive impairment in AD. Cognitive impairment is closely correlated to neurofibrillary tangles (NFT's), synaptic and neuronal loss, dystrophic neurites, and plaque formation [10 11, 12]. The fact that AD involves both hyperphosphorylation of tau and Aβ deposition, makes it more than an amyloidosis and the relationship between NFT's and plaque formation has yet to be revealed. In the past, plaque formation was thought to correlate less strongly to cognitive decline than NFTs but this could have been due to the early appearance of Aβ deposition which makes it difficult to correlate significant cognitive impairment with an initiating factor such as Aβ deposition. With the

onset of Aβ load analysis in brain homogenates of AD patients, a clear correlation of Aβ levels to cognitive decline is evident [11, 12].

Neurofibrillary tangles consist of two fibers twisted around each other to form a rope-like filament called a paired helical filament (PHF). A particular feature of these PHFs is the presence of microtubule-associated, abnormally phosphorylated proteins such as the hyperphosphorylated tau protein. This form of tau could lead to lower levels of microtubule binding, which would destabilize the microtubules and disrupt axonal transport. In turn, this could provoke a cascade of events including the loss or degeneration of cortical connections, impaired synaptic transmission, and the emergence of impaired cognitive function [9]. Recent studies show that the hyperphosphorylation of tau and the formation of NFT's follows the deposition of Aβ [13-15]. This is very important as it shows that these two stages in AD are related and further studies will show the link between the two.

Plaques consist mainly of β-amyloid which is a protein of 40-43 amino acids that is cleaved from amyloid precursor protein (APP) located on chromosome 21 [16, 17]. A gene mutation in this area causes an increase in the ratio of the more toxic 42/43-amino acid form of β-amyloid to the less toxic 40-amino acid subtype of β-amyloid [18]. β-Amyloid deposits occur from seeding of the highly- aggregating form, $A\beta_{42}$, which form diffuse plaques [19, 20]. β-Amyloid aggregates into insoluble β-pleated sheets which form the dense fibrillar core of senile plaques that are thioflavin-S (ThioS) positive and Congo red positive. Factors such as heparan sulfate proteoglycan, promote aggregation and fibrillar formation due to the sulfate moieties on glycosaminoglycans [21, 22].

Plaques have been typed into four different groups: diffuse; primitive; classic; and compact [23]. Diffuse plaques are nonfibrillar, consist mainly of $A\beta_{1-42/43}$ and APP, and contain complement, proteoglycans, ApoE, and α_1-antichymotrypsin. Primitive plaques differ from diffuse plaques as they contain a fibrillar Aβ core and are often surrounded by glial cells which play a role in plaque maturation, dystrophic neurite formation, or the degradation and removal of plaque components. Classic plaques consist of fibrillar, condensed $A\beta_{1-40}$ and $A\beta_{1-42/43}$ core with a corona of dystrophic neurites and are also surrounded by glial cells. Finally, compact plaques are rare and are similar to classic plaques although they lack the corona and glial cells.

There are two hypothesis of plaque formation [23]. The first hypothesis is that plaque types are the result of a successive progression of diffuse plaques to primitive and then to classic, to the final stage of compact plaques. This is based on temporal studies of Down's Syndrome patients that develop AD by the age of 50 [24, 25]. Transition from each plaque form would occur due to the formation of amyloid from diffuse deposits which would provoke the development of dystrophic neurites, followed by the formation of Aβ fibrils.

In support of the theory of plaque evolution, four plaque types were observed in immunohistochemical sections from AD patients around the age of 73 years [26]. Interestingly, diffuse β-amyloid$^+$ plaques were found to be immunogenic and attract and activate microglia with overexpression of IL-1α. This is followed by activation of astrocytes with increased expression of β-APP and release of neurotrophic fragments. Thus proposing that glia could be involved in brain amyloidosis once diffuse plaques are detected. The nomenclature used for describing plaques is slightly different from

Armstrong et al. [23]. beta-Amyloid-immunopositive plaques were classified according to the pattern of beta-amyloid distribution (diffuse vs dense-core) and the presence or absence of dystrophic beta-amyloid precursor protein-immunopositive (beta-APP+) neurites (neuritic vs non-neuritic). Therefore, the plaques observed were: diffuse non-neuritic plaques (diffuse); diffuse neuritic plaques (classic); dense-core non-neuritic plaques (primitive) and dense-core neuritic plaques (compact). Dense-core neuritic plaques are less immunogenic and thus, have fewer IL-1α^+ microglia and therefore, fewer dystrophic β-APP$^+$ neurites.

The second hypothesis of plaque formation is that individual plaque types develop independently and depends on the amount of Aβ deposited and the 'fibro-architecture' of the tissue at the site of deposition. This hypothesis was introduced by Wisniewski et al. after spectral observations of Aβ deposits in AD tissue [27]. Although neither hypothesis is conclusive, the arrival of a multitude of transgenic models and better medical resonance imaging of AD patients should reveal which theory best applies to amyloidosis in AD.

APP processing

β-Amyloid is derived from APP which is constitutively expressed by neuronal and non-neuronal cells in brains of both healthy individuals and AD patients. APP is a single-transmembrane protein with a 590-680 amino acid long extracellular amino terminal domain and an approximately 55 amino acid cytoplasmic tail that contains intracellular trafficking signals [28] (Figure 1). The APP mRNA undergoes alternative splicing to yield 10 possible isoforms of APP. APP695 is the predominant isoform which contains 695 amino acids and lacks the Kunitz protease inhibitor (KPI) domain that is present in two other major isoforms APP751 and APP770 also known as KPI-APPs [29-31]. The APP695

isoform is produced mainly in neurons whereas APP751 and APP770 are found mostly in non-neuronal glial cells and all three are amyloidogenic.

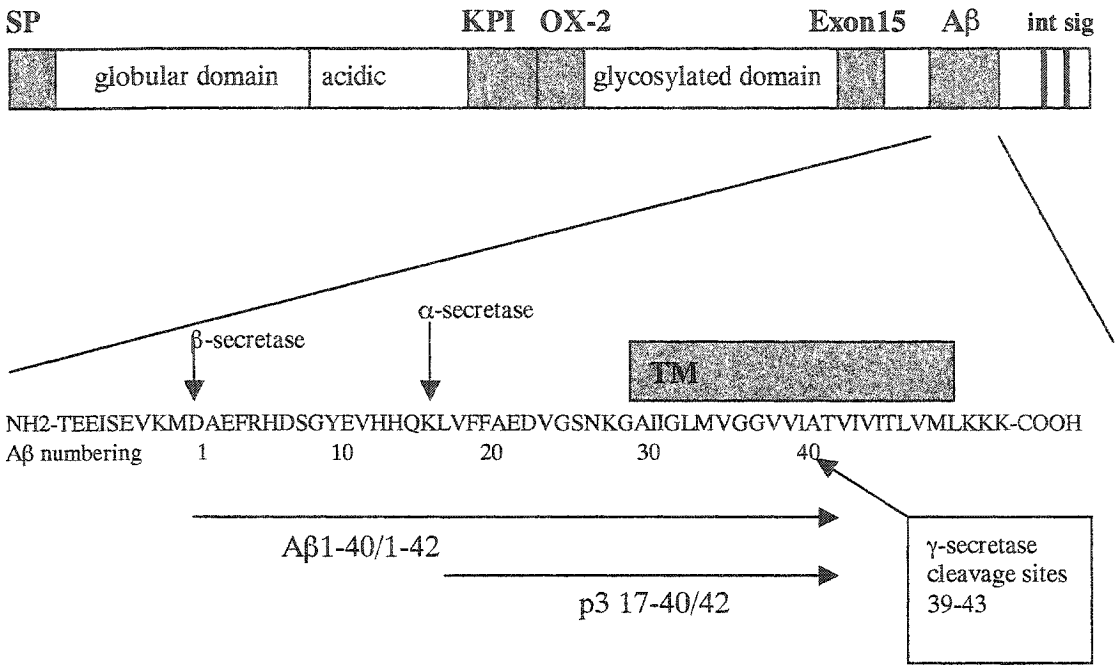

Figure 1 APP 770 structure

The APP770 isoform (and the APP751 isoform) contains a KPI sequence and the OX-2 region (exon with homology to OX-2) unlike the commonly-found APP695 isoform. This figure illustrates the signal peptide region (SP), globular and glycosylated domains, acidic region, Exon 15 (spliced to generate L-APP), the Aβ region also containing part of the transmembrane domain (TM), and the internalization signals (int. sig.). The secretase cleavage sites are also indicated [32].

APP is translocated to the endoplasmic reticulum by its signal peptide and matures through the secretory pathway as it moves through the trans-Golgi network [33]. Protein domains of APP consist of the intracellular, transmembrane, and Aβ domains where the Aβ sequence of APP spans from the middle of the membrane to the extracellular part of the APP molecule. When exon 15 is spliced out, it generates L-APP molecules and reveals an attachment site for chondroitin sulfate proteoglycans where the binding of a glycosaminoglycan chain near Aβ may affect APP processing [34]. APP also contains a globular domain that contains heparin-, zinc-, and copper-binding domains as well as a

glycosylation domain that may be involved in dimerization [35]. The cytoplasmic carboxyl-terminal domains contain internalization and transduction signals which implicate its role as a cell-surface receptor [32]. Although the function of APP is unknown, it has been suggested to be involved in neurite extension or memory, cell-cell/matrix interactions, axonal growth modulation, and regulation of intracellular calcium levels [35-38].

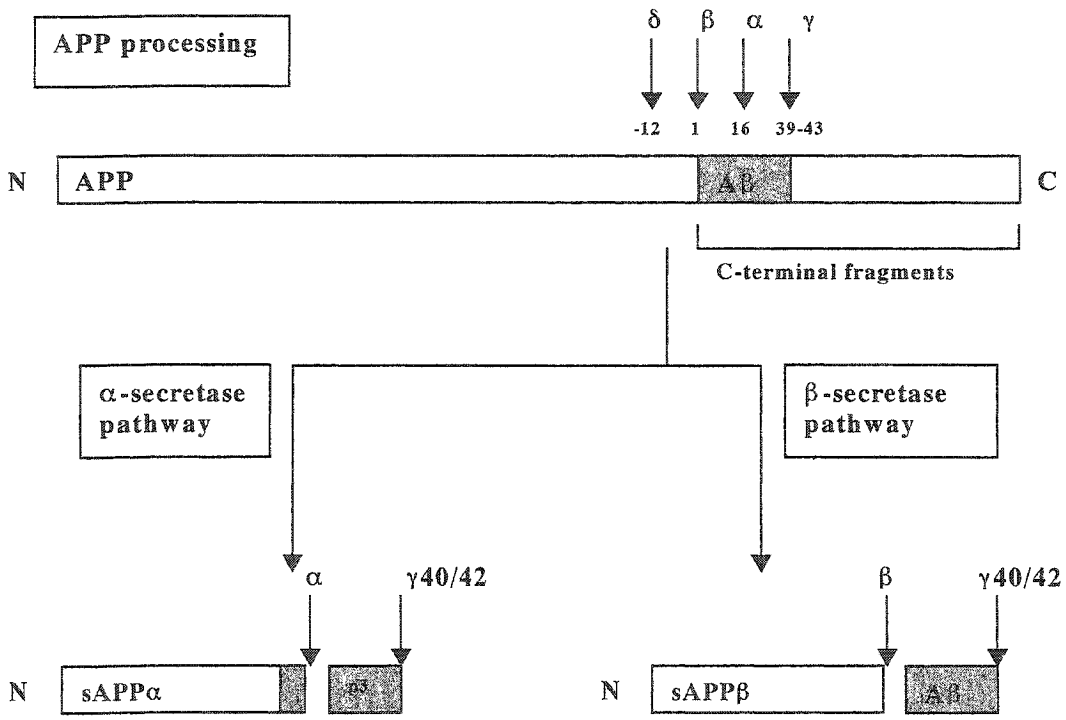

Figure 2 APP secretase pathways

The first step in APP cleavage is by δ-secretase at Thr^{-12} (12 residues from the N-terminus of Aβ), by β-secretase at Met^{1}, or by α-secretase at Lys^{16}. Cleavage by α- or β-secretase is followed by γ-secretase cleavage at residues 39 through 43. The α-secretase pathway generates soluble APPα and p3 and the β-secretase pathway generates soluble APPβ and Aβ including the toxic form, $Aβ_{1-42}$ [39].

There are two major pathways involved in the cleavage of APP [28, 39-41] (Figure 2). In the α-secretase pathway, APP is cleaved at lysine 16 within the Aβ sequence by α-

secretase at the cell surface or within the cell, into soluble amino-terminal fragments of 100-130 kDa long (αAPP) and a 10kDa membrane-associated carboxyl-terminal fragment (αCTF). The αCTF fragment is then cleaved by a γ-secretase into a 3 kDa, truncated N-terminal $A\beta_{17-40}$ fragment, p3, and $A\beta_{17-42/43}$. In this way, the α-secretase pathway precludes the formation of Aβ [42]. In the β-secretase pathway, APP is first cleaved by β-secretase (BACE/Asp2) into βAPP and βCTF (or A4CT). Following this, γ-secretase cleaves the βCTF fragment within its transmembrane region into an Aβ peptide of 40-43 amino acids in length. These Aβ peptides are secreted by cells and can therefore be detected in the plasma and cerebrospinal fluid [43].

The two most common forms of Aβ are $A\beta_{40}$ which comprises 90-95% of secreted Aβ and $A\beta_{42}$ which makes up 10% of the secreted Aβ [43]. A large area of research is focussed on determining whether there is a metabolic control over the production of $A\beta_{40}$ versus $A\beta_{42}$. Presenilins play a role in the γ-secretase-cleavage of Aβ as they have been proposed to be γ-secretases or closely-linked factors to γ-secretase cleavage. Additionally, debate continues over whether there is a γ-secretase specific for $A\beta_{40}$ and for $A\beta_{42}$ or only one that is regulated differently. A proof of their involvement in C-carboxy-terminal APP cleavage comes from PS1 knockout mice that produce less Aβ due to impaired γ-secretase cleavage of APP [44]. Also, FAD-linked mutations in the presenilins provide a link between Aβ and AD in that expression of these mutants in cells favours the production of $A\beta_{42}$ [45]. Furthermore, a recent molecular study has suggested that PS1 is directly involved in the cleavage of APP by γ-secretase [46].

The amino acid sequence of Aβ has been studied to determine functional domains. Many investigators have used the Aβ25-35 sequence due to its potent neurotoxicity. Other important sequences are the aggregation domain Aβ16-20 (KLVFF) [47], the microglial binding and activation domain Aβ10-16 (YEVHHQK) [48], the Aβ13-16 (HHQK) region is required for an initial microglial interaction with plaques through a cell-surface binding site involving heparan sulfate [49], the amnesic domain Aβ18-20 (VFF) [50], and the cell surface-binding domain Aβ31-34 (IIGL) [51] .

Both secretase pathways produce CTFs that vary in size from 8-12 kDa but the β-secretase pathway generates a CTF, C100, that is particularly amyloidogenic and has been shown to be neurotoxic *in vitro* [52]. *In vivo,* C100 leads to similar neuropathology to that seen in AD such as neurodegeneration, cognitive dysfunction, increases in acetylcholinesterase, and abnormalities in synaptic transmission [53-55]. Since C100 is amyloidogenic and can induce AD-like neuropathology, the following hypothesis has been proposed by Neve and others implicating the C100 fragment in neurodegeneration [56]. The brain portion of APP is present as an integral plasma membrane protein that mediates the transduction of extracellular signals into the cell via its C-terminal tail. Therefore, they hypothesize that FAD (familial AD) mutation-induced abnormal accumulation of Aβ-containing C100 in neurons, can cause progressive dysfunction of APP signaling in AD and results in apoptosis. This demonstrates another potential amyloidogenic factor in addition to Aβ.

Genetics of Alzheimer's Disease

AD can be divided into three general clinical categories*: early- and late-onset familial disease* occurs before 50 years of age and later than 65 years of age, respectively and are

linked to specific mutations or chromosomal loci; *late-onset familial disease* occurs after 65 years of age; *and sporadic, late-onset disease* occurs after 65 years of age with no apparent familial association [57]. Approximately 90% of AD cases are of the sporadic, late-onset form.

From genetic approaches, it has been shown that infrequent familial-associated AD (FAD) found in early-onset autosomal dominant AD that begins before 60 years, is associated with gene mutations on chromosomes 1, 14, and 21 [58]. Chromosome 21 contains the amyloid precursor protein (APP) gene where post-translational processing can lead to the production of the neurotoxic Aβ protein. Point mutations in the APP gene on chromosome 21 were discovered in families with early-onset AD [9].

Figure 3 Known FAD mutations in hAPP

Known FAD mutations in hAPP include: double Swedish (KM671NL); Dutch-type (E693Q); Arctic (E693G), Italian (E693K), London mutations at 717 including Indiana mutation (V717F), Iowa (D694N), Florida (I716V), Australian (L723P), and Flemish (A692G) mutations.

Substitution mutations at codon 693 of APP770 have been described as Dutch-type (substitution of Glu-Gln, E693Q), Arctic (substitution of Glu-Gly, E693G), and Italian-type (substitution of Glu-Lys, E693K) [59-61]. Other mutations include missense

mutations at residue 717 in APP770 of Val (Indiana mutation, V717F; London mutations are a collection of all mutations at the 717 site: V717F, V717G, V717I), double mutations at codons 670 and 671 of Lys-Met to Asn-Leu (Swedish double mutation, K670N M671L), mutation at codon 694 of Asp-Asn (Iowa mutation, D694N), mutation at codon 716 of Ile-Val (Florida mutation, I716V), mutation at codon 723 of Leu to Pro (Australian mutation, L723P), and substitution mutation at codon 692 of Ala-Gly (Flemish mutation, A692G) [62-67]. Mutations at codons 717 and 692 lead to the an increase in the production of the more amyloidogenic $A\beta_{1-42}$ and the Swedish mutation results in a 5-8-fold increase in the formation of Aβ [18, 68]. It is interesting to note that mutations near the γ-secretase and α-secretase sites lead to increases in $A\beta_{1-42}$ whereas mutations near the β-secretase leads to large increases in overall Aβ load.

Presenilins 1 and 2 (PS1 and 2) located on chromosomes 14 and 1, respectively, also contain mutations that cause early-onset familial AD. Most mutations occur in the PS1 gene where more than 50 mutations have been reported which account for 25-30% of early-onset cases of FAD [69-71]. The most severe forms of AD arise from mutations in the S182 gene on chromosome 14 since the age of onset can be from 30 to 60 years [69]. PS2 mutations in the STM-2 gene on chromosome 1 with a Asn-Ile substitution at codon 141, account for the early-onset familial AD of the Volga Germa variety [72]. Another PS2 mutation causing autosomal dominant AD is in the Italian pedigree with a PS2 variant Met239Val [73]. These mutations account for only 1-3% of all AD cases, but elucidating the mechanisms involved in these forms of AD may help better understand sporadic AD, which is much more common.

A large portion of the familial and sporadic AD cases are associated with inheritance of the apoE4 allele on chromosome 19 which appears to confer susceptibility to the common form of late-onset AD [74]. It is a 34-kDa glycoprotein which is the major serum protein involved in cholesterol storage, transport, and metabolism, and is produced and secreted in the CNS by astrocytes [75]. Apolipoprotein E has three alleles: apoE2, apoE3, and apoE4. It has been shown that the difference in the apoE genotype alone determines the distribution of age at the time of AD onset [76]. The five commonly inherited apoE genotypes are apoE2/3, apoE2/4, apoE3/3, apoE3/4, and apoE4/4. The mean onset for apoE4/4 and apoE3/4 is much earlier with a mean of less than 75 years whereas apoE2/3 is more than 90 years. Overall, the apoE4 allele increases the risk of developing late-onset AD while the apoE2 allele appears to reduce the risk of late-onset AD [77, 78].

Other genetic risk factors include α-macroglobulin [79], endothelial nitric oxide synthase-3 genes [80], IL-6 [81], and IL-1 [82]. Genetic risk factors are still being uncovered and will become more evident as the neuropathogenesis of AD is understood in its full complexity.

Aβ aggregation and fibrillar formation

The deposition of amyloid has been considered the earliest step in AD pathogenesis and these deposits are toxic to the brain [83]. Amyloid refers to the insoluble protein deposits that are congophilic and exhibit red-green birefringence in the presence of plane polarized light [84]. Thus, determining the way in which Aβ aggregates and forms fibrils is necessary to find therapeutic agents that could potentially delay or prevent AD pathogenesis by disrupting Aβ aggregation or fibril formation.

A simple model of fibril formation is the nucleation-dependent mechanism which is often referred to as 'seeded growth'. The slowest part of the process is the lag time where monomers form dimers which form tetramers and other oligomers (also known as ADDLs or Aβ-derived diffusible ligands), until a highly-unstable nucleus is formed. Monomers and dimers have been termed low molecular weight Aβ (LMW Aβ). Once the nucleus is formed, the equilibrium is favourable for fibril formation as nuclei in solution serve as 'seeds' for fibril growth [85]. Monomer Aβ forms fibrils at physiological pH with agitation in a matter of hours for $A\beta_{42}$ or in a few days for $A\beta_{40}$ [85]. The difference in rate of fibrillization between $A\beta_{40}$ and $A\beta_{42}$ can be explained by the highly aggregating characteristic of $A\beta_{42}$ which allows for rapid fibril formation [83]. The rate of fibrillogenesis is dependent on the equilibrium between the low molecular weight (LMW) Aβ and the protofibril (PF) formation that occurs as an intermediate step [86]. Protofibril formation is followed by irreversible maturation into fibril formation.

Fibrillar and nonfibrillar Aβ can be differentiated by the amount of β-sheet content. LMW Aβ structure is highly disordered but protofibrils and fibrils contain more than 50% β-sheet structure, about 40% random coil structure, and 10% α-helix structure which can be measured by circular dichroism (CD) spectroscopy and thioflavin T (ThioT) assays [86]. Fibrils and protofibrils induce neurotoxicity in a concentration-dependent manner whereas LMW Aβ has no effect on neurons. Hence, fibrillar Aβ is directly neurotoxic and protein structure analysis allows for neurotoxic Aβ species to be defined.

Another study of fiber assembly used electron microscopy and X-ray diffraction to support the ordered assembly from monomer to fiber via a protofilament structure [87]. They found that the amyloid fiber consists of a hollow cylinder composed of five or six

protofilaments arranged in a pentagonal or hexagonal array (respectively). Each protofilament consists of an arrangement of contiguous β-sheet polypeptide chains which are aligned perpendicular to the long axis of the fiber. Furthermore, soluble peptide converted from an α-helical or a random coil structure to a β-structure upon aggregation. This study gives insight into the assembly of fibers and will help to define the role of Aβ fiber-inducing factors such as ApoE (apolipoprotein) and sulfate proteoglycans in the assembly process [21, 88].

Trends are changing from the traditional view of the 'amyloid cascade hypothesis' where fibrillar Aβ is the driving force in neurodegeneration to smaller aggregates [86, 89, 90]. In the past, studies have shown a poor correlation of cognitive loss to plaque formation therefore, researchers have begun to evaluate other forms of Aβ. At high concentrations, oligomers lead to protofibril followed by fibril formation, but at constant, low concentrations, they directly induce memory impairment, synaptic loss and neuronal death [90]. Thus, oligomers are also neurotoxic. Since the steps of aggregation and fibril assembly are still being investigated, there could be many smaller aggregates of Aβ to target for drug therapy [87]. New studies show strong correlations between Aβ load (particularly, $A\beta_{42}$) and cognitive decline [11, 12]. Therefore, early forms of aggregated Aβ could be the key to neuropathology in AD.

Intracellular/Extracellular Aβ

Extracellular Aβ in the form of diffuse or senile plaques, has been widely characterized in AD patients [5, 27, 57, 91]. There has been ongoing debates as to whether plaques or NFTs are the key factors leading to neurodegeneration [13]. As the reconciliation of both plaques and NFTs playing a part in neuropathogenesis in AD is underway, recent studies show that

Aβ load strongly correlated with cognitive decline and that intracellular Aβ is being observed in AD and Down's syndrome patients before plaques or NFTs appear [11, 12, 92, 93]. The fact that Aβ load and not the presence of extracellular senile plaques, correlates strongly to cognitive decline, further supports the implication of intracellular Aβ in AD [94]. Therefore, intracellular Aβ is now being viewed as a preliminary event in the neuropathology of AD.

Once Aβ is produced through the amyloidogenic secretory pathway, it is either stored intracellularly or secreted extracellularly. Intracellular stores of Aβ are formed through γ-secretase cleavage on the endoplasmic reticulum (ER), the Golgi, or through cell surface recycling via the endosomal/lysosomal pathway [28]. A recent study showed that there were accumulations of γ-cleaved $A\beta_{42}$ in human neurons in AD-vulnerable brain regions [92]. Adjacent sections of intraneuronal $A\beta_{42}$ staining and hyperphosphorylated tau staining suggest that neuronal $A\beta_{42}$ staining is more abundant and precedes NFT. In addition, they showed that early $A\beta_{42}$ immuno-reactive senile plaques developed from neurons with large accumulations of $A\beta_{42}$. In a study of amyloid plaques in Down's syndrome patients who invariably develop the neuropathologic features of AD, intraneuronal Aβ-amyloid was found to precede plaque formation [93]. Gyure et al. propose a sequence of events where intracellular Aβ accumulates in neurons and astrocytes, followed by extracellular deposition of Aβ and diffuse plaque formation, and finally, the development of senile plaques and NFTs with microglial activation occurring late in disease progression. Transgenic mouse models have also shown that intracellular Aβ accumulates before plaque formation [95, 96]. Thus, these studies suggest

that intracellular $A\beta_{42}$ accumulations may be the earliest event in Aβ deposition and in the pathology of AD.

As studying anabolism of Aβ is technically easier than catabolism of Aβ, few studies have been done on the cellular degradation of Aβ. An investigation of Aβ catabolism was performed by tracing multiple-radiolabeled synthetic peptides injected into the rat hippocampus [97]. After narrowing down potential peptidases, specific peptidase inhibitors were used to determine the peptidase(s) responsible for intracellular Aβ accumulation. Neural endopeptidase (NEP) which is similar to neprilysin, was found to control the rate limiting step in Aβ degradation. Also, NEP was found to increase the amount of soluble $A\beta_{42}$ which was reflected in the elevated $A\beta_{42}/A\beta_{40}$ ratios as observed in early-onset FAD that is also achieved in the transgenic mice overexpressing a mutated form of PS1 [98]. Finally, after 30 days of NEP treatment, diffuse plaques were observed in the hippocampus and surrounding cortical areas where the inhibitor had been infused. This study is one of the rare ones that directly demonstrate the effect of slowing Aβ catabolism on AD pathology.

Extracellular Aβ deposition has been proposed to be a result of intracellular accumulations of $A\beta_{42}$ since a plaque consists of large concentrations of Aβ which would most likely require some pre-seeding in addition to other factors, to enhance its formation. In a theoretical model suggested by Wilson et al., insoluble, intraneuronal accumulations of $A\beta_{42}$ could be increased by FAD mutations and promote fibril formation [28]. Accumulations of $A\beta_{42}$ would induce apoptotic death in neurons which would then, release the aggregated $A\beta_{42}$. This would seed plaque formation as aggregation of $A\beta_{42}$ occurs rapidly under *in vitro* conditions [99] and in *in vivo* conditions,

proteoglycans, gangliosides, metal ions and other aggregation-inducing molecules are available [100, 101]. Therefore, intracellular Aβ accumulations may not be necessary although they would contribute to extracellular plaque formation.

Most AD cases are late-onset and are not linked to mutations in β-APP, PS-1, or PS-2, which directly cause increases in Aβ but half of these cases have been shown to be linked to the rate of deposition through ApoE [102]. Therefore, studying the removal or degradation of Aβ is key in treating the majority of Alzheimer cases. Secreted (extracellular) Aβ can be degraded by extracellular proteases or by uptake back into the endosomal/lysosomal pathway and intracellular degradation. Frautschy et al. showed that co-infusion of $A\beta_{40}$ with the cysteine protease inhibitor, leupeptin (inhibits lysosomal degradation), resulted in increased extracellular and intracellular Aβ immunoreactivity and exacerbated neurotoxicity [103]. In this way, dysfunctional intracellular degradation can enhance neurotoxicity.

Intracellular $A\beta_{1-42}$ has recently been shown to be neurotoxic by apoptosis through the p53-Bax cell death pathway [104]. Microinjections of $A\beta_{1-42}$ peptide or cDNA-expressing cytosolic $A\beta_{1-42}$ into primary human neurons were highly neurotoxic whereas $A\beta_{1-40}$ and reverse peptides were not. Also, this neurotoxicity was not induced in human primary astrocytes nor in neuronal and non-neuronal cell lines. Since intracytoplasmic accumulation of $A\beta_{1-42}$ has been observed in AD patients and this accumulation has been shown to be neurotoxic, this brings the attention away from plaques and NFTs which could be end events in AD to what may be as yet, the earliest event in AD, intraneuronal Aβ accumulation [92, 105].

Aβ neurotoxicity

Aβ can be directly or indirectly neurotoxic. Direct neurotoxicity arises from Aβ binding to the surface of neurons, inducing radical mechanisms, which eventually leads to neuronal apoptosis. This has been demonstrated when the addition of Aβ to neuronal cultures initiated production of H_2O_2 and lipid peroxidation, suggesting that free radical damage and oxidative stress are involved [106-108]. Direct neurotoxicity due to Aβ, can arise from inside the neuron. As described in the previous section, microinjections of $A\beta_{1-42}$ into primary human neuronal cultures was strongly neurotoxic and induced apoptosis through the p53-Bax cytotoxicity pathway [104]. Thus, leakage of $A\beta_{42}$ from vesicles inside the cytosol, would be directly neurotoxic. Other mechanisms of direct toxicity involve the alteration of ionic homeostasis [109].

Indirect neurotoxicity occurs by the activation of inflammatory cells such as microglia and astrocytes. Activated microglia can induce iNOS expression and release of NO, reactive oxygen species (ROS), the complement cascade that leads to MAC formation and bystander lysis, and the activation of proinflammatory cytokines which promotes chronic inflammation in AD [48, 110-112]. Astrocytes also play a role by producing proinflammatory cytokines and other inflammatory mediators [3].

The form of Aβ that is responsible for neurotoxicity is continuously being investigated. *In vitro* studies have been performed with primarily three different peptide sequences of Aβ, $A\beta_{1-40}$, $A\beta_{25-35}$, and $A\beta_{1-42}$ [110, 113-115]. The $A\beta_{1-40}$ protein is variable in neurotoxicity but its rate of aggregation is much slower than $A\beta_{1-42}$ which makes it easier to work with [99, 115]. Very strong neurotoxicity is induced with the $A\beta_{25-35}$ sequence but as it is only a portion of the whole sequence, it omits many domains such as the microglial

binding domain and the aggregation domain that could be important in studying effects of Aβ on cell cultures [48]. As $A\beta_{1-42}$ levels are increased in AD, are related to cognitive decline, and readily aggregates to form the neurotoxic, fibrillar Aβ, most studies are currently performed with $A\beta_{1-42}$ [11, 12].

Many studies have shown that Aβ neurotoxicity requires Aβ assembly into fibrils whereas the nonfibrillar amorphous form is not neurotoxic [116, 117]. In a recent review by Klein et al., they discuss the view that fibers are no longer the only neurotoxic form of Aβ and that oligomers and protofibrils induce potent neurological activity [90]. They suggest that even in fibrillar preparations used in inducing neurotoxicity, there are amounts of soluble Aβ such as oligomers and protofibrils which are more likely to be responsible for strong neurotoxic responses. Studies have shown that protofibrils cause oxidative stress and neuronal death [86]. Furthermore, the soluble amyloid component has been shown to correlate clearly with disease severity and synaptic loss [118, 119]. Hence, both soluble and fibrillar Aβ are considered to be neurotoxic.

Inflammatory response and AD

Inflammation is the process where cells of the immune system and their products are concentrated to the site of infection. The site of inflammation is marked by tissue damage and complement activation which promotes the release of chemotactic mediators that direct phagocytes to the inflammation site. Microglia are the brain macrophages that are responsible for cleaning-up cell debris and 'infectious agents' through phagocytosis and responding to inflammation with pro-inflammatory cytokine production. Microglia and astrocytes (glial cells) have been found around senile plaques which shows that extracellular Aβ deposits induce an inflammatory response [91, 120-124]. Furthermore, this

microglial-mediated inflammatory response has been shown to contribute to neuronal cell loss and cognitive decline [125]. Hence, 'inflammatory foci' are an accepted feature in the neuropathology of AD.

Thus, the brain is not as immunologically privileged as once thought. Essential immune reactions are involved in response to a wide variety of defined pathogens and toxins in the brain, as well as in neurodegenerative diseases [126]. Additionally, there are many studies that support the mediation of microglia and astrocytes in the immuno-inflammatory response contributing to AD. One aspect to consider when mentioning brain inflammation is the integrity of the blood brain barrier (BBB) which plays an important role in the homeostasis of the central nervous system. Highly specialized cerebral endothelial cells form the anatomical basis of the BBB. These cells regulate the entry of circulating substances into the brain by the tight junctions and specialized transport functions [127]. Cytokines (TNF, IL-1, IL-6) have been shown to induce a disruption of the BBB *in vitro* and in this process, cyclooxygenase activation within the endothelial cells seems to play a key role [128]. de Vries et al. measured the resistance across rat cerebral endothelial cells after treatment with each of the cytokines and in the presence of indomethacin, a cyclooxygenase inhibitor. They propose that TNF upregulates IL-1 that then stimulates the release of IL-6 from cerebral endothelial cells. The cytokine influence on the BBB acts through their ability to activate the cerebral endothelial cells to produce eicosanoids that induce the opening of BBB. So far, there is a lack of evidence for BBB dysfunction in AD and for an immune complex-mediated or cellular immune-mediated response. Therefore, a locally-induced, non-immune-mediated, chronic inflammatory response without any apparent influx of leukocytes from the blood, appears to be the main response involved [129].

Microglia and astrocytes participate in the immune surveillance and defence of the CNS directly or indirectly through the upregulation of molecules that recruit other cells of the immune system, lead to cell activation, and regulate the immune or inflammatory response. These players can lead to cell injury of surrounding glia and neurons if they are not effectively counter-regulated. Both astrocytes and microglia secrete cytokines to activate other neighbour cells, induce proliferation, permeabilize the endothelium to allow other immune cells to enter, destroy infected cells, and regulate the immune/inflammatory response [130]. Astrocytes produce a wider range of molecules (complement factors, acute phase reactants, cathepsin, cytokines, growth factors, etc.) compared to microglia but microglia secrete factors that are directly required for inflammation [3, 131]. Activated microglia are established sources of proinflammatory cytokines IL-1, IL-6, and TNF-α as well as anti-inflammatory cytokines IL-10, IL-1ra, TGF-β, and prostaglandin E2 [132-134]. They also produce chemokines such as MIP-1α, MIP-1β, RANTES, MCP-1, and IL-8. In addition, they produce numerous neurotoxic molecules such as platelet activating factor (PAF), NO, TNF-α, glutamate, reactive oxygen species (ROS) and some undefined neurotoxic factors [133]. The extent to which activated microglia benefit neuronal survival or damage the neuronal network, is still under debate [131, 135].

Microglia and astrocytes produce many neurotoxic factors but nitric oxide (NO) and reactive oxygen species (ROS) appear to be the most neurotoxic [136-139]. NO inhibits growth and can kill intracellular organisms such as bacteria and parasites [140]. This has been shown to be enhanced in the presence of TNF-α and lead to neuronal apoptosis [141, 142]. Neurotoxicity of ROS is mainly due to the production of superoxide anions that act via their respiratory burst system. When microglia phagocytose a particle, the external

membrane invaginates and the free radicals are directed at the internalized product. If invagination fails, the free radicals are directed outside the cell to surrounding host tissue.

There are many functions of IL-1 that may contribute to the pathophysiology of AD. These include the upregulation of the expression [143] and processing of β-amyloid precursor [144]; complement C3 transcription [145]; induction of prostanoid, cytokine, chemokine, and acute-phase protein secretion [146, 147]; increased formation of free radicals and nitric oxide [146, 147]; increased expression of adhesion molecules [146]; promotion of astrogliosis [148]; and inhibition of synaptic strength and long-term potentiation [149]. Additionally, it has been shown that incubation or 'aging' of concentrated $A\beta_{25-35}$ solutions, stimulated human astrocytes to produce IL-1β which in turn, induced IL-6 and IL-8 secretion [150]. This implies that chronic neurodegeneration in AD-affected brain regions may be mediated in part by the ability of Aβ to exacerbate inflammatory pathways. Furthermore, IL-1 activates astrocytes, which leads to their overexpression of neurotrophic cytokine, S100β. Therefore, IL-1 can play a neuroprotective role by upregulating S100β in astrocytes which favours the growth of dystrophic neurites in β-amyloid plaques [151].

Another important aspect of this immune response is the production of IL-6 by activated astrocytes. In a study by Hull et al., they looked for the presence of IL-6 in plaques and found that most IL-6-positive plaques were diffuse (71%) and contained Aβ suggesting that neuritic degeneration is preceded by the presence of IL-6 [152]. In IL-6 transgenic mice overexpressing IL-6 in astrocytes [153], they showed pronounced cerebral abnormalities including astrogliosis, neurodegeneration of hippocampal neurons, and reductions of dendritic arborisation. Interestingly, it has been shown that

neurotransmitters stimulate the synthesis of IL-6 and may therefore explain the induction of IL-6 due to psychological stress [154]. Additionally, basal IL-6 levels increase with normal aging [155]. Therefore, elevated levels of IL-6 could be damaging to neurons.

In terms of sequence of events, elevated levels of IL-1β in microglia leads to an increase in IL-6 production from activated astrocytes which can then act via nuclear proteins to augment the synthesis of α-2 macroglobulin (α2M) or α1-antichymotrypsin (ACT), class II acute phase proteins. ACT is also found to be elevated in AD patients [156]. The acute phase protein α2M promotes neurite growth via the α2M low-density lipoprotein (LDL) receptor [157], acts as a broad range protease inhibitor [158], and as a chaperone protein for a diverse array of proteins such as TNF-α, βFGF, and IL-6 which might affect neuronal function [158]. Furthermore, IL-6 and IL-1 together can increase the synthesis of class I acute phase proteins, CRP and complement C3, in astrocytes [159]. Increases in CRP and C3 enhances the potential for immune-mediated cell lysis and is thus, detrimental to neighbouring cells [160].

Another pro-inflammatory cytokine is TNF-α. Its role as a neurotoxic or neuroprotective factor is still being debated [161-164]. Viel et al. suggest that low levels of TNF-α are protective and high levels of TNF-α are neurotoxic and that this is age-related [165]. These high levels of TNF-α could be induced in the presence of an additional stimuli whereas low levels of TNF-α would persist in the absence of extra stimuli [141]. Interferon regulatory factor (IRF) is inducible by a variety of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-12 and activates one molecular pathway leading to iNOS transcription [166]. Treatment of microglia with a secreted derivative of β-amyloid precursor protein led to activation of NF-κB with ensuing production of TNF-α, IL-1, and

iNOS [167]. This upregulation of TNF-α can lead to iNOS-dependent apoptosis of neurons [141]. TNF-α can also enhance vascular permeability to allow other immune cells to influx to the site of inflammation [168]. Therefore, TNF-α would act as an anti-apoptotic factor (or neurotrophic factor) by default unless it is strongly-induced through co-stimulation as observed in the case of Aβ stimulation, where it would lead to neuronal apoptosis [169].

Astrocytes and microglia may not be the only inflammatory cells involved in AD. Recent findings have shown that neurons have an increased expression of complement factors, some cytokines (IL-1 and IL-6) and cycooxygenase-2 (COX-2) which is not found in glial cells [3, 129, 170]. This shows that neurons themselves, are active in the neuroinflammatory process. The advent of studies on intracellular Aβ within neurons suggests that this could be the starting point of AD neuropathology where an APP mismetabolism could occur. Therefore, intraneuronal accumulation of Aβ leading to neuronal cell lysis could initiate an inflammatory response by activating microglia, astrocytes, and possibly, neurons.

Long-term treatment with non-steroidal anti-inflammatory drugs (NSAIDS) have been suggested to prevent or delay the development of AD [1, 2]. This effect would be due to the NSAID inhibitory effect on COX and prostaglandin H2 synthase which is involved in the first two steps of the synthesis of prostaglandins from the substrate, arachidonic acid. Two isoforms of COX have been identified. COX-1 is expressed in microglial cells constitutively and is involved in the production of prostaglandins and effective in cellular housekeeping functions. The second isozyme, COX-2, is produced in neuronal cells and can be induced by inflammatory stimuli such as IL-1 [170]. NSAIDS have also been shown to regulate gene expression via their interaction with peroxisome proliferator-activated

receptors (PPAR) [171]. One of the three isoforms, PPARγ, is principally involved in suppressing the expression of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 in monocytes and macrophages [172, 173]. Furthermore, PPARγ agonists inhibit the β-amyloid-stimulated secretion of proinflammatory products (IL-6 and TNF-α) by microglia and monocytes as well as COX-2 expression [174]. This indicates that PPARγ agonists may serve as specific therapeutic agents to treat AD rather than NSAIDS that act on both COX isoforms providing these agonists can cross the BBB.

Complement and β-amyloid

The classical complement cascade is involved in AD [175]. It consists of multiple cleavage steps of C1 through to C5b which combines with C6, C7, C8, and multiple C9 molecules to form the membrane attack complex (MAC). Many complement cascade components have been shown to be increased in the AD neocortex and/or shown to be associated with plaques such as C1q from microglia [112], C3 from astrocytes, C4 from neurons, and C5b membrane attack complex [176]. Activated microglia express complement proteins and complement receptors which bind C3 and C4 fragments [177]. C1 is a macromolecular complex that initiates the classical complement pathway cascade of reactions where Clq is the recognition subunit. It has been shown that C1q binds to β-amyloid through its collagen tail instead of binding to the globular-head region where immunoglobulins bind. This interaction is a charge interaction between the positively charged ClqA region from amino acids 16-26 where Arg_{16}, Arg_{19}, and Arg_{20} are responsible for this charge concentration. This region interacts with a negatively charged area on β-amyloid [178]. Upon C1q binding to Aβ, the classical complement cascade is activated, generating MAC. MAC inserts itself into cell membranes which causes cell lysis and the death of

the cell. As this is a non-specific event, many cells proximal to MAC formation can be affected by bystander lysis. Endogenous inhibitors can act at several steps of the complement cascade. Particularly, three are known to inhibit the formation of MAC: vitronectin (S-protein); clusterin (SP-40,40, apolipoprotein J); and protectin (CD59). Vitronectin and clusterin bind to the soluble C5b-7 complex and block its insertion into the membrane whereas protectin binds to C8 and C9 where it inhibits the incorporation and subsequent polymerization of further C9 [175]. Complement is involved in AD but whether this is a major component of AD neuropathology remains unclear.

Treatment of AD

Nonsteroidal anti-inflammatory drugs (NSAIDS) may favourably influence the clinical expression of AD as shown in epidemiological studies [121]. They can have many potential direct and indirect effects on C-mediated inflammation in the brain pertinent to AD [111]. Nimesulide is a selective PGHS-2 antagonist that prevents C3 activation. Other ways of prevention are by the stimulation of C1-inhibitor and inhibition of C5a inducing effects. Indomethacin was found to significantly attenuate microglial response to continuous *i.c.v.* infusion of Aβ [179].

Due to large choline acetyl transferase deficits (more than 50%) in AD brains, cholinergic agent use in therapy is promising. To counteract cholinergic atrophy, nerve growth factors or estrogens have been implemented in therapy [180]. Both tacrine and nicotine have been shown to increase the performance of $A\beta_{25-35}$-injected mice in the Y-maze [181]. Other agents such as donepezil and rivastigmine are now being used in addition to tacrine in AD treatment.

Oxidative stress is also an important part in AD pathogenesis and antioxidants have been shown to protect neurons from Aβ-induced toxicity [108]. In clinical trials, treatment with idebenone, a potent antioxidant, resulted in a clear dose-dependant anti-dementia activity in AD [182]. In a recent *in vivo* study by Yamada et al., repeated administration of two anti-oxidants, idebenone and α-tocopherol, were found to prevent learning and memory deficits in rats that were continuously infused with $A\beta_{1-42}$ [183].

Due to two times higher prevalence of AD in women relative to men and results showing that estrogen replacement therapy in postmenopausal women delayed onset and decreased the risk of AD, studies began on the effects of estrogen on AD pathogenesis [184]. Estrogens have been shown to attenuate excitotoxicity, oxidative injury, Aβ toxicity, to regulate APP metabolism, and to reduce the neuronal generation of Aβ [185]. More recently, replacement therapy with 17-β-estradiol in ovariectomized rats with $A\beta_{1-42}$-induced working memory deficits, showed an improvement in water maze performance [186].

Prevention along the amyloid cascade leads to many potential targets for therapeutic strategies in AD. One target is to obstruct the formation of fibrils using β-sheet breaker peptides such as used in glycosaminoglycan (GAG) mimetics [187]. Short peptides that imitate the GAG side chains of proteoglycans through their binding affinity to the HHQK sequence of Aβ and block fibrillar formation. Another strategy involves inhibiting the β- and γ-secretases which would therefore eliminate the production of Aβ. Small aldehydes have been shown to be effective as γ-secretase inhibitors [188]. An enhancer of α-secretase activity such as muscarinic agonists or AChE inhibitors, would also have a similar effect.

Other possibilities such as cognitive enhancers have been shown to improve Aβ-induced learning and memory impairments (see review, [189]). Over the past few years, methods of vaccination have also been examined. Vaccines have been used in transgenic models to reduce Aβ load by Ig-coating of Aβ plaques for recognition by microglia or through neutralizing Aβ in the plasma which then acts as a sink for Aβ in the CNS. These methods have successfully lowered pathology and cognitive deficiency [190-192]. Unfortunately, recent clinical vaccination trials have had detrimental effects on AD patients [193]. Therefore, the function of and mechanisms involved in APP must be further defined before continuing with this route of treatment.

Human APOE and AD

The involvement of apoE in AD was first suggested by its presence in extracellular amyloid deposits and in neurons in neurofibrillary tangles [194]. Interestingly, it has been found in other amyloid deposits associated with Creutzfeld-Jakob disease, Down's syndrome, and prion diseases. Thus, it has been suggested that apoE may serve as a pathological chaperone for amyloidogenic proteins.

ApoE is a 34 kDa protein that plays a role in plasma lipoprotein metabolism, cholesterol transport, and lipid transport in the nervous system where it directs the lipid uptake [195]. It is a ligand for three lipoprotein receptors: the low-density (LDL) receptor; the LDL receptor-related protein (LRP); and the very low density lipoprotein (VLDL) receptor [196]. ApoE is expressed in many organs but the highest levels are observed in the liver and in the brain where it is synthesized and secreted primarily by astrocytes and microglia in the central nervous system (CNS) [197, 198]. Three common isoforms exist in

humans, apoE2 (cys^{112}, cys^{158}), apoE3 (cys^{112}, arg^{158}), and apoE4 (arg^{112}, arg^{158}) which are products of alleles at a single gene locus on chromosome 19 [195].

ApoE consists of two major domains: the N-terminal domain, which contains the LDL receptor binding region and the C-terminal domain that contains the major lipid-binding region which is the peptide binding site for β-amyloid [88]. Due to the presence of cysteine at position 112 in the N-terminal domain, both apoE2 and apoE3 prefer lipoprotein associations with high density lipoprotein (HDL) in the C-terminal region [195]. On the other hand, apoE4 contains arginine in position 112. Hence, its C-terminal domain associates with VLDL [199, 200]. ApoE4 forms a denser, more extensive matrix of amyloid monofibrils than apoE3 giving evidence of isoform-dependent specificity of amyloid binding and an effect on fibrillogenesis [201 202]. On the other hand, other studies have shown that both lipid free apoE3 and apoE4 decreased fibrillogenesis by inhibiting Aβ seeding [203]. Therefore, the role that apoE plays in fibrillogenesis is still being debated although transgenic mouse models are beginning to elucidate this.

Transgenic mouse models that express mutant forms of human APP (APP^{V717F} or APPsw) causing familial autosomal dominant (FAD) AD, have been bred onto an apoE knockout (-/-) background [204-206]. In $APP^{V717F+/+}$, $apoE^{-/-}$ mice, a major decrease in Aβ deposition and Aβ levels was observed in comparison to $APP^{V717F+/+}$, $apoE^{+/+}$ mice. Also, the hAPP transgenic mice with no apoE expression had no fibrillar plaque formation which strongly supports the role of apoE in promoting fibrillogenesis. Furthermore, a dose-dependent effect was shown as $APP^{V717F+/+}$, $apoE^{+/-}$ mice had intermediate pathology (see review [207]). Studies in hemizygous transgenic mice revealed that apoE promotes the conversion of Aβ to a β-sheet conformation (amyloid) rather than

influencing the total Aβ load in brain homogenates [208]. Using hemizygous transgenic mice in the absence of endogenous mouse apoE ($APP^{V717F+/-}$ and $APPsw^{+/-}$), Holtzman et al. showed that there is a critical species-specific (human versus mouse) and isoform specific role for apoE in the conversion of Aβ to a fibrillar form with ensuing neuritic-plaque formation [209-211]. The presence of apoE led to increased Aβ deposition, fibrillar plaque formation, neuritic dystrophy, and a dramatic effect on cerebral amyloid angiopathy (CAA) where $apoE^{-/-}$ mice had no evidence of CAA [207]. Also, the mice that expressed human apoE4 had more Aβ deposition and neuritic plaque formation in comparison to those with apoE3. Overall, apoE plays a strong role in Aβ fibrillization although how this occurs is not yet clear.

Other isoform effects have also been observed. ApoE3 binds tau protein in neurofibrillary tangles whereas apoE4 cannot. Hyperphosphorylation of tau seen in amyloidosis can therefore inhibit the binding of the N-terminal domain apoE3 to tau. This could mean that apoE3 has protective ability in AD by preventing the hyperphosphorylation of tau seen in neurofibrillary tangles [212]. Furthermore, there are considerable isoform effects on neurite outgrowth. ApoE3 plus β-VLDL increased neurite extension, whereas apoE4 plus β-VLDL markedly decreased neurite branching and neurite extension in the dorsal root ganglion neurons [213]. Furthermore, mice doubly transgenic for the Swedish mutations and one isoform of human apoE, either apoE3 or apoE4, showed that apoE4 accelerated the Aβ deposition in the mouse brain [214]. Thus, apoE3 plays a protective role compared to apoE4.

There are two ways in which ApoE can be taken up by cells: the LDL receptor pathway or by the (heparin sulfate proteoglycans) HSPG/LRP pathway. The second

pathway involves LRP, which is a very large protein in the LDL receptor gene family and consists of two cell-surface molecules. In this pathway, initial binding of ApoE-containing lipoproteins with cell-surface HSPG is required. Following this, ApoE enriched with lipoproteins may then be transferred to the LRP for internalization or the HSPG/LRP may form a complex that is taken up by neurons [215]. The HSPG/LRP pathway appears to be required for maximal apoE3 and apoE2 stimulation of neurite extension and maximal apoE4 inhibition of neurite extension.

Another way in which apoE could influence Aβ deposition is through clearance. ApoE-containing lipoproteins in the CNS could sequester soluble Aβ and alter the metabolic pathway used to remove Aβ from the brain [207]. Removal could occur by LDLR, LRP, VLDLR but *in vitro* evidence suggests that LDLR and LRP may be involved [216, 217]. Clearance of Aβ could also be influenced through apoE/Aβ interactions by affecting transport of Aβ from the brain extracellular space back into the systemic circulation [207]. This is supported by the rapid transport of $A\beta_{40}$ from the CNS to the plasma with an elimination half-life of less than 30 minutes [218] and a role for apoE in a LRP-mediated transport mechanism [219].

Due to recent studies, links are now being observed between inflammation and ApoE expression. Recent studies on $APP^{V717F+/-}$ $ApoE^{-/-}$ transgenic mice showed that the lack of ApoE protein not only dramatically reduced the amount of Aβ-immunoreactive deposits, but a significant reduction in microgliosis and astrogliosis was also observed [208]. They propose that apoE promotes both the deposition and fibrillization of Aβ. In another study, LPS was chronically administered to APP^{V717F} $ApoE^{+/+}$ transgenic mice to induce an inflammatory response [220]. This resulted in an acceleration in ThioS-positive plaque

formation and a marked increase in glial activation in comparison to LPS-treated or vehicule-treated control mice. From this data, they show that inflammation can accelerate amyloid deposition and this requires the expression of ApoE.

Amyloid β-peptide-related animal models of AD

Nontransgenic models involve the injection or continuous infusion of Aβ peptide or amyloid into the brain which leads to neurotoxic effects, loss of learning or memory, microglial activation, and inflammatory response [49, 50, 221, 222].

Transgenic mouse models that express human APP [223], Aβ [224], the C-terminal fragment of APP [225, 226], and the APP genes carrying familial AD mutations [204, 227, 228] have been created to exhibit senile plaques and Aβ-associated neuropathology. Another type of transgenic mouse model overexpresses cerebral IL-6 and shows age-related deficits in avoidance learning and reduced long-term potentiation in the hippocampus [229, 230].

APP transgenic mice carry a transgene overexpressing FAD mutations in hAPP that is driven by a well-activated promoter such as the prion protein (PrP) promoter, platelet-derived growth factor β-chain (PDGFβ) promoter, or Thy-1 promoter. The various APP mutations lead to different patterns of Aβ accumulation. The V717I and V717F mutations enhance γ-secretase-mediated cleavage and result in increased levels of $A\beta_{1-42}$ [231]. Mutations around the α-secretase site at codon A692G show an augmentation in Aβ levels whereas mutations at codon E693G led to increased protofibril formation [60, 232]. On the other hand, the 'Swedish' mutation K670N/M671L is associated with an overproduction of Aβ and a notable increase in $A\beta_{1-40}$ production due to the stimulation of the β-secretase pathway [233].

One of the APP transgenic mice, the PDAPP mouse, was generated using a PDGFβ promoter driven by a human APP minigene encoding the Indiana mutation, APP V717F [204]. It incorporates all three splice variants of APP of 695, 751, and 775 amino acids long. Amyloid deposition begins at 6-9 months and plaques consist mainly of $A\beta_{1-42}$. Immunohistochemical analysis of these mice showed that they had extracellular ThioS-positive Aβ deposits, neuritic plaques, synaptic loss, astrocytosis, and microgliosis. When these mice were crossed with an ApoE knockout mouse, Aβ deposition was decreased although APP expression and Aβ formation was unchanged implying a role for ApoE in amyloid deposition [206]. A transgene for TGF-1β (transforming growth factor) under the control of the GFAP (glial fibrillary acidic protein) promoter in PDAPP mice, showed an increase in Aβ peptide deposition in plaques with an emphasis on cerebrovascular and meningeal deposition [234].

Tg2576 mice contain an $hAPP_{695}$ transgene carrying the 'Swedish' mutation and is driven by the PrP promoter. Tg2576 mice on FVB/N backgrounds, exhibited a high frequency of spontaneous death and behavioural changes such as neophobia and impaired spontaneous alternation in the Y-maze [235]. No signs of AD-like pathology were observed. When these mice were bred onto a different genetic background, C57BL6JXSJL F2, they exhibit AD-like pathology with Congo Red positive amyloid deposits from 9-12 months of age, astrocytosis, and microgliosis [205]. When crossed with mutant $PS1_{M146L}$ transgenic mice, they showed increased levels of total Aβ as well as $A\beta_{42(43)}/A\beta_{40}$ ratios with age and reduced Y maze performance before the apparition of Aβ deposition [236]. Another set of transgenic mice that co-express APP swe and Hu PS1-A246E, were shown to have increased levels of total Aβ and $A\beta_{42(43)}/A\beta_{40}$ ratios with age [237]. Accelerated

amyloidosis is also observed in other hAPP transgenic mouse models that carry a mutant PS1 transgene [238].

Another set of mice, the APP23 mice, contain the $hAPP_{751}$ transgene with the 'Swedish' mutation driven by the Thy-1 promoter [239]. These mice have diffuse and congophilic plaques from 6 months of age, neuronal loss, astrocytosis, and microgliosis. Additionally, mouse background plays a large role in the behaviour and pathology of these mice [235].

A more recent transgenic model exploits the accumulating knowledge on already-developed transgenic models. TgCRND8 mice carry a mutant hAPP transgene with both the double Swedish and Indiana mutations (APP_{695} , KM670/671NL and V717F) under the control of the PrP gene promoter [228]. These mice displayed AD-like pathology with an increase in Aβ load with high ratios of $A\beta_{1-42}$ /$A\beta_{1-40}$ as well as ThioS-positive plaques and cognitive decline starting from 3 months of age [191]. The aggressiveness of the combined double Swedish mutation and the Indiana mutation has been reported earlier in PDGF-β promoter-driven mini-gene transfected mice with high hAPP expression [240]. Contrary to this, APP22 mice that carry both the double Swedish mutation and the Indiana mutation under a Thy-1 promoter, have delayed amyloid deposition compared to APP23 mice that do not carry the Indiana mutation [239]. By combining the double Swedish mutation with the Indiana mutation under the control of a Syrian hamster Prion promoter, a highly aggressive transgenic mouse model was derived compared to other transgenic models (Table 1).

Table 1 Pathology comparisons in hAPP mutant transgenic mice

Transgenic mouse	*Promoter* & Mutation	Plaque onset age in months[1]	Total Aβ load increase[2]	Ratio of Aβ42 /Aβ40[2]	Glia	Behaviour Impairment	Survival up to age in m
PDAPP	*PDGFβ* V717F	6-9 m fib	high 112 pm/g	high 0.68	yes	yes	27 m
Tg2576 (APPsw)	*PrP* K670N M671L	7-8 m fib 12 m diff	high 53pm/g	high 0.26	yes	yes	more than 23 m
APP23	*Thy-1* K670N M671L	6 m diff & fib	N.A.	N.A.	yes	N.A.	more than 12 m
TgCRND8	*PrP* K670N M671L V717F	3 m diff & fib	high 82 pm/g	high 5.1	yes	yes	6 m

[1] Diffuse plaques (diff); Fibrillar plaques (fib)are Congo Red and/or ThioS positive; months (m)
[2] Aβ load and $A\beta_{42}/A\beta_{40}$ ratios are compared at age of plaque onset
N.A. refers to Not Applicable

The ratios of $A\beta_{42}/A\beta_{40}$ at the time of plaque deposition are highest in the TgCRND8 model with a ratio of 5.1. This is similar to what is observed AD patients and Down's syndrome where $A\beta_{42}$ is elevated in the beginning and contributes to early plaque deposition more than $A\beta_{40}$ [19, 20]. At later ages, this ratio diminishes to 2 as $A\beta_{40}$ production becomes important [228]. Due to the fast appearance of plaques and the AD-like pathology, the TgCRND8 mouse model is a good tool for studying Aβ mechanisms and for drug treatment trials.

Transgenic mouse models are useful tools for studying the mechanisms involved in AD pathogenesis. As there are a variety of factors involved in any model, it is difficult to attribute resulting pathology and behavioural traits to a specific FAD mutation since

these models are limited due to the choice of APP isoform, transgene promoter, strain background, and APP mRNA expression.

Thesis strategy

From the literature review, inflammation is clearly involved in the neuropathology of AD. As previously stated, Alzheimer's disease leads to a series of neuropathologies such as neuronal dystrophy, synaptic loss, amyloidosis, and chronic brain inflammation. This can vary in severity and rapidity depending on an individual's genetic background. The magnitude of the inflammatory response may play a role in the different development rates of AD seen in individuals. The phenotypic variations in inflammatory response to Aβ between individuals and in the transgenic mouse models have not been closely examined. Thus, the following hypothesis is proposed:

Hypothesis: Inflammatory response can influence brain amyloidogenesis.

Model: Two strains of mice with major differences in their magnitudes of inflammatory response, were used to determine microglial inflammatory response to Aβ as well as the impact of both mouse backgrounds on the phenotypic variations of hAPP transgene expression in transgenic mice (TgCRND8). The following experimental set-ups were used: primary microglial cultures to study differences in microglial activation to Aβ *in vitro*, TgCRND8 mice to determine when inflammation intervenes in brain amyloidosis, and TgCRND8 mice bred onto two different background strains to reveal the effect of mouse genetic background on the phenotypic effects of inflammatory response during brain amyloidogenesis as well as overall phenotypic variation of the hAPP transgene expression.

Manuscript 1: *Differences in the amyloid-β -induced inflammatory response in microglia from C57BL/6 and A/J strains of mice.* Sherri Dudal, Emil Skamene, Céline Morissette, Patrick Tremblay, and Francine Gervais. Manuscript sent for submission to Journal of Neuroimmunology.

To determine whether the level of brain inflammation in individuals may differ and influence the course of the neuropathogenic process of AD, *in vitro*, primary microglia were isolated from two genetically different inbred strains of mice, A/J and C57BL/6 mice. These mice were categorized as low responders (A/J) or high responders (C57BL/6) to different inflammatory stimuli [241-245]. This manuscript demonstrates that $A\beta_{1-42}$ can induce different microglial activation profiles in the different strains of mice in terms of morphology, nitric oxide release, and TNF-α secretion.

Manuscript 2: *Inflammation occurs early in Aβ deposition in TgCRND8 mice.* Sherri Dudal, Pascale Krywkowski, Julie Paquette, Céline Morissette, Patrick Tremblay, and Francine Gervais. Submitted to American Journal of Pathology.

Through immunohistochemical analysis, a complete study of Aβ deposition, fibrillar plaque formation, and inflammation demonstrates the early induction of inflammation in the process of Aβ deposition. Using an early onset mouse model of AD (TgCRND8) [228], a kinetic analysis of amyloidosis and gliosis showed the strong correlation of inflammation to early Aβ deposition.

Manuscript 3: *Influence of genetic background on survival and plaque formation in TgCRND8 mice.* Sherri Dudal, Giovanna Sebastiani, Emil Skamene, Julie Paquette, Patrick Tremblay, David Westaway, and Francine Gervais.

TgCRND8 mice were backcrossed onto C57BL/6 and A/J strains of mice to analyze whether the high and low inflammatory response to $A\beta_{1-42}$ may influence the brain amyloidogenic process. Mice bred on a low-responder A/J background showed delayed pathology and extended survival time compared to mice bred on a high-responder C57BL/6 background. The mRNA expression of hAPP, protein levels of $A\beta_{1-40}$ and $A\beta_{1-42}$, and immunohistochemical analysis suggest genetic background influences the rate of the Aβ amyloidogenic process.

CHAPTER 2: Manuscript 1 Differences in the amyloid-ß-induced inflammatory response in microglia from C57BL/6 and A/J strains of mice

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Manuscript 1 demonstrates that primary microglia isolated from mice with a high-level inflammatory response (C57BL/6), induced rapid microglial activation in response to $A\beta_{1-42}$ as shown by ameboid (activated) morphology, increased NO levels and very high levels of TNF-α. On the other hand, mice with a low-level inflammatory response (A/J), displayed few ameboid microglia, very strong induction of NO, and moderate increases in TNF-α levels. This difference in microglial activation could have profound effects on the neuropathology of AD.

Abstract

In Alzheimer's disease (AD), the deposition of amyloid-β peptide (Aβ) is associated with the activation of microglia in the vicinity of amyloid plaques. Moreover, Aβ can directly activate microglia. Several models of infection or inflammation have shown that macrophage inflammatory responses were genetically controlled. Using A/J and C57BL/6 strains of mice, which have low and high macrophage inflammatory response to different stimuli, respectively, we show that microglial inflammatory response to Aβ is also strongly modulated by the genetic background. Inflammatory response to Aβ stimulation was compared in microglial cultures from A/J and C57BL/6 mouse strains following treatment with $A\beta_{1-42}$ alone or in combination with interferon-γ (IFN-γ). Morphological analysis, nitric oxide (NO) and pro-inflammatory cytokine measurements indicated that the magnitude of the microglial response to $A\beta_{1-42}$ differed in these two strains of mice. A/J microglia did not show major morphological changes in response to stimulation with IFN-γ alone or in combination with $A\beta_{1-42}$. In contrast, when treated similarly, C57BL/6 microglia adopted an ameboid morphology (activated state) that was corroborated by a decrease in NO production and a marked increase in tumor necrosis factor-α (TNF-α) production following a 48-h stimulation with IFN-γ and $A\beta_{1-42}$. These results indicate that, as previously reported for other macrophage inflammatory stimuli, the magnitude of the microglial inflammatory response to Aβ is strongly influenced by genetic factors. Differences in the microglial response to $A\beta_{1-42}$ between individuals could play an important role in the sequence of neuropathological events associated with the development of AD.

Introduction

Neuropathology of Alzheimer's disease (AD), which is characterized by amyloid-β peptide (Aβ) deposition, is also associated with brain inflammation and neuronal cell death [3]. Activated ameboid microglia surround fibrillar Aβ deposits in the brains of AD patients [246]. Neuronal death can result from the deposition of Aβ directly or indirectly through the effect of pro-inflammatory cytokines, complement factors, nitric oxide (NO) release, or reactive oxygen species (ROS), all of which have been implicated in the inflammatory process.

Aβ triggers a microglial inflammatory response [247]. In co-culture with astrocytes or neurons, resting ramified microglia are characterized by small cell bodies with slender extended processes [248, 249]. Resting microglia have been shown to migrate toward plaques by chemotaxis, proliferate, and attain an activated state characterized by swelling of the cell body, retraction of cellular processes, and ameboid shape with accumulation of cytoplasmic vacuoles [250]. At this stage, microglia are phagocytic and secrete growth factors [251], such as neuroprotective factors, transforming growth factor-β (TGF-β) [135] and β-fibroblast growth factor as well as pro-inflammatory cytokines with neurotoxic activity like ROS [252] and NO [110]. They also secrete a variety of cytokines such as interleukin-1β (IL-1β) [253], interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) which are toxic to some cell types and which can induce complement-mediated killing [79]. NO release constitutes a cytotoxic microglial response aimed at eliminating local cells containing abnormal or foreign proteins. However, NO release has also been shown to inhibit phagocytosis [254]. In AD, the release of NO can thereby inhibit the clearance of $A\beta_{1-42}$

and accelerate plaque formation [255]. The TNF-α pro-inflammatory cytokine released by fully activated microglia has been shown to increase in the brain of AD patients [3].

The rate at which neurodegeneration progresses varies between AD patients [256]. This process is strongly influenced by genetic factors [257, 258]. The level of the inflammatory response within the central nervous system could be a central factor influencing the neurodegenerative process through the release of inflammatory mediators. The characterization of the microglial response to the amyloid in strains of mice such as A/J and C57BL/6 known to differ in the magnitude of their macrophage inflammatory response,[241] may help in elucidating the role of the inflammation in AD. In several *in vivo* and *in vitro* models, the magnitude of the inflammatory response in A/J and C57BL/6 mice was genetically controlled. Therefore, we used A/J and C57BL/6 mouse strains to evaluate the influence of the genetic background on the microglial activation profile following stimulation with Aβ.

We compared the process of microglial activation induced by soluble or fibrillar Aβ in primary microglial cultures isolated from A/J and C57BL/6 mice (referred to as A/J microglia and C57BL/6 microglia, respectively). Parameters of activation were morphological changes as well as NO and TNF-α release into the culture supernatant. The magnitude of the inflammatory microglial response triggered in the presence of $A\beta_{1-42}$ was significantly modulated by the genetic background of A/J and C57BL/6 mice.

When activated in the presence of interferon-γ (IFN-γ) and $A\beta_{1-42}$, most C57BL/6 microglia displayed an altered cell morphology from a resting ramified type to a typically-activated ameboid morphology while A/J microglia maintained a mixed morphology, consisting mainly of ramified and rod-shaped cells. Moreover, at 48 h of

stimulation, C57BL/6 microglia presented NO and TNF-α response profiles which significantly differed from that observed with A/J microglia with an increased TNF-α production in response to IFN-γ and $A\beta_{1-42}$. On the other hand, NO production in A/J microglial cultures in response to IFN-γ / $A\beta_{1-42}$ stimulation was markedly greater than that of C57BL/6 microglia. These observations support the view that the magnitude of the brain inflammatory response to Aβ deposition is associated with the generation of neurotoxic factors, which can modulate the rate of the neurodegenerative process associated with AD. Most importantly, our study identifies two distinct genetic backgrounds where such response differs and could be molecularly characterized.

Materials and Methods

Cell Culture

Microglia were isolated from 2–3-day old mice of the A/J (Jackson Laboratory, Bar Harbor, MA) and C57BL/6 (Charles River Laboratories, Toronto, Canada) strains as described previously [249, 259]. Briefly, brains were carefully stripped of the meninges, cortices were dissected and placed in ice-cold PBS. Cortical tissue was dissociated in high glucose Dulbecco's modified Eagle's medium (DMEM) (4.5 mg/ml) (cellgro®, Mediatech, Herndon, VA) and the cortical tissue was dissociated. Cells were then plated in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) (MBI, Montréal, Canada), and 1% antibiotic /antimycotic solution (Life Technologies, Gibco BRL, Rockville, MD) onto uncoated TC-80 flasks (BDFalcon™, Becton Dickinson, Franklin Lakes, NJ) at a concentration of 1–2 $X10^7$ cells per flask. Yields of 2.4–3.0 $X10^6$ cells/brain were obtained from mice of the A/J and the C57BL/6 strains.

Following 12–14 days of culture, microglial cells were collected from the confluent primary cultures by vigorous shaking on a rotary Lab-line shaker at 180 rpm, 37°C, 5% CO_2 for 2 h. The milieu was collected and centrifuged at 450 *g* for 10 min at room temperature (RT) and the pellet was resuspended in high glucose phenol red-free DMEM, supplemented with 1% FBS (Hyclone Laboratories, Logan, UT), recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF; 0.05 ng/ml equivalent of 25 U/ml; Chemicon International, Temecula, CA), and 1% antibiotic/antimycotic solution. Primary cultures gave microglial yields (8 ± SD) of approximately 16 ± 4% (A/J strain) or 7 ± 3% (C57BL/6 strain). GM-CSF was added for

a 24-h period to partially replace the paracrine effects of co-culturing astrocytes with microglia [3, 248, 260] and to maintain the cells in a semi-resting state [261]. The microglia were plated at 1.5 $X10^5$ cells per chamber in Labtek 8-well chamber slides (Becton Dickinson). The adherent microglia (99% purity was determined by Mac-1 and glial fibrillary associated protein (GFAP) immunostaining) were cultured in high glucose phenol red-free DMEM supplemented with 1% FBS and 1% antibiotic/antimycotic solution. The cells obtained from neonates of multiple litters were pooled for every experiment.

Peptides, Recombinant Protein, and Antibodies

Synthetic human $A\beta_{1-42}$ (American Peptide Company, Sunnyvale, CA) was solubilized in di-methyl sulfoxide (DMSO), monomerized in hexafluoroisopropanol (HFIP), diluted in Tris-HCl, and frozen immediately at -80°C for later use. To obtain fibrils, $A\beta_{1-42}$ (20 μM) was incubated for 24 h at 37°C with constant agitation. The $A\beta_{1-42}$ conformation of monomer/soluble and fibrillar preparations was verified by circular dichroism (CD) and electron microscopy (EM) analysis as described previously (see Figure 4) [187]. Briefly, CD spectra were acquired on a Jasco spectropolarimeter Model J-715 (Jasco Corp., Japan) at 37.5°C in a 0.1-cm path length cell over the wavelength range 190-250 nm with a 1.0-nm bandwidth, 0.1nm resolution, 2s response time and 50 nm min^{-1} scan rate. As for EM analysis, negative staining was performed on 10 μL of each sample which was applied to pioloform-and carbon-coated grids, blotted with filter paper and stained with 1% (w/v) phosphotungstic acid, pH 7. The specimens were examined on a Hitachi H7000 electron microscope with an accelerating voltage of 75 kV. Since monomeric $A\beta_{1-42}$ adopts a fibrillar conformation rapidly at room temperature (RT), $A\beta_{1-42}$ that has not been

pre-aggregated before addition to culture is referred to as soluble $A\beta_{1-42}$ throughout the paper. Recombinant mouse IFN-γ (Life Technologies, Gibco BRL) was diluted in PBS from stock solution to a final concentration of 5 U/μl.

Microglial morphology was observed following staining with a rat anti-mouse CD45 monoclonal antibody (mAb) (1/50) (BDPharmingen, Mississauga, Canada). Microglial cell preparation purity was determined with rat anti-mouse Mac-1 mAb (1/40; BDPharmingen) and GFAP mouse mAb cocktail (1/100; BDPharmingen). A goat anti-rat biotin conjugate (1/200; Chemicon International) was used as secondary antibody with the Very Intense Purple substrate detection system (VIP; Vector Laboratories, Burlingame, CA) followed by bright field microscopic detection. Finally, the interaction of $A\beta_{1-42}$ with the microglial cell surface was monitored using rabbit anti-human $A\beta_{1-42}$ polyclonal Ab (1/1000; Biosource International, Camarillo, CA). Secondary antibodies used for fluorescent microscopic observation of Mac-1, GFAP, and $A\beta_{1-42}$ primary antibodies were goat anti-rat IgG-FITC (1/100; Sigma, St. Louis, MO) and rabbit anti-mouse IgG-FITC (1/100; Sigma).

Immunocytochemistry

Cell culture supernatants were collected for analysis of nitrite levels and TNF-α levels while immunocytochemistry was performed on the cells as described. Cells were fixed with cold methanol for 10 min, washed with PBS, and blocked for 1 h at RT. For GFAP staining, permeabilization was performed with 0.1% Triton X-100™ (Sigma) for 30 min at RT before blocking. The primary antibody was added and cells were incubated at RT for 1 h or overnight at 4°C. Cells were washed and incubated with a biotinylated

secondary antibody for 1 h at RT. Again, the cells were washed and the reaction was revealed using Streptavidin ABC (Vector Laboratories) for 45 min at RT. After washing, a two-fold dilution of VIP substrate was added and incubated accordingly. The slides were mounted and viewed under light and fluorescent microscopy using an Olympus BX40 microscope. Images were captured using a Pro-series camera (DC-330, DAGE-MTI Inc, Michigan, IN) and analyzed with the Image Pro-Plus® Systems software (Version 4.0 for Windows, Media Cybernetics, Silver Spring, MD).

Activation of Microglia

Microglia were stimulated with $A\beta_{1-42}$ by adding 1.0 or 2.5 μM of a soluble or fibrillar preparation of $A\beta_{1-42}$ to the culture. Synergistic effects of $A\beta_{1-42}$ and IFN-γ were determined by adding $A\beta_{1-42}$ solution 1.5 h following priming with 50U of IFN-γ. As a control, 50U of IFN-γ was added to a separate Labtek chamber. The activation was evaluated after 24 h and 48 h of incubation.

Nitrite and TNF-α Levels

NO was measured indirectly by using a sensitive fluorometric nitrite/nitrate quantification assay [262]. Briefly, 100 μl of microglial cell culture supernatant was mixed with 10 μl of 2,3-diaminonaphthalene (0.05 mg/ml) (Molecular Probes, Eugene, OR) in 0.62 M of HCl. After 10 min, the reaction was stopped by adding 5 μl of 2.8 N NaOH and fluorescence was read on a HTS 7000 Plus BioAssay Perkin Elmer plate reader at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. Nitrite levels in cell-free medium were determined in each experiment and subtracted from the value obtained with

cells alone . Concentrations of nitrite were determined from duplicate samples by linear regression using a standard curve with known concentrations of sodium nitrite added to the culture medium and concentrations were expressed as μM per 1.5 $X10^5$ cells.

TNF-α levels in cell culture supernatants were determined using a quantitative sandwich enzyme-linked immuno-sorbent assay (ELISA) kits as recommended by the manufacturer (Biosource International).

Statistical Analysis

All stimulations were run in duplicate or quadruplicates in two separate experiments. For each experiment, duplicate measurements of NO or TNF-α were averaged and results were expressed as the mean ± the standard error of the mean (8 ± S.E.M). Data were first analyzed using a factorial analysis of variance (ANOVA; Sigma Stat, Version 2.0, Jandel Scientific, Chicago, IL) using the presence of $A\beta_{1-42}$, the concentrations of $A\beta_{1-42}$, and the aggregation state of $A\beta_{1-42}$ as factors. The presence of $A\beta_{1-42}$ had a significant effect on the production of NO and TNF-α by microglia ($P < 0.05$) but the concentrations of $A\beta_{1-42}$ (1.0 and 2.5 μM) and the aggregation state (monomer and fiber) had no significant effect ($P \cdot 0.05$). Data were then grouped as unstimulated control microglia, microglia stimulated with $A\beta_{1-42}$ alone, IFN-γ alone, or IFN-γ and $A\beta_{1-42}$ for further analysis. After 24 h and 48 h of stimulation, data from each strain of mice were analyzed using a one-way ANOVA or a Kruskal-Wallis one-way ANOVA on ranks to find differences among the stimulation conditions ($P < 0.05$). Differences between strains of mice for their NO and TNF-α production ($P < 0.05$) were analyzed for each stimulation condition using an unpaired two-tailed *t*-test or a Mann-Whitney rank sum test.

Results

Microglial Culture

Control unstimulated primary A/J and C57BL/6 microglia showed a mixture of morphologies consisting of mainly ramified and some rod-shaped cells (Figure 5). Also, NO and TNF-α production levels were similar in control A/J and C57BL/6 microglial preparation (Figures 6,7).

Aβ 1–42 Stimulation

A/J and C57BL/6 microglia were affected differently by treatment with $A\beta_{1-42}$ alone. $A\beta_{1-42}$ was found to bind equally well to the cell surface of microglia from both strains of mice after 24 h of stimulation with $A\beta_{1-42}$ as determined by immunodetection (data not shown). No major changes were observed in the morphology of both A/J and C57BL/6 microglia when compared to the cell morphology seen in the controls following a 48-h stimulation with $A\beta_{1-42}$ (Figure 5). Moreover, NO release was at control levels in response to $A\beta_{1-42}$ stimulation at 24 h (data not shown) and 48 h of incubation (Figures 6 and 7). In $A\beta_{1-42}$-stimulated A/J microglia, TNF-α levels were also similar to control levels after 24 h and 48 h of incubation. In contrast, TNF-α levels appeared slightly elevated over controls in C57BL/6 microglia when stimulated under the same conditions (24 h and 48 h). As C57BL/6 microglia released modest levels of TNF-α in response to $A\beta_{1-42}$ stimulation and A/J microglia did not secrete NO or TNF-α, they may be activated through different pathways.

IFN-γ Stimulation

A/J and C57BL/6 microglia differed in their response to 50 U of IFN-γ. After 48 h of stimulation, only small changes in A/J microglial morphology were observed in comparison to the unstimulated control culture. In contrast, C57BL/6 microglia responded strongly to IFN-γ with many cells adopting an ameboid morphology (Figure 5).

Production of NO by A/J and C57BL/6 microglia was minimal following a 24 h period of incubation with 50 U of IFN-γ, with nitrite levels of less than 3.5 μM for 1.5 X 10^5 microglia (data not shown). In contrast, following 48 h of incubation with IFN-γ, NO levels were markedly increased (Figure 6). A/J microglia showed a ~7-fold increase in NO release (IFN-γ compared to unstimulated and $A\beta_{1-42}$ alone) upon exposure to IFN-γ ($P < 0.001$) while C57BL/6 microglia presented only a 2.5-fold increase ($P \cdot 0.05$).

TNF-α levels were also measured in the cell culture supernatants following 24-h (Figure 7A) and 48-h treatments (Figure 7B) with 50 U of IFN-γ. A significant increase in TNF-α production by A/J microglia was seen only after 48 h of incubation ($P < 0.001$) (Figure 7B) while C57BL/6 microglia readily responded by producing significantly more TNF-α than A/J microglia ($P < 0.001$) and their unstimulated control ($P < 0.001$) after 24 h of incubation. After 48 h of stimulation, A/J and C57BL/6 microglia presented similar levels of TNF-α, equivalent to that observed in C57BL/6 microglia at 24 h. These results suggest that the induction of TNF-α production in A/J microglia in response to IFN-γ is slow but ultimately reaches levels equivalent to those obtained early in IFN-γ-treated C57BL/6 microglia.

IFN-α and Aβ 1–42 Stimulation

The synergistic stimulatory effect of $A\beta_{1-42}$ with IFN-γ priming on microglial activation was also determined. Cells were pre-treated with IFN-γ for 1.5 h before $A\beta_{1-42}$ were introduced into the cell culture system. Once again, after a 48-h stimulation, the A/J microglia adopted more ameboid morphology in the presence of IFN-γ and $A\beta_{1-42}$ but not to the extent observed by C57BL/6 microglia.(Figure 5). Unfortunately, quantification of microglial morphology was not possible due to the increased mobility of the C57BL/6 microglia.

NO release was also significantly greater in A/J than in C57BL/6 microglia ($P < 0.001$) following treatment with IFN-γ and $A\beta_{1-42}$ for 48 h (Figure 6). However, $A\beta_{1-42}$ had no synergistic effect on the IFN-γ-induced NO release by A/J microglia. Interestingly, the increase (although not significant) in NO release observed when IFN-γ-primed C57BL/6 microglia were incubated with $A\beta_{1-42}$ suggests that $A\beta_{1-42}$ had a small synergistic effect on IFN-γ-induced NO release by C57BL/6 microglia but not on that by A/J microglia. However, the synergistic effect may be masked in A/J microglia if their NO release is already maximal following priming with IFN-γ.

TNF-α production by IFN-γ primed A/J microglia was not significantly affected by the addition of $A\beta_{1-42}$ to the culture (Figure 7). However $A\beta_{1-42}$ had a major impact on the TNF-α production in IFN-γ-primed C57BL/6 microglia after 48 h of incubation ($P < 0.001$). Indeed the TNF-α production doubled compared to that seen in IFN-γ-treated microglia suggesting that $A\beta_{1-42}$ can significantly potentiate the IFN-γ-induced TNF-α production by C57BL/6 microglia but not that by A/J microglia.

Table 2 summarizes the outcome of the different stimulation regimes in both A/J and C57BL/6 microglia at 48 h post-stimulation. It is clear that these microglia have a different induction profiles to stimulation with $A\beta_{1-42}$. These differences most probably reflect the diverse responses that amyloid can induce on cells depending on the genetic background regulating the inflammatory response.

Discussion

During activation, microglia undergo morphological changes from a ramified or rod-shaped morphology with slightly swollen centres and retracted processes to fully ameboid with the concomitant formation of cytoplasmic vacuoles. According to their morphological classification, most primed microglia (slightly enlarged) are not associated with plaques while enlarged and phagocytic microglia with characteristic cytoplasmic debris are generally plaque-associated [124]. When incubated *in vitro* in the presence of aggregated $A\beta_{1-42}$, microglia display an ameboid morphology reminiscent of their appearance around diffuse plaques found *in vivo* [263, 264]. As microglial activation is associated with the release of neurotoxic and inflammatory mediators, their activation could interfere with the function of nearby cells. Therefore, the ability of microglia to rapidly change from a primed to an activated state in response to amyloid, may have a major impact on cell viability in the vicinity of Aβ deposits.

The level of the inflammatory response varies in humans. While some individuals react very strongly to a given stimulus, others develop only a mild response. Such a difference in the magnitude of the inflammatory response has been observed in several animal models of inflammation and infectious diseases [241, 244, 245] strongly suggesting that such variations are genetically controlled. Variation in the amplitude of the microglial inflammatory response could influence the development pace of various neurodegenerative conditions. Indeed, microglia and astrocytes can greatly impact the survival of adjacent neurons following the release of highly toxic products such as ROS, NO, excitatory amino acids, cytokines, and complement components [265].

In AD, the extracellular deposition of fibrillar Aβ peptide has been shown to induce an inflammatory response cascade [3, 133]. Once local $A\beta_{1-42}$ reaches a critical concentration, aggregation would be initiated and recruitment of activated microglia to the site would be enhanced by the inflammatory mediators produced to clear the amyloid plaques. A variation in the robustness of the microglial response to Aβ could modulate the neurodegenerative process and thereby contribute to AD pathogenesis. Interestingly, soluble $A\beta_{1-42}$ can also activate IFN-γ-primed microglia, indicating that Aβ could trigger microglial activation very early in the pathogenesis of AD, even prior to $A\beta_{1-42}$ aggregation and deposition as plaques [266]. This early $A\beta_{1-42}$-mediated activation has been proposed as being a critical pathogenic event in the development of AD [125, 208, 256]. It has also been postulated that microglial activation plays a role in the upregulation of apolipoprotein E (apoE) expression, influencing $A\beta_{1-42}$ fibrillogenesis, deposition, and amyloid plaque formation [209, 256]. Acute induction of inflammation by lipopolysaccharide (LPS) has also been recently shown to contribute to amyloid deposition in an apoE-dependent manner [220, 267].

The microglial activation process in response to $A\beta_{1-42}$ stimulation was compared in primary culture obtained from A/J and C57BL/6 strains of mice known to differ in the magnitude of their macrophage inflammatory response. While A/J are low responders, C57BL/6 mice display a robust response. Morphological changes, NO release, and TNF-α secretion were used to evaluate $A\beta_{1-42}$-induced microglial activation *in vitro*.

Except for modest increases in TNF-α levels in C57BL/6 microglia, $A\beta_{1-42}$ did not significantly affect any of the parameters studied when incubated alone in primary microglial culture, suggesting that $A\beta_{1-42}$ is not capable of strongly activating primary

microglia on its own. This is similar to results reported with other microglia or macrophage cell preparations where cells needed a priming agent such as IFN-γ or endotoxin in order to respond to $A\beta_{1-42}$ stimulation [110].

Primed microglia from the low responder A/J strain responded differently to $A\beta_{1-42}$ than those from the high responder C57BL/6 strain. Indeed, when stimulated with IFN-γ and $A\beta_{1-42}$, A/J microglia responded by a moderate activation with minimal morphological changes, high NO production, and low TNF-α production. Conversely, the activation of C57BL/6 microglia preserved a marked response to the same stimulation with increased mobility and increased ameboid morphology, low NO production, and high TNF-α production.

NO release, as measured by nitrite levels, was increased 2- to 3-fold higher in A/J microglia compared to C57BL/6 microglia after 48 h of stimulation with IFN-γ alone or in combination with $A\beta_{1-42}$. NO release by A/J microglia was mostly induced by IFN-γ since the addition of $A\beta_{1-42}$ to the IFN-γ treated microglia failed to increase the already high NO release, suggesting that $A\beta_{1-42}$ did not potentiate IFN-γ -induced NO production. The difference in the level of NO production between A/J and C57BL/6 microglia has also been observed in microglia obtained from *Nramp-1* (natural resistance-associated macrophage protein-1) resistant and susceptible congenic mouse strains [268]. A/J and C57BL/6 strains of mice are well-known representatives of *Nramp-1* resistant and susceptible strains, respectively [269]. This correlation between *Nramp-1* genotype and NO production following exposure to IFN-γ has also been reported using macrophages from different anatomical sites [270], suggesting that IFN-γ-induced activation of microglia may be affected by the expression of the *Nramp-1* gene.

The significant synergistic effect of IFN-γ and $A\beta_{1-42}$ on TNF-α production by C57BL/6 microglia could be due to the expression of specific cell surface receptors following priming with IFN-γ. Since A/J microglia are not fully differentiated by IFN-γ, they may express different types or levels of receptors on their cell surface. Several cell surface receptors such as the receptor for advanced glycosylation end-products (RAGE) [267], the scavenger type A receptor (SR-A)[267], and the low-density lipoprotein receptor related protein (LRP-1 and LRP-2),[271] bind Aβ either directly (RAGE, SR-A) or indirectly (LRP-1, LRP-2) [219] through ligands such as α2M, ApoE and Apo-J, which act as high affinity transport binding proteins [257, 272]. The expression of receptors for other cytokines such as GM-CSF has also been shown to modulate Aβ-RAGE-mediated microglial activation [273]. A difference in the type, the affinity, or the number of specific receptors expressed on the cell surface may lead to different activation profiles [257]. Characterization of the different potential Aβ receptors on the surface of A/J and C57BL/6 microglia, may provide some insight in the molecular mechanisms regulating the synergistic effect of Aβ and IFN-γ in C57BL/6 microglial cultures. In response to IFN-γ and Aβ, A/J microglia increase their NO production while C57BL/6 microglia display morphological changes and augment their TNF-α production. Understanding the molecular basis behind the differential activation profiles may also help to explain the different effects observed in the NO and TNF-α production.

The activation of microglia and astrocytes by Aβ has also been shown to dramatically upregulate the expression of ApoE, a gene that has been shown to dramatically influence the course of AD [274, 275]. It is therefore equally possible that the potent IFN-γ priming effects seen in C57BL/6 microglia leads to a significant increase in

ApoE release, which in association with Aβ and LRP-1 or LRP-2, triggers significant TNF-α production. ApoE knock-out mice crossed with hAPP Tg mice have a dramatic delay both in the amount of Aβ deposited and in the resulting astrogliosis and microgliosis normally seen in hAPP Tg mice [206, 208, 256]. Furthermore, robust effects of inflammation on amyloid deposition were observed following the activation of microglia by intracranial injection of LPS endotoxin in hAPP Tg mice. Using this paradigm, hAPP apoE +/+ Tg mice revealed a marked acceleration of amyloid deposits while hAPP apoE-/- Tg mice did not have any deposition. These observations support the hypothesis that inflammatory stimuli can markedly accelerate amyloid deposition and indicate that expression of apoE is necessary for this process. The strong inflammatory response seen in C57BL/6 microglia could similarly favour amyloid deposition, thereby promoting neuronal damage.

Studying the *in vivo* microglial response in mouse strains known to differ in their *in vitro* microglial activation process to Aβ will help to determine the impact of the brain inflammatory response on the Aβ deposition process. Depending on their genetic background, microglial cells may promote the process of Aβ brain deposition or, alternatively, its clearance [219]. We are currently comparing the levels of brain amyloid deposition and the inflammatory response *in vivo* between transgenic mice expressing a mutant hAPP transgene on either an A/J or a C57BL/6 genetic background. The magnitude of the inflammatory response to amyloid formation *in vivo* may have a significant impact on the amyloidogenic process in these mice. These studies may also facilitate the identification of genetic factors that influence the pace of progression of AD in humans.

Table 2 Activation profiles of A/J and C57BL/6 microglial cell cultures following 48 h of stimulation

Conditions of Stimulation	Morphology[1]		NO Levels		TNF-α Levels	
	A/J[2]	BL/6[3]	A/J	BL/6	A/J	BL/6
Control	Mixed	Mixed	+[4]	+	+	+
$A\beta_{1-42}$	Mixed	Mixed	+	+	+	++
IFN-γ	Mixed	Mixed & Ameboid	++++	++	++	++
IFN-γ + $A\beta_{1-42}$	**Mixed**	**Mixed & Ameboid**	++++	+++	++	++++

[1]Morphology of the majority of microglia in the culture. Mixed refers to ramified, rod-shaped, and some ameboid cells. Ameboid is indicated when a large increase in this morphology was observed.
[2]A/J: A/J strain of mice.
[3]BL/6: C57BL/6 strain of mice.
[4]+: low level (basal); ++: moderate level; +++: high level; ++++: very high level.

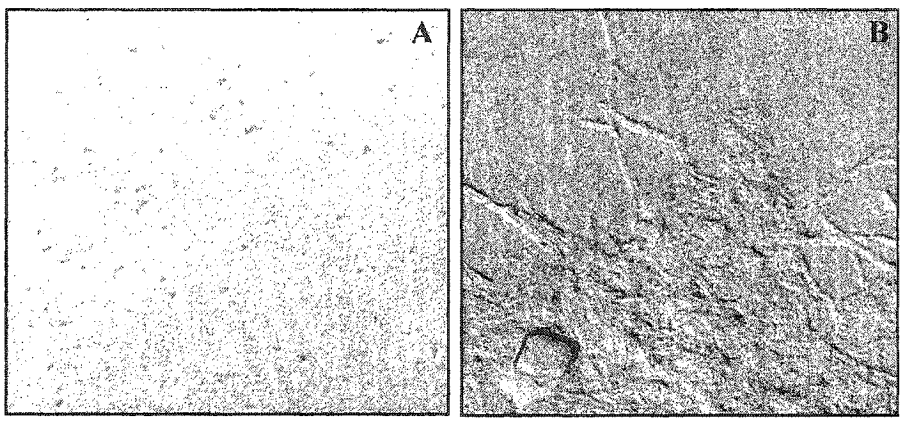

Figure 4 Electron microscopy of both soluble and fibrillar preparations of 20 μM Aβ.

Soluble preparations (**A**) consisted of single-unit structures whereas fibrillar preparations (**B**)were weaven rope-like strands in appearance. Negative staining was performed on 10 μL of each sample which was applied to pioloform-and carbon-coated grids, blotted with filter paper and stained with 1% (w/v) phosphotungstic acid, pH 7.

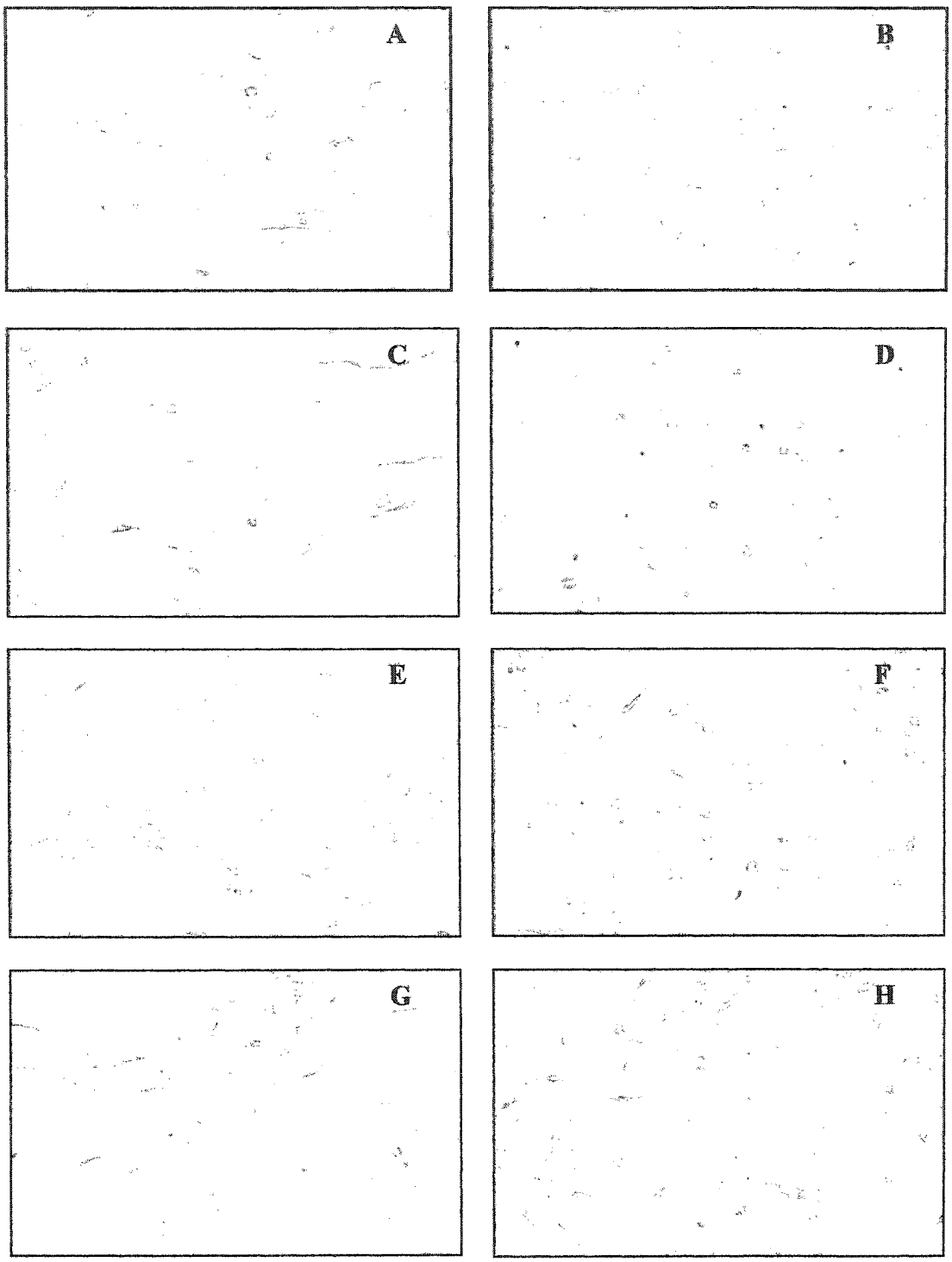

Figure 5 Morphology of primary A/J and C57BL/6 microglia following stimulation

Morphology of primary A/J (left panel) and C57BL/6 (right panel) microglia kept stimulated for 48 h with 1.0 μM $A\beta_{1-42}$ (**A, B**) IFN-γ and $A\beta_{1-42}$ (**C, D**), 50 U of IFN-γ alone (**E, F**), or unstimulated (Control) (**G, H**). Microglia were counterstained with methyl green (100X).

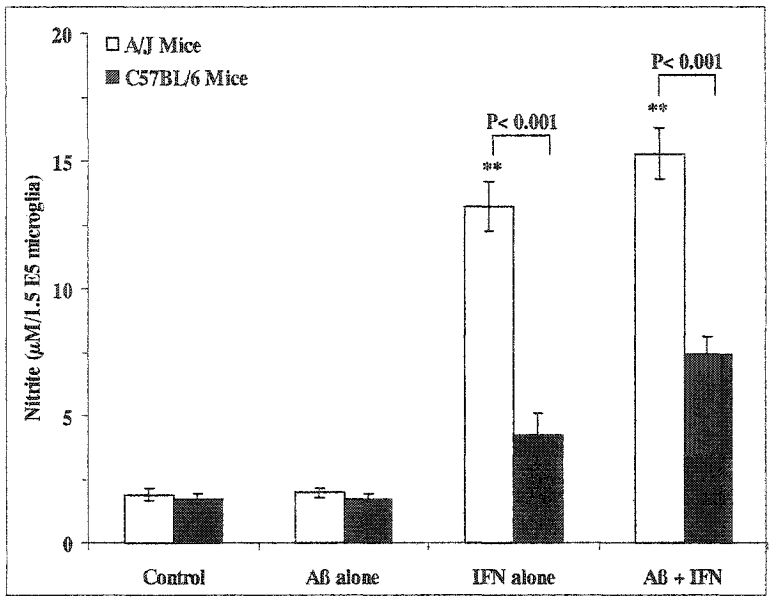

Figure 6 Production of NO as measured by nitrite levels in stimulated microglia isolated from A/J and C57BL/6 strains

Production of NO as measured by nitrite levels in stimulated microglia isolated from A/J and C57BL/6 strains. Microglia (1.5 X 10^5) were kept unstimulated (control) or stimulated with 1.0 and 2.5 μM of $A\beta_{1-42}$ alone (soluble or fibrillar), 50 U of IFN-γ alone, or IFN-γ with 1.0 and 2.5 μM of $A\beta_{1-42}$ (soluble or fibrillar) for 48 h. Values are expressed as a mean from 2 experiments (in duplicates or quadruplicates) with standard error of the mean (S.E.M.). **: within the A/J strain of mice, significantly different from unstimulated control microglia and microglia stimulated with $A\beta_{1-42}$ alone ($P < 0.001$). ++: within the C57BL/6 strain, significantly different from unstimulated control microglia and microglia stimulated with $A\beta_{1-42}$ alone ($P < 0.001$).

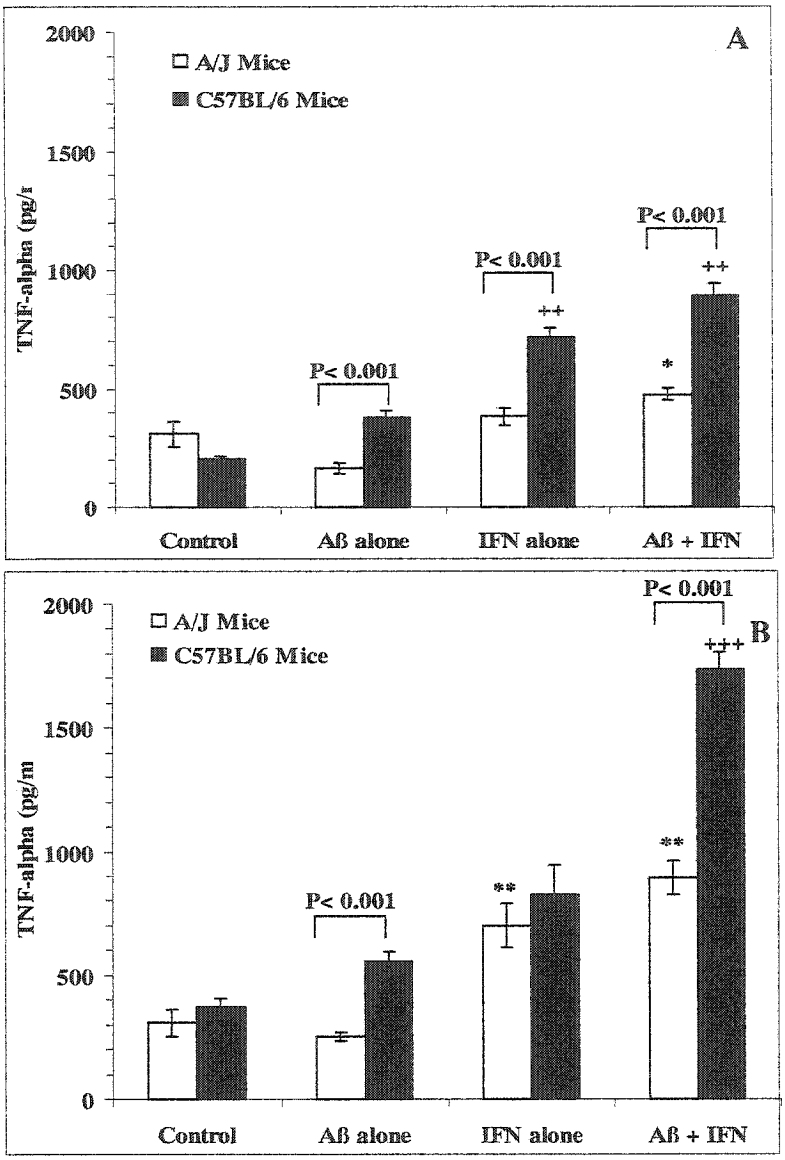

Figure 7 Production of TNF-α in stimulated microglia isolated from A/J and C57BL/6 strains.

Microglia (1.5 X10^5) were kept unstimulated (control) or stimulated with 1.0 and 2.5 μM of $A\beta_{1-42}$ alone (soluble or fibrillar), 50 U of IFN-γ alone, or IFN-γ and 1.0 and 2.5 μM of $A\beta_{1-42}$ (soluble or fibrillar) for **A:** 24 h or **B:** 48 h. Values are expressed as a mean from 2 experiments (in duplicates or quadruplicates) with S.E.M. *: within the A/J strain of mice, significantly different from microglia stimulated with $A\beta_{1-42}$ alone ($P < 0.001$); **: within the A/J strain of mice, significantly different from unstimulated control microglia and microglia stimulated with $A\beta_{1-42}$ alone ($P < 0.001$). ++: within the C57BL/6 strain of mice, significantly different from unstimulated control microglia and microglia stimulated with $A\beta_{1-42}$ alone ($P < 0.001$); +++: within the C57BL/6 strain of mice, significantly different from unstimulated control microglia, microglia stimulated with $A\beta_{1-42}$ alone, and microglia stimulated with IFN-γ alone ($P < 0.001$).

CHAPTER 3: Manuscript 2 Inflammation occurs early in Aβ deposition in TgCRND8 mice

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Manuscript 2: A complete immunohistochemical characterization of the TgCRND8 mice in terms of Aβ deposition, fibrillar plaque formation, microglial activation, and astrogliosis was performed. It demonstrates that inflammation occurs early in Aβ deposition. Age groups from 9 to 20 weeks were examined to follow the early progression of Aβ deposition and its correlation to inflammation in this early-onset model of AD.

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* Both authors contributed equally

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterized by Aβ amyloidosis, neuronal loss, synaptic loss, cognitive decline, and inflammation. An aggressive transgenic mouse model, TgCRND8, carrying a transgene expressing V717F (Indiana) and double K670N M671L (Swedish) mutations in human amyloid protein precursor (hAPP), was used to study the kinetics of brain amyloid deposition in relationship to the appearance of gliosis in aging mice. The neocortices and hippocampal formations from animals aged 9 to 20 weeks, were analyzed for their amyloid burden and their inflammatory status. Both diffuse and compact plaques were detectable as early as 9 weeks of age. Aβ-immunoreactive plaques (4G8-positive) appeared first in the neocortex (retrosplenial, piriform, entorhinal areas) and amygdala, then in the hippocampal formation, and were last observed in the thalamus. Compact plaques (ThioS-positive) with amyloid cores, were seen as early as Aβ-reactive plaques but in lower numbers. The degree of deposition increased progressively with age. Formation of these plaques was concurrent with the appearance of activated microglial cells and closely followed by the clustering of activated astrocytes around the plaques at 14 weeks of age. This TgCRND8 mouse model allows for a rapid, time-dependent study of the relationship between the fibrillogenic process and the inflammatory response during the development of brain amyloidosis.

Introduction

The development of Alzheimer's disease (AD) in humans is characterized by gradual cognitive dysfunction and the cerebral accumulation of senile plaques composed mostly of β-amyloid (Aβ) peptide. Neuropathological examination usually reveals several degenerative features such as neurite dystrophy, neurofibrillary tangles, and neuronal cell loss accompanied by the upregulation of several inflammatory markers [5, 91]. While most AD cases are sporadic (90%), early onset AD can be caused by mutations in amyloid precursor protein (APP), presenilin-1 (PS-1), or in presenilin-2 (PS-2) which generate increases in the levels of total Aβ and in some, increases in the levels of $A\beta_{42}$ relative to $A\beta_{40}$ [238]. Increases in the amount of total Aβ and in particular, the highly-aggregating, neurotoxic form, $A\beta_{42}$, initiates the process of amyloid plaque formation.

The early process of plaque deposition is promoted by high levels of $A\beta_{42/43}$ which is later accompanied by $A\beta_{40}$ deposition [20, 276]. In humans, plaque formation is marked by diffuse plaques (Aβ deposits) with the ensuing appearance of compact plaques that can be detected with thioflavinS (ThioS) and Congo Red due to their β-sheet structural properties [86]. These plaques are often surrounded by activated microglia and reactive astrocytes which play a role in plaque maturation, dystrophic neurite formation, or the degradation and removal of plaque components [23, 277, 278].

Familial Alzheimer's Disease (FAD) mutations identified in AD, have been introduced in transgenic mice to establish models that reconstitute the pathogenic process associated with Aβ amyloidosis [204, 227, 228, 239, 279, 280]. These models display several pathological characteristics of AD such as Aβ-immunoreactive and ThioS-positive plaques, which can be detected at 6 to 9 months of age and are accompanied by

dystrophic neurites and reactive gliosis [238]. These changes are paralleled by significant cognitive deficits [240].

TgCRND8 mice were generated using a hAPP transgene containing both the Indiana (V717F) and double Swedish mutation (K670N M671L), driven by a hamster prion promoter. These mutations (V717F and K670N/M671L) are linked to an increase in the ratio of $A\beta_{1-42}/A\beta_{1-40}$ and in an increase in overall Aβ load, respectively [18, 68]. This transgene induces rapid neuropathological changes with a robust increase in brain Aβ levels and early formation of plaques. A progressive cognitive decline also accompanies the neuropathological changes in these transgenic animals [228].

Non-steroidal anti-inflammatory drug (NSAID) treatment has shown that diminishing inflammation can delay the pathology of AD [1]. In fact, activated microglia and reactive astrocytes have been observed around plaques and the cytokines and growth factors that they release, have been proposed to favour the Aβ deposition process, neuropathological changes, and cognitive decline [91, 120-125]. Therefore, a better understanding of the role of microglia in plaque formation could shed some light onto the mechanisms of Aβ amyloidosis.

Therefore, we followed the accumulation of plaques in TgCRND8 mice in relationship with the appearance of inflammatory foci (CD11b). Rare Aβ-immunoreactive deposits as well as mature ThioS-positive plaques were evident starting from 9 weeks of age. Diffuse and fibrillar plaque formation accelerated in aging animals such that by 20 weeks of age, all animals presented an Aβ plaque load superior to that seen in 14-week-old animals. This progression was paralleled by a similar increase in the inflammatory foci where activated microglial clusters (CD11b-immunolabeling) were

detected at the earliest time-points with both diffuse and fibrillar deposits. Clusters of glial fibrillary acidic protein (GFAP)-reactive astrocytes were first observed around plaques at 14-16 weeks of age, considerably later than plaques and activated microglia. Thus, inflammation markers in TgCRND8 mice are associated with the earliest neurohistological changes. These findings underline the relationship between early inflammatory changes and the progression of AD.

Materials and Methods

I TgCRND8 mice

The transgenic line of mice used are the hAPP TgCRND8 mice which were obtained from D. Westaway [228]. The transgene is driven by a Syrian hamster prion promoter and expresses hAPP containing two types of FAD mutations: double Swedish mutations (K670N/M671L) and the Indiana mutation (V717F). Mice were originally on a 65% C57BL/6, 35% C3H background. These mice were backcrossed for two generations onto an FVB background to create TgCRND8.FVB (N2).

All mice used in the study were genotyped for the human APP transgene by PCR. DNA was isolated from hair follicles as previously described [281]. The human APP transgene cassette was detected by using a transgene-specific PCR reaction of the hamster PrP 3'UTR with the following primers: 5'-GCCTTTGAATTGAGTCCATCAC-GGGCCA-3' and 5'-AGAAATGAAGAAACGCCAAGC GCCGTGACT-3' using standard cycling conditions. The PCR products were separated on a 7% polyacrylamide denaturing gel and visualized by autoradiography.

II Tissue preparation

Mice of 9, 10, 12, 13, 14, 15, 16, and 20 weeks of age were anesthetized and trans-cardially perfused with saline and then, with 4% paraformaldehyde (PFA). Brains were collected, post-fixed in fresh 4% PFA for 3 hours and then transferred to a 30% sucrose solution at 4°C. Once cryoprotected, the brains were frozen in isopentane at -45°C and stored at -80°C until sectioning. Brains were serially sectioned at 30 μm using a cryostat and transferred in cryoprotectant to be stored at -20°C.

III Histochemistry

Free-floating sections were extensively washed before treating them with 3% H_2O_2 for 15 minutes to quench the endogenous peroxidase. Sections for CD11b- and GFAP-immunolabeling were treated with 0.1% Triton X-100 for 30 minutes before blocking. All sections were blocked using 4% goat serum for 45 minutes. Then, sections were immunostained with 4G8, mAb for total Aβ (Senetek, CA; 4G8-biotin; 1/2000), 6E10, mAb for total human Aβ (Senetek, CA; 6E10-biotin; 1/750), CD11b, mAb for microglial activation (Pharmingen, San Diego, CA; 1/1000), or GFAP, a marker for astrocytes (DAKO; 1/3000). Primary antibodies were incubated overnight at 4°C. Secondary biotinylated goat anti-rat antibody for indirect CD11b immunostaining (1/200) or secondary biotinylated goat anti-mouse antibody for indirect GFAP immunostaining (1/400), was incubated at room temperature (20-24°C) for 1 hour. Following amplification with an avidin-biotin kit (Vector Laboratories, Burlington, ON, Canada), the reaction was developed with Ni-3,3'-diaminobenzidine/H_2O_2 (DAB). Alternatively, for direct visualization of fibrillar amyloid, another set of brain sections were mounted on Super Frost slides, air dried, and stained with 1% thioflavinS (ThioS, an amyloid marker for the detection of compact plaques) in distilled water.

Image capture

Microscopy was performed on an Olympus BX40 microscope with a Pro-series camera to capture images for analysis of bright field images and a Spot-RT digital camera (Diagnostic Instruments Inc.) for analysis of ThioS fluorescence and high quality images. Quantitative analysis was performed using an Image Pro-Plus Systems software (Media cybernetics, USA).

Analysis and Statistics

Two sections from two different levels of the brain (-2.0 and -3.4 mm from the bregma, atlas from Franklin and Paxinos) were examined by an analyst blinded for the age and the genotype of the mice [282]. The number of plaques was measured in the neocortex and the hippocampal formation and their total area was determined. This measurement was normalized using the total surface of the region analyzed and expressed as a percentage. For each marker, results of duplicate measurements from each animal were averaged before obtaining the group average.

Using the Pearson product moment correlation, we computed the coefficient of correlation ($R_{pearson}$) to determine the degree of association of each marker with age and to measure the degree of association between the presence of diffuse and compact plaques with that of inflammatory sites (number and amyloid plaque/inflammatory site area (5)). Kruskal-Wallis one-way ANOVA on ranks was computed and the Dunn's method was used to detect any significant difference ($P < 0.05$) in plaque and inflammatory site number as well as amyloid plaque/inflammatory site area (%) from 9-10 weeks of age up to 20 weeks. The same tests were used to detect significant differences among diffuse plaques, fibrillar plaques, and inflammatory sites at each time-point. Mann-Whitney Rank Sum tests and *t*-tests were used to compare the diffuse and mature plaque and inflammatory site number and the amyloid plaque/inflammatory site area (%) in the neocortex and the hippocampal formation.

Results

Progressive accumulation of diffuse and compact cerebral plaques in TgCRND8 mice

The number and size of plaques, the amyloid plaque as well as the inflammatory site area (%) progressively increased in aging animals. Qualitative analysis revealed that amyloid deposition (4G8) was rarely detected at 9 and 12 weeks of age (Figure 8 A,D) while consistent deposition was observed starting at 13-14 weeks of age (data not shown). Aβ deposits were mainly observed in the cingulate/retrosplenial, temporal, and entorhinal areas of the neocortex as well as in the hippocampal formation. Deposition increased sharply at 13 weeks of age (Figure 8 B, E) and continued to increase up to 20 weeks of age (Figure 8 C, F).

The presence of amyloid-containing plaques was determined using ThioS staining. Mature compact plaques were distributed as Aβ-immunoreactive deposits and followed the same trend with few plaques at 9-10 weeks and a sharp increase from 13-20 weeks of age (Figure 8 G, H, I).

By double labeling for Aβ and ThioS, we verified the proportion of Aβ-reactive plaques that contained a core of amyloid fibrils (Figure 9). We found that about 30-50% of all the Aβ-reactive plaques were associated with a ThioS-positive core. Furthermore, the presence of amyloid fibrils within a plaque was independent of its size. While some small plaques clearly contained amyloid fibrils, numerous large plaques observed were 6E10-positive and ThioS-negative.

Early glial response accompanies plaque accumulation in TgCRND8 mice

As inflammation is closely associated with the development of AD, we followed the activation of microglia and astrocytes in the brain of TgCRND8 animals. Rare activated microglia detected by their CD11b immunoreactivity, became evident at 9 weeks of age in the neocortex and hippocampal formation (Figure 8 J). At 13 weeks of age, multiple CD11b foci were clustered in the neocortex and hippocampal formation, as microglia were presumable recruited toward Aβ deposits (Figure 8 K) while numerous large foci were obvious by 20 weeks of age (Figure 8 L).

A robust astrocytic response was also evident. At the early age of 9 weeks, scattered activated astrocytes were first found throughout the neocortex and hippocampal formation. However, at 13 weeks of age, clusters of GFAP-immunoreactive astrocytes began to emerge as the number of plaques became significant. At 16 weeks of age (data not shown), reactive gliosis around plaques was more pronounced and continued to progress through 20 weeks of age. Double labeling for GFAP and ThioS demonstrated that the scattered staining did not coincide with plaques whereas astrocyte clusters were found around plaques (Figure 10). Furthermore, many clusters of activated astrocytes were not associated with a ThioS amyloid core, being presumably around diffuse plaques.

Double immunohistochemical staining with CD11b and ThioS confirmed that amyloid containing (ThioS-positive) plaques were in close association with activated microglial cells (Figure 11). Interestingly, not all clusters of CD11b-positive cells were associated with an amyloid core, indicating that both diffuse and mature plaques were surrounded with activated microglia.

Quantitative analysis of plaque formation and microglial activation

To determine the quantitative relationship between plaque accumulation and inflammation, image analysis from hippocampal and neocortical regions was carried out over a 20-week time-course study. Deposition of diffuse plaques (4G8-positive, ThioS-negative), compact plaques (4G8-positive, ThioS-positive), and microgliosis (CD11b) were all found to increase with age (Figure 12, Table 3). Plaques appeared in the neocortex and the hippocampal formation simultaneously with microglial activation as evidenced by correlation analysis between Aβ deposition and mature plaques and inflammation (Table 3). Although rare at 9-10 weeks (~20-30% of the animals), the vast majority of the animals displayed plaques at 13-14 weeks of age (>95%) (data not shown).

Plaques were increased in both number (Figure 12 A,B) and area (% of area occupied (Figure 12 C,D) from 13 to 20 or 15 to 20 weeks of age for the neocortex and hippocampal formation, respectively ($P < 0.001$). The area (%) of diffuse plaques was greater than that of mature plaques from 13 to 20 weeks of age in both the neocortex ($0.001 < P < 0.005$) and hippocampal formation ($0.002 < P < 0.005$). At 20 weeks of age, the area (%) occupied by diffuse plaques was 4-6 times greater than that occupied by compact amyloid plaques (neocortex and hippocampal formation: $P < 0.003$).

The appearance of inflammatory foci (CD11b) paralleled the increase in Aβ-immunoreactive plaques (number and area (%)), but was slightly delayed, increasing from 15 to 20 weeks of age ($P < 0.001$). Therefore, the number of inflammatory sites and the inflammatory site areas (%) observed as activated microglia, were lower than that of Aß-immunoreactive plaque number ($P = 0.007$) and amyloid burden (%) ($P < 0.001$) at 15.5 weeks of age in the neocortex. In contrast, inflammatory site values were greater

than those of mature amyloid plaque numbers (neocortex: $P = 0.011$; hippocampal formation: $P = 0.007$) and mature plaque amyloid burden (%) (neocortex and hippocampal formation: $P = 0.003$) at 20 weeks of age in the neocortex and the hippocampal formation. Altogether, these observations suggest that TgCRND8 microglial activation closely follows the deposition of Aß into plaques and that their recruitment to both diffuse and compact plaques takes place as early as they form.

When comparing Aß deposition and fibrillization in the neocortex and the hippocampal formation, we observed that diffuse plaque number was greater in the neocortex than in the hippocampal formation from 13.5 and 15.5 weeks of age ($P < 0.001$) but the difference did not reach a significant level at 20 weeks of age (Figure 13). Mature plaques were also in greater number in the neocortex compared to the hippocampal formation, from 15.5 to 20 weeks of age ($P < 0.003$ for both time points). The amyloid plaque and the inflammatory site area (%) were similar in the neocortex and the hippocampal formation (data not shown).

Discussion

TgCRND8 mice express high levels of the double mutant (Swedish / Indiana) APP gene and develop neuropathological lesions at a very young age [228]. They have previously been shown to produce robust levels of $A\beta_{40}/A\beta_{42}$, which accumulate and lead to the formation of Aβ plaques starting at ~10 weeks of age. This is accompanied by age-dependent behavioural deficits [235]. In fact, these mice constitute an aggressive AD mouse model that is rivalled only by bigenic mice harbouring distinct transgenes encoding both mutant hAPP and PS1 [236, 237].

In young TgCRND8 mice, we find early accumulation of Aβ deposits in an age-dependent manner in accordance to previous reports [236, 237, 283-285]. Although rare at 9-10 weeks of age (~20-30% of the animals), the vast majority of the animals display plaques at 13-14 weeks of age (>95%). These observations are similar to those obtained in PDAPP mice where both fibrillar and compact plaques develop simultaneously but at an older age [190, 204]. In contrast, APP23, Tg2576, or doubly transgenic mice (hAPP + PS1), display less plaque heterogeneity and mostly compact plaques with an age of onset between 40-52 weeks [239, 286]. The mechanism by which some plaques develop an amyloid core while others do not is unclear. However, high levels of $A\beta_{42}$ appear to clearly favour the formation of both compact and diffuse plaques. Interestingly, some reports indicate that $A\beta_{40}$ is preferentially associated with compact plaques while $A\beta_{42}$ is found in both types[19, 190, 286]. Other factors such as apolipoproteins, B2 microglobulin, or antichymotrypsinogen can also promote the formation of plaques[210, 287-290].

Double labeling of brain sections to detect Aβ-immunoreactive plaques and fibrillar amyloid deposits (ThioS) revealed that both diffuse and dense core plaques

appeared concomitantly at an early age and in aged mice, both diffuse and compact plaques sometimes contained fibrillar (ThioS-positive) cores. These observations are contrary to the continuum concept which suggests that compact plaques form from diffuse deposits by fibrillization of their core [291, 292] (for a review see Wisniewski)[293]. Our transgenic model supports the concept that two parallel pathways exist and independently lead to the formation of diffuse or compact plaques. This view has been supported by recent data from other transgenic systems where congophilic plaques appear before diffuse ones [239, 285, 286, 294].

Generally, Aß peptide load reflects histological changes where elevated levels of total Aß and high $Aß_{42}$/ $Aß_{40}$ ratios favour plaque formation [294]. In TgCRND8 mice, similar trends were observed where the Aß levels begin to increase around 8-10 weeks of age (approximately 100 ng/g of brain) when the first rare plaques are observed [228]. Also, this increase is primarily due to $Aβ_{42}$ levels as observed by the $Aβ_{42}/Aβ_{40}$ ratio (5.05 ±1.56). Thus, our data supports the idea that elevated levels of Aß, particularly $Aß_{42}$, favour the early development of not only compact but also diffuse plaques.

TgCRND8 mice are similar to the Tg2576 mice in their plaque deposition pattern and in the early increase in Aβ load before plaque appearance. Also, they contain the double Swedish mutations in hAPP695 transgene driven by a prion promoter with the same level of transgene expression but, they lack the Indiana mutation in their transgene. The Swedish mutations generate an increase in overall Aβ load whereas the mutation at V717F, promotes an increase in $Aβ_{42}$ with a decrease in $Aβ_{40}$, causing an imbalance in $Aβ_{42}/Aβ_{40}$ levels [18, 68, 231]. The Tg2576 mice have a high Aβ load but very low ratios of $Aβ_{42}/Aβ_{40}$ at plaque onset of approximately 0.26 ± 0.02 [236]. As TgCRND8 mice have

both a very high Aβ load and high levels of $A\beta_{42}$ compared to $A\beta_{40}$, this could account for the earlier plaque deposition and the quick appearance of fibrillar plaques following diffuse plaque formation, respectively. Thus, TgCRND8 mice are unique as they contain both the Swedish and Indiana mutations. It should be noted that these transgenic mouse models also differ in the background strain used to create the transgenic mice which has been shown to have a strong impact on mouse survival [205, 228, 295].

Other mice have been created with the same FAD mutations in the hAPP695 transgene but the transgene is driven by a different promoter, the PDGF-β promoter [240]. Another recently reported transgenic mouse line designated J20, presents a pathology similar to that of TgCRND8 mice with substantial Aß, high $A\beta_{42}$ / $A\beta_{40}$ ratios, and plaque deposition evident from 2-4 months of age. Despite their early onset of deposition, only 50% of the mice displayed evidence of plaques at 20-28 weeks of age while 95% of TgCRND8 mice present deposits by 14 weeks of age. These findings underscore the role of high $A\beta_{42}$ levels in the seeding process of Aß plaques and explain the early deposition in TgCRND8 mice and the concomitant appearance of both compact and diffuse plaques.

Inflammation is associated with the development of AD in humans[296]. In fact, the use of NSAIDs suggests that inhibiting the inflammatory reaction may improve the prognostic by delaying the onset of AD[125, 297, 298]. Inflammation has been implicated in both the deposition and the clearance of Aß plaques. Although our experiments do not allow us to discriminate in which process microglial cells intervene in TgCRND8 mice, we find that microglial activation is intimately associated with Aß plaques at all stages. In our model, microglial activation was evident early with rare foci of CD11b microglia being detected at 9-10 weeks of age and their progressive accumulation paralleling the

plaque load during aging. These findings corroborate previous reports using other Tg models[263, 264, 286, 299, 300]. CD11b-positive microglial elements were found with or without associated amyloid. The microglial inflammation strongly correlated with diffuse and compact plaque load indicating that microglia are activated by both types of deposits. Tg APP23 and Tg2576 mouse models display activated microglia only in association with compact plaques [263, 264, 284, 300]. These discrepancies could be accounted for by the transgene itself, which expresses different APP alleles under distinct promoters that could modulate microglial activation to Aß plaques. Alternatively, these differences could be explained by the distinct genetic backgrounds used for Tg2576 and APP23 mice. Activation of primary microglial cells by fibrillar $Aß_{42}$ *in vitro* displays different kinetics whether the cells were obtained from an A/J or a C57BL/6 genetic background (see manuscript 1). This suggests that genetic background could modulate the magnitude of the activation process in response to Aß.

Activated astrocytes detected using antibodies against GFAP is another sensitive marker of the inflammatory process associated with neuronal injury. As early as 9 weeks of age, we found a scattered GFAP staining in the neocortex and hippocampal formation. These changes probably represent a response to the toxicity resulting from the high levels of mutant APP transgene, reflected by the high mortality rate in TgCRND8 between 4 to 10 weeks of age (40%) (data not shown). The scattered GFAP staining evolved into a pattern of clustered activated astrocytes by 13-14 weeks of age when acceleration in the deposition process was evident and 90% of the animals displayed multiple plaques. The astrocytic response to the presence of Aß plaques followed that of microglia by about a month (14 weeks of age). Reactive astrocytes were found to surround the core of amyloid

plaques but were not always associated with amyloid cores indicating that astrocytes, like microglia, can be activated by both compact and diffuse plaques.

The presence of hypertrophied activated astrocytes around the perimeter of the plaques has been reported in humans and transgenic mice [286, 301]. Astrocytes have been proposed to play a critical role in the clearance of Aß deposits. However, activated astrocytes could also exacerbate the degenerative process and favour the deposition of Aß into plaques following IL-1ß-induced secretion of chaperones such as antichymotrypsinogen and ApoE, which promote the formation of plaques[210, 256, 287, 288, 302]. Furthermore, activated astrocytes can accelerate the deposition process by upregulating their own expression of APP under the stimulation of IL-1ß[303].

In conclusion, we describe the progressive age-dependent appearance starting at 9-10 weeks of age of both diffuse and compact Aß deposits in TgCRND8 mice. These deposits were always observed in association with an early microglial inflammatory response and a slightly delayed microglial activation that surrounded the deposits (13-14 weeks of age). Importantly, both fibrillar and diffuse deposits appear sufficient to induce this response as the inflammation markers were found in association with both fibrillar and diffuse plaques.

Table 3 Correlation Analysis Results for Amyloid Deposition and Mature Plaques with Inflammation

	Cortex		Hippocampus	
	Plaque Number	Plaque /Inflammatory Site Area (%)	Plaque Number	Plaque /Inflammatory Site Area (%)
Aß Deposition (4G8) - Inflammation	0.910[1] (<<0.001)[2]	0.870 (<<0.001)	0.885 (<<0.001)	0.889 (<<0.001)
Mature Plaques (ThioS) - Inflammation	0.917 (<<0.001)	0.770 (<<0.001)	0.857 (<<0.001)	0.914 (<<0.001)

[1] $R_{Pearson}$: Pearson correlation coefficient for each marker with CD11b

[2] (*P*): Probability associated with $R_{Pearson}$.

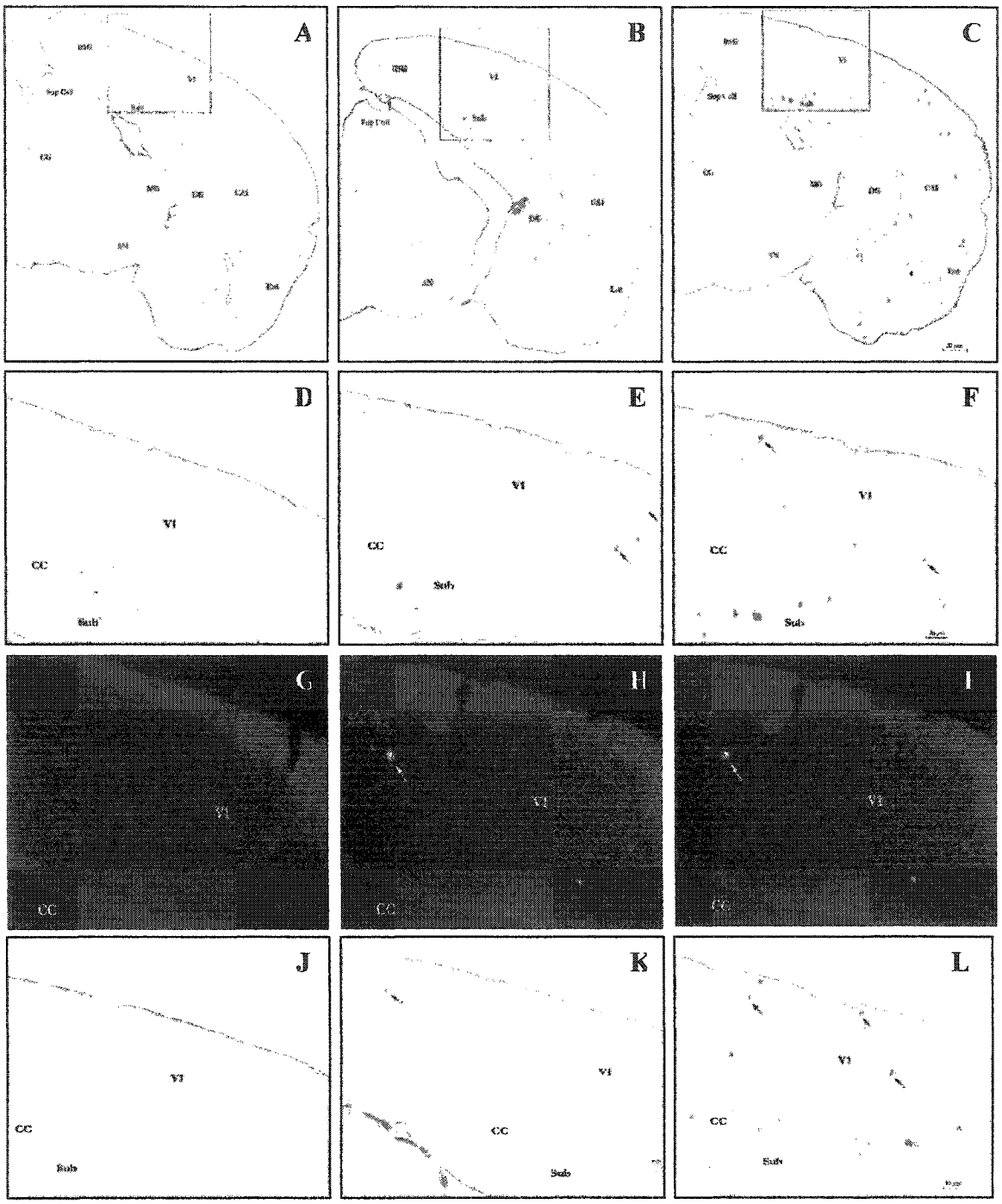


Figure 8 Profile of Aβ deposition and inflammation in the neocortex

Time course of total Aβ deposition 4G8 immunolabeling (Aβ deposits) **A-F**-first two rows, ThioS staining (fibrillar or mature plaques) **G-I**-third row, and CD11b immunolabeling (activated microglia) **J-L**-last row at 9 weeks, 13 weeks, and 20 weeks of age as indicated from left to right. The first three figures of 4G8 immunolabeling are at 12.5X original magnification and all others are at 40X original magnification

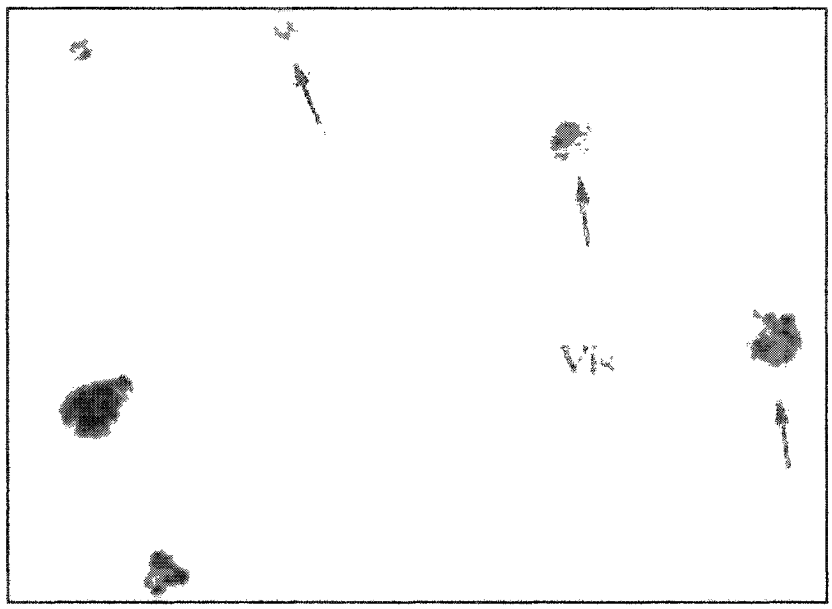


Figure 9 Double labeling of 6E10 and ThioS in TgCRND8 mice at 20 weeks of age

Double labeling of 6E10 (all human APP-derived Aβ, *left*) and ThioS (fibrillar or mature plaques, *right*) showing that not all 6E10-labeled plaques are positive for ThioS at 20 weeks of age. Original magnification 40X.

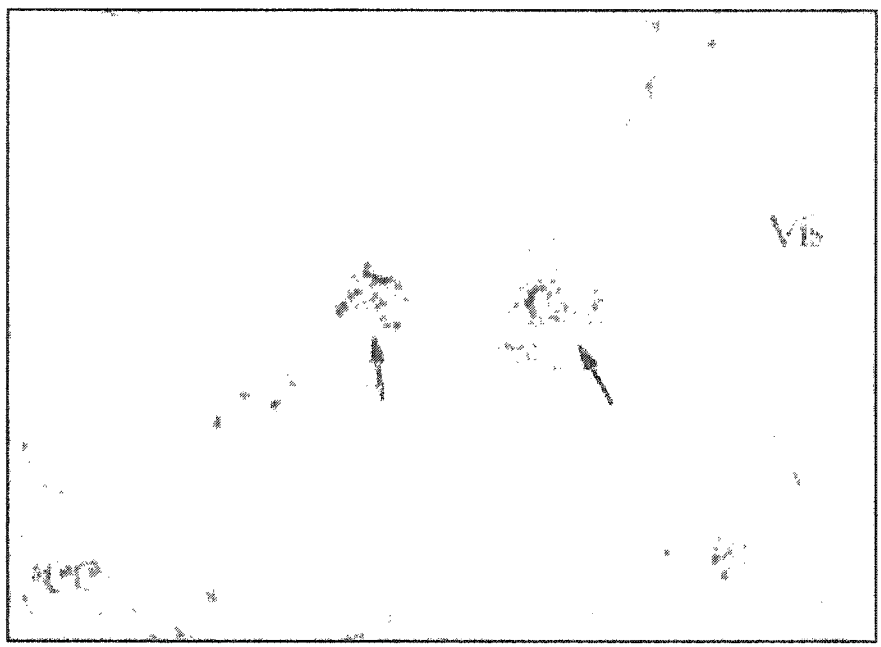


Figure 10 Double labeling of GFAP and 6E10 or ThioS in TgCRND8 mice at 20 weeks of age

Double labeling of Aß deposition and inflammation in the neocortex of mice at 20 weeks of age. A, Total human Aß (6E10, *left*; dark reaction product) and fibrillar Aß (ThioS, *right*; fluorescent labeling). B, Astrocytes (GFAP; dark reaction product) and fibrillar Aß (ThioS; fluorescent labeling). **CC:** corpus callosum; **Vis:** primary visual cortex; **arrow**: double labeled plaques or loci; **arrowhead**: simple labeled plaque. Original magnification: 40X.

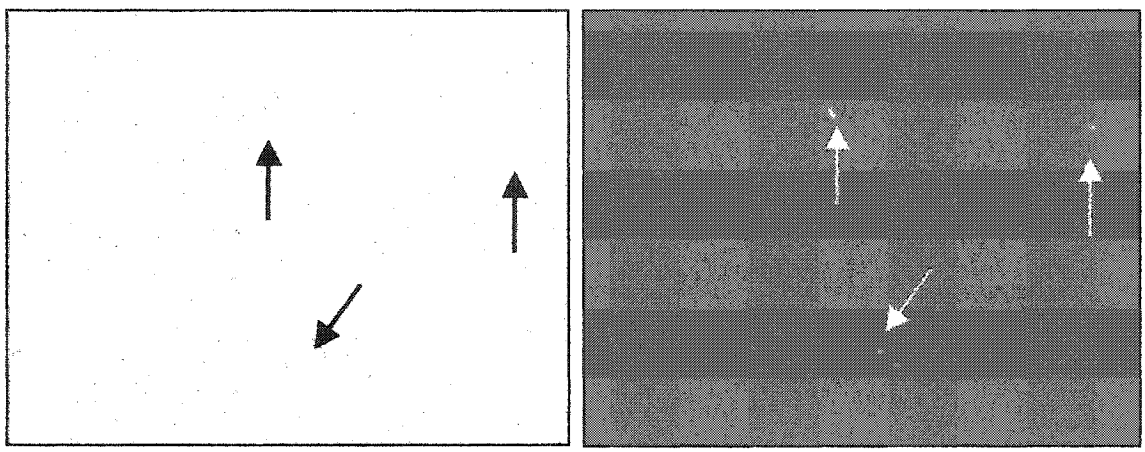


Figure 11 Double labeling of CD11b and ThioS in TgCRND8 mice at 17 weeks of age

Double labeling of CD11b (microglial activation) and ThioS (fibrillar or mature plaques) showing that microglia did not always cluster around ThioS-positive plaques at 17 weeks of age. Original magnification 100X.

Figure 12 Evolution of plaques and inflammation in the neocortex (a) and hippocampal formation as plaque/loci number and area (%).

Plaques (4G8, ThioS) and inflammation site (CD11b) number and area (%) increase with age in the neocortex (A, C) and the hippocampal formation (B, D) in TgCRND8 mice. The *n* for each group is 7 in the 9.5-week-old group (range 9-10 weeks), 6 in 12-week-old group, 10 in the 13.5-week-old group (range 13-14 weeks), 8-9 in the 15.5-week-old group (range 15-16 weeks), and 6 in the 20-week-old group. Results from two coronal sections of the brain are expressed as means ± standard error of the mean (S.E.M.) *: Statistical difference between 4G8 and ThioS at a given time-point ($0.001 < P < 0.015$); +: Statistical difference between 4G8 and CD11b at a given time-point ($0.001 < P < 0.007$); x: statistical difference between CD11b and ThioS at a given time-point ($0.003 < P < 0.011$)

Figure 12A,B Plaque numbers in neocortex and hippocampal formation

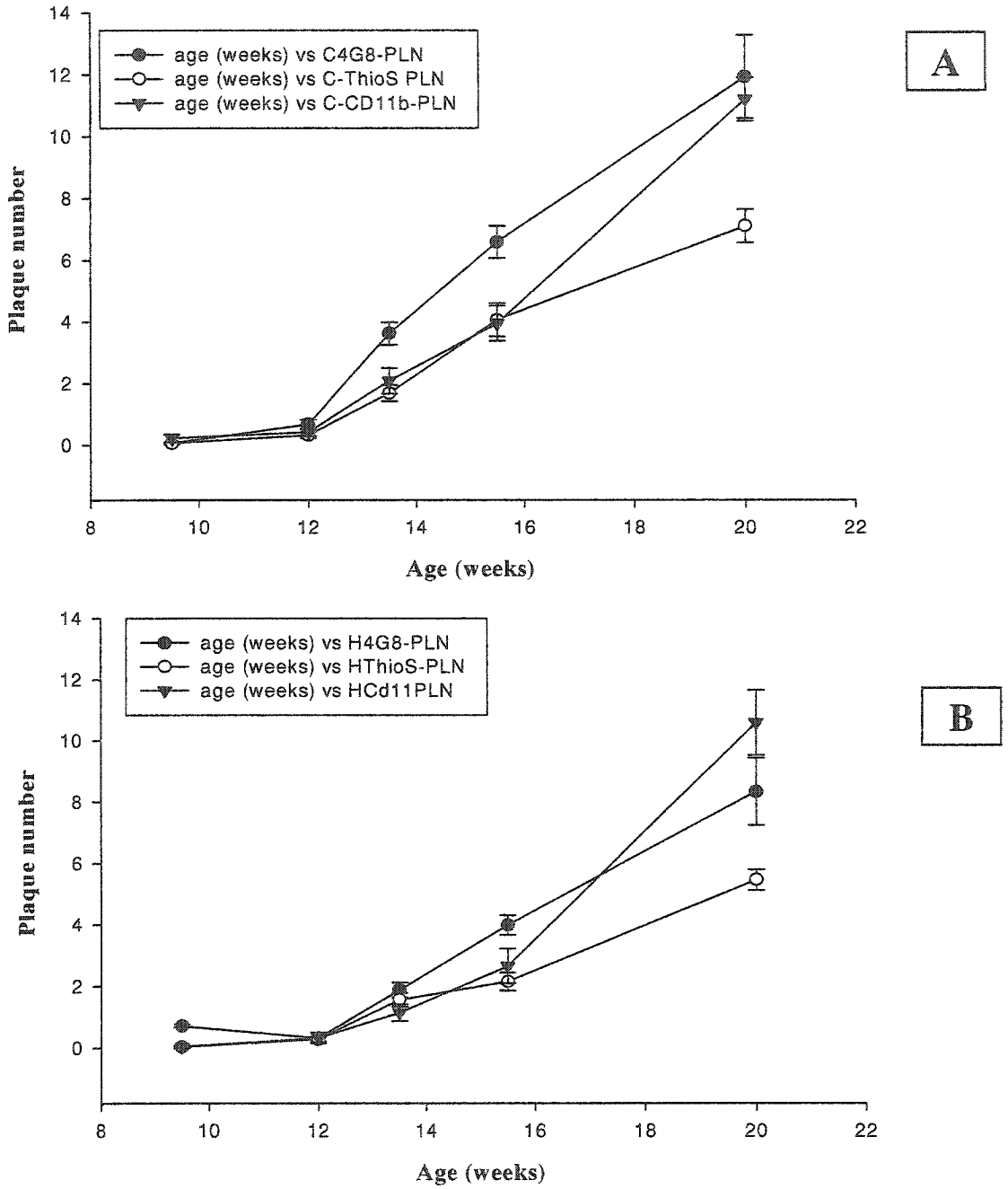
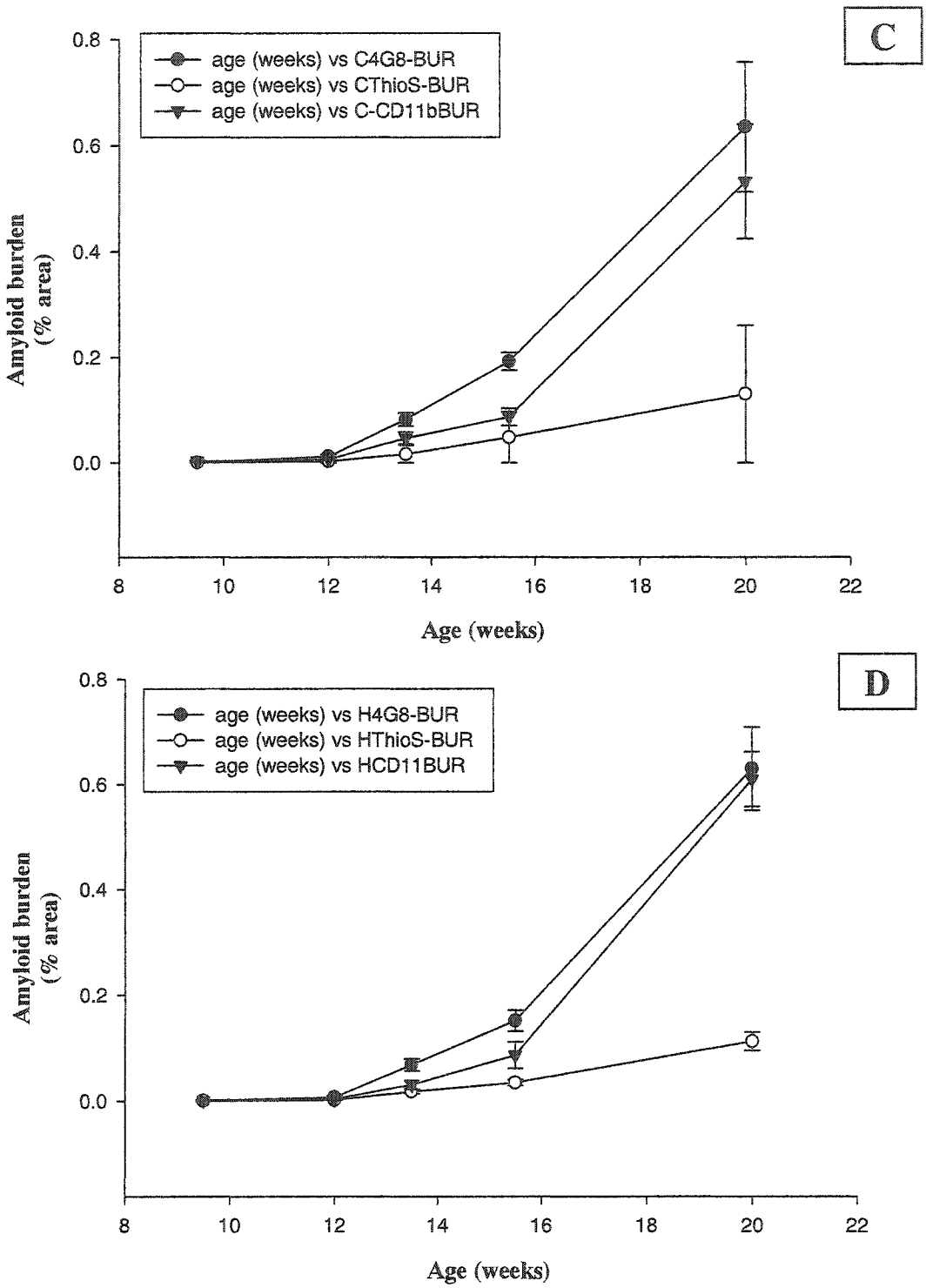


Figure 12C,D % Area occupied by plaques in neocortex and hippocampal formation



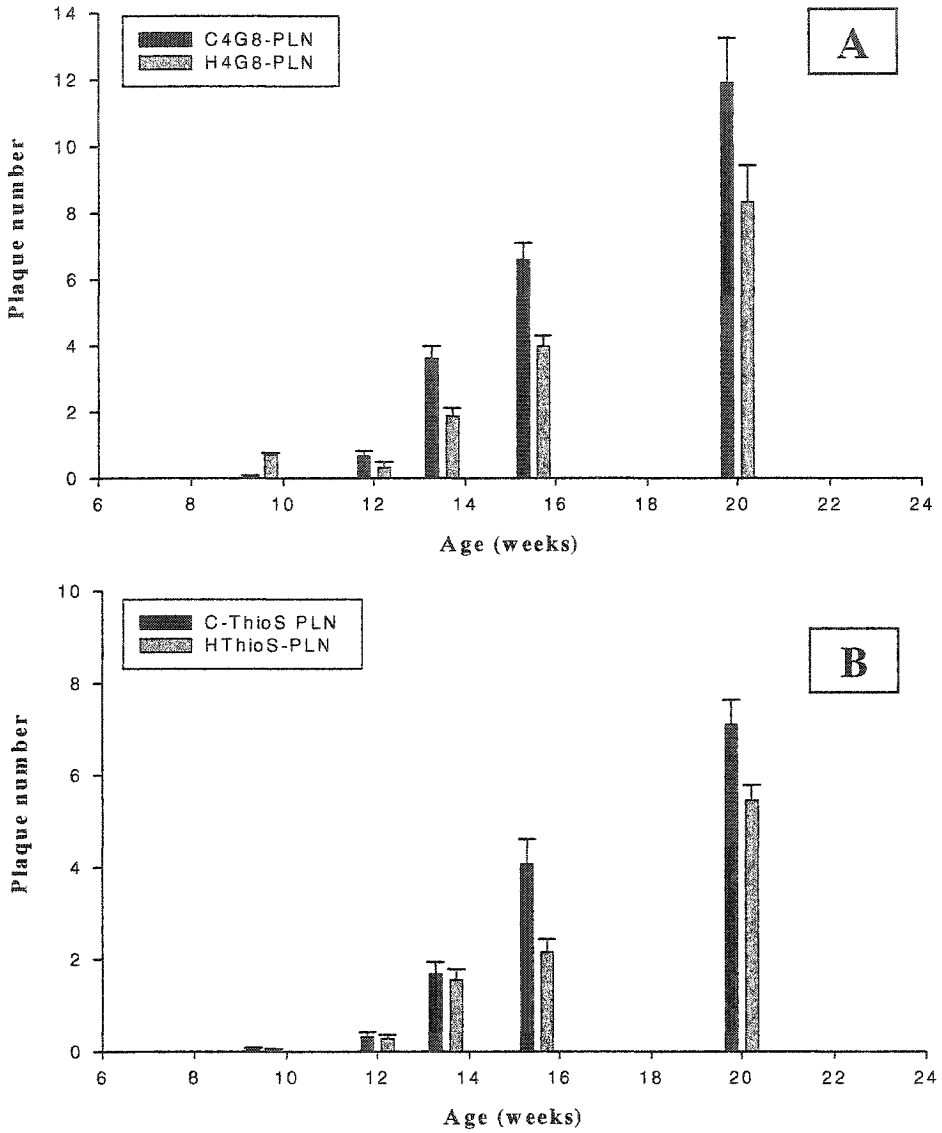


Figure 13 Comparison of neocortex and hippocampal formation.

Number of Aß-immunoreactive plaques (A) and mature plaques (B) in the neocortex of TgCRND8 mice compared to those in the hippocampal formation.

CHAPTER 4: Manuscript 3 Influence of genetic background on survival and plaque formation in TgCRND8 mice

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From the first two manuscripts, it is evident that inflammation does play a role in Aβ deposition and that genetic predisposition can affect the inflammatory response of microglia to Aβ. Therefore, TgCRND8 mice were backcrossed onto the C57BL/6 and A/J strains of mice to determine the effects of these two backgrounds on the amyloidogenic process. The following results show that genetic factors can alter the pathological outcome of these mice in terms of survival and plaque deposition. This may have an impact on various therapeutic approaches for AD patients as individuals vary in their genetic background.

Abstract

Since inflammation plays a role in Alzheimer's disease (AD), inflammatory response to beta-amyloid (Aβ) deposition is becoming recognized as an important factor in AD pathology. To determine genetic background effects of inbred strains of mice, we have taken the TgCRND8 mice which express a double mutant form of the amyloid precursor protein (APP) 695 [228] and bred them onto either an A/J background or a C57BL/6 background. These strains of mice are known to have a low or high inflammatory response to foreign antigens, respectively. As inflammation is a factor in AD neuropathology and plays a role in amyloidosis, phenotypic differences arising from variations in inflammatory response were studied. Mouse background did not have an effect on hAPP transgene-induced hAPP mRNA expression nor on the production of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ measured in brain homogenates by sandwich ELISA. Interestingly, background differences greatly affected the kinetics and magnitude of brain amyloidosis in these mice. Analysis of histochemistry/immunohistochemistry showed a difference in the extent of Aβ diffuse and fibrillar plaque formation in these mice. TgCRND8 mice on an C57BL/6 background deposited 30% more Aβ as shown by 6E10 immunostaining and 30% more thioflavin S-positive Aβ deposits (fibrillar deposits) in comparison to the A/J background. Furthermore, TgCRND8 mice on a C57BL/6 background had a much lower rate of survival than the A/J background which lived well past 24 weeks of age. Both survival and the amyloid deposition process in the brain, were strongly influenced by the genetic background of the mice. Thus, their genetic background was found to have a specific affect on the phenotypic expression of the hAPP transgene expression.

Introduction

Alzheimer's disease (AD) is clinically-rated in measures of cognitive decline and is characterized by neuronal loss, intraneuronal neurofibrillary tangles, dystrophic neurites, and both diffuse and fibrillar plaque formation [5, 6, 10, 12]. An important area of research is dedicated to determining the mechanism of Aβ deposition and fibrillization which, in turn, is responsible for neurodegeneration. Neurotoxic effects of Aβ arise directly through radical mechanisms or indirectly through activation of an inflammatory response leading to neurotoxic factor release [108, 125].

Inflammation is believed to play a key role in neurodegeneration as microglia and astrocytes have been observed around diffuse and fibrillar (senile) plaques [120-123]. Anti-inflammatory therapy with non-steroidal anti-inflammatory drugs (NSAIDS) delay AD [1, 2]. Therefore, inflammation plays a role in neurodegeneration observed in AD.

In *in vitro* studies, microglia have been shown to respond to Aβ stimulation by secreting proinflammatory cytokines [110]. Microglia can phagocytose Aβ *in vitro* in the presence of TGF-β and ex vivo as shown when microglia are added to AD brain tissue containing immunoglobulin-coated Aβ plaques [278, 304]. In *in vivo* models, intracranial injection of Aβ has led to the infiltration of glial cells to the site of deposition [222, 305]. In addition, transgenic mouse models have clearly shown the presence of glial cells around plaques using immunohistochemical analysis [263, 299]. Furthermore, vaccination of transgenic mice reduced levels of Aβ deposition [190, 191, 304]. This reduction in plaque load has been proposed to arise from Fc receptor-mediated phagocytosis by microglia although it is equally possible that Ig sequestration of Aβ in the plasma causes an influx of Aβ from the CNS to the periphery. So far, microglial clearance of Aβ has been described to

occur through receptor-mediated phagocytosis whether it be through the Fc receptor, complement receptor, scavenger receptor (SR), or receptor for advanced glycogen end products (RAGE) [255, 273, 306, 307]. Thus, it is evident that microglia are closely involved in Aβ clearance and degradation.

As microglia are the primary cells in the inflammatory response, differences in the rapidity in which they are activated, the efficiency in which they clear/degrade Aβ and the levels of neurotoxic factors that they secrete, directly implicates them in brain amyloidosis and neurodegeneration. Macrophages from inbred mouse strains have been reported to respond to stimuli with varying degrees of macrophage activation as measured by their secretory products [244, 268, 270, 308]. This is suggested to be related to their genetic background and some genes have been identified to be responsible for differences in macrophage activation in the Nramp1 and H-2 loci [241, 269]. Within the inbred strains of mice studied, C57BL/6 mice showed strong inflammatory response whereas A/J mice were noted for their low inflammatory response as measured by macrophage activation to stimuli.

In AD, phenotype differences arising from the inbred mouse strain background used to create hAPP transgenic lines of mice has been noted although this has not been studied experimentally. Furthermore, no direct relation of individual inflammatory response to amyloidosis has been demonstrated. Therefore, we have taken the TgCRND8 mice and backcrossed them onto C57BL/6 and A/J strains of mice to determine the effect of their differences in inflammatory response on Aβ deposition.

The TgCRND8 mice are characterized by rapid Aβ deposition, gliosis, and neuronal loss starting from 3 months of age, accompanied by cognitive decline [228]. They

express an hAPP transgene containing the double Swedish (K670N/M671L) and Indiana (V717I) mutations driven by the Syrian hamster prion promoter which generates high Aβ loads and increases in the ratio of $A\beta_{42}/A\beta_{40}$. In the previous manuscript, microglial activation was detected at the same time as Aβ deposition and astrogliosis followed the appearance of fibrillar plaque formation (manuscript 2). Microglial activation correlated strongly with fibrillar plaque formation as well as the total Aβ plaque load.

Previous *in vitro* work with microglia from the A/J or C57BL/6 mouse strains, showed that the microglia from the C57BL/6 mouse strain had a much more robust inflammatory response to Aβ stimulation than microglia from the A/J mouse strain (manuscript 1). Therefore, TgCRND8 mice were backcrossed onto the A/J mouse strain (CRND8.A/J) and C57BL/6 mouse strain (CRND8.B6) to determine the phenotypic effects of differences in inflammatory response on brain amyloidosis in an AD mouse model.

In our study, CRND8.AJ and CRND8.B6 mice were used at different ages (8, 13, and 16 weeks) to assess the effect of these two genetic backgrounds on the brain amyloidogenic process as determined by the level of hAPP mRNA expression, the total brain Aß load, and $A\beta_{1-42}$ / $A\beta_{1-40}$ ratio (measured by ELISA), and by the extent of amyloid deposition in the neocortex and the hippocampal formation following 6E10 and ThioS staining. The results clearly show that the genetic make-up influences the phenotypic expression of hAPP transgene with effects seen in survival and in the kinetics of deposition and fibrillization of Aß in the brain. Gliosis of microglia (CD11b) and astrocytes (GFAP) were also analyzed. We found differences only in the rate of plaque deposition and in survival where CRND8.AJ mice had lower plaque loads and higher

survival rates compared to CRND8.B6 mice. Therefore, mouse background strongly influences the phenotype of hAPP expression.

Materials and Methods

Breeding of mice

The hAPP TgCRND8 carrying an hAPP transgene with double Swedish (K670N/M671L) and Indiana (V717I) mutations driven by a Syrian hamster prion promoter, were developed by D. Westaway [228]. To construct the TgCRND8.FVB(N_2).A/J(N_2) line, TgCRND8.FVB(N_2) mice were selectively backcrossed onto an A/J (Charles River) background by choosing $Tg^{+/-}$ and $C5^{-/-}$ breeders from each litter. As A/J and C57BL/6 mice differ in their C5 phenotype, microsatellite markers close to the *Hc* locus, were used to genotype for A/J- or C57BL/6-like inflammatory response characteristics. To our surprise, we found that the FVB strain of mice are $C5^{-}$. The TgCRND8.B6.FVB.B6(N_2) line was created from TgCRND8.B6.FVB mice by selectively backcrossing onto C57BL/6 mice (Jackson Labs). In summary, the mouse background for TgCRND8.FVB(N_2).A/J(N_2) mice consisted of 75% A/J: 22% FVB: 2.5%C3H: 0.5% C57BL/6 and the TgCRND8.B6.FVB.B6(N_2) mice consisted of 82% C57BL/6:17.5% FVB:2.5% C3H. For simplicity, the TgCRND8.FVB(N_2).A/J(N_2) will be referred to as CRND8.AJ and the TgCRND8.B6.FVB.B6(N_2) will be referred to as CRND8.B6.

Genotyping for the transgene and C5

All mice used in the studies were genotyped for human APP transgene by PCR. DNA was isolated from hair follicles as previously described [281]. The human APP transgene cassette was detected by the amplification of the hamster PrP 3'UTR with the following oligonucleotides: 5'GCCTTTGAATTGAGTCCATCA CGGGCCA-3' and 5'-AGAAATGAAGAAACGCCAAGCGCCGTGACT-3'. All mice were also genotyped at

the *Hc* locus (Hemolytic complement C5 gene). Mouse strains that are complement deficient contain a two base pair deletion within the coding region of the *Hc* gene. Primer pairs were designed to amplify a fragment surrounding this 2-bp deletion as described previously [309]. One of the primers was end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and the PCR was performed using standard cycling conditions. The PCR products were separated on a 7% polyacrylamide denaturing gel and visualized by autoradiography.

hAPP mRNA expression

Left brain hemispheres isolated from groups (n=5) of TgCRND8.FVB(N_2).AJ(N_2) mice and TgCRND8.B6.FVB.B6(N_2) aged 8, 13 and 16 weeks of age were used to extract total RNA with the TRIZOL reagent (Invitrogen Canada). Northern blots were prepared with 10 μg of each RNA as described previously [310]. A 2.4 Kb DNA fragment corresponding to the human APP_{695} mRNA sequence was labeled with [α-^{32}P]dCTP using the *redi*prime™II random prime labeling system (Amersham Pharmacia Biotech) and hybridized to the blot in Chruch's hybridization buffer at 68°C for 16 hours. The blot was washed to a final stringency of 0.1 × SSC, 0.1 % SDS for 20 minutes at 65°C and subsequently exposed to BIOMAX MS film for 1-3 hours with 2 intensifying screens at –80°C. A PCR-derived murine GAPDH probe was also hybridized to the blot to verify the integrity and loading of the RNA. Nontransgenic mouse controls from each background were used to show the lack of hAPP mRNA expression.

Rnase Protection Assay

The Ribo-Quant Multi-probe Rnase Protection Assay System (Pharmingen Canada, Mississauga, Canada) was used to compare cytokine expression levels in human APP Transgenic mice on a B6 or A/J genetic background. The mCK-2B multiprobe set (containing template for IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, IFN-γ, MIF, L32 and GAPDH) was labelled with [α^{32}P]-UTP and subsequently hybridized for 16 hours to 3 μg of total brain RNA. After Rnase treatment and purification of the protected fragments, the samples were electrophoreses on a 5% denaturing polyacrylamide gel at a constant power of 30W. The protected fragments were visualized by autoradiography.

Aβ load

The right brain hemispheres that corresponded to the left brain hemispheres used for mRNA analysis, were homogenized according to the protocol by Johnson-Wood, K. et al. [283]. Briefly, right brain hemispheres that had been snap frozen in liquid nitrogen, were weighed to obtain the wet weight. Then, the sample was homogenized on ice with 8X the brain mass of 5.0 M Guanidine-HCl/ 50mM Tris/Cl pH 8.00 using a Wheaton overhead stirrer. The homogenate was mixed for 3.5 hours at room temperature and stored at -80°C.

The brain homogenates were diluted in sterile standard/sample diluent supplied by the Signal-Select β-amyloid ELISA kit that measures human Aβ (Biosource Intl., Camarillo, CA, USA). Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA, USA) was used in all samples and standards at a concentration of 10% of the stock solution. Once the samples were appropriately diluted to fit in the mid-range of the

standard curve, duplicate samples of five mice per time point were analyzed for each mouse background, CRND8.B6 and CRND8.A/J.

Histochemistry

Tissue preparation

Mice of 8, 13, and 16 weeks of age were anesthetized and trans-cardially perfused with saline and then, with 4% paraformaldehyde (PFA). Brains were collected, post-fixed in fresh 4% PFA for 5 hours and then transferred to a 30% sucrose solution which was kept at 4°C. Once cryoprotected, the brains were frozen in isopentane at -45°C and stored at -80°C until sectioning. Brains were serially sectioned at 30 μm on a cryostat and transferred in cryoprotectant to be stored at -20°C.

Immunohistochemistry

Free-floating sections were extensively washed before treating them with 3% H_2O_2 for 15 minutes to quench the endogenous peroxidase. Sections for CD11b- and GFAP-immunolabeling were treated with 0.1% TritonX-100 for 30 minutes before blocking. All sections were blocked using 4% goat serum for 1 hour. Then, sections were immunostained with 6E10, mAb specific for total human APP (Senetek, CA; 6E10-biotin; 1/600), CD11b, mAb for microglial activation (Pharmingen, San Diego, CA; 1/50), or GFAP, an astrocyte marker (Pharmingen; 1/3000). Primary antibodies were incubated overnight at 4°C. Secondary biotinylated goat anti-rat antibody for indirect CD11b immunostaining or secondary biotinylated goat anti-mouse antibody for indirect GFAP immunostaining, was incubated at room temperature (20-24°C) for 1 hour. Avidin-biotin kit (Vector Laboratories, Burlington, ON, Canada) and Ni-3,3'-diaminobenzidine reaction were used. Sections were chosen for staining in the following

sequential order ThioS, 6E10, CD11b, and GFAP to allow homogeneity between animals and between brain levels studied.

Histochemistry

Brain sections were mounted on Super Frost slides and allowed to dry before staining with 1% thioflavinS (ThioS, a labels fibrillar Aβ in plaques by recognizing β-sheet structure) in 0.1 M phosphate buffer.

Image capture

Light and fluorescent microscopy were performed on an Olympus BX40 microscope with a Pro-series camera to capture images for analysis of light field images and a digital camera for analysis of ThioS fluorescence and high quality images. Image Pro-Plus Systems software (Media cybernetics, USA) was used for manipulation and analysis of captured images.

Analysis

Two sections from two different levels of the brain (-2.0 and -3.4 mm from the bregma, atlas from Franklin and Paxinos) were analyzed [282]. The plaque numbers were measured as well as the % area which is the area occupied by the plaque versus the total analyzed area of the neocortex or hippocampal formation as indicated. In terms of CD11b-labeling, the number of foci were measured instead of plaque number.

Statistical analysis

In both backgrounds, results from Aß load and histochemistry were averaged and expressed as mean ± standard error of the mean (S.E.M.). The Mann-Whitney rank sum test and the Student t-test were used to compare Aß load, plaque number, and plaque area (%) measured in both backgrounds. The level of statistical significant difference was $P < 0.05$.

Results

Survival of transgenic mice

Survival of CRND8.B6 dropped by 50% around 13 weeks of age and continued to decrease with age (Figure 14). On the other hand, the CRND8.AJ mice had stable survival rates of 95% from 3 to 16 weeks of age and continued to survive past 50 weeks of age.

hAPP mRNA expression, Aβ load, and Aβ42/Aβ40 ratios

The hAPP mRNA expression as measured by Northern blotting, showed no differences between the two mouse backgrounds between the three age groups i.e. 8, 13, and 16 weeks (Figure 15). Expression of hAPP remained constant.

The Aβ load was measured using a highly sensitive sandwich ELISA in the brain homogenates of CRND8.B6 and CRND8.AJ mice at 8 weeks, 13 weeks, and 16 weeks of age (Table 4). Basal $A\beta_{1\text{-}40}$ levels were observed at 8 weeks of age in both mouse backgrounds of 5 ± 3 ng per g of wet brain weight (ng/g) (Table 4). At 13 weeks of age, the CRND8.B6 mice had 105 ± 24 ng/g and the CRND8.AJ mice had 56 ± 11 ng/g. In the 16-week age group, levels of $A\beta_{1\text{-}40}$ increase to 444 ± 99 in the CRND8.B6 mice and to 636 ± 139 in the CRND8.AJ mice. Although CRND8.AJ mice had a tendency to have a lower amyloid load than CRND8.B6 mice at 13 and 16 weeks of age, the difference did not reach significance level ($P < 0.05$) due to the low number of mice and the inter-mouse variation. No $A\beta_{1\text{-}40}$ was detected in the control, non-transgenic mice of the C57BL/6 strain nor of the A/J strain.

The levels of $A\beta_{1-42}$ were also measured in the brain homogenates of the CRND8.B6 and CRND8.AJ mice (Table 4). At 8 weeks of age, basal levels of $A\beta_{1-42}$ were observed in CRND8.B6 mice (31± 16 ng/g) and in CRND8.AJ mice (12 ± 2 ng/g). Much higher levels of $A\beta_{1-42}$ were seen at 13 weeks of age in both CRND8.B6 mice (1592 ± 242 ng/g) and CRND8.AJ mice (1185 ± 377 ng/g). At 16 weeks of age, large increases in the levels of $A\beta_{1-42}$ were found in CRND8.B6 mice (3711 ± 991 ng/g) and CRND8.AJ mice (3977 ± 1052 ng/g). No significant difference was observed between the two mouse backgrounds. Much higher levels of $A\beta_{1-42}$ were observed in comparison to $A\beta_{1-40}$ in both mouse backgrounds. The total Aβ load of $A\beta_{1-40}$ and $A\beta_{1-42}$ increased from 8 weeks to 13 weeks to 16 weeks as follows: 36 ± 20; 1676 ± 257; and 4156 ± 1077 ng/g in CRND8B6 mice and 17 ± 7; 124 ± 387; and 4613 ± 1137 ng/g in CRND8.AJ mice.

The ratios of $A\beta_{1-42}/A\beta_{1-40}$ were determined per mouse and then averaged within each age group (Table 4). At 8 weeks of age, the average ratio was slightly higher in the CRND8.B6 mice of 7.3 ± 2.7 in comparison to the CRND8A/J mice with 2.1 ± 0.4. The ratios were approximately equal in both backgrounds of mice at 13 weeks of age with 19.2 ± 0.5 in the CRND8.B6 mice and 20.3 ± 2.7 in the CRND8.AJ mice. At 16 weeks of age, the ratios decreased in both backgrounds to 8.2 ± 1.6 in the CRND8.B6 mice and 6.7 ± 1.6 in the CRND8.AJ mice due to increased $A\beta_{1-40}$ production.

Histochemistry

Histochemistry was used for ThioS staining which binds to the β-sheet structure found in fibrillar Aβ deposits. Immunohistochemistry was used for 6E10 (a marker for total

human Aβ, both fibrillar and nonfibrillar deposits), CD11b (a marker specific to activated microglia), and GFAP (a marker used to identify astrocytes). Two levels of brain sections at -2.18 mm bregma and -3.40 mm bregma, were analyzed in 3-4 mice per age group per mouse background. Plaques refer to Aβ deposits. No difference in trends were observed when the results were regrouped by level of brain section therefore, the results from both levels were grouped.

ThioS histochemistry in the neocortex showed that plaques were not observed before 13 weeks of age in CRND8.B6 mice (8 ± 2 plaques) and in CRND8.AJ mice (4 ± 1 plaques) (P = 0.045) (Figure 16). At 16 weeks, twice as many plaques were seen in the CRND8.B6 mice (24 ± 2 plaques) as compared to the CRND8.AJ mice (12 ± 2 plaques) (P = 0.0007). The % of the area occupied by the plaque over the cortical area (% area) followed similar trends at 13 weeks and 16 weeks of age, respectively, with values of 0.009 ± 0.002 plaques and 0.038 ± 0.005 plaques in CRND8.BL6 mice and 0.004 ± 0.001 plaques and 0.017 ± 0.006 plaques in CRND8.AJ mice (At 13 weeks: P = 0.026; at 16 weeks: P = 0.001) (Figure 17).

ThioS histochemistry in the hippocampal formation showed that plaques were first detected at 13 weeks of age in CRND8.B6 mice (3 ± 0.9 plaques) and in CRND8.AJ mice (1 ± 0.5 plaques) (Figure 18). Twice as many plaques were observed in the hippocampal formation of the CRND8.B6 mice (10 ± 1) versus the CRND8.AJ (5 ± 1) mice at 16 weeks of age (P = 0.012). The % area followed similar trends at 13 weeks and 16 weeks of age, respectively, with values of 0.005 ± 0.001 and 0.030 ± 0.004 in CRND8.B6 mice and 0.001 ± 0.001 and 0.010 ± 0.004 in CRND8.AJ mice (Figure 19) (13 weeks : P = 0.0003 ; 16 weeks : P = 0.005).

6E10 immunohistochemistry labeled the total Aβ deposited, both diffuse and fibrillar plaques. In the neocortex at 8 weeks of age, plaques were not observed in CRND8.B6 nor in CRND8.AJ mice. Similar trends in plaque numbers and % area were observed in the 6E10-labeled plaques as seen in ThioS staining (Figure 16). At 13 weeks of age, plaque numbers were higher in CRND8.B6 mice (20 ± 2 plaques) than that found in CRND8.AJ mice (8 ± 2 plaques) (P = 0.002). These numbers increased at 16 weeks with the same trend in CRND8.B6 mice (43 ± 3 plaques) and in CRND8.AJ mice (27 ± 2 plaques) (P = 0.0008). The % area also increased from 13 to 16 weeks of age with approximately one and a half fold higher levels in CRND8.B6 mice (13 weeks 0.059 ±0.006; 16 weeks 0.206 ±0.022) as compared to CRND8.AJ mice (13 weeks 0.034 ±0.009; 16 weeks 0.132 ±0.013) (13 weeks : P = 0.035 ; 16 weeks : P = 0.014) (Figure 17).

In the hippocampal formation, similar plaque numbers of 4 ± 1.5 plaques in CRND8.B6 mice and 5 ± 1.8 plaques in CRND8.AJ mice were seen at 13 weeks of age (Figure 18). Plaque numbers increased in both CRND8.B6 mice (15 ± 3 plaques) and CRND8.AJ mice (11 ± 2 plaques) at 16 weeks of age. The % area followed the same pattern in CRND8.B6 mice (13 weeks, 0.024 ± 0.006; 16 weeks, 0.130 ± 0.031) and CRND8.AJ mice (13 weeks, 0.023 ±0.009; 16 weeks, 0.104 ± 0.010) (Figure 19).

Overall, the CRND8.B6 mice showed a significant increase in amyloid deposition when compared to the CRND8.AJ mice with more diffuse and fibrillar plaques.

Immunohistochemistry with CD11b had large variations in each age group. Microglial activation was evident at 16 weeks of age although no trends between the two different backgrounds could be determined (data not shown).

In Figure 20 and 21, a panel of microimages of the neocortex are shown to portray the evolution of plaques, microglia, and astrocytes at 8 weeks, 13 weeks, and 16 weeks of age in both CRND8.B6 and CRND8.AJ mice. Using 6E10 immunolabeling, a marked increase in plaque labeling was observed at 16 weeks of age, moreso in the CRND8.B6 mice than in the CRND8.AJ mice (Figure 20). Microglia were activated (CD11b) moreso at 16 weeks of age and immunolabeling was much stronger in the hippocampal formation than in the neocortex in both mice (Figure 21). Astrocytes (GFAP) could be seen throughout the neocortex at 8 weeks of age with increasing density at 13 weeks of age and clustering began at 16 weeks of age (Figure 21).

Discussion

Differences in the genetic background between A/J and C57BL/6 strains of mice influence the magnitude of the inflammatory response to different non-peptidic and antigenic stimuli [244, 268, 270, 308]. Microglia obtained from these two strains of mice differ in the magnitude of their response to Aβ stimulation *in vitro*. Phenotypic expression of hAPP transgene may be affected by several genetic factors including the glial response to the brain amyloidosis process. In our study, TgCRND8 mice were backcrossed onto these two mouse backgrounds, A/J and C57BL/6, to determine their effect on the phenotypic expression of the hAPP transgene. Several parameters were assessed: survival, hAPP transgene expression and Aβ protein levels as well as the development of plaques and associated gliosis in the brain at different ages.

We found that only certain aspects of the amyloidogenic process were affected by the genetic make-up. hAPP transgene mRNA expression levels and Aβ protein levels remained unaffected by the mouse genetic background. Interestingly, survival rates were extremely influenced by the genetic background with a high mortality rate seen very early (prior to the initiation of amyloid deposition) in CRND8.B6 mice while the majority of CRND8.AJ mice survived more than one year. CRND8.B6 mice showed signs of aggressive behaviour and often died of seizures.

The loss of mice due to seizures seen early in CRND8.B6 mice is most probably not caused by high Aβ levels since CRND8.AJ mice had similar Aβ levels with almost no animal loss in the first 25 weeks of age. Carlson et al also showed in Tg2576 hAPP transgenic mouse model that survival rates did not correlate with brain Aβ concentration and $A\beta_{1-42}/A\beta_{1-40}$ ratios [295]. The reason for the development of seizures in these mice is

not known. It may be due to the high APP concentration exerting a strong vasoconstrictive effect in certain strains of mice leading to the onset of fatal seizures.

Mouse background has previously been noted to affect the hAPP phenotype in other transgenic models but the effect of the A/J background has never been studied previously. In other models, Tg 2576 mice on a 93.75% C57BL/6 background (N_4) all died at a very young age (65 ± 4 days) [295]. When N_2 offspring of this cross were bred to B6SJL F1 survival rates were drastically improved with an 84% survival rate at 74 ± 3 days of age. The effect of the FVB background on the Tg2576 mice was also studied [311]. This cross led to high premature death in offspring, neophobic or thigmotaxic behaviour. Tg2576 born on a B6XFVB mixed background showed strong astrogliosis. The effect of the B6 and A/J mouse backgrounds on the hAPP transgene is most probably post APP processing since both hAPP transgene mRNA levels and total Aβ brain load are similar in both CRND8.B6 and CRND8.AJ mice.

The kinetics of the brain amyloidosis process showed major differences in CRND8.B6 and CRND8.AJ mice. It is clear that CRND8.AJ mice take significantly more time to develop plaques than CRND8.B6 mice. Whether this difference is the result of a weaker glial inflammatory response, remains to be determined. Studies with long-term NSAIDS treatment would support such an explanation. Indeed, long-term treatment of patients with NSAIDs was found to delay the development of AD in these individuals when compared to a non-treated population [1].

Robust inflammatory response can enhance the Aβ deposition process and promote neuronal loss. Intracellular and extracellular $A\beta_{1-42}$ accumulation are toxic and lead to neuronal cell lysis [104]. Aβ itself as well as lysed neuronal cells, complement and

cytokines all contribute to activate surrounding microglia. These microglia phagocytose cell debris and Aβ deposits, leading to further gliosis via cytokine release (IL-1, TNF-α) [125]. The equilibrium between Aβ clearance by microglia and Aβ build-up and deposition is most probably difficult to maintain [312]. Eventually microglia could contribute to the neuronal loss through the secretion of inflammatory mediators like NO, ROS, complement and pro-inflammatory cytokines (IL-1, TNF-α). Such neuronal loss caused by inflammation could then contribute to Aβ deposition due to an increase in Aβ leakage from lysed neurons.

The slow progression of Aβ deposition seen in CRND8.AJ mice may not be directly linked to their poor microglial inflammatory response to Aβ. The extent of gliosis was not significantly different between CRND8.B6 and CRND8.AJ mice. This could be due to the large inter-individual variation seen following the CD11b straining of brain sections. However, mRNAse protection assays on the left brain hemisphere extracts did not show any differences in various inflammatory markers between CRND8.B6 and CRND8.AJ (data not shown). The rate of deposition may be strongly influenced by a variety of fibrillogenic factors. Several factors like ApoE, perlecan, degree of sulfation of proteoglycan have all been shown to promote Aβ fibril formation both *in vitro* and *in vivo* [21, 207, 209, 313, 314]. Other factors like ApoJ have been suggested as playing a protective role in Aβ amyloidogenic process [315]. Differences in any of these elements may modify the amyloidogenic process by either promoting or delaying its course.

Mice of the A/J strain are known to develop reactive secondary (AA) amyloidosis at a rate that is significantly slower than that seen in C57B46, CBA15 and other strains of mice [316]. A/J mice take significantly longer time to develop the "seeds" or "nucleating

event", which triggers amyloid AA deposition systemically (liver, spleen, kidneys) [317]. However, when A/J mice are injected with seeds, these mice will develop amyloid as fast as other susceptible strains of mice suggesting that A/J mice have a mechanism contributing to delay the early organization of the amyloidogenic protein as protofibrils or fibrils, which would act as seeds. This delay would maintain the animals for a longer period of time in the lag phase of the disease, retarding their entry in the rapid progression of the disease where amyloid fibrils get deposited in organs at a rapid pace.

Using two different models of amyloidosis, A/J mouse background has been shown to markedly slow down the rate of amyloid fibril formation and deposition [317, 318]. In both models, precursor levels (SAA for AA amyloidosis, hAPP, and Aβ for Aβ amyloidosis) could not explain phenotypic differences as these levels were similar to that seen in more susceptible strains of mice. The rate of amyloid deposition may be influenced by promoting factors (ApoE, proteoglycans), by the presence of nucleating agents or seeds (amyloid enhancing factor, AEF), which trigger fibrillogenesis or by other factors, which would act as "natural inhibitors" of fibril formation (ex: ApoJ).

Genetic studies on the phenotypic expression of the hAPP transgene using these different strains of mice may lead to the identification of modifier genes which could play a major role in the time of onset of brain amyloidosis and AD.

Table 4 Aβ load in TgCRND8 mice

Mouse age	8 weeks		13 weeks		16 weeks	
Aβ levels (ng/g of brain)	CRND8.A/J (5)[2]	CRND8.B6 (4)	CRND8.A/J (5)	CRND8.B6 (3)	CRND8.A/J (4)	CRND8.B6 (5)
$A\beta_{1-42}$	12.4 ± 2.4	31.3 ± 15.8	1184.8 ± 377.3	1592.0 ± 242.1	3976.8 ± 1052.1	3711.6 ± 991.0
$A\beta_{1-40}$	5.0 ± 3.8	4.5 ± 4.2	55.4 ± 24.6	105.0 ± 23.8 (4)[3]	636.3 ± 139.0	444.2 ± 99.2
$A\beta_{Total}$	17.4 ± 7.3	35.8 ± 19.9	1240.2 ± 387.5	1675.7 ± 256.9	4613.0 ± 1137.3	4155.8 ± 1077.1
$A\beta_{1-42}$/ $A\beta_{1-40}$	2.1 ± 0.5 (2)[3]	11.5 ± 7.1	20.3 ± 2.7	19.2 ± 0.5	6.72 ± 1.6	8.2 ± 1.6

[1]: Mean ± S.E.M.
[2]: (Number of mice)
[3]: (different number of mice)

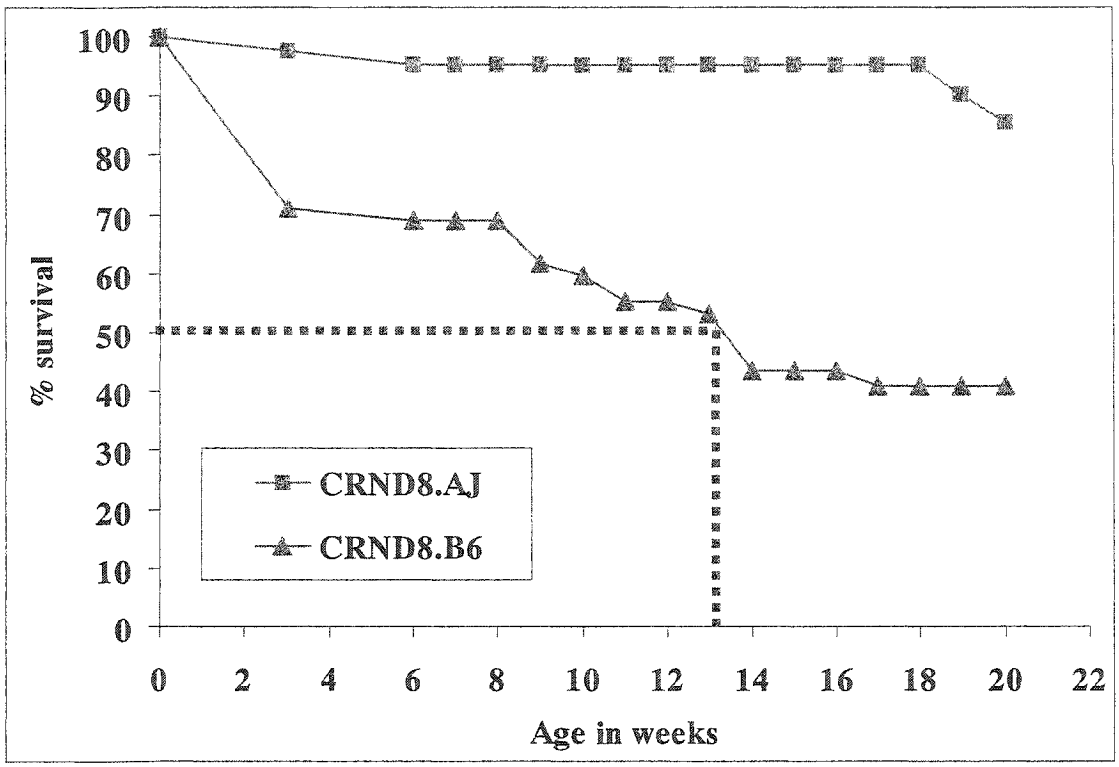

Figure 14 Survival curves for CRND8.AJ and CRND8.B6 mice.

The survival of CRND8.AJ and CRND8.B6 mice from birth through to 16 weeks of age with n=32 and n=45, respectively.

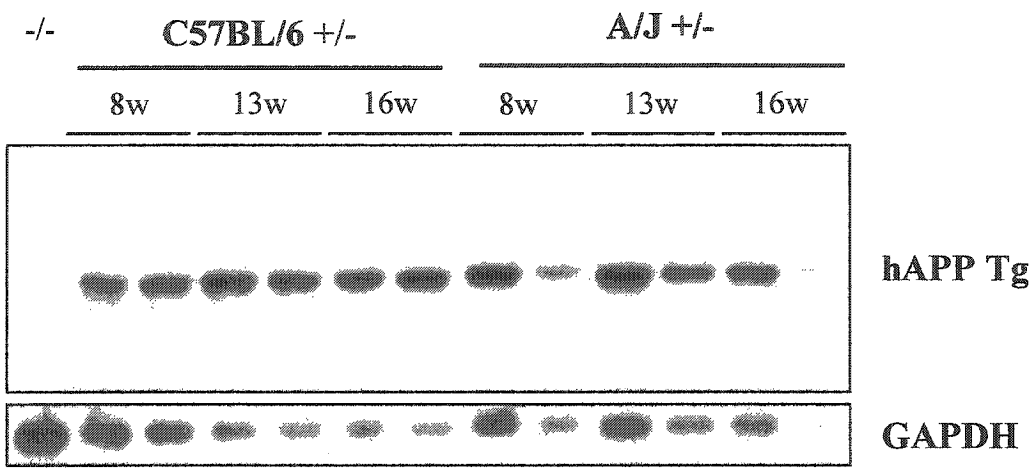

Figure 15 Effect of genetic background on the expression of hAPP transgene mRNA in CRND8 mice.

No differences in hAPP mRNA expression were observed. The upper panel represents a Northern blot of brain RNA that was hybridized with a probe specific for the human APP Transgene. The lower panel depicts the mouse GAPDH control. Lanes 2-7 show hAPP mRNA expression of TgCRND8 C57BL/6 mice and lanes 8-13 show hAPP mRNA expression in TgCRND8 A/J mice. Lane 1 shows non-transgenic control mice. Two representative mice are shown for each time point. Left brain hemispheres isolated from groups (n=5) of CRND8.AJ mice and CRND8.B6 aged 8, 13 and 16 weeks of age were used to extract total RNA with TRIZOL reagent. Northern blots were prepared with 10 μg of each RNA and probed with a 2.4 Kb DNA fragment corresponding to the human APP_{695} mRNA sequence which was labeled with [α-^{32}P]dCTP using the *redi*prime™II random prime labeling system (Amersham Pharmacia Biotech).

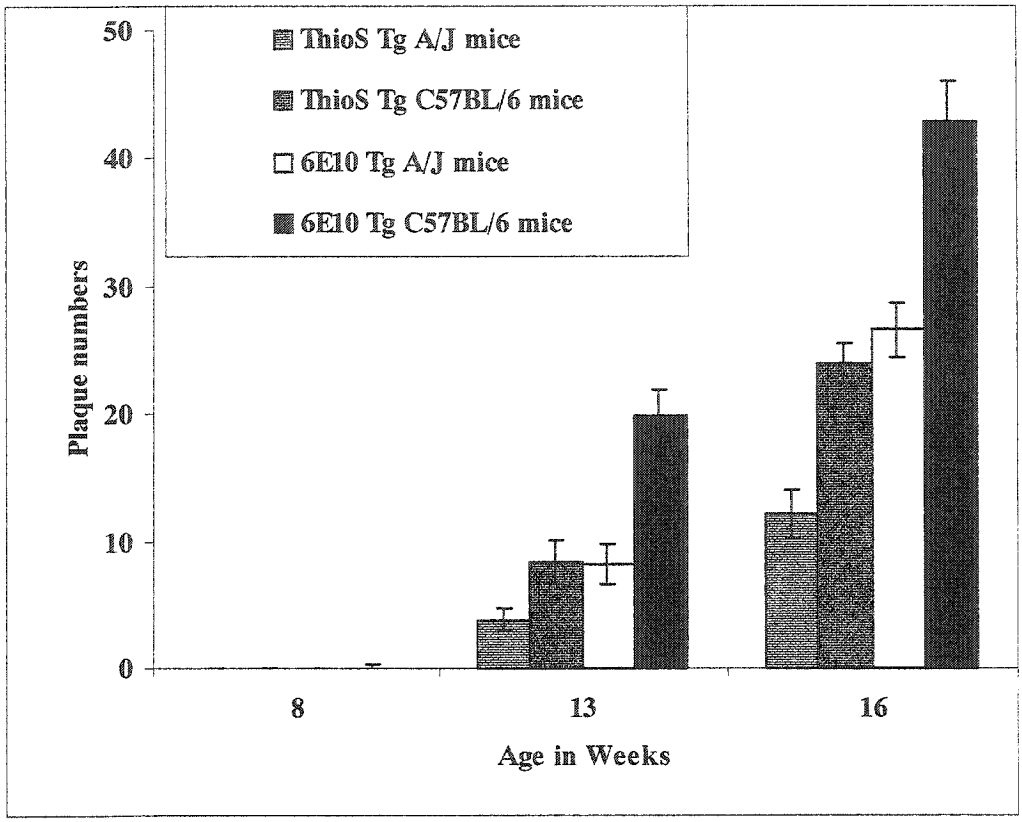

Figure 16 Immunohistochemical analysis of ThioS and 6E10 labeling in the neocortex of CRND8.AJ and CRND8.B6 mice represented as plaque numbers.

Coronal sections (n=3-4 mice, 2 sections per mouse) of 8, 13, and 16 week-old CRND8.AJ and CRND8.BL6 mice were stained with ThioS and 6E10 and analysed in terms of their plaque numbers. Results are expressed as mean ± SEM*: Significant difference between CRND8.AJ and CRND8.B6 mice for ThioS staining. Plaque number: 13 weeks: P = 0.045; 16 weeks: P= 0.0007. Significant difference between CRND8.AJ and CRND8.B6 mice for 6E10 labeling. Plaque number : 13 weeks : P = 0.002 ; 16 weeks : P= 0.008.

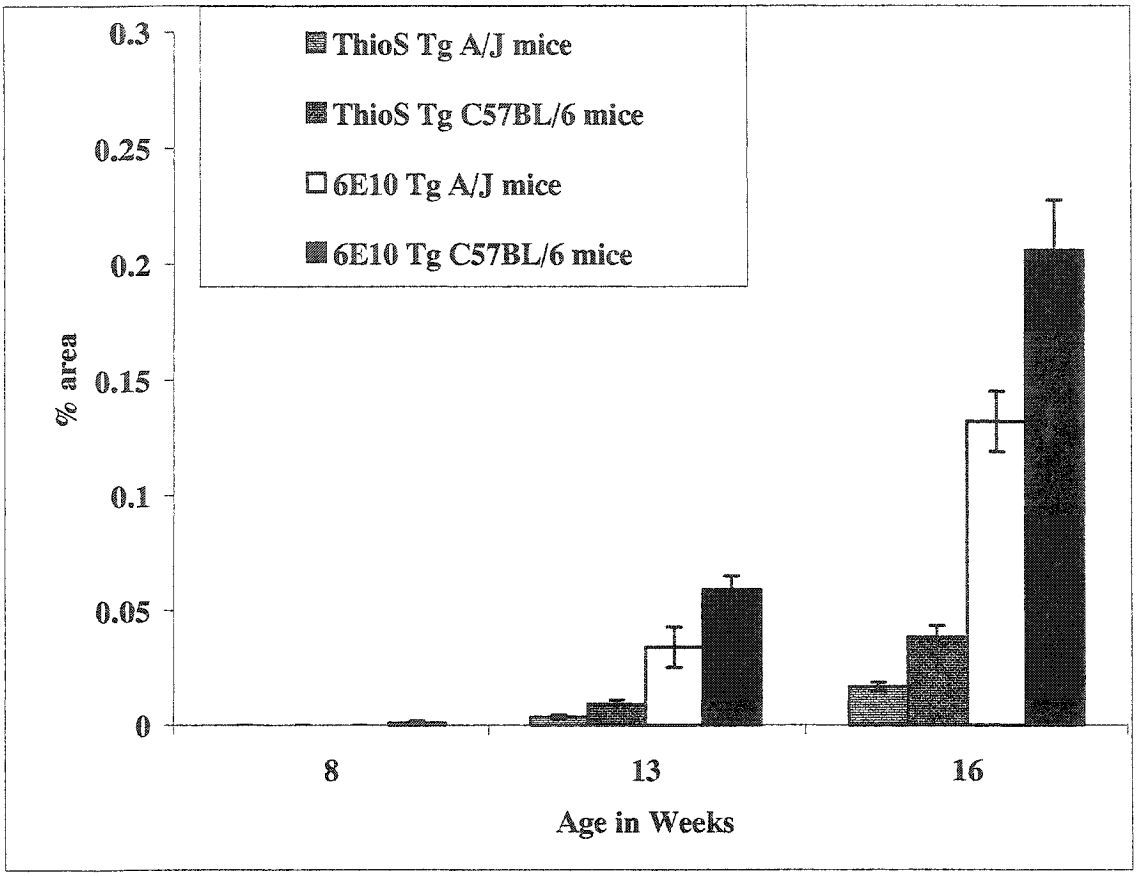

Figure 17 Immunohistochemical analysis of ThioS and 6E10 labeling in the neocortex of CRND8.AJ and CRND8.B6 mice represented as % area.

Coronal sections (n=3-4 mice, 2 sections per mouse) of 8, 13, and 16 week-old CRND8.AJ and CRND8.BL6 mice were stained with ThioS and 6E10 and analysed in terms of their % area. Results are expressed as mean ± SEM*: Significant difference between CRND8.AJ and CRND8.B6 mice for ThioS staining. % area: 13 weeks: P = 0.026; 16 weeks: P = 0.001. Significant difference between CRND8.AJ and CRND8.B6 mice for 6E10 labeling. % area : 13 weeks : P = 0.035 ; 16 weeks : P = 0.014.

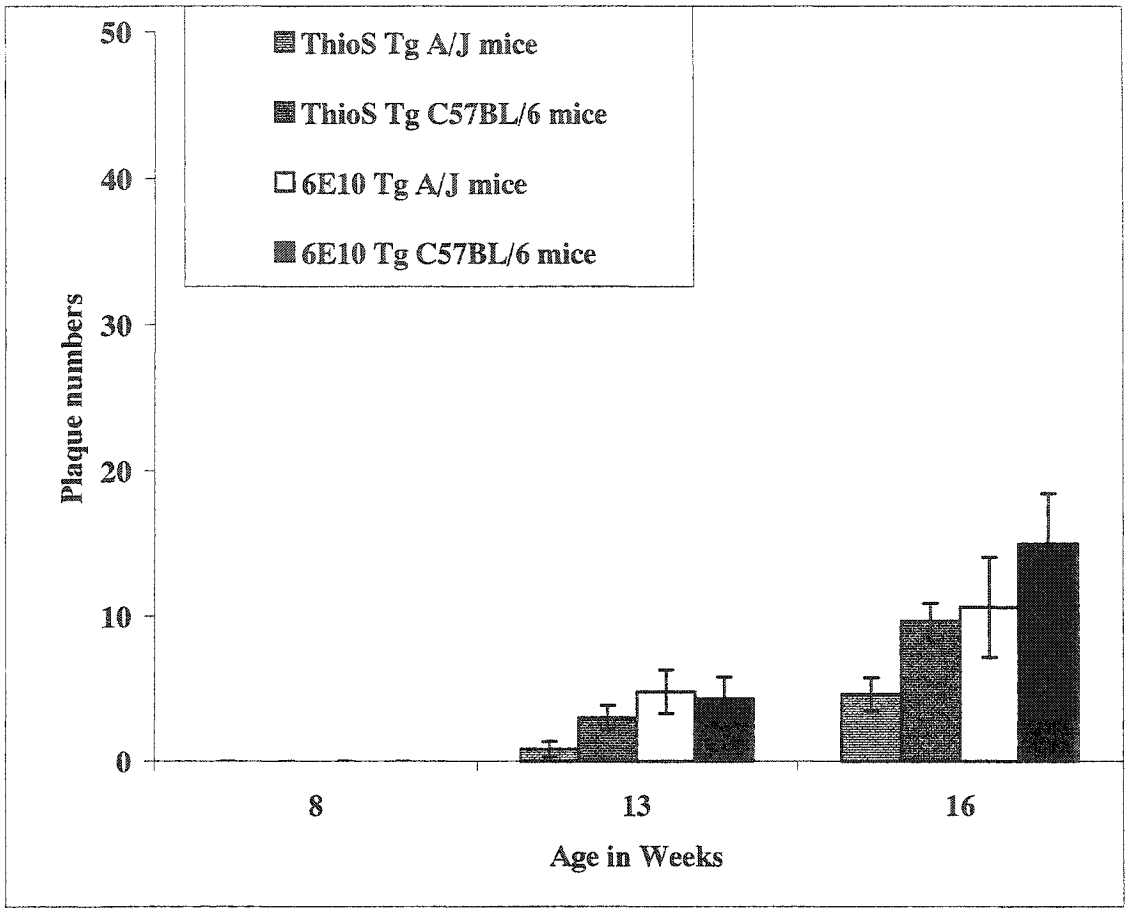

Figure 18 Immunohistochemical analysis of ThioS and 6E10 staining in the hippocampal formation of CRND8.AJ and CRND8.B6 mice represented as plaque number.

Coronal sections (n=3-4 mice, 2 sections per mouse) of 8, 13, and 16 week-old CRND8.AJ and CRND8.BL6 mice were stained with ThioS and 6E10 and analysed in terms of their plaque numbers. Results are expressed as mean ± SEM*: Significant difference between CRND8.AJ and CRND8.B6 mice for ThioS. Plaque numbers: 16 weeks: P= 0.012.

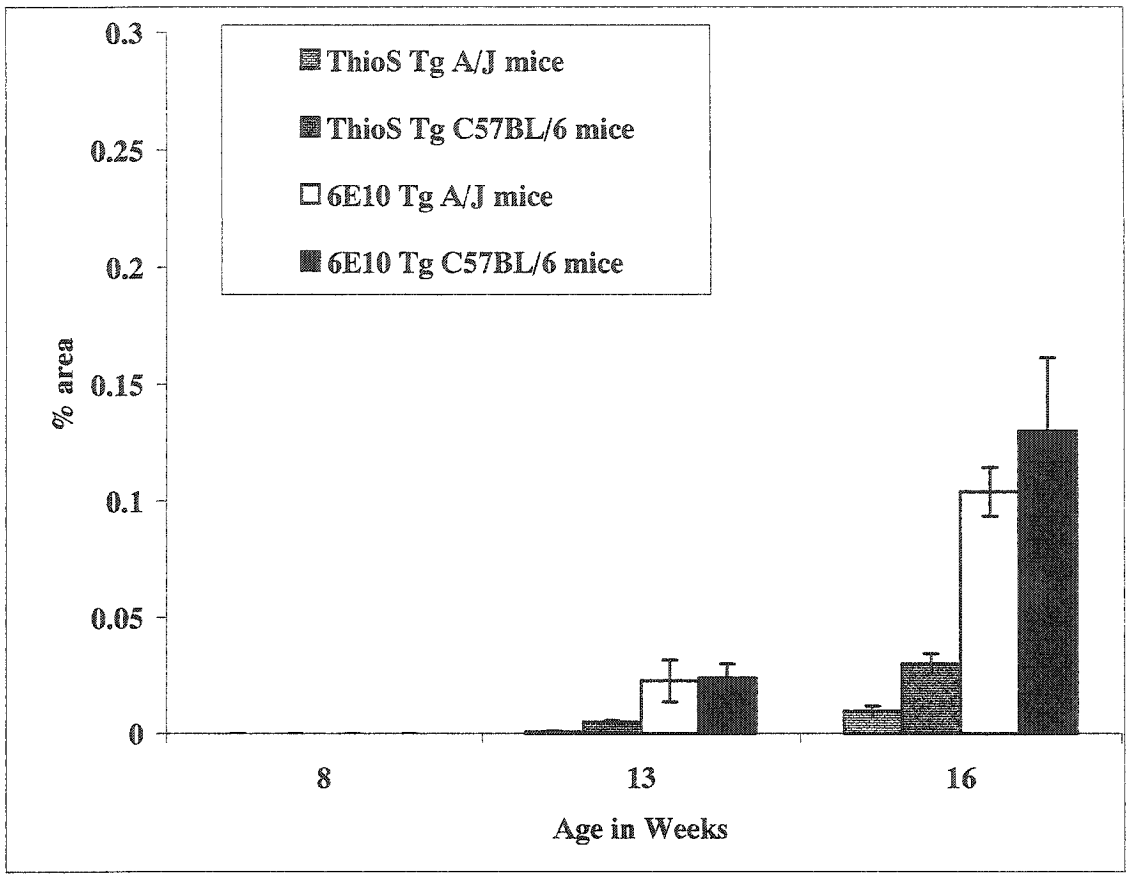

Figure 19 Immunohistochemical analysis of ThioS and 6E10 staining in the hippocampal formation of CRND8.AJ and CRND8.B6 mice represented as % area.

Coronal sections (n=3-4 mice, 2 sections per mouse) of 8, 13, and 16 week-old CRND8.AJ and CRND8.BL6 mice were stained with ThioS and 6E10 and analysed in terms of their % area. Results are expressed as mean ± SEM. Significant difference between CRND8.AJ and CRND8.B6 mice for ThioS. % area: 13 weeks: $P = 0.0003$; 16 weeks: $P = 0.005$.

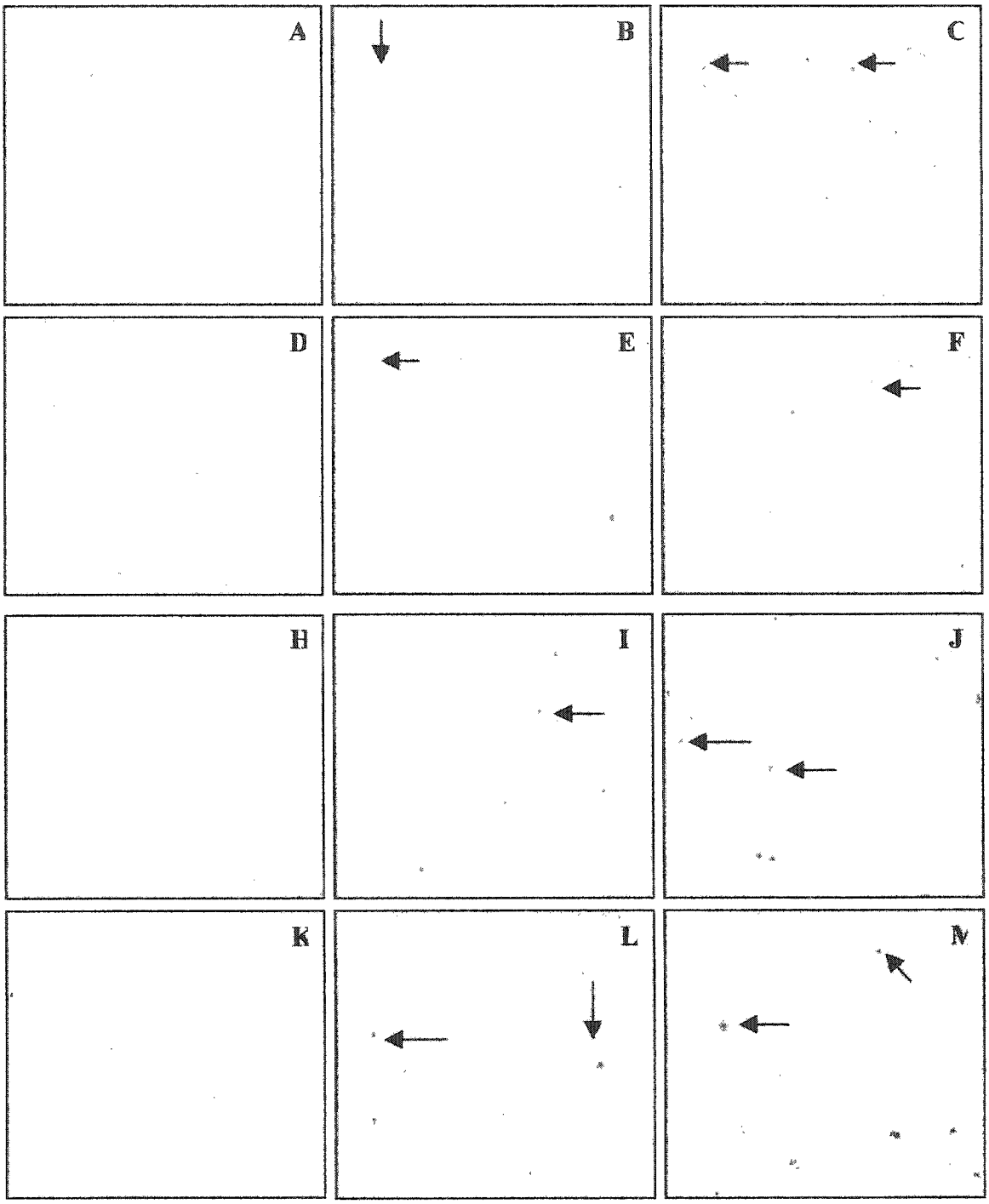

Figure 20 Plaques in coronal sections of CRND8.B6 and AJ mice.

Coronal sections (n=3-4 mice, 2 sections per mouse) of 8, 13, and 16 week-old CRND8.B6 and CRND8.AJ mice were labeled with 6E10 (total human Aβ). The panel of images shows 6E10 staining as a dark purple DAB-Ni reaction product. **RS** retrosplenial neocortex; **Hip** hippocampal formation; **CC** corpus callosum Original magnification 12.5X (first two rows) and 40X (last two rows showing RS only).

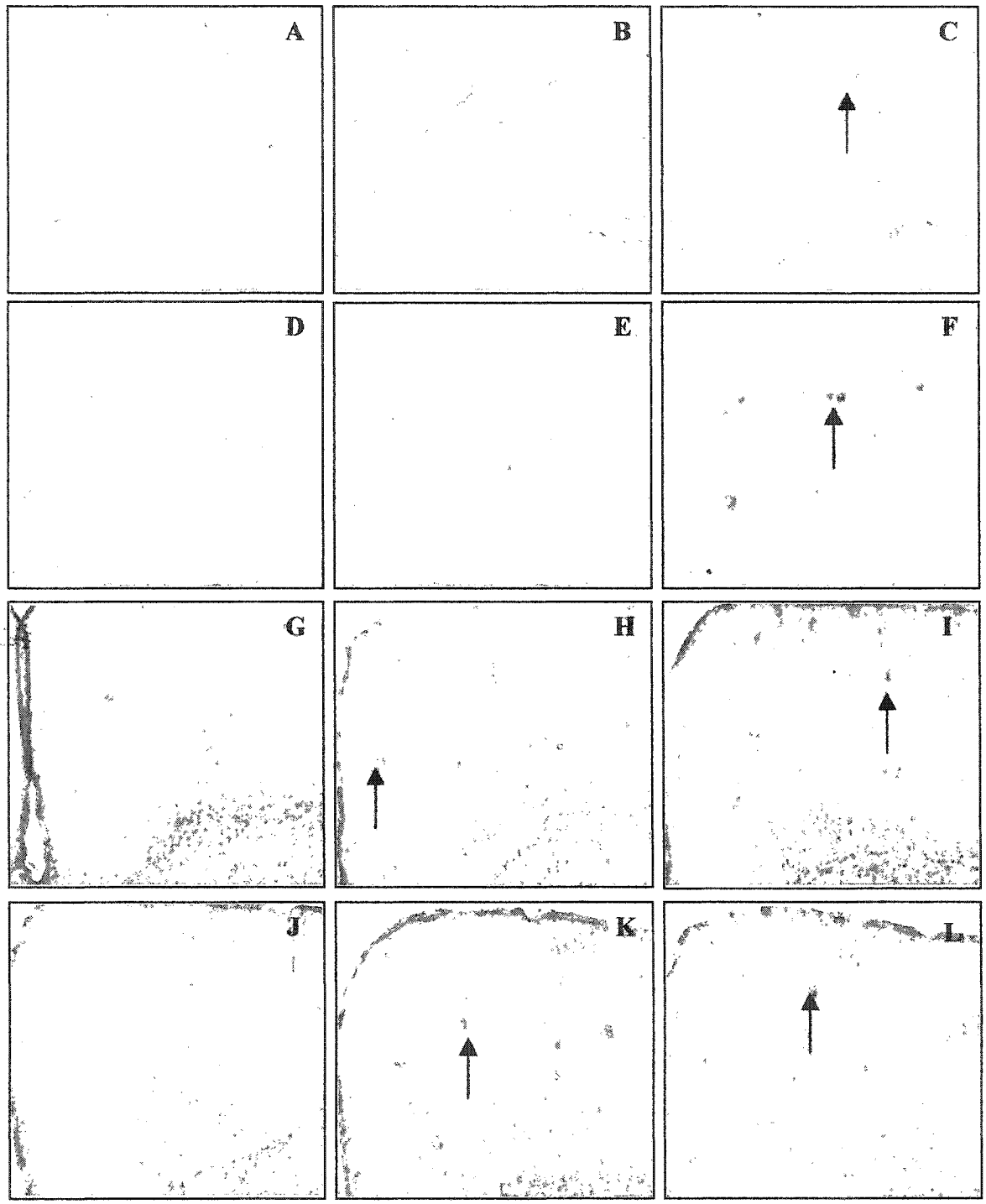

Figure 21 Inflammation in coronal sections of CRND8.B6 and AJ mice.

Coronal sections (n=3-4 mice, 2 sections per mouse) of 8, 13, and 16 week-old CRND8.B6 and CRND8.AJ mice were labeled with CD11b (activated microglia, Hip) and GFAP (astrocytes, RS). The panel of images shows CD11b labeling in the hippocampal formation (Hip) and GFAP immunostaining in the retrosplenial neocortex (RS) as dark purple DAB-Ni reaction products. Magnification 40X.

CHAPTER 5: Discussion

Alzheimer's disease is characterized by many neuropathological and clinical characteristics: plaques, gliosis, NFTs, synaptic loss, neuritic dystrophy, neuronal loss, and cognitive decline [5, 6]. Amyloidosis is the process of Aβ deposition which leads to plaque formation. Levels of Aβ load and $A\beta_{1-42}/A\beta_{1-40}$ ratios increase preceding plaque appearance and thereafter [19, 20, 319]. These Aβ levels are clearly related to cognitive decline which strongly correlates with neuronal loss [11, 12, 320]. Thus, this strong correlation supports the theory that brain amyloidosis is closely implicated in neuronal loss leading to cognitive decline.

Does inflammation play a role in amyloidosis?

Inflammation can influence brain amyloidosis although its importance in amyloidogenesis continues to be debated. Multiple clinical studies with NSAIDS provide unequivocal evidence that reducing inflammation is beneficiary to AD patients and leads to a delay in AD-linked pathology [1, 2, 121]. The presence of glia surrounding plaques in AD brains is widely accepted although their role in Aβ deposition and/or clearance is unclear [26, 123, 124]. Microglial activation is marked by increased production in IL-1 and overexpression of IL-1 is a risk factor for AD [122, 321]. Thus, inflammation is implicated in AD and reducing the inflammatory response does result in diminishing pathology.

Conversely, inflammation can counteract amyloidosis. Microglia are capable of phagocytosing Aβ plaques in human brain slices and degrading ingested Aβ incubated with anti-Aβ antibodies [304]. As these microglia are in an *ex-vivo* culture, this probably does not represent their reaction *in vivo* where many neurotoxic factors are present as well

as other interacting cells. Interestingly, vaccination in hAPP transgenic mice leads to decreases in plaque load [190-192]. There are two suggested mechanisms in which vaccination exerts an effect on Aβ load. Injection of Aβ peptide stimulates an antibody response. Then, anti-Aβ immunoglobulins cross the BBB and bind to plaques. This facilitates Aβ degradation by microglia via antibody-mediated phagocytosis and would most likely stimulate an inflammatory response. Another mechanism of Aβ vaccination is through injecting anti-Aβ antibodies, peripherally, which act as a sink by sequestering Aβ and drawing it out of the CNS and into the plasma for clearance. Recent vaccination clinical trials did not show the beneficial effects observed in mice and an overt CNS inflammatory response was seen in a subset of patients [193]. This could be expected as humans and mice are different in terms of aging, genetic variability, cytokine expression, immune response to toxins, and possibly in BBB integrity as well. In addition, mice used in transgenic mouse models are inbred which reduces their genetic variability that would otherwise be seen in humans. Hence, vaccination can have positive effects in mice, but it should be well-noted that mice serve only as models for human neurodegeneration.

From human studies, it is apparent that brain inflammation is detrimental and therapy should be developed to regulate it. Poorly-regulated inflammation has consequences in the brain where the BBB tries to keep inflammation and the immune response in check. This can begin with microglial activation followed by secretion of complement, cytokines and neurotoxic factors such as NO, ROS, and TNF-α. Recruitment of activated astrocytes and microglia results in increased levels of locally-secreted neurotoxic factors. If the inflammatory response is not suppressed with anti-inflammatory cytokines such as TGF-β or IL-10, this leads to significant neuronal lysis

and eventual neuronal loss [125]. Interestingly, TGF-β also regulates Aβ clearance and production [278]. This seems contradictory since anti-inflammatory cytokines would be thought to have a principal role in diminishing the neurotoxic effects induced by activated microglia and astrocytes. In fact, TGF-β is a multifactorial cytokine involved in increased glial response, accumulation and re-distribution of amyloid to cerebral vessels, and regulation of programmed cell death [322]. Thus, TGF-β-induces amyloid production and glial activation leading to the clearance and redistribution of amyloid to cerebral vessels where accumulation of amyloid can provoke a cerebral haemorrhage. Therefore, even the regulation of inflammation can lead to harmful effects.

Since many cytokines often have multi-factorial roles, they are not ideal candidates for drug therapy. NSAIDS are promising although there are severe gastro-intestinal side effects [323]. Their mechanism of action is through inhibition of COX-1 and COX-2 which blocks prostaglandin production [324]. Prostaglandin production is constitutively-induced by COX-1 and upregulated by inflammatory stimuli through COX-2 activation. As prostaglandins are required for regular physiological function, COX-2 has become the most recent therapeutic target. Anti-inflammatory drugs, NSAIDS, act as agonists for the ligand-activated nuclear receptor peroxisome proliferator-activated receptor γ (PPAR-γ) which is a DNA-binding transcription factor that induces pro-inflammatory cytokine production [174]. PPAR-γ is regulated by agonists such as protaglandin J2 and drugs of the thiazolidione class. Inhibition of PPAR-γ blocks pro-inflammatory cytokines (IL-6 and TNF-α) and COX-2 mRNA expression and is therefore, a good potential therapeutic target.

A subset of NSAIDS has recently been shown to act on APP processing by lowering the $A\beta_{1-42}$ production and not by their anti-inflammatory effects. This has been proposed to occur by shifting the γ-secretase activity to $A\beta_{1-38}$ production or through stimulation of an exopeptidase that converts $A\beta_{42}$ to a shorter Aβ species such as $A\beta_{38}$ [325]. Reduction of $A\beta_{42}$ was shown not to be due to Aβ catabolism, to COX-1 or COX-2 activity, nor to direct effects on APP processing. Thus, NSAIDS may have many mechanisms of action that could lead to specific anti-amyloidogenic therapy strategies.

Another theory claims that NSAIDS act as fibrillogenesis inhibitors rather than anti-inflammatory drugs [326]. They claim that only minute amounts of NSAIDS are required to inhibit fibrillization of Aβ. As only large doses of NSAIDS can delay AD (hence the severity of gastro-intestinal side effects), promising results would have been seen with small doses of NSAIDS if they did act as anti-fibrillogenic factors in the brain. Therefore, it seems unlikely that the effects of NSAIDS lie in their anti-fibrillogenic properties. Thus, the use of specific anti-inflammatory therapy to regulate AD-related inflammation is a viable route for therapy and the possibility of lowering $A\beta_{1-42}$ production with NSAIDS should be explored.

It is reasonable to conclude that inflammation is detrimental to brain amyloidosis in AD patients. Since individuals differ in their genetic background and in their inflammatory response to infection, two mouse strains representing high and low inflammatory responses were chosen to study Aβ-induced inflammation [265, 327]. Inbred mice provide a model with minimal genetic variability. This avoids problems in relating individual mouse samples and experimental reproducibility that could be observed in outbred mouse and human samples. By using two strains of inbred mice, genetic

variability was controlled with a set of known and unknown genetic differences to compare phenotypic differences. These experiments showed that high inflammatory responses due to microglial response to Aβ, led to high levels of neurotoxic factor release such as NO and TNF-α. As primary microglial cultures were used, these responses were directly related to the activation state of microglia. In a microglial population of mainly ameboid (activated) morphology, extremely high increases in TNF-α were observed compared to less-activated microglia. The differences in inflammatory response can be explained by genetic differences between these two mice such as in H-2 genotype, Nramp-1 allele, or an undefined genetic difference in inflammatory gene expression such as IL-1, IL-6, TNF-α, TGF-β, iNOS, or an ROI enzyme [241, 265, 269]. These differences could lead to variations in secretion of cytokines or neurotoxic factors or in the rate of their secretion. Morphological changes from ramified to ameboid has been shown to be time-dependent and this could also explain the differences in activation of these microglia [328]. In this respect, the C57BL/6 microglia (high) respond quickly to stimuli whereas A/J microglia (low) respond more slowly.

Evidence of a delay in amyloidosis due to differences in their genetic make-up was demonstrated in manuscript 3 with TgCRND8 mice backcrossed onto A/J and C57BL/6 strains of mice. In mice with a strong inflammatory response to Aβ (CRND8.B6), accelerated amyloidosis and low survival was observed in comparison to mice with a low inflammatory response (CRND8.A/J). This appeared to be due to a difference in Aβ deposition rate and/or fibrillogenesis as hAPP expression and total Aβ load were similar in both backgrounds. Microgliosis in TgCRND8 mice, occurs as Aβ is deposited and astrogliosis was seen once fibrillar plaque formation is observed

(manuscript 2). Therefore, inflammation is related to extracellular Aβ deposition and if one of the differences between the A/J and C57BL/6 mice is their magnitude in inflammatory response, this response could be responsible for influencing plaque deposition and survival. Further proof is required to associate this difference primarily to inflammation. One possibility is to treat the CRND8.B6 mice with NSAIDS or with anti-TNF therapy and compare plaque reduction and survival rates to CRND8.AJ mice.

Inflammation is clearly involved in brain amyloidosis and can occur by microglial uptake of extracellular Aβ through receptor-mediated phagocytosis. This would be followed by IL-1 and TNF-α secretion, complement factor production, and release of neurotoxic factors such as NO and ROS [125]. Astrocytes can also respond to microglial activation by secreting IL-1 and activating microglia. They later release anti-inflammatory cytokines such as TGF-β to regulate inflammation. Initially, microglia would take up Aβ and degrade it but, as the rate of Aβ degradation is much slower than phagocytosing Aβ, more plaques would form [312]. Eventually, very high levels of Aβ would be toxic to microglia and enhance Aβ deposition in late stages of amyloidosis. This is confirmed by their presence around senile plaques that has been reported repeatedly [121, 123, 124]. Astrocytes could aggravate the situation by releasing ApoE to enhance Aβ clearance, but this would promote Aβ fibrillization instead [207]. Fibrillar Aβ deposits are more difficult for microglial recognition for phagocytosis and digestion and would limit microglial ability for Aβ degradation. The continual but unsuccessful attempts of an inflammatory response to eliminate amyloid would in fact, be deleterious to neurons and promote amyloidosis.

Thus, in CRND8.AJ mice, the delay in plaque progression may be due to the ability of microglia to successfully degrade Aβ at a rate sufficient to compete with the phagocytic uptake of extracellular Aβ. Both microgliosis and astrogliosis would be well-regulated, neuronal loss would be minimal which would in turn, minimize cognitive decline, and survival would increase. Injection of LPS into hAPP V717F apoE$^{+/+}$ transgenic mice, showed that a high inflammatory response and the presence of apoE leads to accelerated amyloid deposition [256], further supporting the detrimental affects of a high inflammatory response as observed in CRND8.B6 mice.

To further test the influence of inflammatory response on amyloidosis, LPS could be injected into TgCRND8 A/J mice before Aβ load increases (6 weeks of age), just before Aβ deposition occurs (11 weeks of age), and once fibrillar plaques are present (13 weeks of age). From this, inflammation would be enhanced at specific moments in amyloidosis and show more convincingly if gliosis can affect Aβ load, Aβ deposition, or fibrillar plaque formation. Results showing a total Aβ load that is unchanged with Aβ deposition with increased fibrillar plaque formation compared to non-LPS-injected transgenic mice, would suggest that indeed microglial inflammatory response to Aβ plays a major role in the pathogenesis of AD. Finally, survival rates would decrease drastically if they prove to be linked to inflammation. LPS injection in Tg2576 mice showed that an induced inflammatory response can amplify degenerative processes including plaque load and supports the effect an inflammatory response can have on brain amyloidosis [329]. Further studies with genome scan analysis of pro-inflammatory and anti-inflammatory genes could specifically reveal an inflammation-related genetic difference although differences in protein pools of inflammatory mediators would require proteomic scans.

If inflammation is not involved in the differences seen between the CRND8.AJ and CRND8.B6 mice, there could be other modifying genes involved. High risk alleles for AD have been identified for ligands and their receptors that are involved in Aβ clearance, such as α_2-macroglobulin, apoE and apoJ [102, 265, 327]. Also, it has been shown that A/J mice have a slower rate of fibrillogenesis in AA amyloidosis compared to other mouse strains, which could be due to fibrillogenic factors and could explain the differences seen in these two mouse strains [316-318]. α_1-Antichymotrypsin plays a role in plaque formation by enhancing the conversion of non-fibrillar forms of Aβ to insoluble Aβ fibers and also has high risk alleles for AD. In addition, other genetic risk factors have been found for insulin-degrading enzyme (IDE) and neprilysin which are involved in Aβ degradation [330-332]. Cholesterol has also been shown to positively correlate to Aβ accumulation [333]. Furthermore, transgenes for superoxide dismutase 1 (SOD-1) and fibroblast growth factor (FGF-β) have been shown to be protective or lethal, respectively, in hAPP transgenic mice [295]. As an SOD-1 transgene has been shown to be protective to the lethal effects of the hAPP transgene in FVB/N and C57B6j mice, this implies that oxidative damage may be responsible for the premature death of these mice. This could be an interesting perspective to look at in the CRND8.B6 mice which show high mortality early-on around 6 weeks of age. Crossing these mice with SOD-1 transgenic mice would elucidate whether the high mortality is due to oxidative damage arising from lethal hAPP transgene effects and/or whether this is linked to their inflammatory response.

There may be other genetic factors related to Aβ production, clearance, and degradation that have not yet been determined. Neuronal survival could differ between individuals in terms of apoptotic efficacy. Rapid apoptosis of neurons containing $A\beta_{42}$

would allow a faster removal of $A\beta_{42}$ and cell debris, before large $A\beta_{42}$ aggregates form and a robust inflammatory response begins. Glutamine synthase expression also has potential as Alzheimer patients have decreased glutamine synthase expression compared to normal individuals [334]. Glutamine synthase is required for the conversion of glutamate to glutamine within astrocytes and its inhibition leads to amnestic effects [335]. High levels of glutamate are neurotoxic and can lead to neuronal loss. Interestingly, COX-2 induction promotes increases in glutamate that are high enough to induce seizures and neuronal cell death [336]. Thus, equilibrating glutamate levels through glutamine synthase could be another therapeutic target.

The CRND8.B6 mice die mainly from seizures. Transgenic mice expressing murine TNF-α specifically in the CNS, suffer from seizures and histopathological analysis of these mice revealed gliosis and infiltration of the CNS parenchyma by CD4+ and CD8+ T cells [337]. Neuronal apoptosis can also be induced by endotoxic shock through TNF-α [141](Bock et al., 1998). Therefore, as CRND8.B6 mice died from seizure and *in vitro* studies (manuscript 1) showed that $A\beta_{1-42}$ induced high levels of TNF-α in C57BL/6 microglia, there may be a link between hAPP expression and mortality due to seizures. In conclusion, high hAPP mRNA expression generating Aβ production would be followed by production and release of TNF-α from activated microglia, provoking seizures and neuronal cell death. This would happen even prior to Aβ organization into fibrillar deposits since death is seen even prior to the appearance of Aβ deposition. An interesting aspect to study would be to determine how CRND8.AJ mice attain prolonged survival and moderate TNF-α levels and whether this is due to anti-fibrillogenic factors, lower levels of fibril-forming factors, or some other mechanism.

The possibility of T cell-mediated response

In Alzheimer's disease literature, the fact that microglia can become professional APCs and induce an immune response has attracted interest in the past few years. It has been thought that the BBB is a tight barrier whose function, in part, is to prevent strong immune responses in the brain. This suggests that an immune response would play a limited role in infection or brain trauma. In fact, T cells have been found in the brain and are involved in immune responses to infection [338-340]. In AD, few studies have revealed the presence of T cells in the brain and their numbers are quite sparse [341]. Furthermore, Aβ is a self-antigen and would not be presented through MHC. Therefore, cell-mediated responses probably don't play a large role in brain amyloidosis.

So far, there hasn't been much support for an effector T cell response to Aβ. Microglia have been shown to induce CD4 T cell function leading to the production of IFN-γ and TNF-α which would promote a Th 1 immune response [342]. Studies have shown increased levels of CD8-positive lymphocyte derived soluble CD8 (sCD8) antigen in the periphery of AD patients [343]. As CD8-positive lymphocytes decreased in AD patients, this would imply that they play an early role in AD pathology [344]. On the other hand, CD8-positive lymphocytes increased in vascular dementia patients. An explanation could be due to the cerebral blood vessels being the interface between the CNS and the peripheral immune system which would allow for direct contact of the peripheral immune system with the amyloid plaques in the parenchyma. Thus stimulating the systemic production of CD8 T cells.

Due to the lack of evidence showing that T cells are involved in brain amyloidosis, more proof is needed to support a role of a cell-mediated immune response. Thymectimizing hAPP transgenic mice before the onset of brain amyloidosis followed by

immunohistochemical analysis could give some insight into this. Naturally, inflammation would be the prominent response to Aβ as their presence is well-documented around senile plaques and due to the fact that they are readily on-hand in the CNS, comprising about 12% of brain glial cells [345].

T cells could be involved in an autoimmune response to Aβ as it is a self-antigen, but so far, no evidence of this has been found. Usually, an autoimmune response is thought to be detrimental, leading to tissue destruction. A very interesting review by Schwartz and Moalem describes the potential protective effects of an autoimmune response in the CNS [346]. In the optic nerve, T cell response to CNS injury was much different than the sciatic nerve (a model of the PNS) due to low MHC class II antigen expression, pronounced FasL expression and the elimination of infiltrating lymphocytes through cell death. This allowed for the containment of the lesion site to keep the CNS in an 'immune-privileged state'. Since cell regeneration does not occur in the CNS, it is very important to limit tissue damage. Thus, neuroprotective effects of an autoimmune T cell response are necessary to controlling immune responses in the CNS.

Possibly, through the tight control of T cell responses and the fact that they don't proliferate in the brain, detrimental effects linked to an autoimmune response are not observed in brain amyloidosis. It would be expected that when Aβ, a self-antigen, is used for vaccination, there would be the risk of an autoimmune response. Levels of autoantibodies to Aβ were low in AD patients and could not be correlated to developing dementia [347]. This could be linked to the neuroprotective effects of the autoimmune response where the response is kept at low-levels to preserve the CNS and prevent tissue damage. An autoimmune response has not been recorded in any hAPP transgenic mice

that have been vaccinated with Aβ peptide. Furthermore, only beneficial effects have been observed in these mice such as amyloid plaque load reduction and prevention of memory loss [190-192]. The lack of an autoimmune response could be explained by the fact that hAPP is not a real self-antigen for the mouse and the formation of amyloid plaques would otherwise not normally occur. In contrast, vaccination in human clinical trials were detrimental and induced mortality. If there is an involvement of a T cell response in brain amyloidosis, this should not be overlooked as it could be a very potent therapeutic tool for early treatment of AD patients.

What is the neurotoxic factor and where is it?

A lot of debate has arisen from the form of Aβ that should be used for *in vitro* studies. Due to the amyloid hypothesis, fibrillar Aβ, found in senile plaques, was considered *the* neurotoxic substance responsible for pathology observed in AD. In the last few years, small toxic oligomers, ADDLs (Aβ-derived diffusible ligands), have been discovered and proposed as preliminary forms of amyloid that are as or more neurotoxic than fibrils [90]. These forms of Aβ have been studied *in vitro* but it would be very difficult to isolate them from the brain, as they rapidly become fibrillar aggregates. Comparisons have been made between amyloid in human brains and that found in the transgenic mouse models [348, 349]. Many differences were found including the lengths and ratios of $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$, post-translational modifications of Aβ which were greater in human brains, plaque structures varied, and the ratios of soluble: dimeric: oligomeric: fibrillar Aβ were different. Transgenic mice tended to have more soluble Aβ compared to human brains which is most likely due to the complex post-translational modifications possible in humans and not in mice. The early stages of amyloidosis in humans, are more difficult to study as

clinical evaluation is not always performed early on and post-mortem samples are not as easy to obtain compared to late-stage AD cases. Therefore, transgenic models are better for studying the evolution of early amyloidosis.

Transgenic mouse models provide insight into early amyloidosis and the resulting neuropathological events. Intraneuronal Aβ accumulation is considered to be a preliminary event before extracellular Aβ deposition [350]. It can be argued that intraneuronal accumulation of Aβ in transgenic mice is related to their inability to perform post-translational modifications that are otherwise carried-out in humans [95]. This is always a possibility although, one of the earliest pathological observations recorded in AD brains, is neurons containing swollen lysosomal granules that stain with Aβ [92]. This hints to early intracellular Aβ accumulation in neurons. Additionally, intracellular Aβ accumulation precedes plaque formation in Down's patients [93]. Structural comparisons of intraneuronal Aβ between humans, transgenic mice, and *in vitro* models would be necessary to validate experiments concerning early Aβ accumulation.

Cultured primary neurons isolated from hAPP transgenic mice provide a model to study how intraneuronal Aβ accumulation occurs and how it is released extracellularly [351]. One thing to consider is the difference in protein post-translational events between humans and mice. Furthermore, neurotoxicity of intracellular Aβ was observed in human primary neuronal cultures but not in human neuronal and non-neuronal cell lines [104]. It was not observed in isolated hAPP transgenic mouse neuronal cultures either. This implies that human primary neuronal cultures have a factor required for p53-Bax-induced cell death or that they are very sensitive to $A\beta_{1-42}$ accumulations. Of course, $A\beta_{42}$ might normally be in vesicles and not directly in the cytosol as performed in these experiments.

The authors suggest that their model of intracellular toxicity would represent leakage of $A\beta_{42}$ into the cytosol from vesicles. In light of this, the best model for studying secretion mechanisms and inflammatory response to intracellular Aβ would be cDNA-expressing cytosolic $A\beta_{1\text{-}42}$ in primary human neuronal cultures (model A). On the other hand, primary hAPP transgenic mouse neuronal cultures (model B) would be better suited for studying hAPP processing mechanisms and an inflammatory-mediated response to intracellular Aβ as the former model induces 70% neurotoxicity within 24 hours and would not allow enough time to study cell-cell interactions.

Co-cultures of transfected human neurons (model A) with homologous human microglia would be a very interesting way to look at inflammatory responses to Aβ-induced neurotoxicity. Measuring inflammatory mediators (cytokines, NO, ROS), receptor upregulation (Fc, SR, RAGE, MHC II), addition of inhibitors (phagocytosis inhibitors, COX-2 inhibitors, PPAR-γ agonists), addition of fibril-inducing factors (ApoE), and mRNA analysis could all lead to a better comprehension of inflammatory mechanisms involved in amyloidosis. Using primary hAPP transgenic neurons (model B) provides a model to study inflammatory-mediated responses. These hAPP-expressing neurons could be placed in co-culture with murine CD8+ T cells to determine if neuronal cytotoxicity is induced. As mentioned, cytokines, neurotoxic factors, and receptor upregulation could be analysed. Microglia could also be co-cultured to determine if they respond to Aβ secreted by these neurons, although more $A\beta_{40}$ than $A\beta_{42}$ is secreted by neurons. There are many co-culturing systems possible with endless factors to be studied.

Transgenic mouse models represent AD

Transgenic mouse models are a tool for understanding the neuropathological events linked to amyloidosis and to AD. They have elucidated AD-linked human pathology observations and portray the following sequence of events. The earliest event known is activation of lipid peroxidation (LPO) [352, 353]. It occurs before detection of Aβ in the brain but could occur as small concentrations of Aβ accumulate inside neurons which are below ELISA detection limits. Consecutively, the order of events is: large intraneuronal accumulation, increases in extracellular Aβ, Aβ deposition, gliosis, ThioS-positive plaque formation, tau hyperphosphorylation, NFT formation, significant neuronal loss and death. Studies of human AD brains support this sequence of events. On the other hand, not any one transgenic model has shown all of these events and a kinetic analysis of the sequence of events has not been rigorously studied in each model.

The principal hAPP transgenic mouse models (Tg2576, APP23, PD-APP, and TgCRND8) have all shown increases of Aβ in brain homogenates before *or* at the same time as Aβ deposition which occurs before *or* at the same time as fibrillar plaque formation [235, 238]. One difficulty in interpretating the results is how the plaque is detected whether it is with an anti-Aβ antibody to label Aβ deposits or with ThioS or Congo red to detect fiber formation. Another problem, is the treatment of brain homogenate samples to measure Aβ load with ELISA. Brain homogenates can be solubilized in SDS (soluble Aβ), guanidium-HCl (soluble Aβ and some aggregates), 70% formic acid (most fibrillar and soluble Aβ), or 99% formic acid (total Aβ). Therefore, it is difficult to compare Aβ and plaque load in the different transgenic models due to the analytical methods used. Plaques would have to be identified and quantified in a uniform manner and soluble and

fibrillar fractions of brain homogenates would need to be separated to give a clearer picture of each transgenic model.

Recently, the relationship between tau and amyloidosis has been illuminated. Double transgenic mice expressing mutant tau and hAPP have NFTs and plaque formation [15]. In addition, $A\beta_{42}$ fibril injection into mutant tau transgenic mice caused an acceleration in NFT formation away from the site of injection [14]. These studies finally link amyloidosis and tauopathy [13]. Transgenic hAPP mouse models show tau hyperphosphorylation but have lacked in displaying NFTs and neuronal loss. The NFTs occur in the neuronal cell body, amyloid plaques are found at the projection sites of the same neuron, and $A\beta_{42}$ injection induces tau hyperphosphorylation [14]. Thus, $A\beta_{42}$ accumulation at neuron projection sites incites tau hyperphosphorylation followed by NFT formation in the cell body of the same neuron. The question remains how extracellular Aβ can induce tau hyperphosphorylation and if intracellular Aβ accumulations can do the same. Most transgenic mouse models probably don't survive long enough for NFT formation to occur as the plaque deposition is too high and induces neurotoxicity directly or indirectly through inflammation. There is most likely a link missing between tau hyperphosphorylation and NFT formation that is present as a result of tau mutations. Maybe the link between amyloidosis and NFTs will only be found in human neurons, as their sensitivity to $A\beta_{42}$ is much higher than that of mice. Determining the connection between Aβ and NFT formation will be a breakthrough in AD.

Conclusion

Although transgenic mouse models do not exactly display all the neuropathological characteristics of AD, they are required to understand portions of brain amyloidosis and tauopathy mechanisms. An important aspect of amyloidosis is the inflammatory response of microglia and the resulting production of neurotoxic factors and cytokines. High levels of TNF-α were observed in C57BL/6 microglia compared to A/J microglia and these levels are neurotoxic and can induce seizure activity. CRND8.B6 mice had a low survival rate due to inflammation-induced seizure activity whereas CRND8.AJ mice survived much longer and had reduced plaque load. This underlines the importance of genetic background on not only inflammatory response, but in amyloidosis-related diseases such as AD. Furthermore, a genome scan of these two transgenic mice will allow the discovery of modifying genes influencing amyloidosis and survival.

Inflammation plays a role early in amyloidosis around the time of Aβ deposition. Since the level of inflammatory response controls the rate of amyloidosis, the timing of inflammation early in Aβ deposition has important consequences. Therapeutic agents against inflammation delay AD-linked pathogenesis and specific therapy to diminish microglial activity and make them moderately responsive, will aid in slowing down plaque progression. Combination drugs could then be used to decrease soluble Aβ production (γ-secretase inhibitors), reduce soluble Aβ loads in the brain (peripheral anti-Aβ vaccination approach), neutralize plaques and stop their fibrillization (Aβ aggregation blockers), and maybe, one day, eliminate neurodegeneration associated with AD.

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Appendices

Ethics certificates for working with animals in the studies described in the thesis. Refer to the following pages.

April 12, 2002

RE: Circumstances concerning thesis defense

Dear Sir/Madam;

I would like to inform you on my present circumstances so that I can facilitate my presence for the date set for oral defense as I am currently residing in France. As my date will fall in summer, I need to reserve a plane ticket as early as possible so please keep this in mind in terms of giving me some advance on organizing my arrival.

Thank you for your comprehension.

Sincerely,

Sherri Dudal
student number:

Dr. Francine Gervais

CENTRE FOR RESEARCH IN NEURODEGENERATIVE DISEASES

Tanz Neuroscience Building

Su

RE: Approval of co-authors

To the co-authors;

I will be submitting a thesis by manuscript titled *Influence of mouse genetic background on hAPP transgene-induced brain amyloidosis and inflammatory response to beta-amyloid protein* to McGill University. To do this, I require your authorization for the publication of the following manuscripts in my PhD thesis:

1) Differences in the amyloid-ß-induced inflammatory response in microglia from C57BL/6 and A/J strains of mice. Sherri Dudal, Emil Skamene, Céline Morissette, Patrick Tremblay, and Francine Gervais

2) Inflammation occurs early in Aβ deposition in TgCRND8 mice. Sherri Dudal, Pascale Krywkowski, Julie Paquette, Céline Morissette, Patrick Tremblay, David Westaway, and Francine Gervais

3) Differences in inflammatory response alters survival and plaque formation in TgCRND8 mice. Sherri Dudal, Giovanna Sebastiani, Emil Skamene, Julie Paquette, Patrick Tremblay, David Westaway, and Francine Gervais.

Please read the titles and authors for the mentioned manuscripts and sign and print your name at the bottom of this letter in agreement for their publication in my thesis. All signatures must be original and no fax copies are accepted. Thank you for your rapidity in signing this letter and mailing it as follows:

Att. Dr. Giovanna Sebastiani

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Sincerely,

Sherri Dudal

signature

David Westaway

University of Toronto

Neurochem Inc

April 12, 2002

RE: Approval of co-authors

To the co-authors;

I will be submitting a thesis by manuscript titled *Influence of mouse genetic background on hAPP transgene-induced brain amyloidosis and inflammatory response to beta-amyloid protein* to McGill University. To do this, I require your authorization for the publication of the following manuscripts in my PhD thesis:

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Att. Dr. Giovanna Sebastiani

Sincerely,

Sherri Dudal

signature

Francine Gervais
name

April 12, 2002

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print name

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Att. Dr. Giovanna Sebastiani

Sincerely,

Sherri Dudal

signature

Julie Paquette

April 12, 2002

RE: Approval of co-authors

To the co-authors;

I will be submitting a thesis by manuscript titled *Influence of mouse genetic background on hAPP transgene-induced brain amyloidosis and inflammatory response to beta-amyloid protein* to McGill University. To do this, I require your authorization for the publication of the following manuscripts in my PhD thesis:

1) Differences in the amyloid-ß-induced inflammatory response in microglia from C57BL/6 and A/J strains of mice. Sherri Dudal, Emil Skamene, Céline Morissette, Patrick Tremblay, and Francine Gervais

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print name April 15th, 2002

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Neurochem Inc.

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Sherri Dudal

signature 18/04/2002

Francine Gervais
name

Neurochem Inc.

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Francine Gervais
name