Neuroinflammation in Alzheimer's Disease: a therapeutic target

by

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Abstract

Alzheimer's disease (AD), the most common dementia, is an age-related neurodegenerative disorder characterized by memory loss, extracellular β-amyloid (Aβ) peptide deposits and intracellular neurofibrillary tangle formation. Current animal models of AD center on the precipitating role of Aβ deposition in a cascade of biological events that ultimately lead to neurodegeneration and dementia. However, increasing evidence shows that the amount of measurable amyloid deposition is a relatively weak correlate of cognitive function impairment among AD patients or individuals at risk for AD. Furthermore, autopsy studies reveal that older individuals with significantly elevated amyloid levels have no symptoms of AD. Together, these findings suggest that Aβ is necessary but perhaps not sufficient to cause the AD syndrome. Neuroinflammation has been implicated in cognitive aging for years. Neuroinflammatory response consists of activated microglia and astrocytes and the release of proinflammatory markers. Acute inflammation plays a critical role in brain function against insults. Neuropathological and neuroimaging studies have demonstrated that uncontrolled glial activation and neuroinflammation in the AD brain may contribute independently to neural dysfunction and cell death.

This thesis shows that both microglial and astroglial activation occurred in paralleled with the pattern of learning deficits rather than amyloid pathology in an APP/PS1 transgenic mouse model of AD. These findings suggest that neuroinflammation might directly contribute to the development and progression of cognitive deficits in APP/PS1 mice. In the context of significant amyloid deposition, the results of the current study suggest that mechanisms underlying the inflammatory process might be an important therapeutic target for AD.

Finally, this work presents a chronic administration of quetiapine in APP/PS1 transgenic mice resulted in a marked change in microglial and astrocyte activation, proinflammatory cytokine

levels, and an improvement in behavioural performance. The beneficial effects of quetiapine occurred when there were only marginal changes in levels of total Aβ, suggesting the anti-inflammatory effect of quetiapine could account for the cognitive improvement observed in APP/PS1 transgenic mice. Moreover, we confirmed that quetiapine significantly reduced $A\beta_{1-42}$ induced secretion of proinflammatory cytokines in primary cultured microglia. Both *in vitro* and *in vivo* experiments demonstrated that quetiapine ameliorated proinflammatory cytokine increases via suppression of NF-κB pathway activation. Since risk factors for the development of inflammation are modifiable, these findings suggest intervention and prevention strategies for the clinical syndrome of AD.

Preface

Unless otherwise indicated, the author, Dr. Shenghua Zhu, created all text, figures, and data in this thesis.

Chapter 1

A large portion of this chapter has been submitted as a review for publication. I am first author on the submission and wrote the first draft. Dr. Jun-Feng Wang provided editorial assistance.

Some of the material covered in section 1.1.5 was adapted from a published Master thesis (**Zhu, S.**, 2011) in which I was first author. Dr. Jun-Feng Wang and Dr. Xin-Min Li provided editorial assistance.

Chapter 2

This work has been previously published (**Zhu, S.**, Wang, J., Zhang, Y., He, J., Kong, J., Wang, J.-F., and Li, X.-M., 2017). I was first author on the paper. Dr. Jun-Feng Wang and Dr. Xin-Min Li were the principal investigators. I wrote the first draft of the paper.

For this work and the rest of the work, I was the primary individual responsible for maintaining both transgenic colonies including both APP and PS1 during my PhD training. APP and PS1 cross breeding, tail sampling and genotyping analyses were performed by the author, with assistance from Ms. Rhonda Kelly. Brain tissues were collected by the author with assistance from Dr. Junhui Wang, Dr. Jue He, Dr. Ruiguo Zhang.

I performed all the behavioural experiments and statistical analyses shown in this thesis. The author performed both coronal sections and immunochemistry. All procedures were approved by

the University of Manitoba Animal Care Committee (Protocols: 07-053). Dr. Wang and Dr. Li provided the initial idea for the project and editorial expertise for the manuscript.

Chapter 3

Part of this work has been published (**Zhu, S.**, Shi, R., Li, V., Wang J., Zhang, R., Tempier A., He, J., Kong J., Wang, J.-F., and Li, X.-M., 2014). The rest work has been presented at my annual progress presentation. I was first author on the paper. Dr. Jun-Feng Wang and Dr. Xin-Min Li were the principal investigator. I wrote the first draft of the paper.

For this work and the rest of the work, I was the primary individual responsible for maintaining C57BL mouse colony. The author performed primary microglia culture and drug preparation. I performed all the *in vitro* experiments shown in this thesis. All procedures were approved by the University of Manitoba Animal Care Committee (Protocols:13-013).

Chapter 4

The author performed approximately 95% of the behavioural experiments shown in this thesis. Dr. Junhui Wang assisted with the remaining 5% of the experiments when I was unable to perform them due to scheduling conflicts. This work has also been previously published (**Zhu, S.**, Shi, R., Li, V., Wang J., Zhang, R., Tempier A., He, J., Kong J., Wang, J.-F., and Li, X.-M., 2014). I was first author on the paper and wrote the first draft. Dr. Wang and Dr. Li were the principal investigator on the project, and as such provided the initial idea for the project and editorial expertise for the manuscript.

The author performed all the rest ELISA, western blots and immunochemistry. All procedures were approved by the University of Manitoba Animal Care Committee (Protocols:09-035).

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List of Abbreviations

Aβ	Beta Amyloid
AICD	APP Intracellular Domain
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
APD	Antipsychotic Drugs
APP	Amyloid Precursor Protein
APP-CTFs	Carboxyl-terminal Fragments
APOE	Apolipoprotein E
BACE1	β-site APP-cleaving Enzyme 1
BACE2	β-site APP-cleaving Enzyme 2
BBB	Blood-Brain Barrier
CNS	Central Nervous System
COX	Cyclooxygenase
CTE	Chronic Traumatic Encephalopathy
CTFα	Carboxyl-Terminal Fragment α
CTFβ	Carboxyl-Terminal Fragment β
DA	Dopamine
EAE	Autoimmune Encephalomyelitis
ER	Endoplasmic Reticulum
FAD	Familial Alzheimer's Disease
FTD	Frontotemporal Dementia
GFAP	Glial Fibrillary Acidic Protein
HDLs	High-Density Lipoproteins
IL-1β	Interleukin-1β
LDH	Lactate Dehydrogenase
MCP	Monocyte Chemotactic Protein-1
MS	Multiple Sclerosis
NF-κB	Nuclear Factor kappa B
NFTs	Neurofibrillary Tangles

NMDA	N-methyl-D-aspartate
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
PBS	Phosphate-buffered Saline
PBS-T	PBS with 0.1% Tween-20
PD	Parkinson's disease
PDGF	Platelet Derived Growth Factor
PKB	Protein Kinase B
PS1	Presenilin 1
PS2	Presenilin 2
Q5	Quetiapine 5 mg/kg/day
ROS	Reactive Oxygen Species
SAD	Sporadic Alzheimer's Disease
sAPPα	Secretory APPα
sAPPβ	Secretory APPβ
SOD	Superoxide Dismutase
Tg	Transgenic
Non-Tg	Non-Transgenic
TNFα	Tumor Necrosis Factor-α
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
VLDLs	Very-Low-Density Lipoproteins

Acknowledgements

Throughout my MD and PhD journey, I have been most fortunate to have the support and guidance of many brilliant minds, who have cultivated both my academic and personal development and, as a result, helped me become the person who I am today. Ergo, I feel my PhD training encompasses much more beyond this thesis.

First and foremost, I would like to extend my gratitude to my supervisor, Dr. Xin-Min Li, for his mentorship. Dr. Li has given me enormous freedom to venture my research interests and taught me to think critically yet creatively, and to work quickly yet efficiently. With his continuous encouragement I was able to pursue a career in both research and clinical medicine. Dr. Li's steadfast support and intellectual guidance, even after his relocation to the University of Alberta, have been invaluable over the years and I feel incredibly privileged to have him as my supervisor and mentor.

I would also like to thank my co-supervisor, Dr. Jun-Feng Wang, who has become my primary supervisor since Dr. Li's relocation. Owing to his kindness and generosity I was able to re-establish my experiments at his laboratory at the Kleysen Neuroscience Research Institute. I would especially like to thank him for his understanding to provide the continuity of supervision throughout the rest of my training. His open-door policy, patience, expertise in neuroscience, and certainly his keen ear to my ideas were inspiring.

I would also like to thank the members of my advisory committee, Dr. Jiming Kong and Dr. Donald Miller, for their support and advice throughout my degree. Their membership in my committee made the annual progress report a meeting that I always looked forward to for their intellectual contributions and insightful comments. I would especially like to thank my MD/PhD program director Dr. Mark Nachtigal and former director Dr. Kent T. HayGlass, both of whom

have been approachable for advice on topics ranging from my academic transition, to career planning, to balancing research activities and clinical duties. Many thanks.

I would also like to thank my colleagues in both laboratories, both past and present, who not only embraced my ideas but also shared memorable friendships. A special thanks to Dr. Yanbo Zhang and Dr. Junhui Wang for their critical feedbacks whenever needed, and to Hua Tan for assisting in ordering supplies. Also, thank you to Ying Wang, Zhu Zhou, and Veni Bharti for being great bench mates.

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family. I would like to dedicate this dissertation to my wife for sharing this amazing journey with me, and to my son for being such a bundle of joy and laughter.

Dedication

For my wife,

Ruoyang Shi

for sharing this amazing journey with me.

For my son,

Nathaniel Geoffrey Chu

for being such a bundle of joy and laughter.

Chapter 1

General introduction

1.1 Alzheimer's disease

Alzheimer’s disease (AD) was first described by Alois Alzheimer in 1906 in the brain of a demented female patient, Auguste D., about 60 years of age. It is the most common form of dementia in the elderly, comprising up to 70% of all cases. Patients with AD display predominantly memory loss, followed by a gradual decline in other cognitive functions, namely aphasia (loss of language), apraxia (loss of motor activities), agnosia (loss of object recognition), and executive functioning (Tariot, Profenno, & Ismail, 2004) (DSM-V, American Psychiatric Association 2013). While the symptoms can vary in severity and chronology, they mirror a gradual expansion of degenerative change in the brain (Braak & Braak, 1991). Each year we can expect to see more AD cases than the previous year because age is the greatest risk factor for AD. The chance of developing AD doubles every five years after age 65 (Mayeux, 2010). The prevalence increases from 1% among those 60 to 64 years old to up to 40% of those aged 85 years and older (von Strauss, Viitanen, De Ronchi, Winblad, & Fratiglioni, 1999). In Canada, the prevalence of AD is one in 20 in those over 65 years of age ("Canadian study of health and aging: study methods and prevalence of dementia," 1994), making it one of the top leading causes of death for older Canadians (Kung, Hoyert, Xu, & Murphy, 2008). The number of AD patients will almost double in less than 20 years, straining families, social systems, and the already burdened healthcare system.

Pathologically, AD is a progressive neurodegenerative disorder characterized by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles (NFTs) and massive

neuronal cell and synapse loss at specific sites (Selkoe, 2002; Storey & Cappai, 1999; Suh & Checler, 2002; Van Gassen & Annaert, 2003). The amyloid plaques are composed of fibrillar β-amyloid (Aβ) peptide and the intracellular NFTs largely consist of hyperphosphorylated tau protein.

Aβ peptide is generated from a larger transmembrane amyloid β precursor protein (APP) following a sequential cleavage by two important enzymes: β-secretase (β-site APP-cleaving enzyme, BACE) and a presenilin (PS)-dependent γ-secretase complex (Figure 1.1) (Fukumoto et al., 2004; Lazarov et al., 2005). β-secretase mediates the initial step of Aβ production by β-cleavage of APP, producing secretory APPβ (sAPPβ) and membrane-bound CTFβ (C89/C99) (Fischer, Molinari, Bodendorf, & Paganetti, 2002). Subsequent cleavage of CTFβ by γ-secretase results in increased production of amyloid peptide Aβ, eventually leading to amyloid deposition. The increased extracellular accumulation of Aβ peptide is believed to be the critical change that initiates AD pathogenesis (J. A. Hardy & Higgins, 1992). Despite the robust expression of the APP gene, resulting in high level of APP protein *in vivo*, Aβ production through the "amyloidogenic" pathway is still a rare event under normal conditions (Y. Li, Zhou, Tong, He, & Song, 2006). The majority of APP is cleaved in the non-amyloidogenic pathway first by α-secretase, instead of β-secretase, producing a secreted form of APP, secretory APPα (sAPPα), and C-terminal fragment C83 (CTFα) (Ashe & Zahs, 2010; Ma et al., 2007). The CTFα can be further cleaved by γ-secretase, generating extracellular fragment p3 and intracellular APP intracellular domain (AICD) (Edbauer et al., 2003; S. H. Kim, Ikeuchi, Yu, & Sisodia, 2003).

The exact cause of AD is not yet fully understood. AD appears to develop when the combined effects of various risk factors cross a certain "threshold" and overwhelm the natural self-repair mechanisms in the brain, thus reducing the brain's ability to maintain healthy nerve cells.

Various hypotheses have been suggested to explain the biological basis of its etiology and pathogenesis of AD.

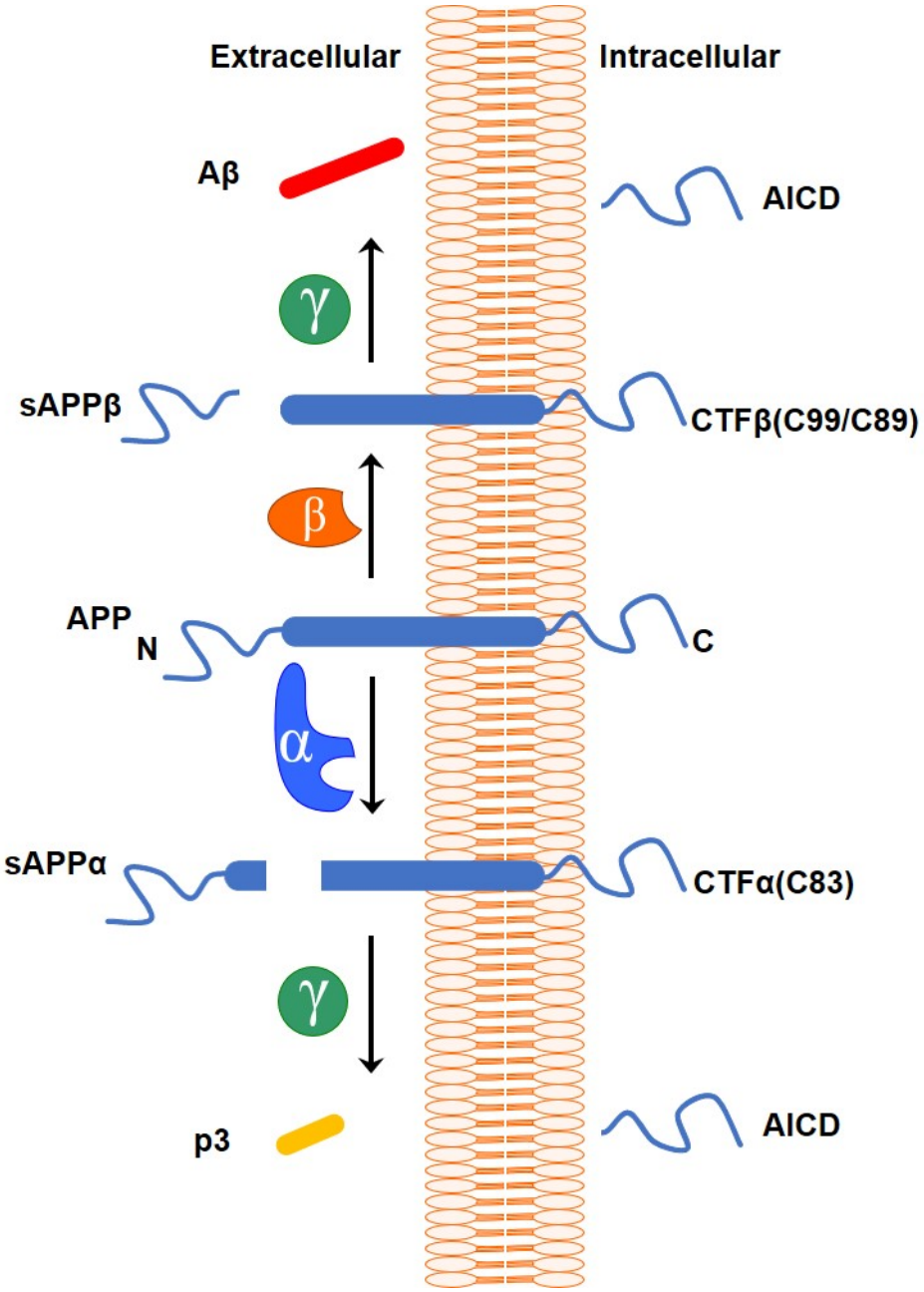


Figure 1. 1: APP processing.

APP can be cleaved in two pathways: the more common, non-pathogenic, non-amyloidogenic pathway (bottom), or the pathogenic, amyloidogenic pathway (top). Under normal conditions, the majority of APP is cleaved within the Aβ domain by α-secretase to produce sAPPα and membrane-bound C83. C83 can be further cleaved by γ-secretase, producing extracellular fragment p3 and intracellular AICD. In the pathogenic pathway, APP is instead cleaved by β-secretase at the start of the Aβ domain, producing sAPPβ and membrane-bound CTFβ (C89/C99). Cleavage of CTFβ by γ-secretase yields pathogenic Aβ fragments, and intracellular AICD.

1.1.1 Amyloid β precursor protein (APP)

APP is a single-pass type I transmembrane protein with large extracellular domains (Figure 1.1). Only APP generates amyloid fragments due to its sequence divergence at the internal Aβ site. It is encoded by a single gene on chromosome 21 and alternate splicing of the APP transcript generates 8 isoforms, of which three are major isoforms: APP695, which is expressed predominantly in the central nervous system (CNS), and APP751 and APP770, which are more ubiquitously expressed (Bayer, Cappai, Masters, Beyreuther, & Multhaup, 1999). Among the three major isoforms, APP695 is the only isoform lacking an extracellular Kunitz Protease Inhibitor domain and is mainly expressed in the neurons of the brain. After N-glycosylation in the endoplasmic reticulum (ER) and O-glycosylation in the Golgi, APP is transported by fast axonal transport to synaptic terminals (Koo et al., 1990).

It is crucial that most APP must be transported to the cell surface as part of its processing. However, this step is very rapid as only a small proportion of APP is detected at the cell surface when compared to the total cellular quantity at any point in time. The half-life for APP at the cell surface is less than 10 minutes (Koo & Squazzo, 1994; Koo, Squazzo, Selkoe, & Koo, 1996). On

the cell surface, APP can be proteolyzed between Lys^{16} and Leu^{17} within the Aβ domain by α-secretase, precluding the formation of amyloid peptide and producing a soluble fragment, sAPPα, and a membrane bound 10 kDa CTFα. Alternatively, cell surface APP can be reinternalized through clathrin-coated vesicles into the endosomal compartment containing the proteases BACE1 and γ-secretase, which results in the production of Aβ. It is unclear why some surface APP is internalized into endosomes and some proteolyzed directly by α-secretase.

1.1.2 β-secretase (BACE1)

β-site APP cleaving enzyme 1 (BACE1), β-secretase *in vivo*, is a type I integral membrane protein. BACE1 is encoded on chromosome 11 and most highly expressed in brain and pancreas yet its expression is tightly regulated at the transcriptional level (R. Yan et al., 1999). Under normal conditions, BACE1 expression is very low because of its weak promotor activity, which results in only a very small fraction of APP undergoing amyloidogenic pathway (Y. Li et al., 2006; Zhou & Song, 2006). However, BACE1 gene expression can be enhanced by Sp1 and oxidative stress, leading to an increased production of Aβ (Christensen et al., 2004; Tong et al., 2005). Although β-site APP cleaving enzyme 2 (BACE2) is the homolog of BACE1, its neuronal expression is close to being undetectable (Acquati et al., 2000; Lin et al., 2000; R. Yan et al., 1999). Furthermore, overexpression of BACE2 decreases Aβ production, suggesting that BACE2 is not functionally homologous to BACE1, instead it shares certain similarities to α-secretase (X. Sun, He, & Song, 2006).

The best characterized substrate of BACE1 is APP protein. The major cleavage site for BACE1 is located between Met^{596} and Asp^{597} of the APP695 isoform, producing sAPPβ and C-terminal fragment C99 (C-terminal fragment β (CTFβ)). In addition, BACE1 also cleaves at a minor site located between Tyr^{10} and Glu^{11} of Aβ producing a lower level C-terminal fragment

C89 both *in vivo* (Masters et al., 1985; Naslund et al., 1994) and *in vitro* (Haass et al., 1992). Following the BACE1 cleavage, the APP C-terminal fragment C99 is then cleaved by the γ-secretase complex at several sites generating Aβ peptides from 37 to 42 amino acids, of which Aβ40 and Aβ42 are the most common forms. Unlike α-secretase located at the cell surface, BACE1 is localized in endosomes. As a result, Aβ is produced intracellularly and quickly exported into the extracellular space following vesicle recycling or degraded in lysosomes (Greenfield et al., 1999; Perez, Squazzo, & Koo, 1996; Wertkin et al., 1993). Of note, the human APP Swedish mutation (APP*Swe*), in which Lys^{595}/Met^{596} (KM) is mutated to Asn^{595}/Leu^{596} (NL) where they are adjacent to BACE1 cleavage site, increases the activity of β-secretase and Aβ production, resulting in early-onset Alzheimer's (Citron et al., 1992).

1.1.3 γ-secretase complex

γ-secretase complex is an atypical multimeric protease that cleaves several single-pass type I transmembrane proteins at sites within their transmembrane domains. It is an integral membrane protein composed of presenilin 1 (PS1) or presenilin 2 (PS2), nicastrin (Nct), anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2) (Bergmans & De Strooper, 2010). This structure complex is essential for the sequential intramembranous proteolysis. The presenilin proteins, PS1 and PS2, were the first two identified components of the γ-secretase complex and play crucial roles in intramembranous cleavage, whereas the other three subunits are involved in the process of assembly, maturation and stability of the γ-secretase complex (Dang et al., 2015; LaVoie et al., 2003; Y. Li et al., 2014). APP and Notch receptors are the best studies substrates of γ-secretase (De Strooper et al., 1999; J. Hardy & Selkoe, 2002; Wolfe et al., 1999). Both substrates are of great physiological and pathological importance.

Missense mutations in PS1 and PS2 are another major cause of early onset AD (Mullan, Houlden, et al., 1992; Schellenberg et al., 1992; St George-Hyslop et al., 1992). These presenilin mutations accelerate β-amyloid deposition in familiar AD (FAD) patients' brains (L. Liu, Herukka, Minkeviciene, van Groen, & Tanila, 2004) and in model mice (Borchelt et al., 1996; Scheuner et al., 1996), both of which suggest the presenilin proteins play a major role in APP processing. While a single mutation in PS2 does not display amyloid plaques in the brains of model mice, these transgenic mice with mutant PS2 found to have elevated both Aβ42 and Aβ43 in their brains (Oyama et al., 1998). Furthermore, Aβ production is completely inhibited in $PS1^{-/-}$ $PS2^{-/-}$ cells, indicating that PS1 and PS2 are absolutely required for enzymatic active site of γ-secretase (Herreman et al., 2000; Z. Zhang et al., 2000). However, overexpression of full length PS1 does not increase enzymatic activity of γ-secretase as expected, which suggests there are other limiting factors reconstituting its activity (Thinakaran et al., 1997). These modifying factors contribute to the high molecular weight of γ-secretase. As a result, a number of γ-secretase modifiers including Nct, Aph-1, Pen-2 and transmembrane emp24-like tracking protein 10 (TMP21) have been identified with an aim to negatively regulate the activity of γ-secretase, which subsequently modifies Aβ peptide generation as potential AD therapeutics (F. Chen et al., 2006). Unfortunately, little success has been achieved in clinical trials, partially due to the complexity of substrate recognition and catalytic processing of γ-secretase (Kumar et al., 2018).

1.1.4 The genetics of Alzheimer's disease

Only a minority of AD patients, less than 5% of total AD cases, has a familiar form of AD (FAD). Yet research on AD has been greatly stimulated by the identification of these causative autosomal dominant mutations in three genes encoding APP, PS1 and PS2, all of which play important roles in the process of amyloid production. The rest (> 95%) of AD cases are sporadic,

meaning that the disease does not follow Mendelian inheritance. Yet sporadic AD (SAD) also displays significant heritability. The most well-known and strongest genetic risk factor is the inheritance of the *ε4* allele of the *apolipoprotein E* (*APOE*) gene (Strittmatter et al., 1993).

The first ever genetic mutations causing FAD were discovered in the APP gene, whose mutations promote the generation of Aβ by favouring proteolytic processing of APP (Chartier-Harlin et al., 1991; J. Hardy, 1992; Mullan, Crawford, et al., 1992). Since then, over 32 different mutations within APP have been identified in 85 families. Interestingly, most of these mutations are located at either the cleavage sites of secretase or the APP transmembrane domain on exons 16 and 17. Full detailed information regarding APP mutations is available in the NCBI database and the Alzheimer Disease Mutation Database (www.molgen.ua.ac.be/ADMutations). Yet, these dominantly inherited missense mutations in APP only account for about 10% - 15% of mutations identified in FAD (Bird, 2008). The average age of onset for patients carrying the APP mutations is in the mid-40s and -50s (Bekris, Yu, Bird, & Tsuang, 2010).

Both PS1 and PS2 are accountable for almost 90% of identified FAD mutations, many of which increase the deposition of β amyloid by enhancing the processing of APP to form amyloidogenic Aβ (Scheuner et al., 1996). In contrast to the pathogenic mutations in APP which are situated around the secretase cleavage sites, mutations in PS are scattered throughout the whole protein at close to 20% of the amino acid residues including cytosolic, transmembrane and extracellular domains (Shen & Kelleher, 2007). These nearly “random” alternations which are sufficient to cause AD again highlighting the importance of PS in γ-secretase.

Late-onset sporadic AD also has a significant genetic component. The most common risk is conferred by the *APOE ε4* allele. The frequency of the *APOE ε4* allele is 10% to 20%, but it varies between ethnic groups (C. C. Liu, Liu, Kanekiyo, Xu, & Bu, 2013). The *APOE* gene is

located on chromosome 19 and comprises 4 exons which encode a 299 amino acids protein. The *APOE* gene encodes three protein isoforms, designated as ApoE2, ApoE3, and ApoE4 which only differ at two amino acid sites at position 112 and 158 respectively (Bekris et al., 2010). The most frequent protein variant is ApoE3. Both E2 and E3 are the predominant apolipoprotein of high-density lipoproteins (HDLs) in the brain, whereas the changed structure causes the E4 isoform to preferentially bind to the very-low-density lipoproteins (VLDLs) (Mahley, Weisgraber, & Huang, 2006). The exact mechanism underlying the isoform specific toxic effects of ApoE has yet not been fully understood. The risk for AD increases threefold in individuals carrying one copy of the *APOE ε4* allele and up to 15 folds for homozygous individuals. The most compelling hypothesis regarding its toxicity is related to the isoform specific binding ability to Aβ. Studies have shown that lipidated ApoE binds to soluble Aβ and facilitates Aβ internalization by glial cells in an isoform dependent manner (C. C. Liu et al., 2013). Thus, the increased risk conferred by ApoE may be explained by its effect on Aβ clearance or cellular uptake.

1.1.5 Transgenic models of Alzheimer's disease

Understanding the potential roles of APP and PS genes in AD has led to several animal models that have been useful to study the pathogenesis underlying AD. Over the last decade, the development of transgenic mouse models of AD has mainly focused on mimicking NFTs and amyloid plaques. Such transgenic models have provided a very useful platform for understanding the significances of Aβ deposits and tau phosphorylation in the AD pathogenesis, as well as their relationship with other pathological features such as neuroinflammation characterized by both astrogliosis and microgliosis. The following section discusses the utility of Aβ mouse models, and their validity in studying AD.

The first transgenic mouse model of AD, which was designed with the platelet derived growth factor (PDGF) promoter expressing V717F (with valine at residue 717 substituted by phenylalanine) APP, was developed by Games et al. in 1995 and shows Aβ amyloid plaques comparable to those in the brains of human AD patients. The PDGF promoter-driven mice (PDAPP) express human Aβ40 and Aβ42 that are 5 to 14 times higher than endogenous mouse Aβ (Games et al., 1995). The PDAPP V717F transgenic mouse progressively develops many of the pathological features of AD, including numerous amyloid plaques, synaptic loss, hippocampus and corpus callosum shrinkage, astrocytosis and microgliosis (Games et al., 1995; Kobayashi & Chen, 2005). In addition, PDAPP mice demonstrated significant memory impairments on several behavioural tests, such as the Morris water maze, radial arm maze, object recognition and fear conditioning tasks compared to age matched controls (Kobayashi & Chen, 2005). The cognitive deficits in the older PDAPP mice are well correlated with increased Aβ levels and reductions in the hippocampus-to-brain ratio. However, similar cognitive decline is also seen in young (3-4 months) animals in which there are no apparent Aβ depositions or reductions in hippocampus (Kobayashi & Chen, 2005).

One year after Games *et al*. developed the PDAPP mouse, a similar transgenic mouse model, which over-expresses the Swedish double mutant form of APP695, was introduced as the Tg2576 (K670M/M671L) mouse (Hsiao et al., 1996). Tg2576 mice over-express the 695-amino acid isoform of human Alzheimer APP containing a Lys670 --> Asn and Met671 --> Leu mutations. Similar to the PDAPP transgenic mouse, heterozygous Tg2576 mice produce a moderate fivefold increase in Aβ40 and a 14-fold increase in Aβ42/43 compared with wild type endogenous murine Aβ (Hsiao et al., 1996). In addition, Tg2576 mice develop amyloid plaques

in the cortex, hippocampus and cerebellum. The learning and memory impairments in spatial reference and alternation task do not appear until 9 to 10 months old (Kobayashi & Chen, 2005).

Shortly after the introduction of Tg2576, Sturchler-Pierrat et al. constructed two transgenic mouse lines developing pathological features reminiscent of AD: APP22 and APP23 (Sturchler-Pierrat et al., 1997). APP23 (K670N/M671N) model produces higher levels of human APP than Tg2576 model, resulting in an early formation of amyloid plaques in the neocortex and hippocampus. In APP23 transgenic mice, elevated tau phosphorylation and mild cortical neuronal loss are demonstrated biochemically in 6 month old animals and increase with age. This is the first APP transgenic mouse model to produce the tau pathology (Calhoun et al., 1998; Sturchler-Pierrat et al., 1997). However, APP22 mice that express human APP with the combined Swedish and London (V717I) mutations do not develop plaques until 18 months of age (Sturchler-Pierrat et al., 1997).

Another mutant mouse line, TgCRND8, exhibits dysfunction in both β-secretase and γ-secretase activities, and has a very aggressive rate of Aβ deposition because it carries both the Indiana (V717F) and the Swedish (K670N/M671L) mutations (Chishti et al., 2001). Plaque formation begins at around 9 weeks in the hippocampus and cortex.

The first mouse model to examine the role of mutant PS1 (M146L) was produced by Duff et al. in 1996. Presenilin mutations caused Aβ accumulation similar to that found in FAD patients' brains (L. Liu et al., 2004), as it affects the activity of γ-secretase. γ-secretase is an atypical multimeric membrane protein complex comprised of at least four subunits including PS1 or PS2, Nct, Aph-1 and Pen-2 (Qing et al., 2008). While a single mutation in PS2 does not produce Aβ plaques in mouse brains, mutant PS2 transgenic mice have shown elevations in Aβ42/43 (Oyama et al., 1998). Similarly, transgenic animals over-expressing human PS1 show high levels of

Aβ42, but amyloid depositions or behavioural alterations do not develop in these animals (Kobayashi & Chen, 2005).

The PS1 knockout mice quickly die after birth, suggesting that PS1 plays an important role in development. PS1 deficiency disrupts early embryogenesis, which results in severe skeletal abnormalities and prenatal death. Severe neuronal loss and hemorrhages are found in the brains of these mice (Shen et al., 1997). Today, a few types of PS1 and PS2 transgenic mice survive past birth and can be used to investigate the biological role of presenilins.

The first double transgenic mouse model of AD was produced to modulate the amyloid accumulation by crossing human PS1 mutation with Tg2576 transgenic mice, also known as the APP/PS1 double transgenic mouse model. Crossing the M146L mutation in PS1 with the Tg2576 transgenic mouse significantly accelerates the rate of Aβ deposition in the brain, and produces 5 times higher fibrillogenic Aβ42 species by 6 months of age than age matched controls (Holcomb et al., 1998). However, stereological methods demonstrated no significant neuronal loss (J. He et al., 2009).

Another multi-gene APP/PS1 mouse model, known as the PSAPP mouse model, was developed by Dineley *et al.*. The PSAPP is developed by crossing a different mutation in the human PS1 (A246E) with the Tg2576 mouse. In these mice, amyloid depositions occur at 7 months old, much earlier than in the Tg2576 mice (Dineley, Xia, Bui, Sweatt, & Zheng, 2002).

There are many other multi-gene transgenic mice models that can also offer insight into the mechanism of AD. A mouse model combining Tg2576 with a nitric oxide synthase 2 knock-out model demonstrates Aβ plaques, tau hyperphosphorylation and neuronal loss in mouse brains (Colton et al., 2006). A model of vascular dementia, APPSwDI (K670M/N671L, E693Q, and D694N), shows a rapid progression of Aβ deposition particularly associated with cerebral

microvascular accumulation (Davis et al., 2004). It has been suggested that the increased rate of cerebral accumulation is due to deficient clearance of Dutch/Iowa mutant Aβ from brain across the blood-brain barrier (Davis et al., 2006). This is one of the first models to mimic the deficiency of Aβ clearance, which closely relates to what happens in human AD patients.

Ultimately, these transgenic mouse models of AD provide researchers with opportunities to investigate the underlying mechanisms of AD, and develop therapeutic agents which effectively target these processes.

1.2 Amyloid cascade hypothesis

The data suggest that abnormal processing of APP and the toxicity of the Aβ peptide are central to the development of dementia in the elderly. The original 'amyloid cascade hypothesis' was first formulated almost three decades ago (J. A. Hardy & Higgins, 1992). The prevailing amyloid hypothesis posits that accumulation of Aβ peptides, particularly Aβ42 which is more hydrophobic and aggregation prone, is the primary influence driving a pathological cascade resulting in neurodegeneration in AD (J. Hardy & Selkoe, 2002). There is genetic and biochemical evidence to support the amyloid cascade hypothesis. The most well documented fact is that mutations of APP cause neuropathological changes associated with AD including both amyloid plaques and neurofibrillary tangles, thus leading to the clinical features of AD in early onset FAD. Also, the *APOE* genotype is related to the degree of deposition of Aβ peptide in the cerebral cortex before AD symptoms arise (Polvikoski et al., 1995). Additionally, both *in vitro* and *in vivo* models expressing a mutant form of the human *APP* gene result in increased Aβ generation consistent with that in AD patients (Ding et al., 2008; J. He et al., 2009; Hsiao et al., 1996; Qing et al., 2008). The evidence suggests that APP processing may start at an early stage in the pathogenesis of AD, however, the precise role of Aβ in AD pathology remains an open

question because amyloid plaques may accumulate up to a decade before there is any observable AD related symptoms in clinical populations (G. P. Morris, Clark, & Vissel, 2014).

In spite of extensive basic and clinical research investigating mechanisms responsible for amyloid deposition, there remains no FDA approved treatment that effectively alters Aβ pathology in clinical populations. Furthermore, there are neurodegenerative dementias well described in the absence of amyloid pathology such as frontotemporal dementia (FTD) emphasizing that amyloid accumulation is not an obligatory feature of neurodegeneration or dementia. As stated previously, patients may exhibit amyloid deposition for up to 10 years before any overt diagnosis of AD. As a result, it is not surprising that neither the number of amyloid plaques in brains nor the regional distribution of amyloid plaques correlates well with the pattern or degree of cognitive function impairment that patients experience clinically (Schmitz et al., 2004). Consistently, some transgenic AD mice overexpressing mutant human APP often do not display clear-cut neuronal loss along with the progressive Aβ deposition (Games et al., 1995; Hsiao et al., 1996). The aforementioned discrepancies in the pathophysiology of AD suggests that other pathological mechanisms instead of Aβ may trigger the onset of the disorder, as well as drive the progression of the disease.

1.3 Inflammation in Alzheimer's disease

In addition to amyloid deposition and NFTs, the inflammatory response has been documented in multiple studies of postmortem tissues of AD patients and in animal models of AD (Griffin et al., 1989). Acute inflammation in the brain is a well established cellular cascade in response to infection, toxin, and other insults. However, the prolonged process seen in the AD brains is characterized by disruption of the equilibrium of anti-inflammatory and proinflammatory signaling resulting in chronic inflammation, known as neuroinflammation (Grammas, 2011).

This chronic neuroinflammation is attributed to the activation of glial cells, and the release of numerous cytokines and chemokines. However, this observed sustained neuroinflammatory response is not exclusive to AD. Many studies have also demonstrated microglial activation and elevated inflammatory markers in the brain of patients with Parkinson's disease (PD) (Herrero, Estrada, Maatouk, & Vyas, 2015; Q. Wang, Liu, & Zhou, 2015), traumatic brain injury associated with chronic traumatic encephalopathy (CTE) (Faden & Loane, 2015), amyotrophic lateral sclerosis (ALS) (McCombe & Henderson, 2011), and multiple sclerosis (MS) (W. W. Chen, Zhang, & Huang, 2016). It is increasingly accepted that a sustained immune response is one of core features of neurodegenerative disorders (Amor et al., 2014; Amor, Puentes, Baker, & van der Valk, 2010; Glass, Saijo, Winner, Marchetto, & Gage, 2010; Griffin, 2006; Wyss-Coray et al., 2002).

Recent evidence on neuroinflammation has provided new understanding of the sustained inflammatory response in the brain of patients with AD. For a long time, it was thought to be merely reactive to the neuronal loss occurring in the affected brains. However, extensive research has now demonstrated that a persistent immune response in the brain also played an important role in both Aβ and NFT pathologies. Research has even suggested that the inflammatory response may act as a potential bridge between the initial Aβ pathology and later development of NFT pathology (Garwood, Pooler, Atherton, Hanger, & Noble, 2011; Kitazawa, Yamasaki, & LaFerla, 2004). The following sections will highlight some of the recent data indicating the role of neuroinflammation in AD and its potential role in driving Aβ pathology and progression.

1.3.1 Overview

The first observation of the association between immune responses and Aβ pathology dates back to the 1980s (Griffin et al., 1989; Rogers, Luber-Narod, Styren, & Civin, 1988). Rogers *et al*. found immune-related proteins and cells located within close proximity to Aβ plaques. Since then, several large epidemiological and observational studies demonstrated that chronic use of anti-inflammatory therapies reduced the risk for developing AD in patients with rheumatoid arthritis (Beard, Waring, O'Brien, Kurland, & Kokmen, 1998; Breitner et al., 1994). With the development of animal models of AD, a number of preclinical studies have replicated the observation utilizing transgenic animal models and demonstrated that anti-inflammatory treatment can reduce Aβ pathology (McGeer & McGeer, 2007). However, a meta-analysis based on several clinical trials on the effects of non-steroidal anti-inflammatory drugs (NSAIDs) in AD showed variable outcomes without convincing evidence of benefit (Miguel-Alvarez et al., 2015).

Despite the inconclusiveness of human trials, research on neuroinflammation has never ceased its progress. Increasing evidence has demonstrated that uncontrolled glial activation and neuroinflammation in the AD brain may contribute independently to neuronal dysfunction and cell death (Akiyama et al., 2000; Wyss-Coray & Mucke, 2002). Evidently, the severity of glial activation correlates well with both the extent of brain atrophy (Cagnin et al., 2001) and cognitive decline (Parachikova et al., 2007) in patients with AD. The majority of transgenic rodent models of AD also exhibited substantial reactive gliosis and accumulation of activated astrocytes in affected brain regions (Noble, Hanger, & Gallo, 2010; Schwab, Klegeris, & McGeer, 2010). Interestingly, these neuroinflammatory features are often observed before the first appearance of tangle pathology in a tau transgenic mouse model (Garwood, Cooper, Hanger, & Noble, 2010; Schindowski et al., 2006). More importantly, the degree of activation of

inflammatory cells also correlates with neuronal death and other pathological development in the tau transgenic mouse models of AD (Garwood et al., 2010; Parachikova, Vasilevko, Cribbs, LaFerla, & Green, 2010).

Both preclinical data and various epidemiological studies serve as the bedrock of support for neuroinflammation playing a major role in the pathogenesis of AD. Unlike the amyloid hypothesis, neuroinflammation is not typically considered to cause AD on its own but rather functions as a potential risk factor associated with AD. Despite that large epidemiological studies have shown a possible association between suppression of systemic inflammation and reduced risk for AD (in t' Veld et al., 2001; Vlad, Miller, Kowall, & Felson, 2008). The diverse physiological functions of glial activation might confound the interpretation of experimental investigations and/or clinical observations related to AD pathology, which could explain the variable outcomes from human trials.

1.3.2 Microglia and astrocytes

Microglia represents a large portion of cells in the central nervous system (CNS). They are exclusively distributed in brain and spinal cord, which represent about 5–20% of the total glial cell population (Lawson, Perry, Dri, & Gordon, 1990). Microglia function as resident immune cells of the brain and constantly monitor the cerebral microenvironment to resist pathogens and heal injuries (V. H. Perry, Andersson, & Gordon, 1993). Microglia in a healthy brain are inactive and in a “resting” state. In this state, its cell somas are small and stationary with cell processes extended (Glenn, Ward, Stone, Booth, & Thomas, 1992). They are constantly surveying their surrounding environment and communicating with neurons and other glial cells such as astrocytes via many different signaling mechanisms (Davalos et al., 2005). Upon insults such as invasion, injury, or disease, microglia transition into an activated state causing a number of

morphological changes including retraction of processes and enlargement of the cell somas, and migration (Davalos et al., 2005).

The role of microglia in the development and/or progression of AD is somewhat controversial. It is hypothesized that the activation of microglia is driven by the presence of Aβ in AD. Activated microglia respond to Aβ and migrate to the amyloid plaque resulting phagocytosis of Aβ. The microglia mediated phagocytosis of Aβ is considered to be one of the key mechanisms of the initial defense in the brain against the toxic accumulation of Aβ (Wyss-Coray et al., 2003; H. Zhang et al., 2014), which is believed to be a protective mechanism, at least in younger animals or at the early stage of the disease (Hickman, Allison, & El Khoury, 2008). However, these activated microglia become enlarged and are no longer able to process Aβ after prolonged periods of stimuli (Hickman et al., 2008). Studies have demonstrated that the sustained activation of microglia reduces their efficiency for binding and phagocytosis of Aβ and decreases Aβ degrading enzyme activity of microglia, which in turn leads to an accumulation of amyloid plaques (Krabbe et al., 2013; Michelucci, Heurtaux, Grandbarbe, Morga, & Heuschling, 2009). On the contrary, the ability of microglial to produce proinflammatory cytokines remains unchanged after they are activated (Hickman et al., 2008). As a result, as the disease progresses, the overall clearance of Aβ decreases while the immune response continues simultaneously, which damages neurons and contributes to neurodegeneration, subsequently leading to the activation of more microglia (Hickman et al., 2008).

In addition to the influence of microglia, the important role of astrocytes in modulating Aβ-induced neuronal death has also recently been illustrated (Jana & Pahan, 2010). Astrocytes are the major glial cell subtype and the most numerous cells in the CNS which greatly outnumber neurons by 10-100 folds (Raff, Abney, Cohen, Lindsay, & Noble, 1983). Similar to microglia,

astrocytes possess a number of important structural and physiological functions (Nagele, Wegiel, Venkataraman, Imaki, & Wang, 2004). Astrocytes are important partners of neighboring cells, including neurons, epithelial cells in the vasculature, and other glial cells. They actively constitute the so-called “tripartite synapse” with neuronal cells (Volterra & Meldolesi, 2005). Their interaction with neurons at the synapse modulates synaptic function and plasticity. More interestingly, they only play structural or metabolic supportive functions, but are also active participants in the regulation of neuronal activity in the brain. One of the central functions of astrocytes is the release and uptake of gliotransmitters/neurotransmitters in the neuronal synaptic cleft. Moreover, like microglia, astrocytes are essential for host defense mechanisms and respond to a variety of stimuli.

Both microglia and astrocytes release a myriad of pro- and anti-inflammatory cytokines, including interleukins (ILs), interferons (IFNs) and tumor necrosis factors (TNFs), as well as chemokines, a family of small proinflammatory cytokines that includes macrophage inflammatory proteins and monocyte chemoattractant proteins (Whitney, Eidem, Peng, Huang, & Zheng, 2009). The evaluation of these cytokines has become another important part of AD inflammation investigations.

1.3.3 Proinflammatory signaling: IL-1β

Interleukin-1β (IL-1β) is a member of the interleukin 1 family of cytokines and is a key mediator of the inflammatory response. It is a proinflammatory cytokine and has been described as a “master regulator” in the brain inflammatory cascade because it is able to regulate the expression of other proinflammatory cytokines, including TNFα and IL-6 (Basu, Krady, & Levison, 2004). It is upregulated early in AD patients which is believed to be crucial for amyloid deposition and disruptions to IL-1β can delay the onset of neuroinflammation and neurodegeneration (Akiyama

et al., 2000). IL-1β binds to specific IL-1β receptor which is expressed throughout the whole brain but dentate gyrus and pyramidal cells in the hippocampus have the greatest concentration. Levels of IL-1β are significantly elevated in both prefrontal cortex and hippocampus in the brains of AD patient, which are areas affected in the early stage of AD (Farrar, Kilian, Ruff, Hill, & Pert, 1987).

In addition to its proinflammatory properties, IL-1β has been shown to directly regulate the synthesis of APP in glial cells. It promotes amyloidogenic processing of APP through upregulation of γ-secretase complex activity via the activation of protein kinase C (Liao, Wang, Cheng, Kuo, & Wolfe, 2004). The ability of IL-1β to increase Aβ burden and plaque deposition creates a forward feed cycle with further activation of microglia and more IL-1β production, resulting in chronic self-sustaining inflammatory reactions, leading to more neuronal damage (Paradisi, Sacchetti, Balduzzi, Gaudi, & Malchiodi-Albedi, 2004; V. H. Perry, Nicoll, & Holmes, 2010).

1.3.4 Proinflammatory signaling: TNFα

TNFα is another important cytokine which is produced by many different types of cells in the body. Similar to IL-1β, monocytic lineage cells including macrophages, astroglia and microglia, are the primary synthesizers of TNFα (Pfeffer et al., 1993). It plays a central role in both initiation and regulation of the inflammatory cascade during the acute phase reaction (Akiyama et al., 2000; Fillit et al., 1991). The release of TNFα recruits more immune cells in response to a challenge via increasing vascular endothelia adhesion molecules (R. T. Perry, Collins, Wiener, Acton, & Go, 2001).

TNFα exerts its biological functions by directly binding to two transmembrane receptors: TNF receptor 1 (TNFR1), also known as p55 or p60, and TNF receptor 2 (TNFR2), also known as p75

or p80 (Granic, Dolga, Nijholt, van Dijk, & Eisel, 2009). TNFR1 is constitutively expressed in most cells, whereas TNFR2 is highly selectively expressed in cells of the immune system. TNFα binding to TNFR1 activates a large number of inflammatory responses. The intracellular domain of the TNFR1 then recruits several adaptor proteins forming a complex which subsequently activates the IkappaB kinase (IKK) complex. The activation of IKK complex is responsible for the translocation of nuclear factor kappa B (NF-κB) subunits into the nucleus triggering apoptotic gene transcription (Z. J. Chen, 2005; Sorriento et al., 2008).

In addition to activation of the NF-κB pathway, TNFα can directly affect Aβ production by upregulating the production of β-secretase and/or increasing γ-secretase activity (Liao et al., 2004). Conversely, deletion of TNFR1 in APP transgenic mice resulted in reduced microglial activation and less amyloid plaques (P. He et al., 2007).

1.3.5 Proinflammatory signaling: NF-κB

NF-κB belongs to a family of inducible transcription factors and has been identified to regulate a large array of genes involved in various processes of the immune and inflammatory responses (Oeckinghaus & Ghosh, 2009). The mammalian NF-κB family is composed of five structurally related units, namely NF-κB1 (also known as p50/p105), NF-κB2 (also known as p52/p100), RelA (also known as p65), RelB and c-Rel (Zheng, Yin, & Wu, 2011). These members can form various homo- or hetero-dimer to mediate transcription of target genes by binding to a specific DNA element and/or κB enhancer (S. C. Sun, Chang, & Jin, 2013). These proteins normally stay inactive in the cytoplasm by a family of inhibitory proteins such as IκB family members. To date, IκBα is the most well studied member in the IκB family.

NF-κB can be activated via different mechanisms including ligands of various cytokine receptor and aforementioned TNFR1 signaling pathway. The primary mechanism involves the

degradation of IκBα triggered through phosphorylation by the IKK complex (Oeckinghaus & Ghosh, 2009). As previously mentioned, the IKK complex can be activated by TNFα binding to TNFR1 triggering a serial signaling pathway intracellularly. The activated IKK complex phosphorylates IκBα at two N-terminal serines, which leads to the degradation of IκBα in the proteasome by an ubiquitin-dependent pathway. Without the presence of inhibitory proteins in the cytoplasm, the NF-κB dimers become active and bind to specific DNA elements resulting in the transcription of target genes (Hayden & Ghosh, 2008). The activated NF-κB translocates to the nucleus which leads to expression of a number of inflammatory genes including cyclooxygenase (COX), IL-1β and TNFα (X. Zhang et al., 2009).

Given its central role in mediating proinflammatory gene induction, NF-κB has been extensively studied in AD. Activated NF-κB was found in both neurons and glial cells surrounding amyloid plaques in the brains of AD patients (Kaltschmidt, Uherek, Volk, Baeuerle, & Kaltschmidt, 1997; Mattson & Camandola, 2001). In addition, a number of DNA binding sites by NF-κB have been identified to be near the BACE1 promoter, which suggests NF-κB in turn directly regulates BACE1 transcription, resulting in a vicious cyclical loop of exacerbating both neuroinflammation and amyloid pathology in AD (Sambamurti, Kinsey, Maloney, Ge, & Lahiri, 2004). *In vivo* studies have shown that modulating NF-κB activity with either a direct NF-κB inhibitor or certain NSAIDs resulted in lower amyloid burden (Eriksen et al., 2003; Tobe et al., 2003).

1.4 Quetiapine and AD

Quetiapine (*Seroquel*®) is a novel atypical antipsychotic drug that was approved for the treatment of patients with schizophrenia (Purdon, Malla, Labelle, & Lit, 2001; Velligan et al., 2002). Clinically, quetiapine is also used to treat psychosis in AD as well as dysfunctional

cognition in Parkinson's disease (Juncos et al., 2004; Madhusoodanan, Shah, Brenner, & Gupta, 2007). Pharmacologically, it is a dibenzothiazepine, and competitively antagonizes both dopamine-2 (D2) and serotonin-2 (5-HT2) receptors, but has a much higher affinity for 5-HT2 receptors than D2 receptors (Gefvert et al., 2001). In addition to the high binding ratios of 5-HT2:D2 receptors, quetiapine also has affinity for 5-HT1A, 5-HT6, histamine-1 (H1), α1- and α2-adrenergic receptors. It also has some moderate inhibitive ability on serotonin reuptake (Hirsch, Link, Goldstein, & Arvanitis, 1996).

The neuroprotective effects of quetiapine have been extensively studied for the past 2 decades. *In vitro* experiments have demonstrated that the neuroprotection of quetiapine along with other atypical antipsychotics may be partially due to their antioxidant properties (Bai et al., 2002; H. Wang, Xu, Dyck, & Li, 2005). Until recently, one study showed that quetiapine, but not other antipsychotics (ziprasidone, perospirone), significantly inhibited both NO generation and TNFα release from activated microglia culture (Bian et al., 2008), suggesting quetiapine may have an anti-inflammatory effect via inhibition of microglial activation. Interestingly, Kim *et al.* (H. Kim et al., 2012) have also demonstrated that quetiapine treatment significantly suppressed IL-6, IL-17 and Prostaglandin E2 (PGE_2) in collagen-induced arthritis in mice.

In our previous *in vivo* studies, quetiapine decreased the accumulation of activated astrocytes and microglia in a cuprizone induced demyelination model of multiple sclerosis (MS) (Y. Zhang et al., 2008), modulated immune responses in an experimental autoimmune encephalomyelitis (EAE) model (Mei et al., 2012), and inhibited NF-κB p65/p50 expression in the brains of ischemic mice (Bi et al., 2009). Although quetiapine has some beneficial effects on cognition in AD mice (Zhu et al., 2013), there is no data published with respect to its effect on glial activation and neuroinflammation in AD mice.

1.5 Overall goals and hypotheses of this research

The overall **goals** of this thesis are to further characterize the relationship between neuroinflammation, amyloid pathology and cognitive changes in the progression of AD, thereby expanding our understanding of the role of neuroinflammation in AD pathogenesis and to investigate the effects of quetiapine on the inflammatory process in the APP/PS1 mouse model.

The main hypothesis is that neuroinflammation, resulting from uncontrolled glial activation, modulates the progression of AD. We hypothesize that the presence of glia (microglia and astrocytes) mediates the Aβ induced neurotoxicity in primary culture and that the release of soluble inflammatory factor(s) from microglia and/or astrocytes may accompany these events. We further hypothesize that a possible anti-inflammatory agent, quetiapine, reduces glial inflammatory responses and associated neuronal loss *in vitro*. We will next examine that the effects of quetiapine on gliosis/neuroinflammation and AD-like behaviours in the APP/PS1 mouse model.

The **objectives** of this project were to:

I. Determine the relationship between neuroinflammation and AD-like behaviours using the amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mouse model of AD. Monitoring of changes in gliosis/neuroinflammation will be conducted using morphological and biochemical analysis.

II. Determine the influence of glia (microglia and astrocytes) in mediating Aβ-induced neuronal death. Identify whether the release of inflammatory mediators from glia is associated with Aβ-induced neuronal death.

III. Examine whether the atypical antipsychotic drug, quetiapine, suppresses these glial inflammatory responses in Aβ treated glial enriched primary cultures and reduces or

abolishes glial mediated Aβ-induced neuronal death. Determine the mechanisms by which quetiapine inhibits these soluble inflammatory factors in Aβ treated glial enriched primary cultures.

IV. Determine whether or not quetiapine provides beneficial effects in the APP/PS1 transgenic mice through its anti-inflammatory effects. We will examine the effects of quetiapine on gliosis/neuroinflammation and AD-like behaviours in the APP/PS1 transgenic mice.

Chapter 2

The role of neuroinflammation and amyloid in cognitive impairment in an APP/PS1 transgenic mouse model of Alzheimer's disease

2.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is mainly characterized by memory loss. Although the exact mechanisms of AD have not been fully understood, amyloid β protein (Aβ) is believed to be the causative agent of Alzheimer's pathologies (J. Hardy & Selkoe, 2002). In addition to extracellular Aβ plaques and intracellular neurofibrillary tangles, neuroinflammation also is an early pathological manifestation in AD brains (Akiyama et al., 2000).

Robust activation of microglia is often in close association with amyloid plaques which are also surrounded by reactive astrocytes (Ho, Drego, Hakimian, & Masliah, 2005; Itagaki, McGeer, Akiyama, Zhu, & Selkoe, 1989). Nevertheless, the diverse physiological functions of glial activation might be a double-edged sword complicating AD pathologies and clinical presentations. Initial glial activation is believed to be one key defensive mechanism of the brain eliminating the toxic Aβ (H. Zhang et al., 2014). Yet, this process becomes notably vicious by creating chronic and self-sustaining inflammatory reactions as disease progresses, leading to further neurotoxic damage (Paradisi et al., 2004; V. H. Perry et al., 2010). Epidemiological studies in human have indicated that increased inflammatory responses increased the risk of developing a sporadic late-onset AD (Eikelenboom et al., 2012). Whereas a long-term suppression of inflammation may reduce risk for AD and possibly delay the onset of the disease

(in t' Veld et al., 2001; Vlad et al., 2008). In animal studies, inhibition of proinflammatory signaling of the cytokines interleukin 12 (IL-12) and interleukin 23 (IL-23) in APP/PS1 mice has been shown to reduce glial activation and cognitive decline (Vom Berg et al., 2012). All this evidence suggests that neuroinflammation characterized by frank microglial and astroglial activation and subsequent upregulation of proinflammatory mediators might be a crucial event in the pathogenesis of AD (Wyss-Coray & Mucke, 2002).

Although it is well documented that neuroinflammatory processes exist in the brains of AD patients (Heneka, O'Banion, Terwel, & Kummer, 2010), whether the progression of neuroinflammation is closely related to cognitive decline has not been fully elucidated. Since these underlying mechanisms rarely occur in isolation, a better understanding of the exact contribution of neuroinflammation to cognitive decline along with the ongoing amyloidogenic process in AD may be beneficial in terms of the prevention and treatment of this devastating disease. To clarify the relationships across behavioural changes, amyloid deposition, and neuroinflammation in AD, a double transgenic mouse model expressing both mutant human amyloid precursor protein (APP) and mutant presenilin 1 (PS1) was employed. Both amyloid pathologies and behavioural impairments in 6 and 9 month old transgenic mice have been described in our previous study (J. He et al., 2009). In the present study, we systematically characterized the changes in behavioural abnormalities, brain amyloid pathologies and neuroinflammation in the APP/PS1 double transgenic mice between the ages of 2 to 22 months.

The APP/PS1 double transgenic mice by crossing the PS1 mouse with the Tg2576 transgenic mouse show significantly accelerated rate of Aβ deposition in the brain, and produce more of the fibrillogenic $A\beta_{1-42}$ species which are 5 times higher by 6 months of age than age matched controls (Holcomb et al., 1998). Moreover, these double transgenic mice also exhibit substantial

reactive gliosis and accumulation of activated astrocytes in affected brain regions. Robust activation of microglia has also been found in and around the area of amyloid plaques in these mice (Ho et al., 2005). However, there is no study that has thoroughly characterized the link between gliosis/neuroinflammation, amyloid pathology and animal behaviours in this AD model. A detailed understanding of the time course of these events during their progression could unveil the important question of how neuroinflammation contributes to the pathogenesis of AD.

2.2 Methods

2.2.1 APP/PS1 double transgenic mice and genotyping

The AD mouse model used was the $APP_{K670N/M671L}$/$PS1_{M146L}$ double mutant mouse, was generated by crossing single transgenic mice expressing human mutant $APP_{K670N/M671L}$ (Hsiao et al., 1996) and mutant $PS1_{M146L}$ (Duff et al., 1996). All animals received standard husbandry care during testing, including cage enrichment (PVC pipe, half a Nestlet square and/or shredded paper, and paper hut) and *ad libitum* access to food and water. All testing occurred during the light cycle. The genotypes of each mouse was determined by PCR using genomic DNA isolated from tail biopsies at weaning (21 days of age). Mice were used in pairs of age-matched transgenic or wild-type (WT) littermates at 2, 3, 5, 9, 12, and 22 months of age. All procedures with animals were performed as per the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Manitoba.

2.2.2 Y maze test

Spatial short-term memory in mice was assessed by the Y maze test as previously described (Xiao et al., 2008; Zhu et al., 2012). The Y maze consisted of three arms diverging 120° from the central point, each 5 cm wide and 35 cm in length, and shielded with 10 cm high walls (Fig. A.2). The mice were individually placed at the end of one arm and allowed to move freely through the maze during an 8 min session. Mice tended to explore the maze systematically, entering each arm in turn. The ability to alternate required that the mice know which arm they had already visited. The sequence of arm entries was recorded visually. Spontaneous alternation behaviour was defined as the entry into all three arms on consecutive choices in overlapping triplet sets. The percent spontaneous alternation behaviour was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus 2) multiplied by 100.

Mice whose total entrance number was less than 15 times during the test were not taken into the final data.

2.2.3 Morris water maze test

Spatial learning and memory was evaluated by the Morris water maze (R. Morris, 1984). Water maze testing occurred in a circular and galvanized pool (120 cm diameter × 50 cm height) with a white interior (Fig. A.3). The water temperature was kept at 20-23°C. The surface area of the water pool was divided into four equal quadrants with one visual cue for each quadrant. Mice received hidden platform trials from Day 1 to Day 4 for 4 days, and a probe trial on Day 5. In the hidden platform (acquisition) trials, the clear platform (10 cm in diameter) was placed in one of the four maze quadrants and submerged 1 cm below the water surface in a fixed position (30 cm away from the side wall) in the northwestern/third quadrant. Its location was kept constant at the same (target) quadrant throughout entire acquisition trials process. Mice were required to find the hidden platform using only distal spatial cues available in the behavioural room. A different starting position was used on each trial. They were given 60 seconds (s) to climb onto the hidden platform. Once the mice reached the platform, they were allowed to stay on it for 10 - 20 s. Then, they were taken out, dried, and placed into a separate cage for approximate 60 min before the next trial (Ding et al., 2008; Qing et al., 2008). If mice could not locate the platform within 60 s, they were guided to the platform. On reaching the platform, mice were allowed to remain on it for 10 s. In between each animal trial, the water tank was cleaned by a strainer and stirred to erase olfactory traces of the previous animal. All spatial memory scores were determined by assessing the average latency time (in seconds) and path length of a mouse reaching the hidden platform.

In the probe trial, the platform was removed from the water pool and mice were allowed to swim for 60 s to assess memory consolidation 24 h after the last hidden platform trial. The time spent in the target quadrant and the number of target area (the previous platform location) crossings were analyzed as measures of degree of spatial memory retention. Tracking of animal movement was achieved with a camcorder mounted above the tank and the data was analyzed by an observer blinded to which experimental group each animal belonged.

2.2.4 Brain tissue processing

At the completion of behavioural testing, animals were anesthetized and perfused with ice-cold 0.01 M phosphate-buffered saline (PBS, pH 7.4). Brains (excluding cerebellum, pons, and medulla oblongata) were divided sagitally. The right hemisphere was separated into the following segments: frontal, middle, and posterior cortex, hippocampus, striatum and then snap frozen and stored at -80°C for protein and other biochemistry analyses. The left hemisphere was post-fixed with freshly depolymerized 4% paraformaldehyde (PFA) in PBS for an additional 48 h at 4°C, and then was equilibrated overnight in 30% sucrose (J. He et al., 2009; Qing et al., 2008). Serial coronal sections were cut at 30 µm thickness using a freezing sliding microtome (Leica Microsystems, Wetzlar, Germany) for immunohistochemistry and histology analysis.

2.2.5 Immunohistochemistry

Sections were first incubated with 0.3% H_2O_2 in 0.01 M PBS for 30 min at room temperature to quench endogenous peroxidase activity, then blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 hour, and then incubated overnight at 4°C with anti-beta amyloid 17-24 (4G8) mouse mAb (1:500; Covance, Princeton, NJ), anti-mouse CD11b rat mAb (1:500; AbD Serotec, Raleigh, NC), anti-glial fibrillary acidic protein (GFAP) mouse mAb (1:1000; Sigma, St. Louis,

MO), and anti-NeuN mouse mAb (1:200; Millipore, Billerica, MA) respectively. After rinsing, sections were incubated with appropriate biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA) at room temperature for 1 hour. Staining was achieved with the avidin biotin complex kit (Vector Laboratories, Burlingame, CA) and visualized with 3,3-diaminobenzidine (DAB) chromogen (ThermoFisher Scientific, Waltham, MA). The immunohistochemical controls were performed as above but without addition of the primary antibodies. No positive immunostaining was found in any of the controls.

2.2.6 Immunofluorescence

Double labelled fluorescent staining was used to examine the expression of CD11b and Aβ plaques. Free floating sections were first blocked and then incubated overnight at 4°C with anti-beta amyloid 17-24 (4G8) mouse mAb (1:500; Covance, Princeton, NJ) and anti-mouse CD11b rat mAb (1:100; AbD Serotec, Raleigh, NC). After washing, sections were incubated with appropriate fluorescence-conjugated secondary antibodies for 1 hour at room temperature. Finally, Hoechst 33342 (Calbiochem, Billerica, MA) was used for nuclear staining. Slides were then sealed with the fluorescent mounting medium (DakoCytomation, Inc., Carpinteria, CA). Immunofluorescent costaining was evaluated with a fluorescent microscope (Olympus).

2.2.7 Congo red staining

The presence of amyloid in tissue sections was investigated after being stained with a commercially available amyloid stain, the Congo Red kit (Sigma–Aldrich, St. Louis, MI), according to the manufacturer's instruction. Quantifications were performed blindly in four coronal sections per animal, spaced 180 μm apart. The total number of amyloid plaques in the cortex and the hippocampus was counted manually under a Zeiss Imager-A1 microscope at 100×

magnification. The size of the total counted area was collected using a Zeiss Imager-A1 microscope and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The plaque number was reported as the total number of plaques counted in the cortex and hippocampus (J. He et al., 2009). The size of plaques was presented as the average area fraction positive to Congo red staining of the cortex and hippocampus (Toledo & Inestrosa, 2010).

2.2.8 Thioflavin T staining

In addition to Congo red staining, Aβ plaques were also identified by Thioflavin T staining. Brain sections were stained with 1% Thioflavin T solution and then visualized using a fluorescent microscope (Olympus).

2.2.9 Image analyses

Immunohistochemical slides were viewed with an Axio-Imager M2, and the Zen software for image acquisition (Carl Zeiss, Jena, Germany). The plaque number was reported as the total number of plaques counted in frontal cortex and hippocampus after Congo red or Thioflavin T staining. The percentage of the area occupied by 4G8, GFAP, and CD11b in the brain was respectively calculated for each age group using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The threshold of detection was kept consistent between each analysis.

2.2.10 Neural cell counts

Neural cell counts of cortical areas were performed at a magnification of 10× on 30 μm thick sections stained with NeuN antibody. All counts of sections from the same mouse were averaged to derive the number of neurons per mouse. All counts were performed in a blinded fashion.

2.2.11 Statistical analysis

All results were expressed as means ± standard error of the mean (S.E.M.). Analyses were performed using a one-way or two-way analysis of variance (ANOVA) followed by a Newman-Keuls *post hoc* test for multiple comparisons. A two-tailed *t*-test for independent samples was used for two-group comparisons. Differences were considered significant at $p < 0.05$. Specific details on the statistical analyses used for each test can be found in the figure captions.

2.3 Results

2.3.1 Spatial learning and memory deficits in APP/PS1 mice

Spatial short-term working memory was evaluated using a Y maze test in 3, 5, 9 and 12 month old mice. At all ages except 3 month old, APP/PS1 mice exhibited impaired short-term memory as they showed lower percentage of spontaneous alternation than age-matched WT mice, and the deficit seemed greater in older mice but there was no statistical significance (Fig. 2.1A). General locomotor activity was examined by counting total number of arm entries in the Y maze test. There was no difference between transgenic and WT mice in any of the age group (Fig. 2.1B).

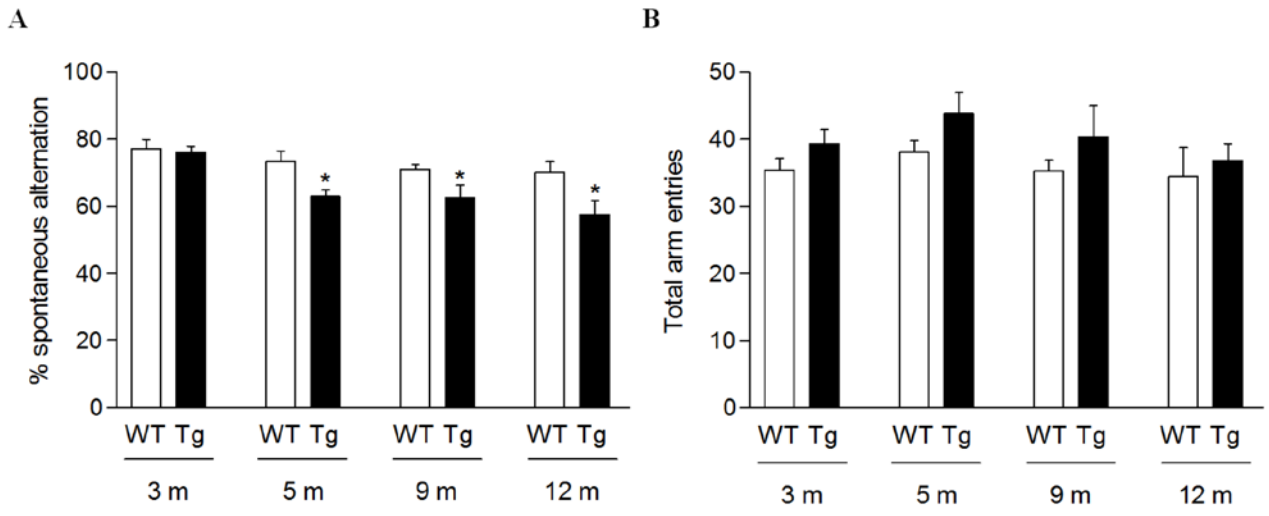


Figure 2. 1: Short term working memory in the 3, 5, 9, and 12 month old APP/PS1 and WT mice in a Y maze test. (A) Spontaneous alternations of mice in Y maze test. (B) Total arm entries of mice in Y maze test. Data are expressed as means ± S.E.M. n = 9-15 mice per group. *$p < 0.05$ vs. age-matched WT mice.

Spatial learning was assessed by the escape latency in the acquisition trials using the Morris water maze test. The mean escape latencies are shown in Figure 2.2A. There was no significant difference in escape latency over all training days between WT and APP/PS1 mice at 3 months old [genotype effect: $F(1,64) = 0.11$, $p > 0.05$; training effect: $F(3,64) = 43.84$, $p < 0.001$; genotype × training interaction: $F(3,64) = 0.59$, $p > 0.05$]. In contrast, the escape latency was longer in 5 months old transgenic mice compared with their counterparts, especially on the training day 4 [genotype effect: $F(1,72) = 2.86$, $p = 0.0951$; training effect: $F(3,72) = 17.58$, $p < 0.001$; genotype × training interaction: $F(3,72) = 0.26$, $p > 0.05$]. Similar results were observed in 9 and 12 months old APP/PS1 mice compared with their age-matched WT [9 months old: genotype effect: $F(1,80) = 5.59$, $p = 0.0205$; training effect: $F(3,80) = 9.50$, $p < 0.001$; genotype × training interaction: $F(3,80) = 0.58$, $p > 0.05$; 12 months old: genotype effect: $F(1,76) = 3.90$, $p = 0.0518$; training effect: $F(3,76) = 9.43$, $p < 0.001$; genotype × training interaction: $F(3,76) = 0.61$, $p > 0.05$].

Spatial memory retention was assessed by the time spent in the target quadrant (the platform located quadrant during the acquisition trials) and the number of target area (the previous platform location) crossings in the probe test. As shown in Figure 2B, all APP/PS1 mice except the 3 month old spent less time in the target quadrant compared with their age-matched WT mice. However, no significant difference was observed in crossing times (Fig. 2.2C).

There was no significant difference between APP/PS1 and their WT littermates in the mean swimming speed across all age groups ($p > 0.05$, respectively; data not shown). This indicates that there were no observable motor deficits associated with the water maze test and the impaired escaped latency in APP/PS1 mice was not due to motor dysfunctions.

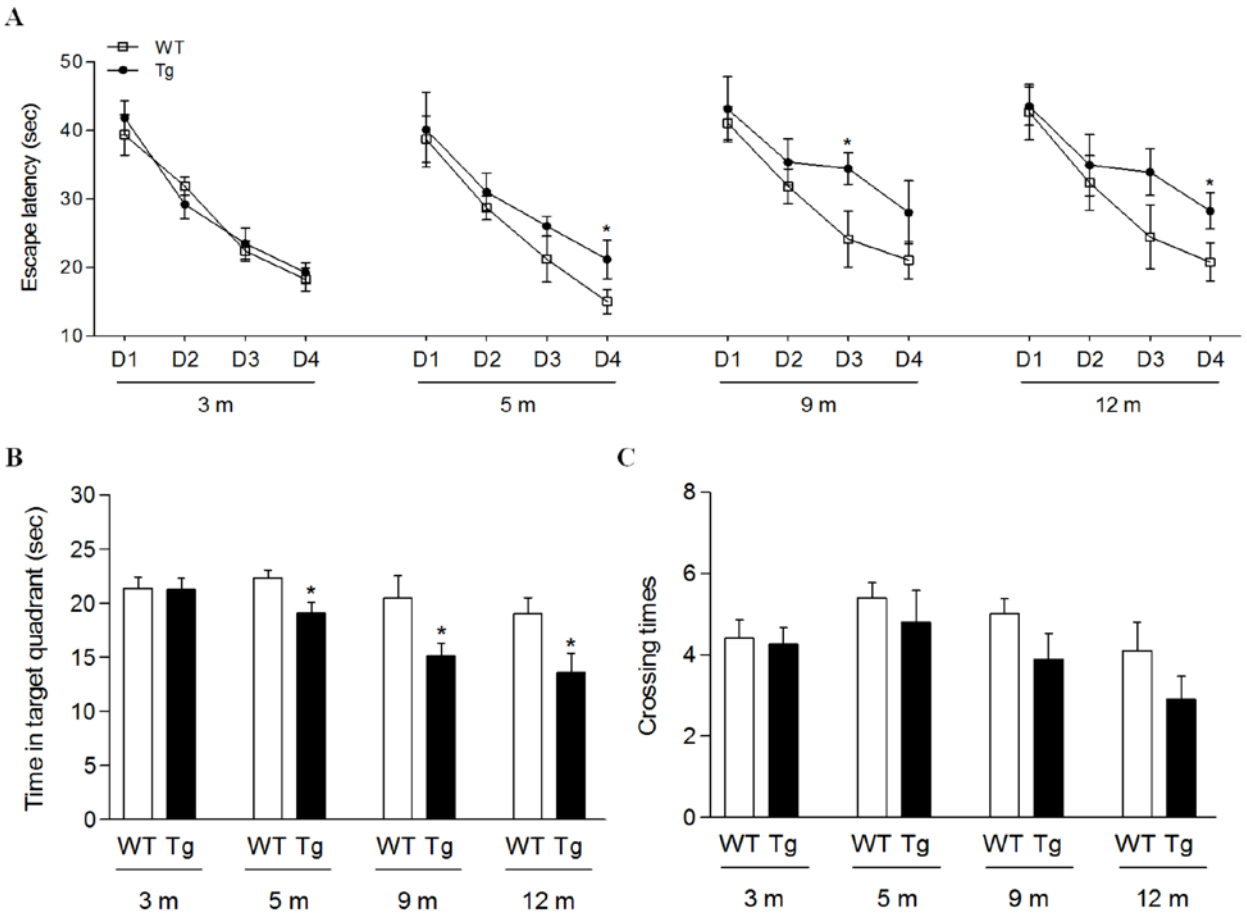


Figure 2. 2**:** Spatial memory in the 3, 5, 9, and 12 month old APP/PS1 and WT mice in a Morris water maze test. (A) Escape latency of mice in the hidden-platform test of the Morris water maze. (B) Percentage of time spent searching for the target (trained) quadrant in the probe test of the Morris water maze. (C) Number of target area (training plat-form area) crossing in the probe test. Data are expressed as means ± S.E.M. n = 8-13 mice per group. *$p < 0.05$ vs. age-matched WT mice.

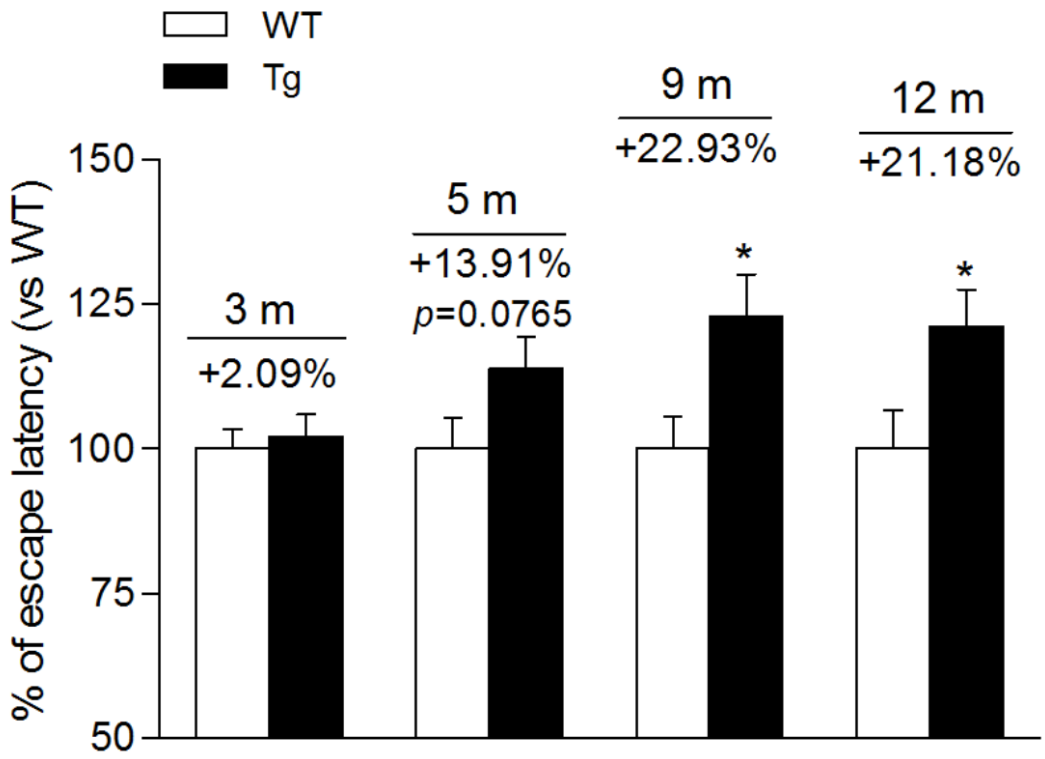


Figure 2. 3: Percentage of cognitive impairment (increase in the mean latency to reach the hidden platform in all trials of all sessions during acquisition in the Morris water maze) of APP/PS1 mice at the age of 3, 5, 9, and 12 months vs. age-matched WT mice which were defined as 100%. Data are expressed as means ± S.E.M. n = 32-52 per group. *$p < 0.05$ vs. age-matched WT mice.

APP/PS1 mice have been reported to exhibit an age-dependent deterioration in the overall performance in a water maze test (Izco et al., 2014). Thus, we averaged escape latencies to reach the hidden platform of APP/PS1 mice in all trials for each day during acquisition and compared them to their age-matched WT mice which were defined as 100%. The differences in the overall performance between APP/PS1 and their counterparts progressively increased up to the age of 9 months but then plateaued at the age of 12 months ($p < 0.0036$, Fig. 2.3) and the averaged latencies were larger in the transgenic mice (2.09% at 3 months $p > 0.05$, 13.91% at 5 months $p = 0.0765$, 22.93% at 9 months $p < 0.05$, 21.18% at 12 months $p < 0.05$, Fig. 2.3).

2.3.2 Cerebral Aβ deposition in APP/PS1 mice

Aβ plaque deposits in the cortex and hippocampus were assessed by 4G8 immunohistochemistry (Fig. 2.4A) and Congo red staining (Fig. 2.4B) in 2-22 months old APP/PS1 mice. Sections stained with the two different methods showed similar plaque profiles. Consistent with the previous report (J. He et al., 2009), amyloid plaques were observed in transgenic mice as young as 3 months of age (Fig. 2.4A) and no amyloid plaques were observed in the cortex or hippocampus of WT mice (data not shown). Both the number of plaques and plaque area fraction were progressively increased in a nearly linear fashion between 3 and 22 months of age (Fig. 2.4A, C and D). Morphologically, the majority of plaques appeared small and compact at 5 months of age in sections stained with Congo red or Thioflavin T (Fig. 2.4B). 4G8 immunostaining showed increased immunoreactivity of diffuse plaques with advancing age as 4G8 antibody stained both compact and diffuse Aβ.

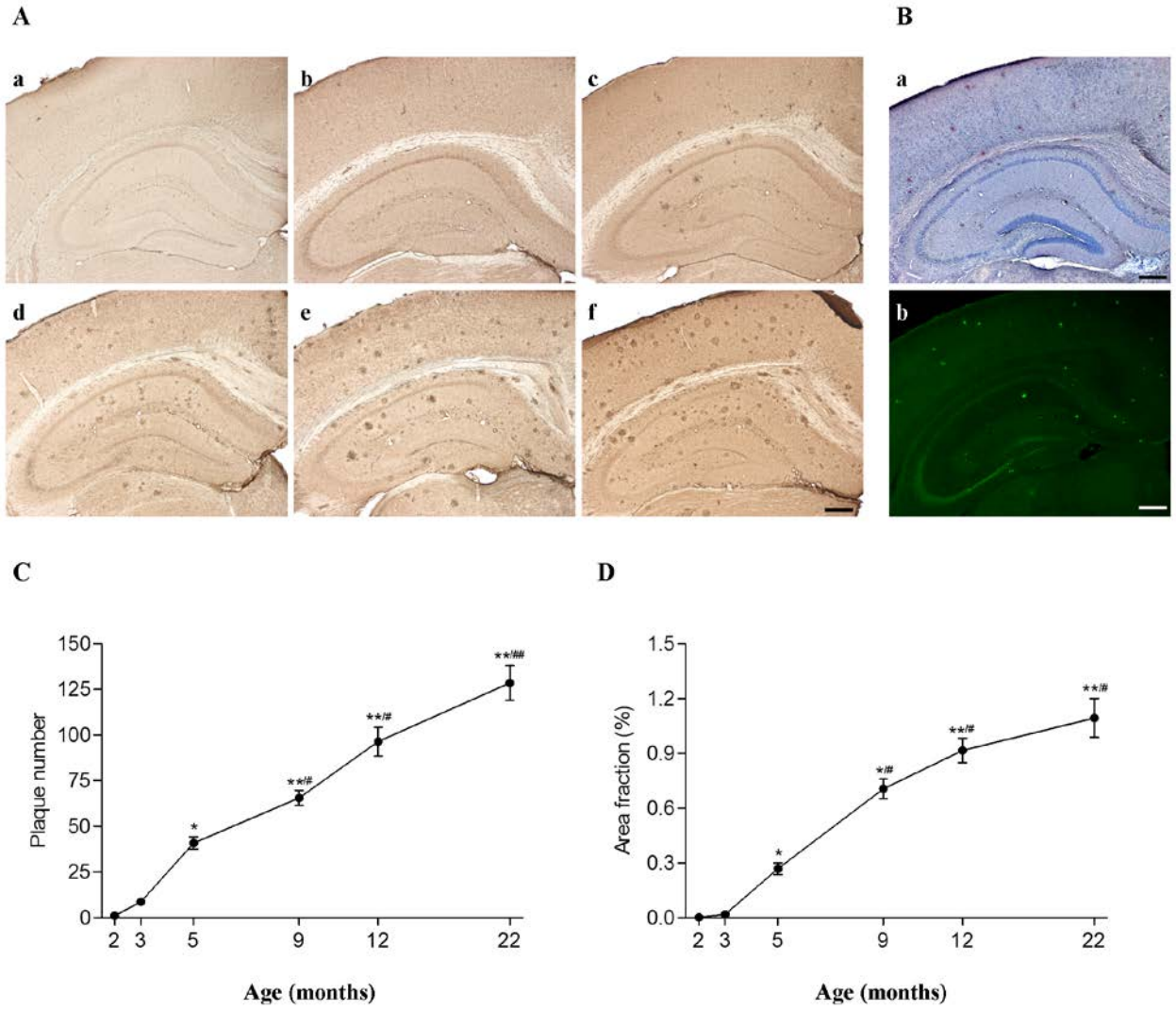


Figure 2. 4: Amyloid deposition with age in APP/PS1 mice. Coronal sections from mice at the age of 2, 3, 5, 9, 12, and 22 months were labeled with an Aβ-specific monoclonal antibody 4G8 or Congo red or Thioflavin-T. (A) Representative illustrations of amyloid plaques stained with 4G8 at the age of 2 (a), 3 (b), 5 (c), 9 (d), 12 (e), and 22 (f) months were shown. Scale bar represents 500 μm. (B) Representative staining of brain sections from 5-month old mice with congo red and Thioflavin-T. Scale bar represents 500 μm. (B) and (C) showed the quantification of amyloid plaques in cerebral cortex and hippocampus after 4G8 immunohistochemistry. Data were representative of 3-9 sections from 3-6 animals. One-way ANOVA followed by a Newman-Keuls

post hoc test. *$p < 0.05$, **$p < 0.01$ vs. 3 month old group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. 5 months old group.

2.3.3 Early glial response accompanies amyloid plaques

Serial adjacent sections with the same brain reference position used for Aβ deposition studies were evaluated for activation of microglia and astrocytes using anti-CD11b and anti-GFAP antibodies, respectively. Although rare, occasionally CD11b positive microglia appeared in 2 month old APP/PS1 mice. Small clustered CD11b positive microglia were first consistently detected in both cortex and hippocampus of 3 month old transgenic mice and the CD11b immunoreactivity was increased in both the number and size of clusters (Fig. 2.5A, a-f). Quantitative analyses revealed that the progressive increase of area faction occupied by CD11b positive staining shared a similar linear trend as the increase of area faction of Aβ in APP/PS1 mice between 2 and 12 months of age (Fig. 2.4C and Fig. 2.5B). Double labeling with CD11b and 4G8 confirmed that activated microglia were in close associated with amyloid plaques in most cases (Fig. 2.5A, g-j) However, the rate of increased CD11b positive staining in transgenic mice between 12 and 22 months of age was significantly slower compared to the rate of Aβ accumulation in the same period of time.

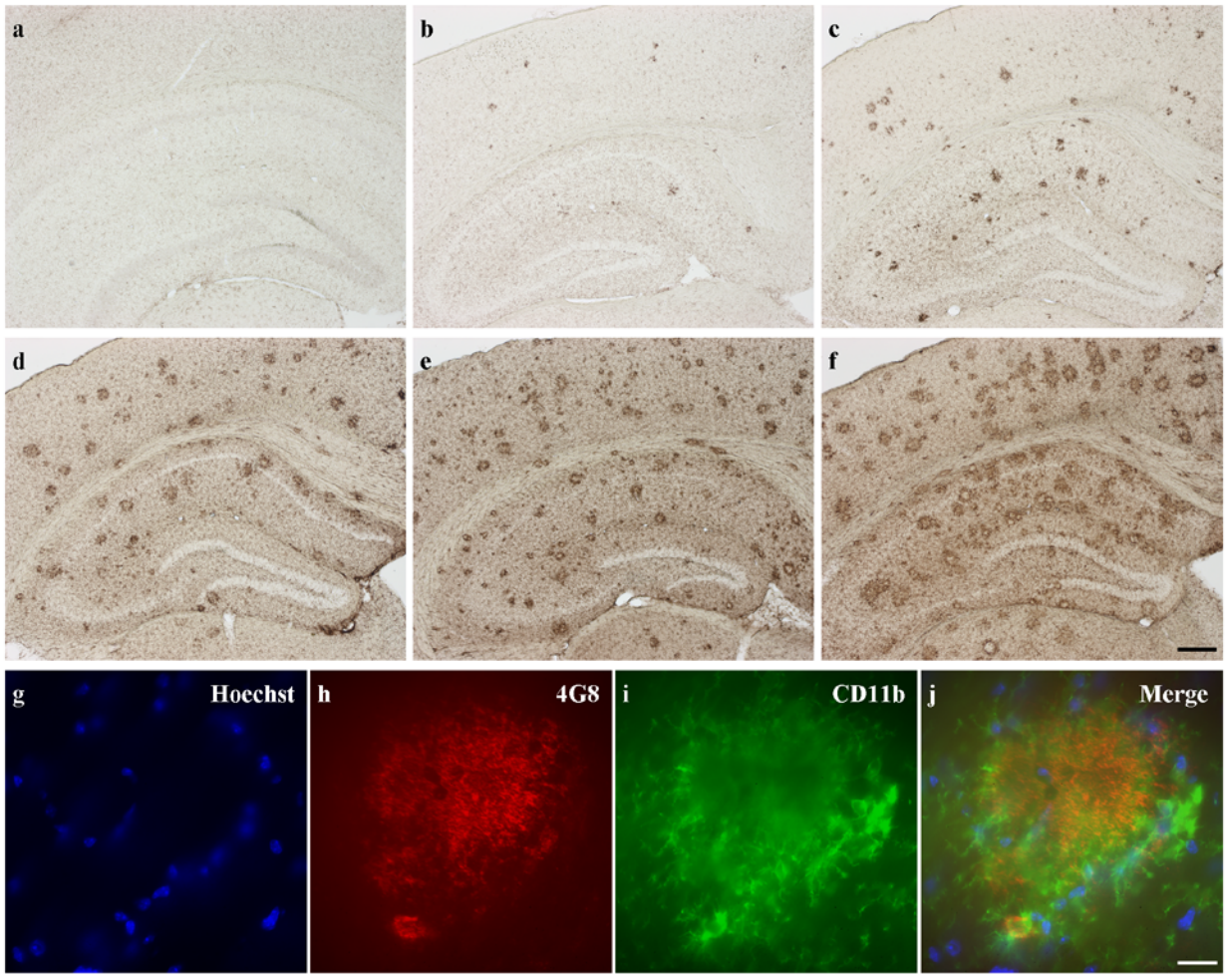
Comparable levels of GFAP positive cells in both cortex and hippocampus were observed between APP/PS1 and WT mice at 2 months old, whereas a notable increase in GFAP positive astrocytes was first detected in the frontal cortex of APP/PS1 transgenic mice compared with age-match WT counterparts at 3 months old (Fig. 2.6A). More interestingly, cortical GFAP staining of 3 and 5 months old transgenic mice (Fig. 2.6A, d and f) appeared to be focal and scattered, which may suggest that at the early stage of disease activated astrocytes were confined to smaller areas which are most likely to be around amyloid plaques. As the disease progresses, GFAP positive cells in the frontal cortices were becoming largely diffuse in 9 month old transgenic mice and older (Fig. 2.6A, h, j, and i), suggesting these diffuse astrocytes were not

associated with amyloid plaques as disease progresses. Quantification of GFAP immunoreactivity in the cerebral cortex showed GFAP positive staining was quickly increased between the age of 2 and 9 months and then leveled off from 12 months to 22 months old (Fig. 2.6B).

2.3.4 No apparent neuronal cell loss in APP/PS1 mice

To further investigate the density of neurons in APP/PS1 mice, adjacent sections with the same brain reference position were stained for the general neuronal nuclear marker NeuN. As shown in Figure 2.7, the density of NeuN-positive neurons in cortex was nearly indistinguishable between transgenic and age-matched WT mice. These data provided evidence that this APP/PS1 double transgenic mouse model does not cause any appreciable neuronal cell loss in cortex despite the presence of robust amyloid plaques, suggesting that memory deterioration might not be directly caused by neuronal degeneration.

A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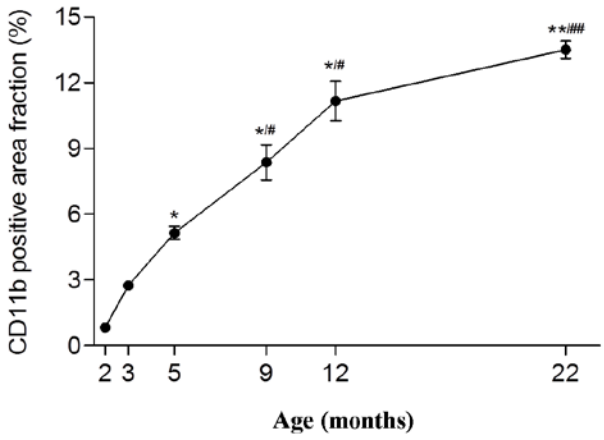
B


CD11b positive area fraction (%)
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3
6
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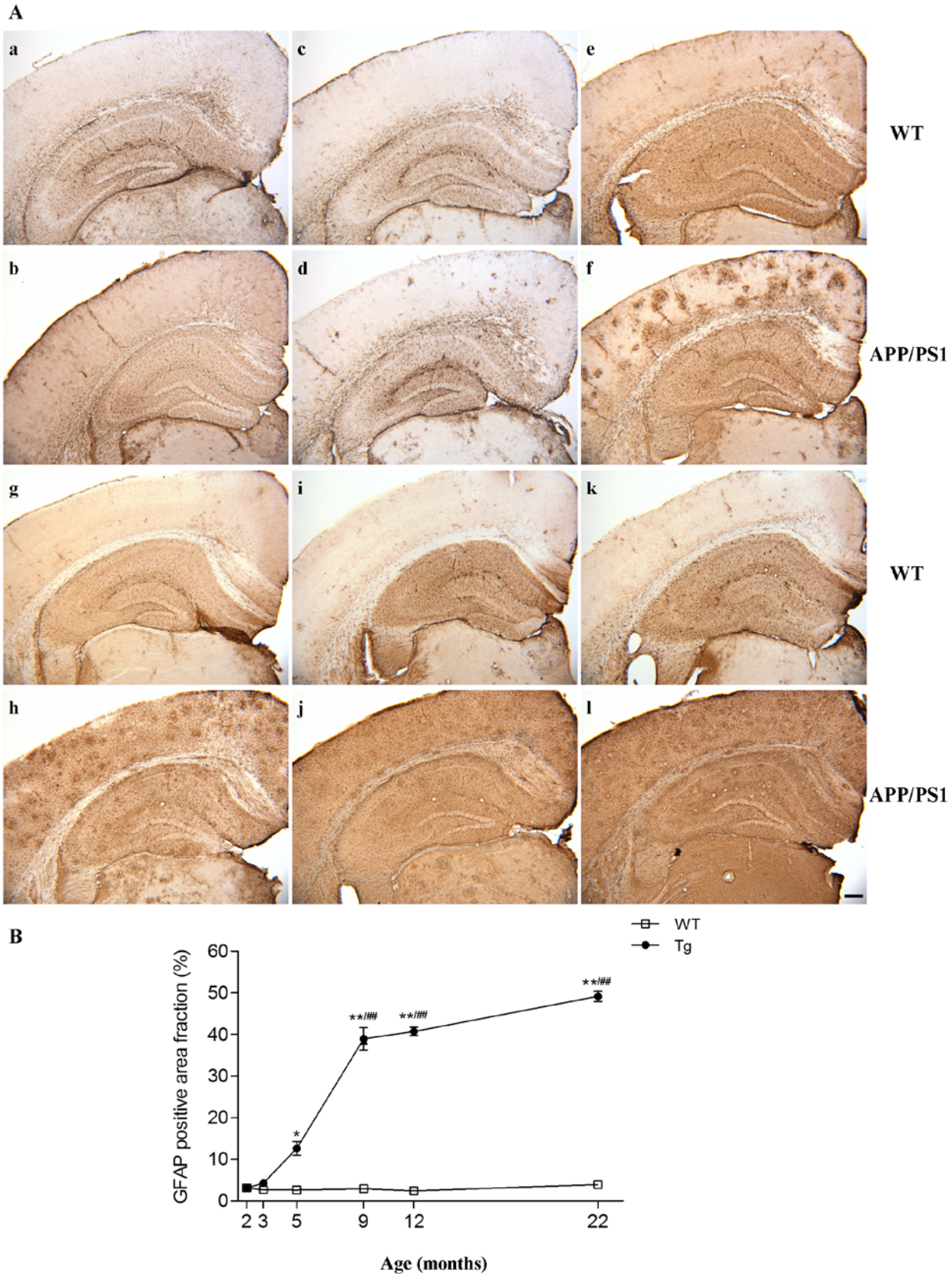
Figure 2. 5: Time-course of microglial activation in cerebral cortex and hippocampus of APP/PS1 mice. Coronal sections from mice at the age of 2, 3, 5, 9, 12, and 22 months were labeled with an anti-CD11b antibody. (A) Representative immunohistochemical staining with CD11b at the age of 2 (a), 3 (b), 5 (c), 9 (d), 12 (e), and 22 (f) months are shown. (g-j) double labeled fluorescent staining of amyloid plaque (4G8; red) and microglia (CD11b; green) in the hippocampus of mice at 12 months of age. Scale bar represents 500 μm in a-f and 20 μm in g-j. (B) Quantification of CD11b positive areas in cerebral cortex and hippocampus. Data are expressed as means ± S.E.M. n = 3-6 animals per group. One-way ANOVA followed by a Newman-Keuls *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 3 month old group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. 5 month old group.


A
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GFAP positive area fraction (%)
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2 3 5 9 12 22
Age (months)
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Figure 2. 6: Astrocyte activation in cerebral cortex and hippocampus of APP/PS1 and WT mice. Coronal sections from mice at the age of 2, 3, 5, 9, 12, and 22 months were labeled with an anti-GFAP antibody. (A) Representative immunohistochemical staining with GFAP at the age of 2 (a, b), 3 (c, d), 5 (e, f), 9 (g, h), 12 (i, j), and 22 (k, l) months are shown. Scale bar represents 200 μm. (B) Quantification of GFAP positive areas in cerebral cortex and hippocampus. Data are expressed as means ± S.E.M. n = 3-6 animals per group. One-way ANOVA followed by a Newman-Keuls *post hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 3 month old APP/PS1 mice; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. 5 month old APP/PS1 mice.

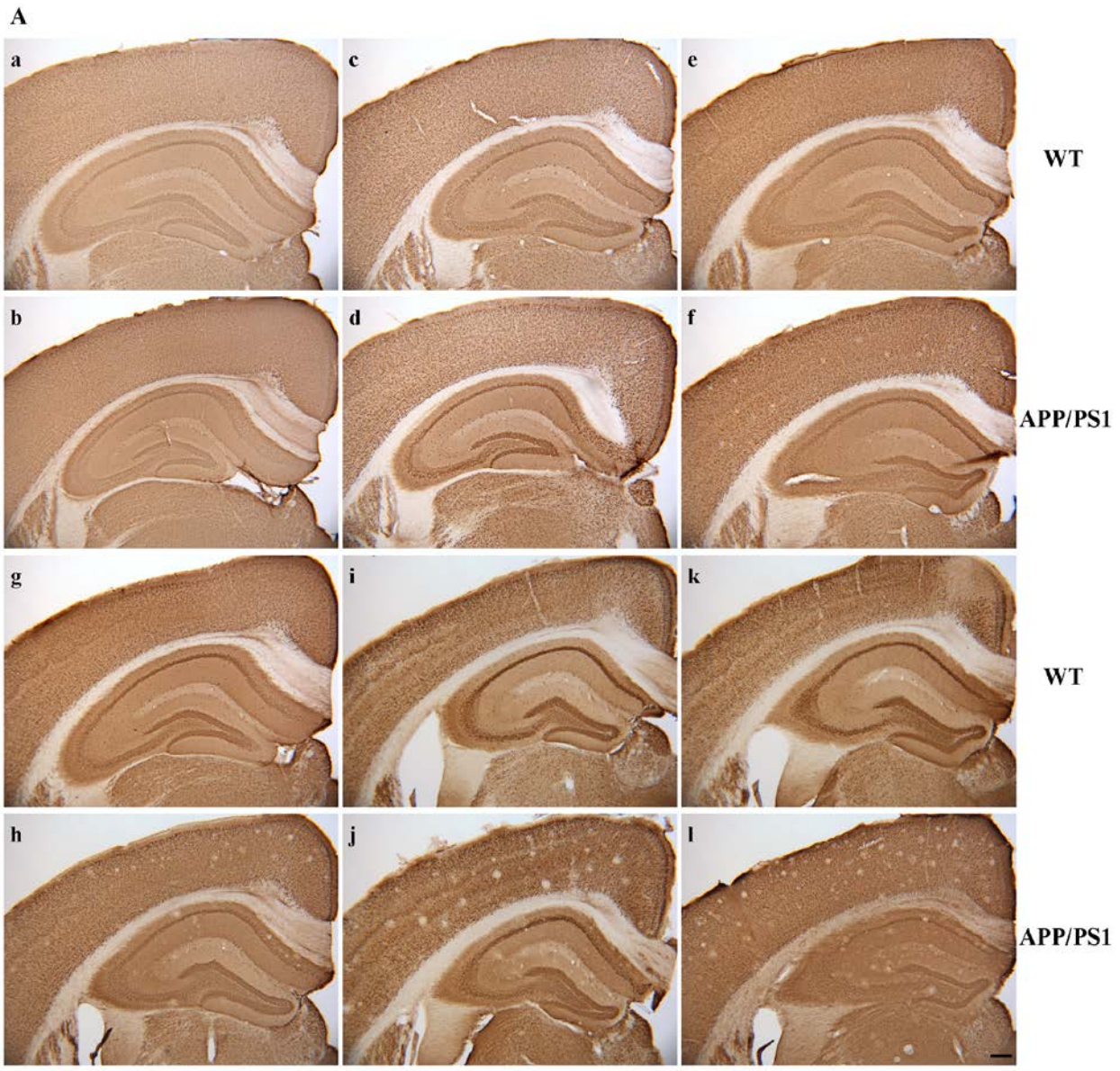


Figure 2. 7: No neuronal cell loss in cerebral cortex and hippocampus of APP/PS1 compared age-matched WT mice. Coronal sections from mice at the age of 2, 3, 5, 9, 12, and 22 months were labeled with neuronal marker NeuN. Representative immunohistochemical staining with NeuN at the age of 2 (a, b), 3 (c, d), 5 (e, f), 9 (g, h), 12 (i, j), and 22 (k, l) months are shown. Scale bar represents 200 μm. No statistical significance was detected in total number of NeuN positive cells in cerebral cortex and hippocampus.

2.4 Discussion

Since Aβ plaques are considered to be the major neuropathological feature in AD brains and cognitive decline is the most prominent clinical presentation in AD patients, an extensive amount of research has been done on investigation of the causative relationship between amyloid accumulation and cognitive decline. On the contrary, it remains unclear whether neuroinflammatory processes are actually involved in the development and progression of the cognitive impairments in AD. Given the classical amyloid-centered hypothesis faces a number of challenges mainly evidenced by that the severity of amyloid burden in the brain does not correlate well with the degree of cognitive impairment in AD patients (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011), a better understanding of the role of neuroinflammation in AD might provide a new promising strategy in preventing or delaying AD.

We first screened the spatial learning and memory abilities in 3, 5, 9 and 12 month old mice. Spatial short-term working memory was impaired in all APP/PS1 transgenic groups except the 3 month old group. However, the deficits did not get greater with age suggesting that the spatial working memory decline is not a function of age. Consistently, spatial learning and retention memory was also impaired in three transgenic age groups except the 3 months group, as the transgenic mice exhibited longer latencies to reach the platform during acquisition trials and spent less amount of time in target quadrant during the probe trial. However, after averaging out the escape latency of all four age groups of APP/PS1 mice in all trials during acquisition and comparing them with age-matched WT groups, the differences in the overall performance between transgenic mice and WT animals were not exactly progressively exacerbated. It was initially increased from 3 to 9 months old and then plateaued between the age of 9 and12 months, which again confirmed that the degree of spatial learning memory deficits was not completely

age-dependent. Furthermore, this finding is consistent with some clinical studies reporting that old age has been associated with a slower rate of change in cognitive function (Bernick, Cummings, Raman, Sun, & Aisen, 2012; Jacobs et al., 1994). Interestingly, earlier animal studies have shown that age-dependent spatial memory deterioration in APP/PS1 mice using the Morris water maze or T maze (Filali & Lalonde, 2009; Izco et al., 2014). This observed discrepancy among different animal models might be due to differences in mouse genetic backgrounds and transgenes, which also reveal some of the challenges in finding a perfect animal model of AD.

Amyloid plaques have been long believed to be the primary driving force in the development of AD as per the classical amyloid hypothesis. To analyze amyloid pathology in this transgenic mouse model, we have utilized three different Aβ staining methods. We found amyloid plaques as young as 3 months of age, which is in agreement with previous reports (Izco et al., 2014; Ruan, Kang, Pei, & Le, 2009). The majority of plaques were small and compact in 5 month old in transgenic mice and the morphology of plaques gradually became bigger and more diffuse as animals aged. Quantification of the number of plaques and plaque area fraction revealed that the accumulation of Aβ was increased with age, suggesting Aβ increase is indeed a function of age in this APP/PS1 double transgenic mouse model of AD, which is also consistent with previous observations (Garcia-Alloza et al., 2006; Ruan et al., 2009).

Therefore, it is reasonable to argue that Aβ may not be the only player driving the cognitive deficits based upon our above observations on the change of behavioural abnormalities and amyloid plaques with age. Although memory impairments observed in 5 month old transgenic mice coincides with the dramatic increase in amyloid, the rate of cognitive decline slowed down as the animal aged from 9 months old onwards whereas the increase of Aβ plaques is still a function of age. This is not the first time that we have observed this discrepancy that amyloid

pathology does not correlate well with the degree of cognitive impairments. For example, single APP transgenic mice start to show cognitive impairment well before the appearance of amyloid plaques (G. Chen et al., 2000). Thus, various hypotheses have been suggested to explain this incongruity.

There is increasing evidence supporting the important role of neuroinflammation in AD (Akiyama et al., 2000; Wyss-Coray & Mucke, 2002). In the central nervous system (CNS), microglia and astrocytes are the major types of glial cells which greatly outnumber neurons. They possess a number of important structural and physiological functions (Nagele et al., 2004). Although glial accumulation is one of the earliest pathological manifestations in AD brains (Wyss-Coray et al., 2003), many studies on AD might have underestimated their critical roles. In the present study, clustered microglia were first detected in both cortex and hippocampus of APP/PS1 mice at 3 month old and double staining the brain tissues demonstrated that these activated microglia were closely associated with amyloid plaques, suggesting that microglial activation is an early event in response to Aβ. Coincidently, we also found early astrocyte activation which mainly existed in cortex at the same age in transgenic mice. Activated microglia and astrocytes have been reported to be able to internalize and degrade Aβ (Wyss-Coray et al., 2003; H. Zhang et al., 2014), suggesting the early response of microglia and astrocytes may be beneficial in facilitating the clearance of Aβ. However, their capacity for clearing Aβ is believed to be much limited or even reduced as disease progresses (Hickman et al., 2008). Meanwhile, continuous uncontrolled glial activation still maintains their abilities to produce excessive multiple cytokines and chemokines such as interleukin 1β (IL-1β), tumor necrosis factor α (TNFα), monocyte chemotactic protein-1 (MCP-1), and nitric oxide (NO), which in turn stimulate and even hasten the progression of AD (Paradisi et al., 2004; V. H. Perry et al., 2010).

Furthermore, blocking the proinflammatory signaling of IL-12 and IL-23 by genetic ablation of the common subunit p40 ameliorates glial activation and cognitive decline in the APP/PS1 mice (Vom Berg et al., 2012).

Given the biphasic functions of glial activation may complicate the interpretation of pre-clinical results and clinical observations related to AD pathology, it is difficult to examine whether neuroinflammation significantly relates to cognitive decline in AD. In present study, we found that the microgliosis and astrogliosis reach a plateau in a similar way to the degree of spatial learning memory impairments, whereas the amyloid load increased linearly throughout the disease course. These results imply that there might exist a critical threshold for amyloid burden and beyond this point accumulation of amyloid plaques does not cause further memory loss. They also suggest that reactive glia might contribute to the memory loss in AD, arguing against the traditional belief that glial activation is merely a straightforward secondary response to plaques. Our findings are in line with earlier clinical studies which have shown that the severity of microglial activation correlates with the extent of brain atrophy (Cagnin et al., 2001) and cognitive decline (Parachikova et al., 2007). Furthermore, these results are also consistent with recent animal studies in which the amount of activated astrocytes in entorhinal cortex showed a close correlation with memory impairments in an APP/tau transgenic mouse model of AD (DaRocha-Souto et al., 2011). Overall, our data indicate that neuroinflammation might be involved in the pathogenesis of cognitive deficits in AD, and reactive glia might act in concert with other factors such as Aβ and neurofibrillary tangles contributing the development and progression of the cognitive impairments in AD.

Yet another concern arises from that neuronal loss has been thought to produce the cognitive deficits in AD (Cummings, Vinters, Cole, & Khachaturian, 1998). To confirm, brain tissues were

stained with the general neuronal nuclear marker NeuN. We found no evidence of any appreciable neuronal cell loss in the layer II of cortex in this APP/PS1 transgenic mice despite the presence of robust amyloid plaques. This finding is further substantiated by previous reports also showing that some transgenic AD mouse models often do not exert clear-cut neuronal cell loss along with the ongoing progressive Aβ accumulation (Onos, Sukoff Rizzo, Howell, & Sasner, 2016; Soto et al., 2016; Webster, Bachstetter, Nelson, Schmitt, & Van Eldik, 2014; Wirths & Bayer, 2010). This evidence suggests the importance of neuronal cell dysfunction rather than the loss of neurons in the pathogenesis of cognitive deficits in AD mice. Besides, astrocytes are involved in the regulation of neuronal activity in the brain as they structurally constitute the "tripartite synapse" with neuronal cells and functionally are involved in the release and uptake of gliotransmitters/neurotransmitters in the synaptic cleft (Volterra & Meldolesi, 2005). Therefore, glial activation and their released inflammatory mediators could directly contribute to the neuronal dysfunction in AD, which eventually leads to the cognitive impairments.

In conclusion, the present study demonstrated that glial activation was paralleled well with the pattern of learning deficits rather than amyloid in APP/PS1 mice. These findings provided evidence suggesting that neuroinflammation might directly contribute to the development and progression of cognitive deficits in APP/PS1 mice, which could help develop novel intervention and prevention strategies for AD. Nevertheless, the precise relationship between neuroinflammation and cognitive decline is complex and requires further research in order to be fully understood.

Chapter 3

Quetiapine reduces proinflammatory cytokine levels in $A\beta_{1-42}$ stimulated primary microglia

3.1 Introduction

Apart from classic hallmarks, extensive evidence has demonstrated that robust activation of microglia has been found in and around the area of amyloid plaques in the AD brain (Ho et al., 2005; Itagaki et al., 1989). This amyloid deposition-provoked microglial activation begins secretion of a plethora of proinflammatory products, including reactive oxygen species (ROS), cytokines, and neurotoxins (Floden, Li, & Combs, 2005). The prolonged and unregulated inflammatory response contributes to the progression of disease marked by neuronal loss and cognitive decline in both preclinical and clinical observations (Akiyama et al., 2000; Zhu et al., 2017).

A variety of *in vitro* studies have documented that Aβ fibrils are able to directly stimulate microglia which leads to the increased production of tumor necrosis factor α (TNFα) (Floden et al., 2005; Tan et al., 2000), which might resemble the physiological conditions *in vivo*. The binding of TNFα to its transmembrane receptor TNFR1 activates a large number of intracellular inflammatory responses, including the activation of nuclear factor kappa B (NF-κB) pathway (Z. J. Chen, 2005; Sorriento et al., 2008). The activated NF-κB translocates to the nucleus, resulting in turn in expression of a number of inflammatory genes including cyclooxygenase (COX), IL-1β, and more TNFα (X. Zhang et al., 2009). This results in a vicious cycle of further glial activation and neurotoxic damage through generating chronic self-sustaining inflammatory reactions.

Quetiapine (Seroquel®) is a novel atypical antipsychotic drug that was approved for the treatment of patients with schizophrenia, and has been demonstrated to have superior therapeutic effects over other antipsychotic drugs on cognitive symptoms displayed by patients with schizophrenia (Purdon et al., 2001; Velligan et al., 2002). Clinically, quetiapine is also used to treat psychosis in AD as well as cognition in Parkinson's disease (Juncos et al., 2004; Madhusoodanan et al., 2007). It has been found that quetiapine can reduce microglia activation in the brains of cuprizone induced demyelinating mice (Y. Zhang et al., 2008), modulate immune responses in an experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) (Mei et al., 2012). In addition, *in vitro* studies have shown that quetiapine inhibits NO generation and TNFα release from activated microglia (Bian et al., 2008). However, the underlying mechanism by which quetiapine regulates microglial activation remains elusive.

In an effort to link Aβ fibril deposition with microglial activation and potential anti-inflammatory effects of quetiapine, we used an *in vitro* approach that used Aβ fibril stimulated primary mouse microglia.

3.2 Methods

3.2.1 Animals and treatments

Female C57BL mice were purchased from Charles River Laboratories Inc. (St. Constant, QC, Canada). The mice were group housed and maintained on a 12-h light/12-h dark cycle with food and water for a 1 week of acclimation period. All mice were treated according to the guidelines established by the Canadian Council on Animal Care and all procedures were approved by the Animal Care Committee at the University of Manitoba.

Quetiapine was obtained from AstraZeneca Pharmaceuticals (Macclesfield, UK) and dissolved into 20 mM of dimethyl sulfoxide (DMSO) and then diluted into 2 mM of PBS for experiments. The concentrations of quetiapine were 0.1, 1, 10, 50, 100, 500, 1000 μM.

3.2.2 Aβ fibril preparation

Synthetic peptides corresponding to amino acids 1-42 of human Aβ protein were purchased from American Peptide (Sunnyvale, CA, USA). Preparation of $A\beta_{1-42}$ fibril was performed as previously described (Floden et al., 2005). In brief, Aβ peptides were dissolved in distilled water (final concentration, 1 mM) and incubated for 1 week at 37°C before use to induce fibril formation. The concentrations of Aβ42 were 0.01, 0.1, 1, 5, 10, and 50 μM.

3.2.3 Mouse primary microglia culture

Microglial cultures were prepared from mixed glial cultures, as described previously (Kauppinen et al., 2008). Briefly, cortices were dissected from 1-day-old C57BL mouse pups in Hanks' Balanced Salt Solution (Invitrogen). Cells were dissociated by mincing, followed by incubation in trypsin for 25 min at 37°C with agitation. After centrifugation for 5 min at 1000 rpm, the cells were resuspended with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with 10%

FBS. Cells were plated on 75 cm^2 flasks at a density of 1.5×10^7 cells per flask and maintained in a 37°C, 5% CO_2 incubator. The medium was changed every 3 to 4 days. After 2 weeks *in vitro*, microglia were harvested by shaking the flasks at 200 rpm on a rotary shaker for 4 h at 37°C. Cells were collected, washed, and seeded at a density of 1×10^6 cells per ml. The purity of the microglial cultures was tested by immunocytochemical staining for Iba1 (1:500; Abcam, Cambridge, MA, USA), a microglia marker, and for GFAP (1:1000; Sigma, St. Louis, MO), an astrocyte marker. The purity of microglial cultures was found to be ~95%. Also, the cellular morphology was carefully investigated under phase contrast microscope.

Primary microglia were pretreated with quetiapine (0 to 1000 μM) for 1 h, and then exposed to 0 to 50 μM Aβ in the presence of the same concentrations of quetiapine for 6 h, 12 h, and 24 h.

3.2.4 Cell viability assay

Cell viability was assessed using the lactate dehydrogenase (LDH) leakage assay. Cells were plated in 96-well plates at a density of $1.0–1.5 \times 10^5$ cells per well. At the endpoint of each treatment, the supernatant of the cell culture was collected. Cells were lysed with the lysis buffer at 37°C for 30 min. Both the samples of supernatants and cell lysates were prepared per the manufacturer's instructions in the LDH-Cytotoxicity Assay Kit II (BioVision, Milpitas, CA, USA). The optical density was measured at 450 nm on a Wallac VICTOR3 microplate reader (Perkin Elmer Life Sciences, Waltham, MA, USA). Cell death rate was calculated as percentage over the respective control as per the manufacturer’s protocol.

3.2.5 Quantification of proinflammatory cytokines by ELISA

The proinflammatory cytokines IL-1β and TNFα in the supernatant of cultured microglia were measured using commercial ELISA kits (Invitrogen, Camarillo, CA). Assays were performed according to the manufacturer's instructions. The levels of IL-1β and TNFα were corrected for the dilution factor, and the final value in each group was expressed in picograms per milliliter.

3.2.6 Hoechst staining

Hoechst staining was performed at room temperature around 22°C. Cells were washed twice with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde (PFA) for 30 minutes. Cells were again washed twice with PBS and permeabilized with 0.2% Triton X-100 for 30 minutes. Finally, cells were incubated for 5 minutes in Hoechst 33258 solution to stain nuclei. Images were taken with a fluorescence microscope (Olympus, Japan).

3.2.7 Immunocytochemistry

Primary microglia were plated on culture slides (BD Science, NJ, USA). After treatment cultured microglia were washed twice with PBS and fixed with 4% paraformaldehyde for 30 minutes. After washing twice with PBS, the cells were permeabilized with 0.2% Triton X-100 for 10 minutes and then incubated overnight with anti-NF-κB p65 (1:100) antibody (Santa Cruz) at 4°C. After washing, the cells were incubated with Alexa Fluor 594-conjugated secondary antibody (1:200, Invitrogen). Then, the cells were incubated with Alexa Fluor 488-conjugated phalloidin (Invitrogen) at room temperature for 50 minutes. Finally, the cells were stained with Hoechst 33342 (Calbiochem, ON, Canada) for 5 minutes at room temperature. The cover slips were mounted on glass slides. Images were taken with a fluorescence microscope (Olympus, Japan).

3.2.8 Statistical analysis

All results are expressed as means ± S.E.M. Analyses were performed using both one-way and two-way ANOVA followed by Newman-Keuls *post hoc* test for multiple comparisons. A two-tailed *t*-test for independent samples was used for two-group comparisons. Differences were considered significant at $p < 0.05$.

3.3 Results

3.3.1 Validity of primary microglia culture

Morphological examination under the phase contrast microscope showed small cells with extended processes which is consistent with resting microglia morphology (Fig. 3.1a). The purity of microglial cultures was found to be over 95% with a specific microglia marker, Iba1, as shown in Fig. 3.1b. There was minimal detectable signal from astrocytes which were stained with GFAP antibody.

3.3.2 Cytotoxicity of quetiapine and Aβ fibril in primary microglia culture

To exclude non-specific effects of quetiapine and Aβ fibril on microglial cells, LDH assay was performed to observe cell death rate of primary mouse microglial cells treated with quetiapine and Aβ fibril at various concentrations.

Cytotoxicity of quetiapine was measured with LDH assay in primary cultures of microglia. Quetiapine at concentrations of 0.1, 1, 10, 50, and 100 μM had no toxic effect on cultured primary microglia (Fig. 3.2a) after 6, 12 and 24 hours of incubation. Quetiapine at the concentration of 500 and 1000 μM showed significant toxicity against the primary microglia even at 6 hours of treatment, suggesting an ideal concentration for quetiapine should below 100 μM given the uncertainty of where it starts to become toxic between 100 and 500 μM. Our previous studies have shown quetiapine at concentration below 1 μM failed to provide any protective effect against aging in a spontaneous cell death model (J. Wang et al., 2014), which narrows the ideal concentration range of quetiapine to between 1 and 100 μM.

On the contrary, treatment with Aβ fibril for 6 hours did not exert overt toxic effects against microglia even at a 50 μM concentration. Although treatment with Aβ at 50 μM for 12 hours

might have increased toxicity, no statistical significance was achieved. However, although treatment with Aβ at 0.01 – 10 μM for 24 hours did not produce any toxic effects, treatment with Aβ at 50 μM for 24 hours significantly increased toxicity (Fig. 3.2b). Hence, the duration of treatment should be less than 24 hours without causing cell death. For the rest experiments, we arbitrarily chose a 6 hour treatment for this study.

3.3.3 Dose dependent release of proinflammatory cytokines in microglia treated with fibril Aβ

Our previous results showed that fibril Aβ had no significant effect on cell viabilities at various concentrations under 50 μM (Fig. 3.2b) for less than 24 hours treatment. However, proinflammatory cytokine IL-1β released into medium was increased after fibril Aβ stimulation in a concentration dependent manner. As shown in Figure 3.3, one-way ANOVA followed by Newman-Keuls *post hoc* test analysis revealed that exposure of microglia to Aβ increased the secreted IL-1β levels by about 2-fold at 10 μM concentration whereas the level of secreted IL-1β increased almost 5 times at 25 μM concentration. For the rest experiments, we arbitrarily chose fibril Aβ at 25 μM concentration for the rest experiments.

3.3.5 Quetiapine reduces proinflammatory cytokine levels in fibril $A\beta_{1-42}$ treated primary microglia

To investigate the effect of quetiapine on the inflammatory response induced by $A\beta_{1-42}$ *in vitro,* primary microglia were pretreated with quetiapine (10 μM) for 1 h and then with $A\beta_{1-42}$ (25 μM) for 6 h. The amount of proinflammatory cytokine IL-1β and TNFα secreted into the culture medium from primary microglial cells was examined by ELISA. As shown in Figure 3.4a, two-way ANOVA followed by Newman-Keuls *post hoc* test analysis revealed that exposure of

microglia to Aβ increased the secreted IL-1β levels by about 5-fold while quetiapine significantly attenuated Aβ-induced IL-1β secretion. Similar trend was seen in the results of TNFα (Fig. 3.5b). The level of TNFα was significantly increased after Aβ treatment. Although this up-regulation tended to be decreased in the presence of quetiapine, this difference did not reach statistical significance.

3.3.5 Quetiapine attenuates the translocation of NF-κB p65 *in vitro*

It has been reported that Aβ stimulates NF-κB activation by inducing nuclear translocation (Huang et al., 2012). The immunostaining for p65 in primary microglial cells showed that p65 was mainly located in the cytoplasm of untreated cells and $A\beta_{1-42}$ treatment induced a translocation of p65 from the cytoplasm to the nucleus, while quetiapine significantly attenuated the p65 translocation induced by $A\beta_{1-42}$ (Fig. 3.5b). These findings suggest that quetiapine might inhibit neuroinflammation via suppressing the NF-κB p65 pathway.

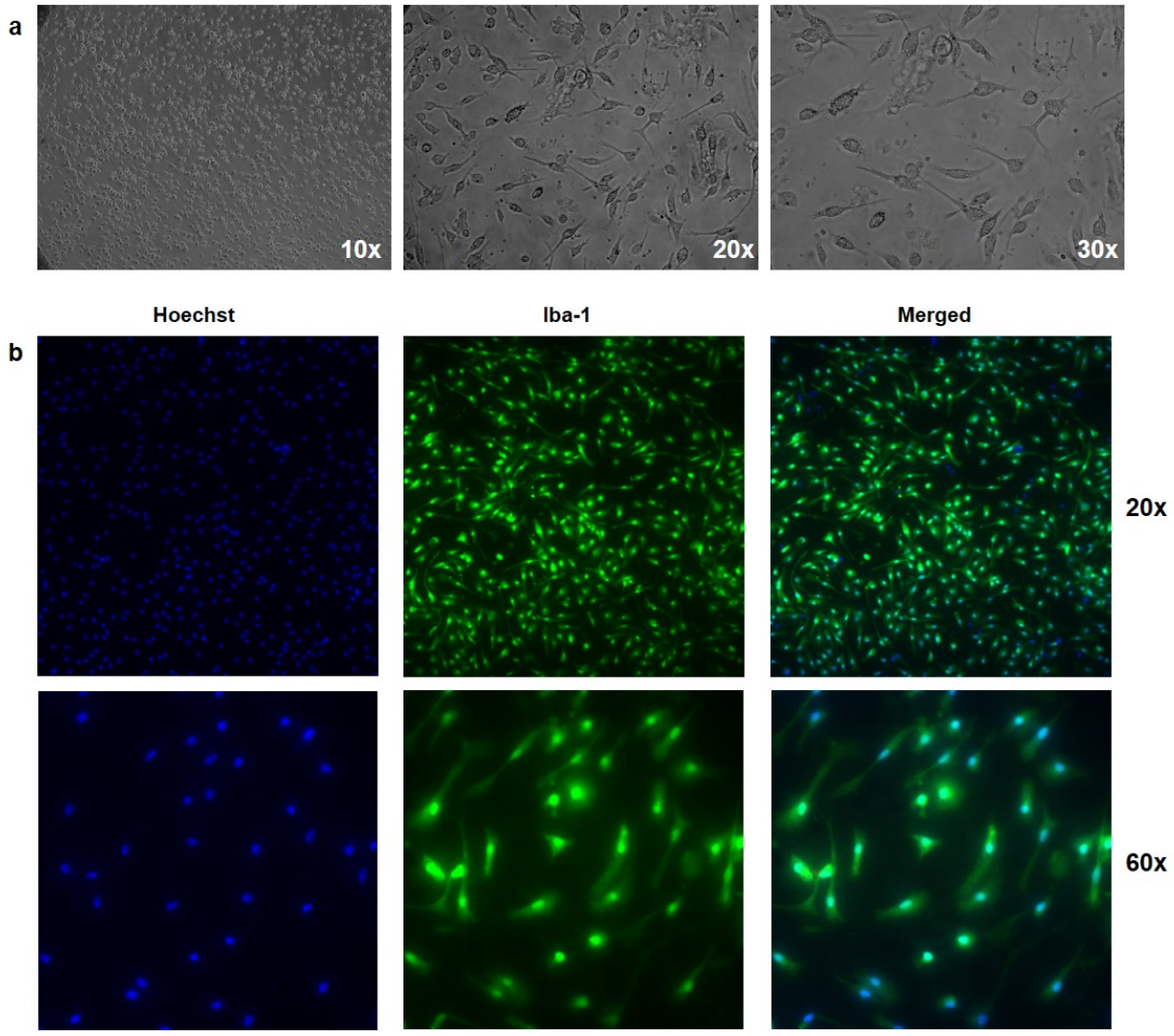


Figure 3. 1 The morphology and purity of primary microglial cultures.

(a) Representative image of microglia culture captured under phase contrast. (b) Representative immunocytochemistry showing Iba1 positive cells in the primary microglia culture. Fluorescent images (20x and 60x magnification): blue, hoechst; green, Iba1.

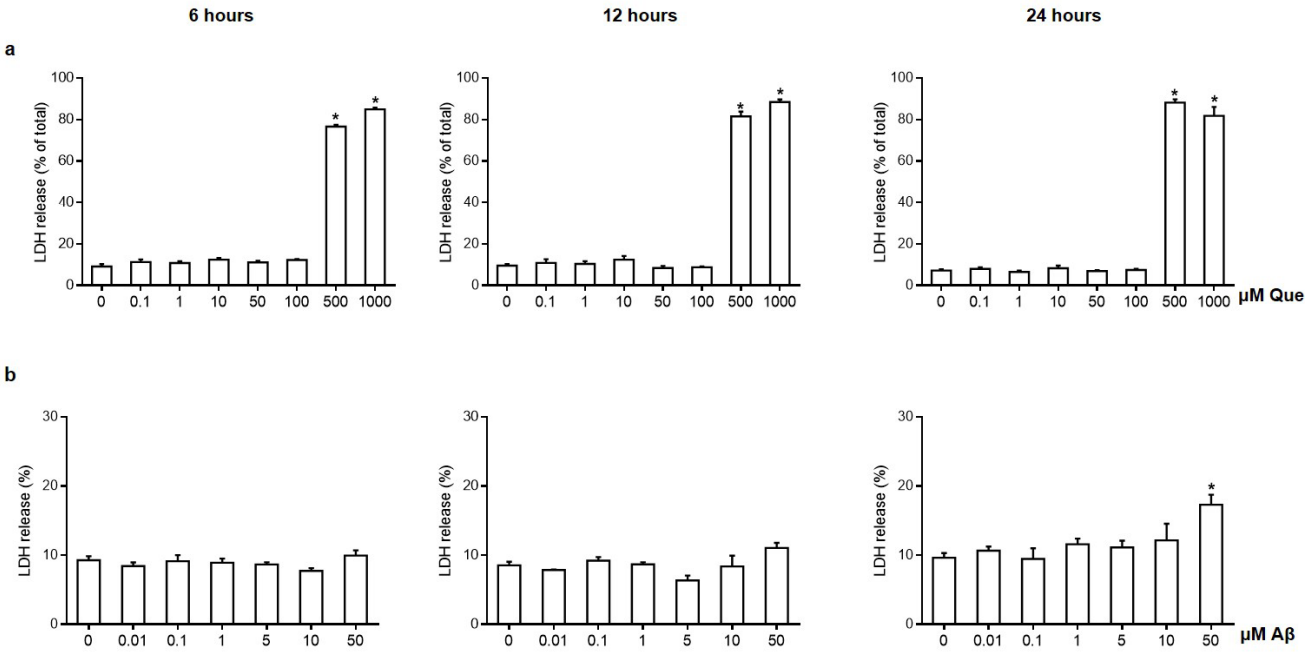


Figure 3. 2 Cytotoxicity of quetiapine and soluble Aβ was measured with LDH assay in primary cultures of microglia.

(a) LDH analysis in primary microglia culture treated with quetiapine at various concentrations for 6, 12, and 24 hours. One-way ANOVA showed quetiapine had significant cell toxicity at concentration above 100 μM. (b) LDH analysis in primary microglia culture treated with fibril Aβ at various concentrations for 6, 12, and 24 hours. One-way ANOVA showed soluble Aβ toxicity increased with duration of treatment. Data are expressed as means ± S.E.M. n = 4. *$p < 0.05$ vs control.

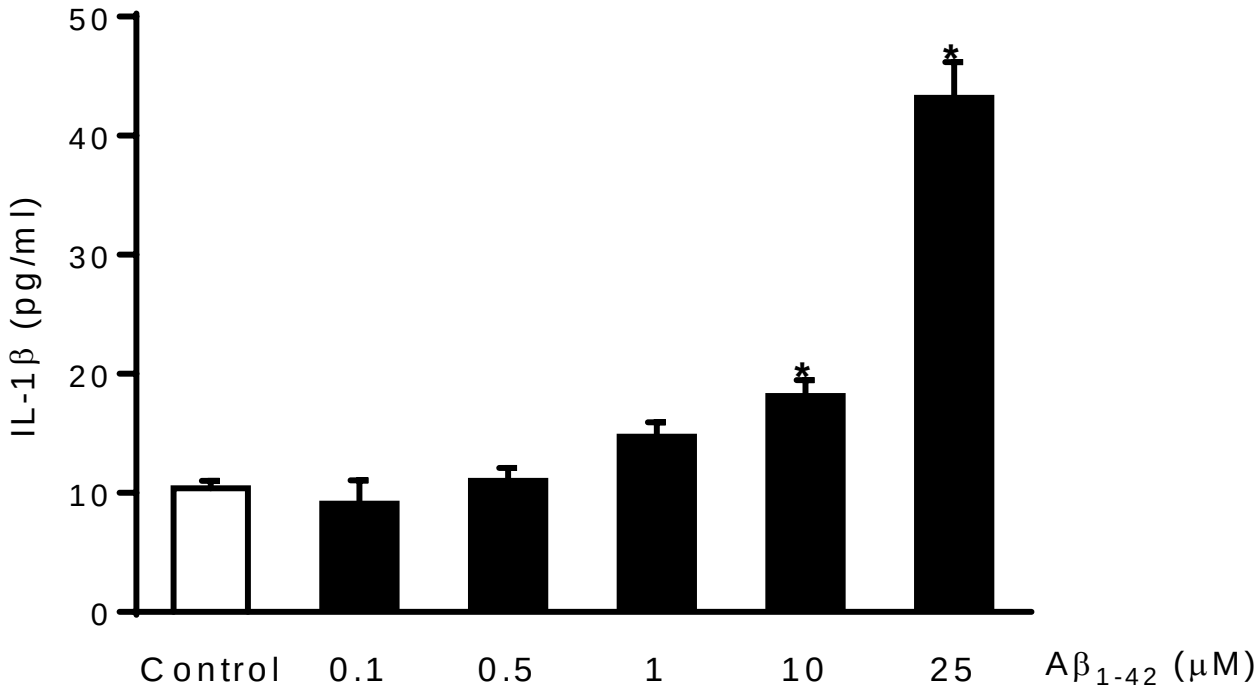


Figure 3. 3 Dose dependent release of IL-1β in primary microglia treated with Aβ for 6 hours. ELISA analysis of IL-1β. One-way ANOVA showed the release of IL-1β in microglia gradually increased with the dose of Aβ. Data are expressed as means ± S.E.M. n = 4. *$p < 0.05$ vs control.

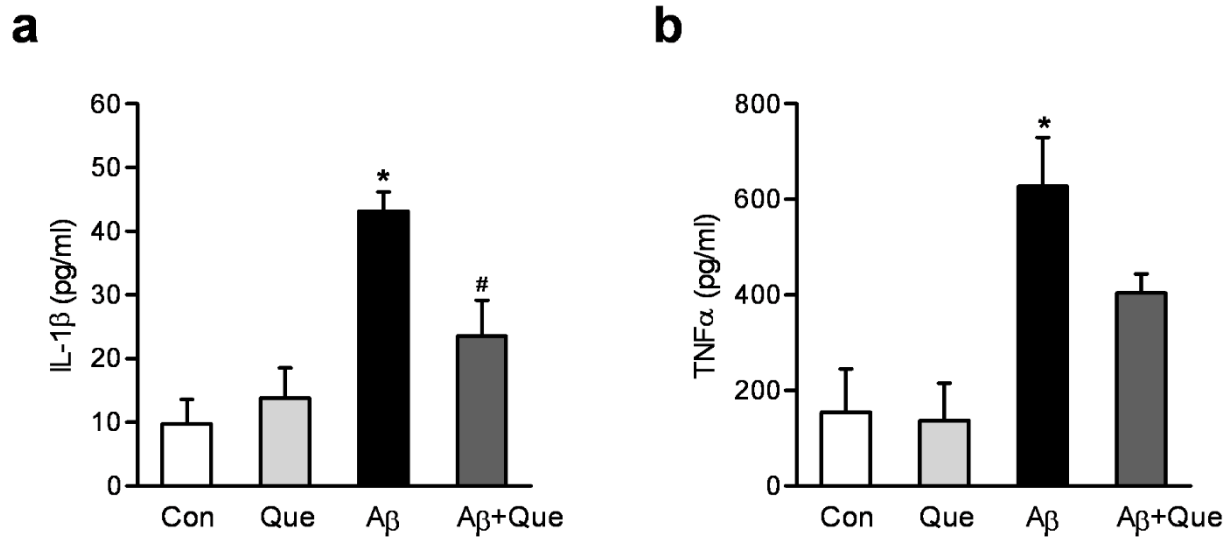


Figure 3. 4: Quetiapine reduces proinflammatory cytokines in $A\beta_{1-42}$ treated primary microglia. Primary microglia were pretreated with quetiapine (10 μM) for 1 h and then with $A\beta_{1-42}$ (25 μM) for 6 h. (**a**) ELISA analysis of IL-1β. Two-way ANOVA showed quetiapine significantly attenuated Aβ-induced IL-1β increase. (**b**) ELISA analysis of TNFα. Two-way ANOVA showed exposure of microglia to Aβ increased secreted TNFα levels. No statistical significance was detected after quetiapine treatment. Data are expressed as means ± S.E.M. n = 4. $^{*}p < 0.05$ vs. Con; $^{\#}p < 0.05$ vs. Aβ.

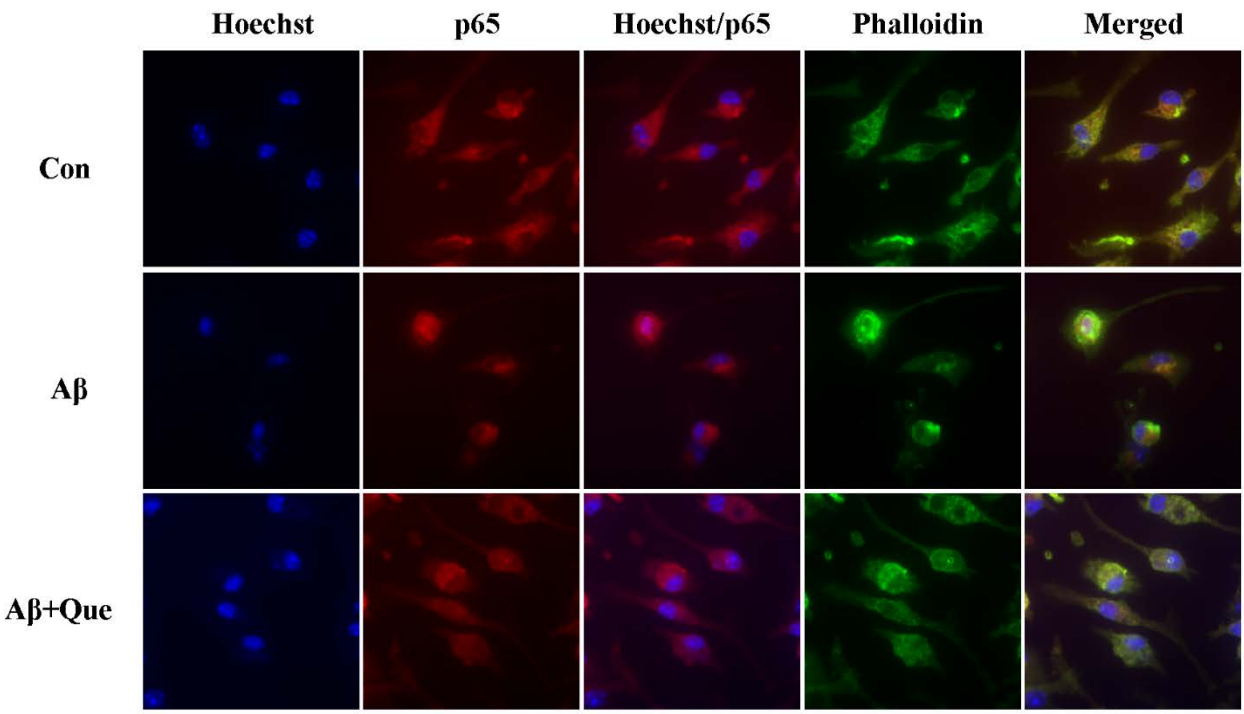


Figure 3. 5**:** Quetiapine inhibits the activation of NF-κB p65 pathway *in vitro*.

Representative immunocytochemistry showing the effect of quetiapine on $A\beta_{1\text{-}42}$ induced NF-κB p65 nuclear translocation in primary microglia. Fluorescent images (100x magnification): blue, hoechst; red, p65; green, phalloidin.

3.4 Discussion

In the present study, using an *in vitro* system of $A\beta_{1-42}$ stimulated primary mouse microglia culture, we found that Aβ stimulated microglia secrete IL-1β in a concentration dependent manner and quetiapine attenuated microglial activation by significantly reducing the production of TNFα and IL-1β. More importantly, our *in vitro* study has also demonstrated that NF-κB p65 is translocated into nucleus following $A\beta_{1-42}$ stimulation, while quetiapine treatment can reverse this translocation in the primary mouse microglia culture. Based on our findings, we propose that Aβ activated microglia release proinflammatory cytokine such as IL-1β and TNFα to initiate the NF-κB signaling pathway, which is capable of further driving the inflammatory response and leading to a vicious forward feeding loop, resulting in neuronal death. It is encouraging to see that quetiapine produces anti-inflammatory effect to break the self sustained cycle by directly inhibiting the translation of the NF-κB into nucleus.

A variety of studies have shown that activated microglia is accumulated around amyloid plaques and that proinflammatory cytokines and chemokines are excessively released in the AD brain, which may contribute to neuronal death and degeneration in this disease (Lucin & Wyss-Coray, 2009). It also has been well known that higher inflammatory levels significantly increase the risk for cognitive impairment (Rosano, Marsland, & Gianaros, 2012). In the present study, the effects of quetiapine on Aβ-induced inflammation were tested on primary microglia culture. We found that quetiapine drastically decreased $A\beta_{1-42}$-induced release of both IL-1β and TNFα in microglial culture. More importantly, our study has also proved that there are no non-specific effects of quetiapine or fibril Aβ as the doses we used in this study did not affect the cell viability. Since these proinflammatory mediators in turn further activate microglia creating a self-perpetuating vicious cycle by which inflammation induces further neuronal damage

(Paradisi et al., 2004; V. H. Perry et al., 2010), blocking these cytokines by quetiapine possibly alleviate the chronic propagating inflammation associated with AD, which could help protect neurons and eventually attenuate behavioural impairment in preclinical experimental or even in clinical trials.

In regard to the specific mechanisms by which quetiapine affects microglia, we demonstrated that quetiapine can modulate NF-κB activation. NF-κB is a transcription factor that has been involved in multiple cellular behaviors including cell differentiation, survival, apoptosis, as well as immune and inflammatory responses (Q. Li & Verma, 2002; Vallabhapurapu & Karin, 2009). It has been reported that quetiapine can suppress the production of proinflammatory cytokines and inhibit LPS-activated NF-κB pathway in microglial cultures (H. Wang et al., 2015). Quetiapine administration also inhibits NF-κB p65/p50 expression levels in mice subjected to global cerebral ischemia (Bi et al., 2009). In the present study, we also demonstrated that quetiapine inhibited Aβ-induced translocation of NF-κB p65 in primary mouse microglia culture, indicating that the inhibitory effect of quetiapine on microglial activation may due to suppression of NF-κB activation.

Collectively, these studies and our results suggest that microglia play an important role in controlling extracellular cytokine concentrations via the NF-κB pathway in response to extracellular Aβ. More importantly, it also provides a great piece of evidence demonstrating the anti-inflammatory effect of quetiapine in addition to its pharmacological characteristics. Furthermore, our results lay the foundation for the next objective to investigate whether glial activation and proinflammatory cytokine releasing can be modulated by quetiapine through regulating the NF-κB pathway in an APP/PS1 humanized knock-in mouse model of AD.

Chapter 4

Quetiapine attenuates glial activation and proinflammatory cytokines in APP/PS1 transgenic mice

4.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to dementia. Extracellular β-amyloid (Aβ) plaques, intracellular neurofibrillary tangles, and massive neuronal cell and synapse loss represent the main pathological hallmarks in AD brains (Selkoe, 2002; Storey & Cappai, 1999; Storey, Katz, Brickman, Beyreuther, & Masters, 1999). Apart from these classic hallmarks, increasing evidence has demonstrated uncontrolled glial activation and neuroinflammation in AD brain may contribute independently to neural dysfunction and cell death (Akiyama et al., 2000; Wyss-Coray & Mucke, 2002). Robust activation of microglia has been found in and around the area of amyloid plaques in the AD brain, and reactive astrocytes have been shown to form a halo surrounding the amyloid plaques (Ho et al., 2005; Itagaki et al., 1989). Additionally, numerous proinflammatory factors have been reported to be elevated in both patients with AD and transgenic animal models of AD (Akiyama et al., 2000; Griffin et al., 1989; Ruan et al., 2009). Whether alleviation of neuroinflammation will offer therapeutic benefit for AD remains unclear. Epidemiological studies show a possible association between suppression of inflammation and reduced risk for AD (in t' Veld et al., 2001; Vlad et al., 2008). Therefore, drugs targeting neuroinflammation might provide benefits for the prevention and treatment of this devastating disease.

In the CNS, microglia and astrocytes are the major type of glial cells and activation of these cells has been involved in all neurodegenerative diseases (Wyss-Coray & Mucke, 2002).

Nevertheless, the diverse physiological functions of glial activation might complicate the interpretation of experimental investigations and clinical observations related to AD pathology. For example, glial phagocytosis of Aβ is considered to be one key mechanism of the initial defense of the brain against the toxic accumulation of Aβ (H. Zhang et al., 2014). As the disease progresses, continuous glial activation by Aβ release excessive multiple cytokines and chemokines such as tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β), monocyte chemotactic protein-1 (MCP-1), and nitric oxide (NO), which leads to a vicious cycle of further glial activation and neurotoxic damage through generating chronic self-sustaining inflammatory reactions (Paradisi et al., 2004; V. H. Perry et al., 2010). This process may stimulate and even accelerate the progression of AD.

The nuclear factor kappa B (NF-κB) is a transcription factor that is involved in regulating immune and inflammatory responses (Kucharczak, Simmons, Fan, & Gelinas, 2003; Q. Li & Verma, 2002). The mammalian NF-κB family consists of RelA/p65, RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) (Zheng et al., 2011). These proteins can form homo- or heterodimers which often are held captive in cytoplasm remaining inactive. The activated NF-κB translocates to the nucleus which leads to expression of a number of inflammatory genes including cyclooxygenase (COX), IL-1β and TNFα (X. Zhang et al., 2009). NF-κB signaling has been proven to be involved in AD. Enhanced immnunoreactivity was observed in neurons surrounding amyloid plaques in the brains of AD patients (Kaltschmidt et al., 1997). In addition, activated NF-κB has been found in microglia of patents with AD (Mattson & Camandola, 2001). In *in vitro* studies, NF-κB can be activated by Aβ in both neuronal and microglial cells (Huang et al., 2012). Together, these findings suggest that activation of NF-κB plays an important role in mediating neuroinflammation in AD.

Quetiapine (Seroquel®) is a novel atypical antipsychotic drug that was approved for the treatment of patients with schizophrenia (Purdon et al., 2001; Velligan et al., 2002). Clinically, quetiapine is also used to treat psychosis in AD as well as cognition in Parkinson's disease (Juncos et al., 2004; Madhusoodanan et al., 2007). In animal studies, quetiapine decreases the accumulation of activated astrocytes and microglia in demyelinated sites followed by cuprizone administration (Y. Zhang et al., 2008), modulates immune responses in an experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) (Mei et al., 2012), and inhibits NF-κB p65/p50 expression in ischemic mice (Bi et al., 2009). *In vitro* studies have shown that quetiapine inhibits NO generation and TNFα release from activated microglia (Bian et al., 2008). Although quetiapine has some beneficial effects on cognition in AD mice (Zhu et al., 2013), there is no data published with respect to its effect on glial activation and neuroinflammation in AD mice. In the present study, we wanted to address the issue of whether glial activation and proinflammatory cytokine increases could be modulated by quetiapine through regulating the NF-κB pathway in an APP/PS1 humanized knock-in mouse model of AD.

4.2 Methods

4.2.1 Animals and treatments

APP/PS1 double transgenic and non-transgenic mice were generated from mating between single transgenic mice expressing human mutant $APP_{K670N/M671L}$ (Hsiao et al., 1996) and mutant $PS1_{M146L}$ (Duff et al., 1996), and chosen by the genotyping results of PCR. The age- and sex-matched wild-type (Con) mice were used as the controls. All mice had free access to food and water under controlled laboratory conditions. All procedures with animals were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committee at the University of Manitoba.

Quetiapine (Que) was obtained from AstraZeneca Pharmaceuticals (Macclesfield, UK). The drug was dissolved in sterile water and delivered to mice at a dose of 5 mg/kg/day for 8 months, starting from the age of 4 months. The doses were chosen referred to our previous report (J. He et al., 2009). APP/PS1 double transgenic mice and wild-type littermates were randomly assigned into four groups: non-transgenic + water (Con), non-transgenic + quetiapine 5 mg/(kg day) (Que), transgenic + water (Tg) and transgenic + quetiapine 5 mg/(kg day) (Tg+Que).

Table 4. 1: Number of mice in each group

Number of female and male control and APP/PS1 transgenic mice used to generate behavioural testing data.

	Con	Q5	Tg	Tg+Q5
12 month				
Male	4	5	3	3
Female	6	4	4	4
Total	10	9	7	7

4.2.2 Open field test

Spontaneous exploratory activity and anxiety-like behaviour were assessed in the open field test. The open field test consisted of a 36" × 36" bare square box divided into 20 outer border squares with 35 cm high walls and 16 inner zone squares (Fig. A.4). The test procedure was the same as that previously described by He et al. (J. He, Xu, Yang, Zhang, & Li, 2005). Mice were placed in a particular corner of the arena and were tracked using ANY-Maze^{TM} Video Tracking Software (version 4.63) with a digital camera. Mice were allowed to explore the maze for 5 min, after which they were returned to their home cage. The maze was cleaned with 75% ethanol wipes before commencing testing with the next mouse. The total distance moved in the arena during the 5 min was analyzed as a measure of locomotor activity. Time spent in the Inner zone of the open field was taken as a measure of anxiety-like behaviour

4.2.3 Object recognition test

Non-spatial memory of mice was measured using the object recognition test as previously described (Clark, Zola, & Squire, 2000). Mice were placed into a 40 cm (width) x 40 cm (width) x 23 cm (height) Plexiglas square box (Fig. A.5). It consisted of three sessions: habituation, training, and retention (J. He, Yang, Yu, Li, & Li, 2006). During the training session, mice were individually placed in the activity box for 10 min of free exploration, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners. During the retention session for the short-term memory test, mice were placed back into the same box 1 h later containing one of the previous objects (A1 or A2) and a novel object (B) for a 5 min testing session. During the retention session for the long-term memory test, animals were subsequently placed back into the same box 23 h after the short-term memory test (24 h after the training session) for 5 min of free exploration, where object B was replaced by a novel object C. During the retention session, the time spent exploring the novel object (B or C) was used to measure memory function. Object exploration was considered as a mouse's nose touched the object or was facing and within 2 cm to the object (Oh et al., 2010). Exploratory activity of each object was recorded for both training and testing sessions using ANY-Maze^{TM} Video Tracking Software (Stoelting, USA) and analyzed off-line with the experimenter blinded to treatment and genotypes.

4.2.4 Tissue processing

After the above behavioural tests, animals were anesthetized and perfused with phosphate-buffered saline (PBS, pH 7.4). The hemispheres were separated by cutting at the midline. The cortex and hippocampus from the right hemisphere were separated and used for biochemistry analyses. The left hemisphere was post-fixed in 4% paraformaldehyde in PBS, and then

cryoprotected in 30% sucrose in PBS (J. He et al., 2009). Finally, the left hemisphere was cut into 30 μm thick coronal sections.

4.2.5 Immunohistochemistry

Six free-floating sections from each animal were first incubated with 0.3% H_2O_2 in 0.01 M PBS for 30 min at room temperature to quench endogenous peroxidase activity, then blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 1 h, and then incubated overnight at 4°C with anti-glial fibrillary acidic protein (GFAP) mouse mAb (1:1000; Sigma, St. Louis, MO) and anti-ionized calcium binding adapter molecule 1 (Iba1) rabbit pAb (1:500; Wako Chemicals, Richmond, VA). After rinsing, the sections were incubated with appropriate biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Staining was achieved with the avidin biotin complex kit (Vector Laboratories, Burlingame, CA) and visualized with 3,3-diaminobenzidine (DAB) chromogen (ThermoFisher Scientific, Waltham, MA). Slides were viewed with an Axio-Imager M2, and the Zen software for image acquisition (Carl Zeiss, Jena, Germany). The immunohistochemical controls were performed as above, but with the omission of the primary antibodies. No positive immunostaining was found in any of the controls.

4.2.6 Western blotting

Protein samples were resolved on 12% SDS-PAGE mini-gels under reducing conditions. They were then electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) non-fat dried milk in TBST buffer and were probed at 4°C overnight with following antibodies: a rabbit polyclonal anti-C-terminal APP (1:3000) antibody (Sigma, St. Louis, MO, USA), a rabbit polyclonal anti-PS1 (1:1000) antibody (Cell Signaling Technology,

Danvers, MA, USA), a mouse monoclonal anti-GFAP (1:1000) antibody (Sigma), a mouse monoclonal anti-NF-κB p65 (1:500) antibody (Santa Cruz Biotechnology, CA, USA), a mouse monoclonal anti-β-actin (1:5000) antibody (Santa Cruz), and a mouse monoclonal anti-GAPDH (1:1000) antibody (Abcam, Cambridge, MA, USA). Blots were then incubated at room temperature for 2 h with corresponding peroxidise-conjugated secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences, NJ, USA). Band densities were quantified using the Bio-Rad Laboratories Quantity One Software (Hercules, CA, USA). All target proteins were normalized to β-actin or GAPDH, and then standardized to the corresponding control group.

4.2.7 Quantification of Aβ and proinflammatory cytokines by ELISA

The levels of total Aβ40 and Aβ42 were measured using the Human Aβ ELISA Kits, following the manufacturer's protocol (Invitrogen-Biosource, Camarillo, CA). Each sample was assayed in duplicate at appropriate dilutions so that relative luminescent units fell within the range of standard curves.

The proinflammatory cytokines IL-1β and TNFα in brain were measured using commercial ELISA kits (Invitrogen, Camarillo, CA). Assays were performed according to the manufacturer's instructions. The levels of IL-1β and TNFα in brain were corrected for total protein of tissue and dilution factor, and the final value in each group was standardized to the control group.

4.2.8 Statistical analysis

All results are expressed as means ± S.E.M. Analyses were performed using a two-way ANOVA followed by Newman-Keuls *post hoc* test for multiple comparisons. A two-tailed *t*-test

for independent samples was used for two-group comparisons. Differences were considered significant at $p < 0.05$.

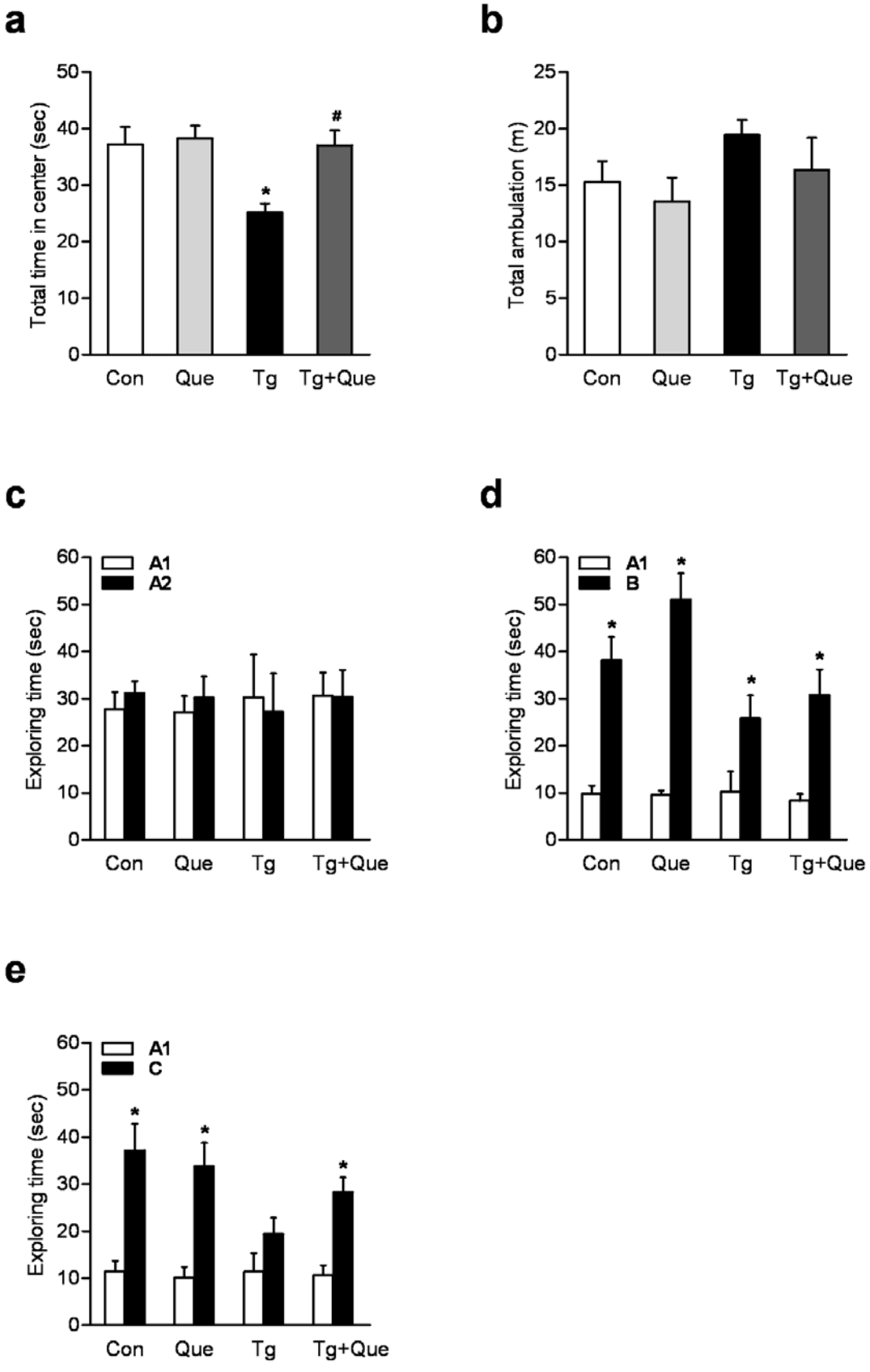
4.3 Results

4.3.1 Quetiapine improves behavioural performance of APP/PS1 mice

The open field test was used to measure locomotion, exploration and anxiety-like behaviour. Two-way ANOVA analysis conducted on the data for total time spent in the central area of the field showed that genotype [$F(1, 28) = 6.13$, $p < 0.05$], and quetiapine [$F(1, 28) = 5.80$, $p < 0.05$] produced a significant change on the time spent in the center (Fig. 4.1A) and there was an interaction between genotype and quetiapine [$F(1, 28) = 4.10$, $p = 0.0526$]. A *post hoc* analysis indicated that the time spent in the center in transgenic mice was less than that in control mice, which demonstrated an anxiety-like phenotype that developed in AD mice at 12 months of age. Quetiapine treatment significantly improved the decreased interaction with the center zone in transgenic mice (Fig. 4.1a). To evaluate whether quetiapine or genotype significantly influenced results, general locomotor activity was examined by looking at total distance travelled in the open field test. There were no significant differences in the total distance travelled across all the groups (Fig. 4.1b).

The object recognition task measures non-spatial visual-discrimination memory in the rodents and takes advantage of the mouse's unprompted nature to prefer exploring novel objects in its surroundings (Kamei et al., 2006). In the training session, mice spent equal amounts of time on each of the two identical objects (Fig. 4.1c), indicating that the two objects were equally preferred. In addition, the total amount of time spent exploring the objects (A1 + A2) was similar in all mice, suggesting that genotype and quetiapine had no effect on the levels of attention and motivation of these mice for the objects. During the 1 h retention session, all mice spent more time exploring the novel object B (Fig. 4.1d), indicating that transgenic mice exhibited no defects in memory for novel objects measured 1 h after training. During the 24 h retention test,

non-transgenic mice treated with water or quetiapine were still able to discriminate between the familiar object and a novel object C (Fig. 4.1e), exploring the latter for a significantly longer time. As expected transgenic mice had no memory for the novel object C (Fig. 4.1e), showing impaired long term memory. In contrast, transgenic mice treated with quetiapine spent more time exploring the novel object C (Fig. 4.1e), implying quetiapine treatment significantly improved long term memory impairment in transgenic mice.


a
Total time in center (sec)
50
40
30
20
10
0
*
#
Con
Que
Tg
Tg+Que
b
Total ambulation (m)
25
20
15
10
5
0
Con
Que
Tg
Tg+Que
c
Exploring time (sec)
60
50
40
30
20
10
0
A1
A2
Con
Que
Tg
Tg+Que
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Exploring time (sec)
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Tg
Tg+Que

Figure 4. 1**:** Quetiapine improves behavioural performance in APP/PS1 mice. (**a**) Total time spent in the center, and (**b**) the total distance travelled in an open field test. (**c**) The exploration time of mice on identical objects (A1 and A2) in the object recognition test. *t*-test showed that all groups of mice demonstrated equal total exploration time for each of the identical objects in the training session. (**d**) The exploration time of mice on a familiar object (A1) and a novel object (B) in a retention trial 1 hour after training. *t*-test showed that all mice spent more time exploring the novel objective. (**e**) The exploration time of mice on a familiar object (A1) and a novel object (C) in a retention trial 24 hours after training. *t*-test showed that transgenic mice exhibited an impaired ability to discriminate between the familiar object and a novel object C, while transgenic mice treated with quetiapine spent more time exploring the novel object C. Data are expressed as means ± S.E.M. n = 7-10 mice per group. $*p < 0.05$ vs. Con; $^{\#}p < 0.05$ vs. Tg.

4.3.2 Quetiapine marginally affects total Aβ40 and Aβ42 levels in APP/PS1 mice

To understand why quetiapine improved the behaviour of APP/PS1 mice, we next assessed the effects of quetiapine in aged APP/PS1 mice on total Aβ levels. Quantitative Aβ ELISA revealed a significant reduction of total $A\beta_{40}$, but not $A\beta_{42}$, in the cerebral cortex of quetiapine treated APP/PS1 mice ($p < 0.01$, Fig. 4.2 a and b). In the hippocampus of APP/PS1 mice, quetiapine also showed a tendency to decrease total $A\beta_{40}$ and not $A\beta_{42}$, but it was not statistically significant ($p = 0.0504$, Fig. 4.2 c and d). Given the important role of APP and PS1 on Aβ production during APP processing, the expression of full length APP (APP-FL) and PS1 was determined by Western blot. As shown in Figure 4.2e, quetiapine had no influence on APP expression or processing, because the steady-state levels of APP-FL or PS1 were not altered by the treatment. These results suggest that quetiapine treatment may be capable of reducing certain Aβ species. However, this marginal effect of quetiapine on Aβ production cannot fully explain its beneficial effects in APP/PS1 mice on behavioural performance.

4.3.3 Quetiapine attenuates microglial activation and reduces proinflammatory cytokine levels in APP/PS1 mice

Neuroinflammation is reflected in AD and its transgenic models brain as elevated inflammatory cytokines and chemokines, and accumulation of activated microglia, particularly occurring around amyloid plaques (Matsuoka et al., 2001). We thus examined whether the activation of microglia was ameliorated by quetiapine treatment. The density of microglia was accessed by using the immunostaining of Iba1 antibody. Two-way ANOVA analysis showed that genotype [$F(1, 25) = 26.79$, $p < 0.0001$] and quetiapine [$F(1, 25) = 3.96$, $p = 0.0577$] produced significant changes on microglial cell density, and that there was an interaction between genotype and quetiapine [$F(1, 25) = 7.61$, $p = 0.0107$]. A *post hoc* analysis indicated that the

Iba1-positive cells per mm^2 were significantly increased in the brains of APP/PS1 mice compared with that in brains of non-transgenic mice. Quetiapine treatment decreased microglia density in transgenic mouse brains (Fig. 4.3a).

To further confirm inhibitory inflammation of quetiapine *in vivo*, levels of proinflammatory cytokines IL-1β and TNFα in both cortex and hippocampus were determined. As shown in Figure 4b, two-way ANOVA analysis conducted on the data for the level of IL-1β in cerebral cortex showed that genotype [$F(1, 18) = 21.56$, $p = 0.0002$] and quetiapine [$F(1, 18) = 11.33$, $p = 0.0034$] produced significant changes in the IL-1β level, and there was an interaction between genotype and quetiapine [$F(1, 18) = 3.03$, $p = 0.099$]. A *post hoc* analysis indicated that IL-1β was significantly increased in the cerebral cortex of APP/PS1 mice compared with that in the cortex of non-transgenic mice. Quetiapine treatment greatly attenuated the increase of IL-1β in the cortex of transgenic mice (Fig. 4.3b). Similar results were seen in the hippocampus. But the difference between transgenic mice and transgenic mice treated with quetiapine did not reach statistical significance ($p = 0.062$, Fig. 4.3b). However, there was no significant difference about the level of TNFα in both cerebral cortex and hippocampus across all the groups.

4.3.4 Quetiapine inhibits activation of astrocytes in APP/PS1 mice

In brains of AD patients (Mancardi, Liwnicz, & Mandybur, 1983) and transgenic AD mice models (Wirths et al., 2010), activated astrocytes that are mainly cells that respond to the neuroinflammation process are often observed in and around the area of amyloid plaques (Itagaki et al., 1989; Matsuoka et al., 2001). Initially, we evaluated the reactivity of astrocytes in the transgenic mouse model of AD. There was a notable increase in activated astrocytes positive cells in the frontal cortex and hippocampus of APP/PS1 transgenic mice (Fig. 4.4A, c) compared with age-match non-transgenic counterparts (Fig. 4.4A, a). In contrast, the GFAP

immunoreactivity was remarkably decreased in the quetiapine treated APP/PS1 transgenic mice (Fig. 4.4A, d) compared with the non-treated counterparts (Fig. 4.4A, c). This reduction was apparent both in the frontal cortex and hippocampus, indicating that astrogliosis was reduced after quetiapine treatment. More interestingly, while GFAP positive cells in the frontal cortices of transgenic mice (Fig. 4.4A, g) was largely diffuse, cortical GFAP staining of quetiapine treated transgenic mice (Fig. 4.4A, h) appeared to be focal, which may suggest that activated astrocytes within quetiapine treated brains are confined to smaller areas than in the brains of non-treated transgenic animals. Quantification of cell number in the cerebral cortex showed the GFAP-positive cells were significantly greater in transgenic mice compared to transgenic mice treated with quetiapine (Fig. 4.4B).

To confirm the immunohistochemistry results, Western blot was conducted to quantify the expression level of GFAP in cortical tissues. Two-way ANOVA analysis showed that genotype [$F(1, 14) = 80.41$, $p < 0.0001$] and quetiapine [$F(1, 14) = 5.80$, $p = 0.0304$] produced significant changes on the GFAP expression level, and that there was an interaction between genotype and quetiapine [$F(1, 14) = 14.87$, $p = 0.0018$]. A *post hoc* analysis indicated that the protein level of GFAP was significantly increased in the cortex of transgenic mice compared with that in the cortex of non-transgenic mice. Quetiapine treatment prevented the up-regulation of GFAP protein content in transgenic mouse brains (Fig. 4.4C). Taken together, these observations confirm the finding that quetiapine treatment suppresses the prolonged astrocytes activation associated with AD progression.

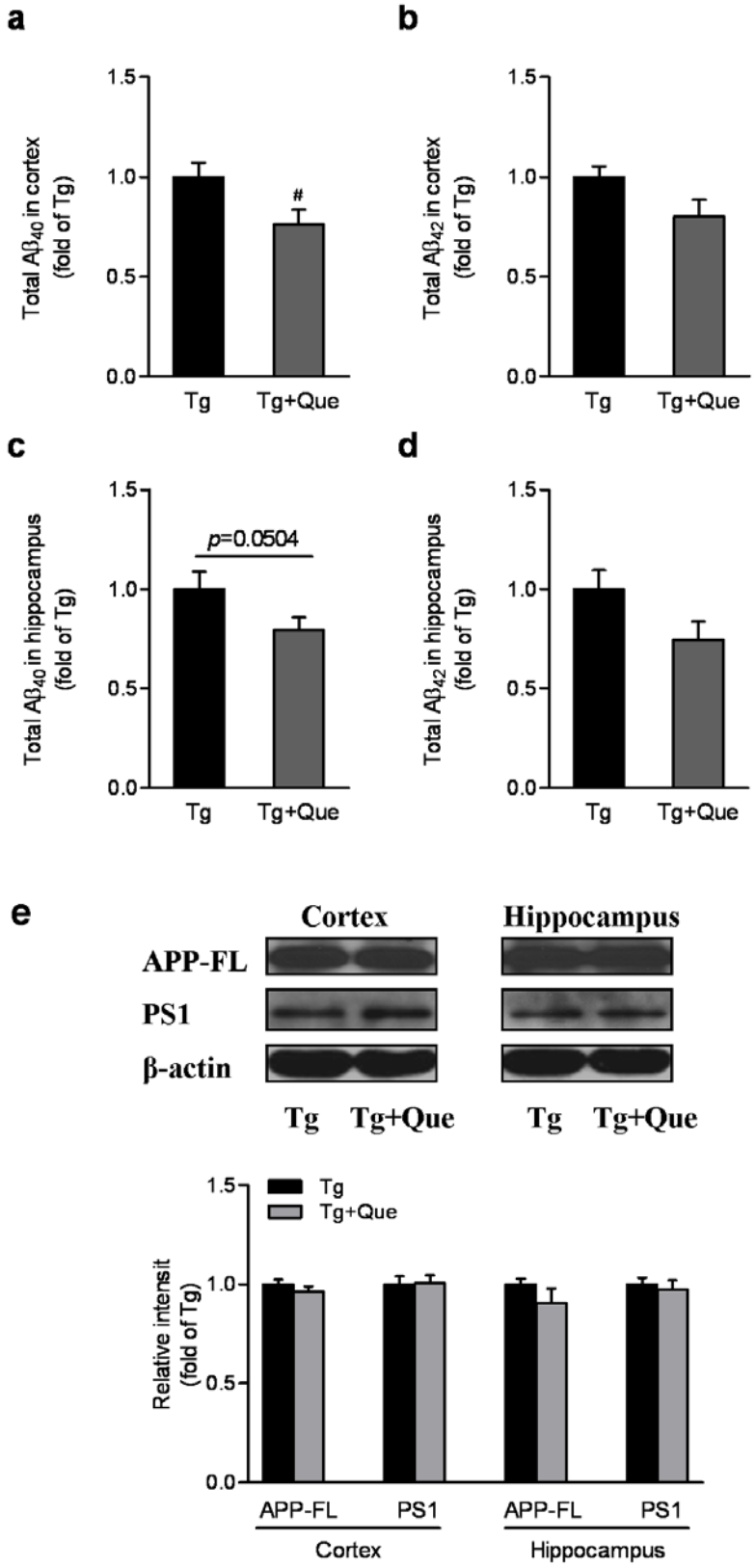
a
b
c
d
e
Total Aβ40 in cortex (fold of Tg)
Total Aβ42 in cortex (fold of Tg)
Total Aβ40 in hippocampus (fold of Tg)
Total Aβ42 in hippocampus (fold of Tg)
1.5
1.0
0.5
0.0
#
p=0.0504
Tg
Tg+Que
Cortex
Hippocampus
APP-FL
PS1
β-actin
Relative intensit (fold of Tg)
APP-FL
PS1

Figure 4. 2**:** Quetiapine marginally affects total $A\beta_{40}$ and $A\beta_{42}$ levels in APP/PS1 mice. (**a**) Total $A\beta_{40}$ and (**b**) total $A\beta_{42}$ in the cortex of transgenic mice. *t*-test showed a significant reduction of total $A\beta_{40}$, but not $A\beta_{42}$ in the cerebral cortex after quetiapine treatment. (**c**) Total $A\beta_{40}$ and (**d**) total $A\beta_{42}$ in the hippocampus of transgenic mice. (**e**) Immunoblot analysis of APP and PS1 in both cortex and hippocampus following the treatment. Quantification of APP-FL and PS1 was shown in the graph. No statistical significance was detected. Data are expressed as means ± S.E.M. n = 4-6 in each group. $^{\#}p < 0.05$ vs. Tg.

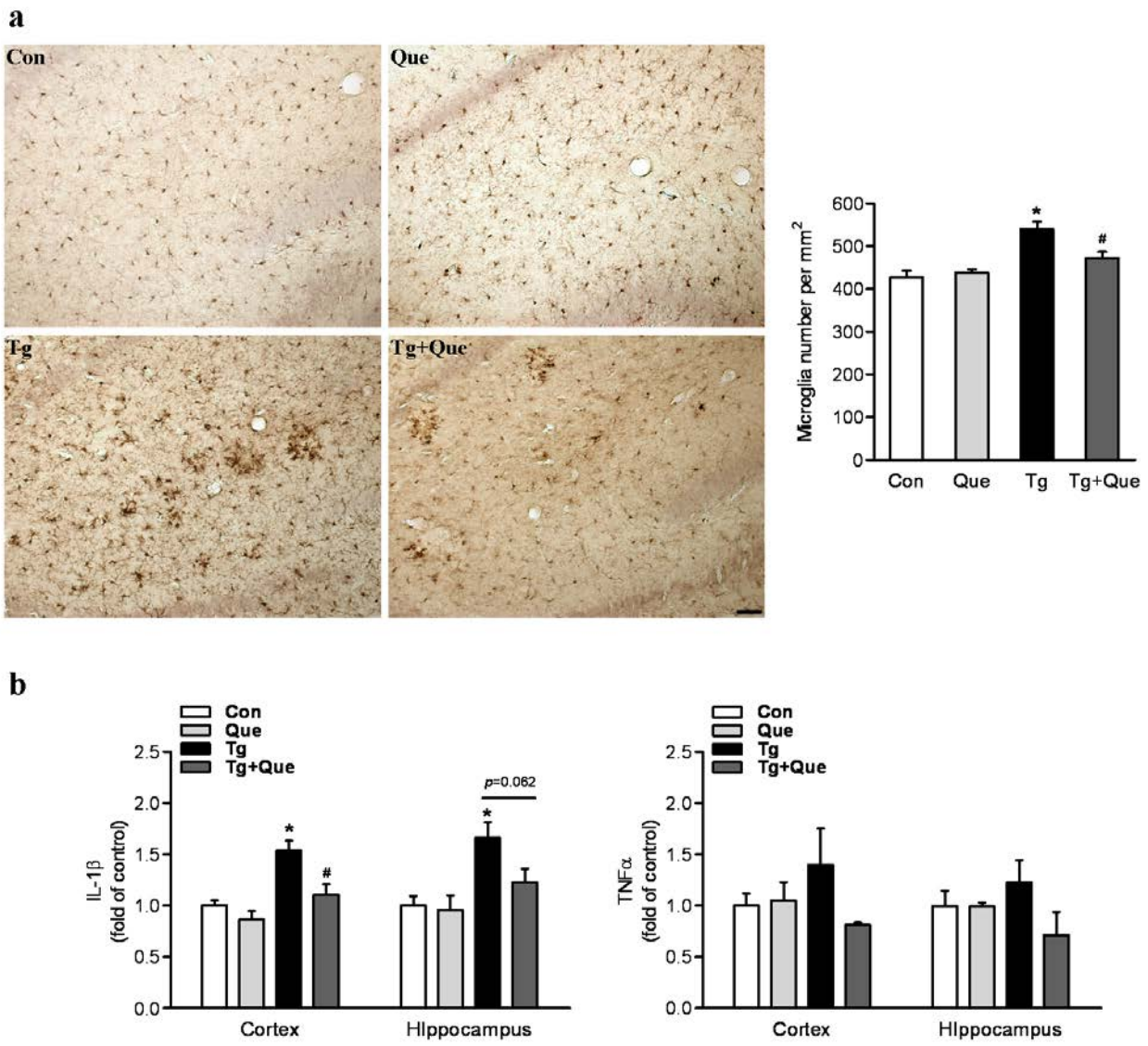

Figure 4. 3**:** Quetiapine attenuates microglial activation and reduces proinflammatory cytokines in APP/PS1 mice. (**a**) Representative immunohistochemical staining with Iba1 in hippocampus following the treatment. The scale bar represents 50 μm. Quantification of the number of Iba positive cells was shown in the graph. Two-way ANOVA showed microglial cell density was increased in transgenice mice and decreased following quetiapine treatment. (**b**) ELISA analysis of selected proinflammatory cytokines. Two-way ANOVA showed quetiapine treatment greatly attenuated the increase of IL-1β in the cortex of transgenic mice. No statistical significance was

detected in the level of TNFα. Data are expressed as means ± S.E.M. n = 5-8 mice per group. *p < 0.05 vs. Con; $^{\#}p$ < 0.05 vs. Tg.

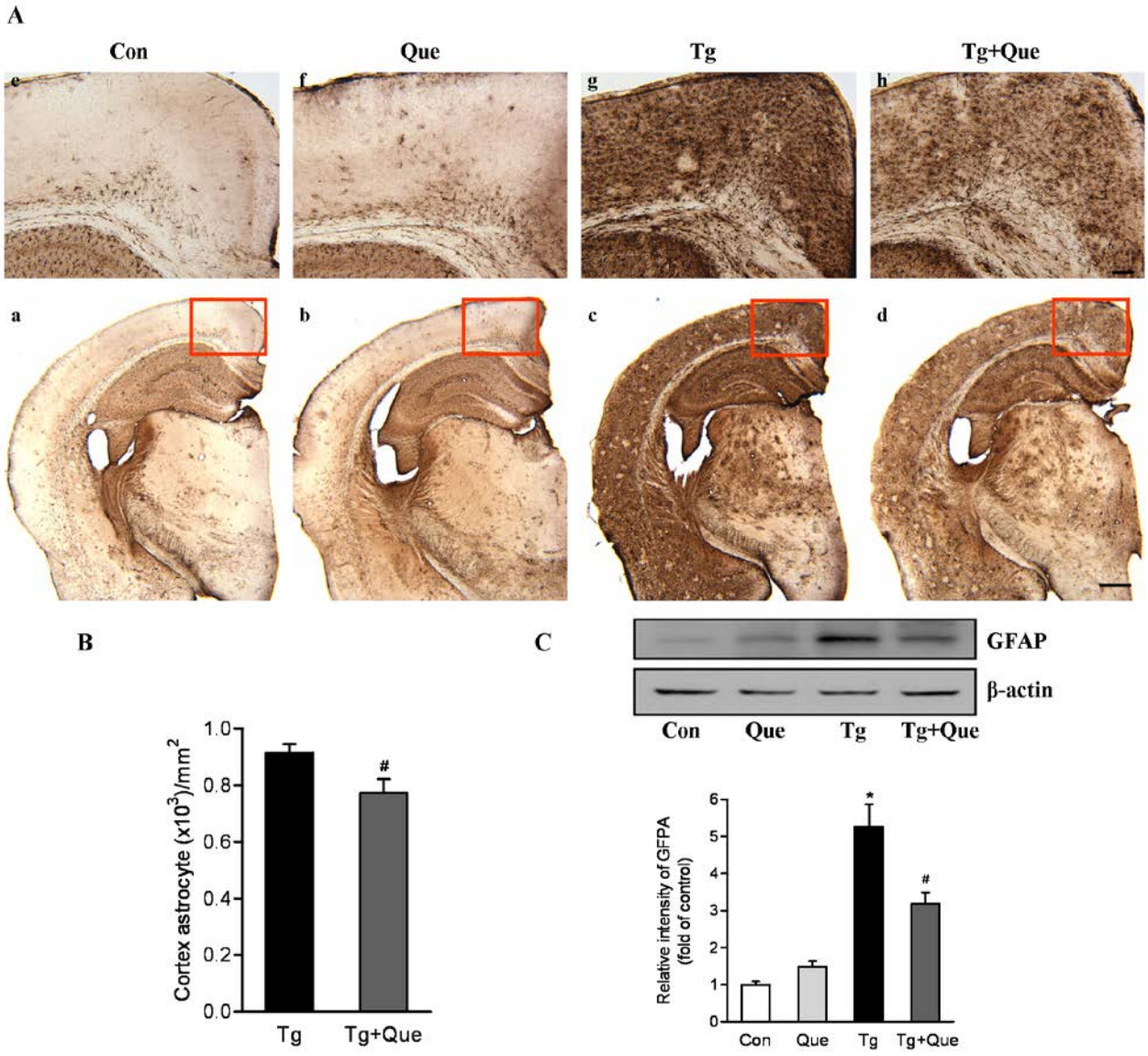

Figure 4. 4**:** Quetiapine inhibits activation of astrocytes in APP/PS1 mice. (**A**) Representatvie immunohistochemical staining using GFAP antibody indicated reduced astroglial cell densities in brain sections of treated transgenic mice compared with untreated transgenic mice. Upper panel shows the higher magnification of the field in red frame.The scale bars represent 100 μm (upper pannel) and 500 μm (lower panel). (**B**) Quantification of cell number in the cerebral cortex showed the GFAP-positive cells were significantly greater in APP/PS1 transgenic mice compared to APP/PS1 transgenic mice treated with quetiapine. (**C**) Immunoblot analysis of GFAP in cerebral

cortex. Quantification of GFAP was shown in the graph. Data are expressed as means ± S.E.M. n = 4-5 mice per group. *$p < 0.05$ vs. Con; $^{\#}p < 0.05$ vs. Tg.

4.3.5 Quetiapine suppresses the expression of NF-κB p65 *in vivo*

To elucidate the possible mechanism of quetiapine in suppression of inflammation, the NF-κB p65 signaling pathway, which has been implicated in microglial activation and neuroinflammation, was studied. The expression of p65 in both cortex and hippocampus was determined by Western blot analysis. As shown in Figure 4.5, two-way ANOVA analysis showed that genotype [$F(1, 18) = 17.86$, $p < 0.001$] and quetiapine [$F(1, 18) = 5.79$, $p < 0.05$] produced significant changes on the p65 expression level in cerebral cortex, and that there was an interaction between genotype and quetiapine [$F(1, 18) = 9.76$, $p < 0.01$]. A *post hoc* analysis indicated that the protein level of p65 was significantly increased in the cortex of APP/PS1 transgenic mice compared with that in the cortex of non-transgenic mice. Quetiapine treatment significantly attenuated this increase in transgenic mice (Fig. 4.5). Similar results were also observed in the hippocampus. These results indicated that quetiapine treatment could inhibit the activation of NF-κB p65 in APP/PS1 transgenic mice.

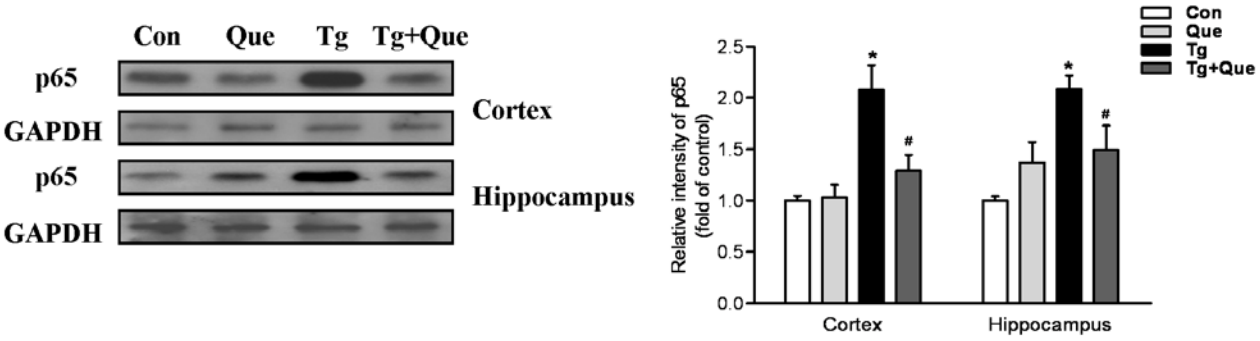

Figure 4. 5**:** Quetiapine inhibits the activation of NF-κB p65 pathway *in vivo*. Immunoblot analysis of p65 in both cortex and hippocampus following the treatment. Quantification of p65 was shown in the graph. Two-way ANOVA showed quetiapine treatment significantly attenuated this increase of p65 in both cortex and hippocampus of transgenic mice. Data are expressed as means ± S.E.M. n = 6-9 mice per group. $*p < 0.05$ vs. Con; $^{\#}p < 0.05$ vs. Tg.

4.4 Discussion

A chronic administration of quetiapine in APP/PS1 transgenic mice resulted in a marked change in microglial and astrocyte activation, proinflammatory cytokine levels, and an improvement in behavioural performance. These beneficial effects of quetiapine occurred when there were only marginal changes in levels of total Aβ, suggesting that the anti-inflammatory effect of quetiapine may account for the majority of cognitive improvement in APP/PS1 transgenic mice. Moreover, we confirmed that quetiapine significantly reduced $A\beta_{1-42}$ induced secretion of proinflammatory cytokines in primary cultured microglia. Furthermore, both *in vitro* and *in vivo* experiments demonstrated that quetiapine ameliorated proinflammatory cytokine increases via suppression of the activation of NF-κB pathway.

The primary clinical presentation of AD is progressive cognitive decline. As AD progresses, a number of neuropsychiatric symptoms, including depression and anxiety, are exhibited (Garcia-Alberca et al., 2008). Twelve month old APP/PS1 transgenic mice showed higher anxiety levels than non-transgenic controls, as seen in decreased time spent in the center of the open field box. Quetiapine reduced heightened anxiety in transgenic mice with no significant effects on general locomotor activity. This suggests that quetiapine may have some level of anxiolytic effect. APP/PS1 mice showed non-spatial visual-discrimination memory deficits indicated by a lower exploration time of the novel object after 24 h training in the object recognition test. This long term retention memory deficit was significantly improved in APP/PS1 mice treated with quetiapine for 8 months, suggesting that this treatment paradigm was effective in improving the non-spatial memory.

The behavioural improvement following quetiapine treatment may be associated with its effects on Aβ pathology according to the amyloid hypothesis (J. Hardy & Selkoe, 2002).

However, the effect of quetiapine on levels of total Aβ was unexpectedly marginal. Only certain species in certain brain regions, such as total $A\beta_{40}$ in cerebral cortex, were significantly reduced following the treatment of quetiapine. This is inconsistent with our previous report with respect to the effect of quetiapine on Aβ pathology. He et al. have reported that quetiapine treatment has significantly decreased total $A\beta_{40}$ and $A\beta_{42}$ production (J. He et al., 2009). Various factors, such as age, therapeutic time window and duration of treatment, could be involved in showing this difference. For example, in this present study, total Aβs were measured at relatively old (12 month old) APP/PS1 transgenic mice, whereas they were evaluated in much younger mice in our previous report. Additionally, we started quetiapine administration after the onset of overt amyloid pathology beginning at the age of 4 months as opposed to 2 months old in the previous study. Given amyloid plaques account for the majority of total Aβs, growing evidence has shown that the severity of amyloid plaques in the brain does not correlate well with the degree of cognitive impairment in AD patients (Schmitz et al., 2004). Therefore, we reasoned that the capacity of quetiapine to improve behavioural performance might be related to the anti-inflammatory effects of quetiapine.

Numerous studies show the presence of a number of markers of inflammation in the AD brain: accumulation of activated microglia occurring mainly around amyloid plaques accompanied by excessive or dysregulated release of proinflammatory cytokines and chemokines which contributes to neuronal death and degeneration (Lucin & Wyss-Coray, 2009). It has been well known that higher inflammatory levels are related to higher risk of cognitive impairment (Rosano et al., 2012). In the present study, the activation of microglia observed in the hippocampus of APP/PS1 transgenic mice, as well as a strong increase of IL-1β but not TNFα compared with the non-transgenic mice, was greatly reduced following quetiapine treatment,

suggesting that quetiapine could have anti-inflammatory effects. To confirm, quetiapine's anti-inflammatory effects were tested on primary microglia culture which was activated by Aβ as shown in Fig. 3.4. In agreement with our animal findings, quetiapine drastically decreased the release of both IL-1β and TNFα in microglial culture treated with $A\beta_{1-42}$. Furthermore, our study has also shown that the doses of $A\beta_{1-42}$ (25 μM) used in this study did not affect microglial cell viability (Data not shown). As these proinflammatory mediators in turn further activate microglia creating a self-perpetuating vicious cycle by which inflammation induces further neuronal damage (Paradisi et al., 2004; V. H. Perry et al., 2010), blocking these cytokines by quetiapine possibly alleviate the chronic propagating inflammation associated with AD, which could help protect neurons and eventually attenuate behavioural impairment. Thus, quetiapine could ameliorate behavioural deficits through inhibiting brain inflammation in APP/PS1mouse model of AD.

Apart from microglia, astrocytes are also recruited during the inflammation process. Astrogliosis process has already been considered as another feature of AD and there are many studies showing that it is an important source of oxidative stress in AD patients (Paradisi et al., 2004; Wyss-Coray & Mucke, 2002). Quetiapine, on the other hand, has been well studied for decreasing the increase of reactive astrocytes in different animal models of global ischemia (B. Yan et al., 2007), cuprizone induced schizophrenia (Y. Zhang et al., 2008), and MS (Mei et al., 2012). To date, there has been no report on the effects of quetiapine on astrogliosis in APP/PS1 mice. In the present study, astrocyte numbers and GFAP expression in the cerebral cortex of APP/PS1 mice were significantly reduced by chronic administration of quetiapine. This effect seemed to be mainly due to the decrease of diffusely distributed astrocytes, since both transgenic and transgenic mice treated with quetiapine showed comparable numbers of astrocyte clusters.

NF-κB is known to be a critical regulator of inflammation by acting as an essential transcription factor for induction of COX2, iNOS, IL-1β and TNFα (X. Zhang et al., 2009). It has been shown that Aβ can directly stimulate microglia through NF-κB signaling pathway resulting in increased secretion of cytokines, chemokines and adhesion molecules (Wyss-Coray & Rogers, 2012). In turn, some proinflammatory cytokines activate NF-κB and lead to a detrimental cycle of neuroinflammation and neurodegeneration. Moreover, studies have shown that NF-κB is activated in both glial cells and neurons in the brains of AD patients, as well as in cultured neurons and glia following Aβ stimulation (Huang et al., 2012; Kaltschmidt et al., 1997; Mattson & Camandola, 2001). Suppression of NF-κB ameliorates astrogliosis in APP/PS1 transgenic mice (X. Zhang et al., 2009). More importantly, our previous study has shown that quetiapine decreased p50/p65 expression levels in mice subject to global cerebral ischemia (Bi et al., 2009). Therefore, to further understand the molecular mechanism of the effects of quetiapine on the expression of IL-1β and TNFα and subsequent glial activation, the expression of NF-κB subunit p65 were analyzed in brains using Western blots. Consistent with previous reports, the present study showed increased expression of NF-κB p65 subunit in both cortex and hippocampus of APP/PS1 transgenic mice. Quetiapine effectively ameliorated the activation of NF-κB in these mice, suggesting the effects of quetiapine against the increased levels of proinflammatory cytokines may be in part attributed to its ability of inhibition of NF-κB p65 expression. Moreover, the activation of NF-κB requires it to translocate from the cytosol to the nucleus, and binds to its cognate DNA binding sites leading to expression of inflammatory mediators (Kucharczak et al., 2003). Our *in vitro* study has demonstrated that p65 are translocated into nucleus following $A\beta_{1-42}$ stimulation, while quetiapine treatment can reverse this translocation.

Many inflammatory mediators such as IL-1β, TNFα, COX and iNOS are believed to play a vital role in the inflammatory process of AD since they have been reported to be elevated in the plasma, brains and cerebrospinal fluid of both patients with AD and transgenic animal models of AD (Akiyama et al., 2000; Blum-Degen et al., 1995; Galimberti et al., 2006; Griffin et al., 1989; Ruan et al., 2009). IL-1β and TNFα represent downstream targets which are regulated by the transcription factor NF-κB in the inflammatory cascade, which is an attractive candidate as a therapeutic target. Furthermore, NF-κB has also been directly implicated in APP processing. The activity of the β-secretase-1 (*BACE1)* promoter is controlled by a NF-κB-dependent pathway in the presence of excessive Aβ (Buggia-Prevot, Sevalle, Rossner, & Checler, 2008), while inhibition of NF-κB signaling pathway can enhance α-secretase activity, which is responsible for the benign, non-amyloidogenic processing of APP (Lee et al., 2009). Therefore, suppressing NF-κB signaling pathway should not only effectively inhibit individual proinflammatory mediators such as IL-1β and TNFα in AD, but also reduce Aβ production. Despite our finding that quetiapine only showed a minimal effect on total Aβ production in 12 month old transgenic mice, levels of the soluble forms of $A\beta_{40}$ and $A\beta_{42}$ were significantly reduced following quetiapine treatment in another study (Zhu et al., 2013). The exact mechanism by which quetiapine specifically reduces only soluble Aβs is yet unknown, but its result is significant, since soluble Aβ specifically is believed to be the primary driver of AD-related pathogenesis, resulting in glial activation, synapse loss, and neuronal cell death (J. Hardy & Selkoe, 2002; Tanzi & Bertram, 2005). We believe that quetiapine may be an efficacious and promising treatment for AD because of its multiple effects including suppressing the NF-κB pathway to reduce inflammation and soluble Aβs.

Overall, the findings reported here confirmed that glial activation and proinflammatory cytokine overproduction as a common pathophysiologic mechanism and potential therapeutic target in AD. This study is the first description revealing that quetiapine improves behavioural performance while attenuating microglial and astrocyte activation in APP/PS1 transgenic mice, and reduces proinflammatory cytokine levels *in vivo* and *in vitro*, which might be related to its inhibition of NF-κB activation.

Chapter 5

Conclusions and future directions

5.1 Overall conclusions

The overall goals of this thesis were to further characterize neuroinflammation through an investigation using an APP/PS1 transgenic mouse model of AD, to expand our understanding of the role of neuroinflammation in AD pathogenesis, to understand how neuroinflammation contributes to mouse behaviours, to examine the potential anti-inflammatory property of quetiapine, and finally to establish anti-inflammatory effects of quetiapine on transgenic mouse behaviours.

In examining the role of neuroinflammation on AD pathogenesis, this thesis largely focused on the chronological behavioural consequences of an APP/PS1 double transgenic mouse model of AD in the context of both amyloid pathology and neuroinflammation. This thesis is the first work which examined the behavioural changes in relation to the pathological changes including amyloid plaques and neuroinflammation at various age points. It has been shown that uncontrolled glial activation and neuroinflammation in AD brain may contribute independently to neural dysfunction and cell death. It is not surprising that both microglial and astroglial activation correlate well with the pattern of learning deficits rather than amyloid pathology in the APP/PS1 transgenic mouse model of AD.

This thesis was also the first work to establish the anti-inflammatory effects of quetiapine in both *in vitro* and *in vivo* systems. Here it is shown that quetiapine suppressed the inflammatory markers including cytokines in both primary microglia culture and AD mouse brains. Microglia and astrocyte activation in AD mouse brains were significantly attenuated by quetiapine

treatment. Most significantly, quetiapine improves behavioural performance in transgenic mice compared to controls, thus it provides valuable information for evaluating future anti-inflammatory agents on AD.

5.2 Significance of the research

This thesis presented several novel findings that could significantly impact the field of AD research, including:

1. A systematic behavioural characterization using a battery of cognitive tests in an APP/PS1 double transgenic mouse model, and the first time the *in vivo* chronological consequences of the interaction between amyloid pathology and neuroinflammation;

2. Evidence that neuroinflammation in the form of glial activation is relevant in the progression of AD;

3. Evidence that quetiapine may not affect AD pathogenesis through its well characterized neuroprotective role in our previous studies, but instead through its anti-inflammatory effects involving the suppression of glial activation and proinflammatory cytokine via NF-κB pathway, providing a potential therapeutic target for future study;

4. Evidence that quetiapine improves behavioural performance with only marginal effects on the amyloid pathology.

5.3 Strengths and weaknesses

5.3.1 Chapter 2: The role of neuroinflammation and amyloid in cognitive impairment in an APP/PS1 transgenic mouse model of Alzheimer's disease

This chapter presented for the first time a chronological relationship between the behavioural outcomes and both neuroinflammation and amyloid pathology in a very widely used AD transgenic model, an APP/PS1 double transgenic mouse model.

One of the major strengths of this study was that multiple tests were used to measure the same type of behaviour, hence ensuring the observed results were not test-specific. For example, learning and memory were examined with both Y maze and Morris water maze. The goal of this study was to investigate the chronological changes in behaviours at different stages of the AD mice which can be translated into the human counterpart. Mice in this study were examined at a very wide age range. We were fortunate enough to exam animals at youth (3 months), young adult (6 months), mid-life (9 months), and late-life (12 months). We did have animals reach a very late stage of life at 22 months old, unfortunately, the number of animals survived at this age in both control group and transgenic group was small and their behavioural performance was confounded by their motor ability. As a result, we decided not to include the behavioural data of 22 month old in the final published paper. By choosing multiple time points we were able to examine age-related changes in behaviour.

Another highlight of this chapter was high inter-test validity with a well-controlled age matched cohort from the same litter at each time point. Instead of re-testing animals at different age point in consideration of animal housing limitations as well as operational costs, all the behavioural tests were only administered once per age group in both control group and transgenic groups. It was presumed that learning and memory tests can be heavily influenced by

the number of testing times regardless of the length of gap time. As shown in our previous work, experience did have an effect on the performance specifically in the Morris water maze test (data was not shown). This meticulous experimental design required a significant amount of work and planning to maintain a sizable number per group at different age group. Despite this, we were still unable to generate meaningful data once animal reached the age of 22 months old.

Lastly, because of the experimental design, we were able to collect brain tissues at different age point right after the completion of behavioural tests. Admittedly, a correlation analysis will further strengthen our observation. In future experiments, it would be prudent to implant a new identifying method such as subcutaneous transponders injection at the beginning of experiments.

5.3.2 Chapter 3: Quetiapine reduces proinflammatory cytokine levels in $A\beta_{1-42}$ stimulated primary microglia

This chapter presented the anti-inflammatory properties exerted by quetiapine via inhibition of activation of the NF-κB pathway in microglia that was stimulated by fibril Aβ. These results furthered our understanding of quetiapine on its beneficial effects on the inflammatory pathway beyond its conventional pharmacological receptor affinity profile. It also lays the foundation for our next chapter in order to investigate whether its anti-inflammatory property would provide beneficial effects in an AD transgenic mouse model.

One of the strengths of this chapter is the cell toxicity studies conducted on both quetiapine and fibril Aβ in primary mouse microglia culture. There have been a variety of studies on effects of quetiapine in many different cells line, primary neuronal culture, and primary astrocyte culture (J. Wang et al., 2014). We have characterized the effects of quetiapine at various concentrations on the cell viability of primary mouse microglial culture at different treatment times. These

results provided a better understanding in our laboratory regarding the effects of quetiapine in primary microglial culture, which could be readily used for future experiments involving other inflammatory markers.

Yet, a concern may rise from the question of the physiological effects on how activated microglia stimulated by Aβ affects neuronal function. We were able to delineate the quetiapine was able to attenuate the elevated release of cytokines in Aβ activated microglia in our *in vitro* system, however, it will not fully explain the beneficial effects of quetiapine *in vivo* as it might be confounded by the direct effects of quetiapine on neurons. In order to fully appreciate that the beneficial effects of quetiapine comes directly from its anti-inflammatory effects on activated microglia, an *in vitro* system utilizing Aβ stimulated conditioned media from the primary mouse microglia would better characterize the effect of quetiapine. My concurrent project during my PhD training has shown that conditioned media from the primary mouse astrocyte pretreated with quetiapine effectively protected GABAergic neurons against aging-induced spontaneous cell death (J. Wang et al., 2014). This study has provided evidence that quetiapine exerts its beneficial effects via glial conduit, further suggesting its anti-inflammatory effects on microglia results in its protective effects in the animal study. In future experiments, it would be very important to include conditioned media from microglia to exclude non-specific effects of quetiapine on neurons.

5.3.3 Chapter 4: Quetiapine attenuates glial activation and proinflammatory cytokines in APP/PS1 transgenic mice

This chapter presented exciting results including that chronic administration of quetiapine in APP/PS1 transgenic mice resulted in a marked change in microglial and astrocyte activation, proinflammatory cytokine levels, and an improvement in behavioural performance. Consistent

with our *in vitro* results, the beneficial effects of quetiapine come from its anti-inflammatory properties via suppression of the activation of NF-κB pathway given it has no effects on number of amyloid plaque and minimal effects of levels of total Aβ.

One of the strengths of this chapter is the behavioural studies. Instead of using Y maze or Morris water maze, the object recognition test was employed to ensure again the observed results were not test-specific. Unlike both Y maze and Morris water maze assessing spatial memory, the object recognition task measures non-spatial visual-discrimination memory. Once again, these results indicate the effects of quetiapine were consistent and not related to the tests utilized. In addition, anti-anxiolytic effects of quetiapine were examined with the open field suggesting the effects of quetiapine on behavioural outcome be multidimensional.

In consideration of the duration of treatment, it was decided to treat animal up to 12 months old. It was a decision based upon both our preclinical data in Chapter 2 and clinical trials using NSAIDs. In our animal studies, the rate of cognitive decline slowed down as animals aged from 9 months old onwards whereas the increase of Aβ plaques rather than neuroinflammation is still a function of age. Large epidemiological studies demonstrated that the reduced risk for developing AD in patients using anti-inflammatory therapies requires prolonged and chronic use of medication. Clinical trials of short-term use of NSAIDs failed to provide convincing results. Therefore, a study of chronic administration of quetiapine was designed to examine its potential effects in the APP/PS1 transgenic mouse model of AD.

5.4 Potential applications in future research

An estimated 500,000 Canadians are currently diagnosed with AD, of those, 71,000 are under the age of 65. The number of people living with AD or a related dementia in Canada is expected to reach 1.1 million by 2035, if nothing improves in prevention and treatment. This thesis has

established a better understanding of the role of neuroinflammation in the pathogenesis of AD using an APP/PS1 transgenic mouse model. More importantly, targeting the inflammatory process using a potential anti-inflammatory agent which is quetiapine provided an improved behavioural outcomes without significantly affecting the Aβ pathology in APP/PS1 transgenic mice. It provided further evidence of how inflammatory changes contribute to the presentation of AD and, in the context of significant amyloid deposition, suggests that mechanisms underlying pro-inflammatory cytokine release might be an important target for therapy. Since risk factors for the development of inflammation are modifiable, these findings suggest intervention and prevention strategies for the clinical syndrome of AD.

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Appendix

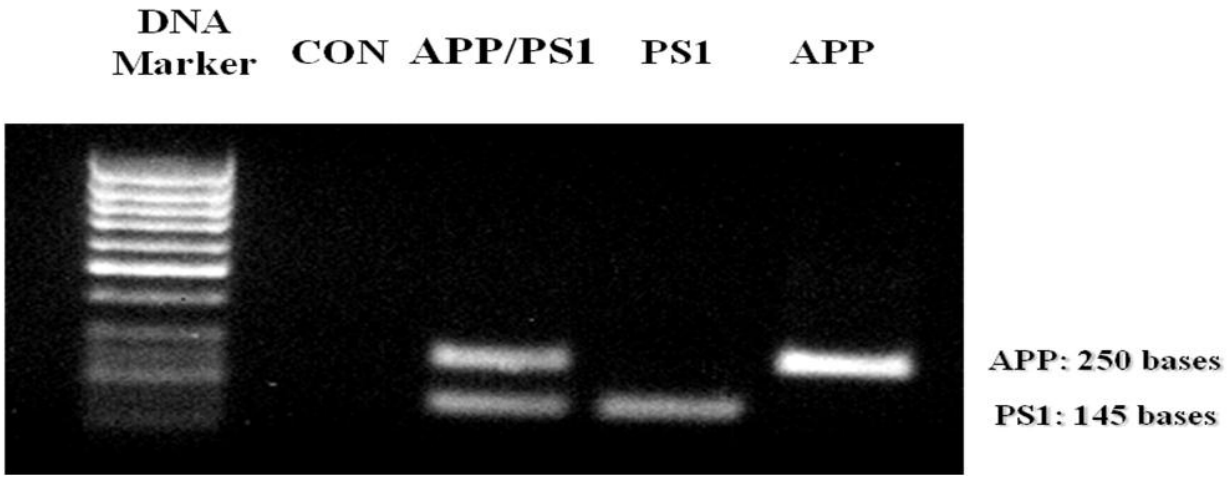

Figure A. 1**:** PCR of complementary DNA obtained from double APP/PS1 mouse carrier and single transgenic APP or PS1 mice carriers

Con: Control or non-transgenic mice; APP/PS1: APP/PS1 double transgenic mice; APP: APP single transgenic mice; PS1: PS1 single transgenic mice.

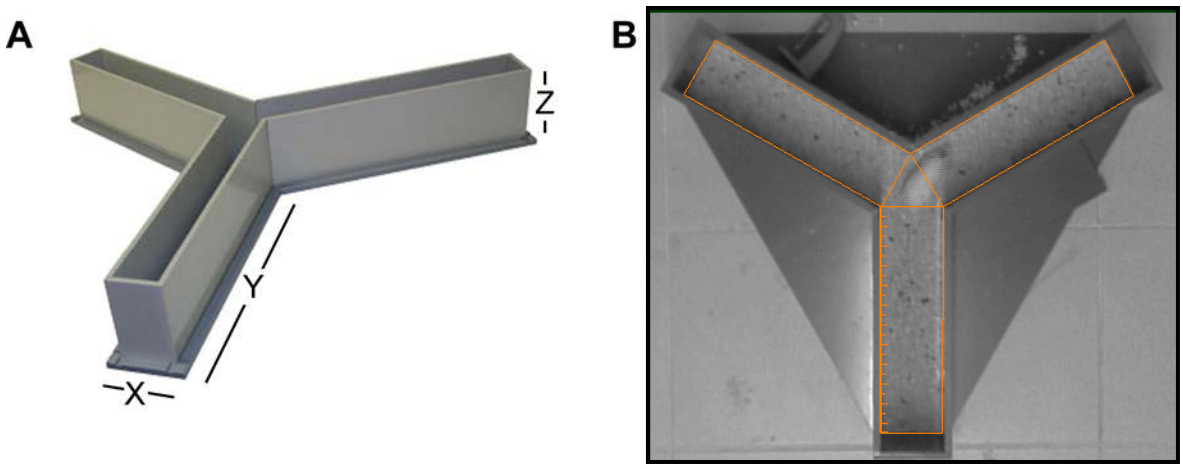

Figure A. 2: Y maze apparatus

(**A**) Image of the Y maze apparatus with specifications. x = 5 cm; y = 35 cm; z = 10 cm (image © Stoelting Co., used with permission). (**B**) Calibration image as viewed in ANY-Maze™ Video Tracking Software (version 4.63). Orange lines represent zone boundaries between 3 arms. The center zone is undefined.

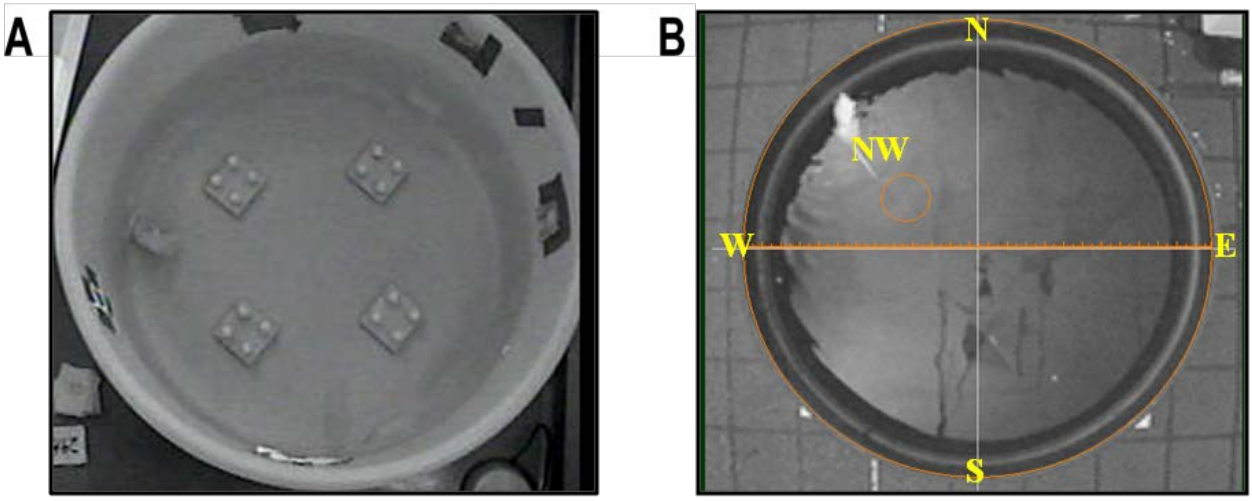

Figure A. 3: Water maze apparatus

(**A**) Image of the water maze pool as used for hidden platform trials. The 10 cm platform was submerged 1 cm below the water surface. The starting direction of the mouse varied across trials (NE, SW, SE), while the platform was kept constant at the same location. As shown, the platform is in the NW quadrant. (**B**) Calibration image as viewed in ANY-Maze^{TM} Video Tracking Software (version 4.63). Orange lines represent zone boundaries. The platform zone was defined as one location: NW. Tracking was achieved through a camera above the pool. During the probe trial, the platform was removed. As shown, the platform (the blue area) is in the NW quadrant.

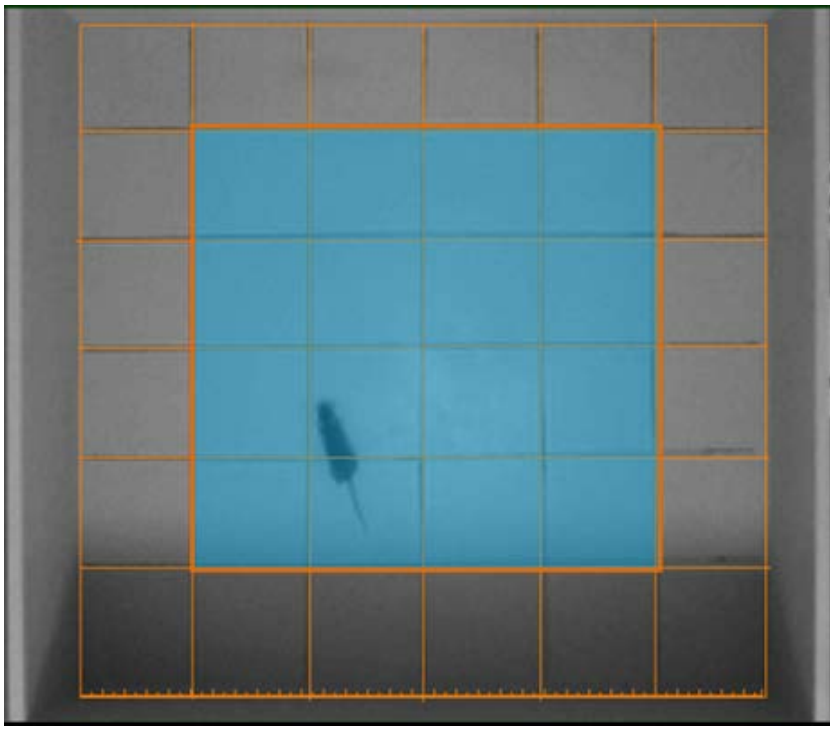

Figure A. 4: Open field apparatus

Calibration image as viewed in ANY-Maze^{TM} Video Tracking Software (version 4.63). The open field apparatus consists of a bare square box (36" by 36") with 35 cm high black walls. The open field box is partitioned into 36 equal sized squares, and divided into 20 outer border squares and 16 inner zone squares. A tracking camera views the box from above. Orange lines are zone boundaries. The blue area is defined as the Inner zone, while the perimeter is defined as the Outer zone.

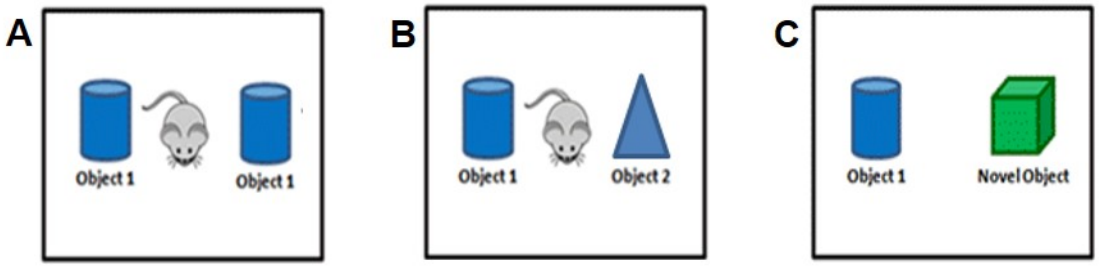

Figure A. 5: Object recognition test

The object recognition test apparatus consists of a 40 cm (width) x 40 cm (width) x 23 cm (height) Plexiglas square box. It consisted of three sessions: habituation (A), training (B), and retention (C). During the training session, mice were individually placed in the activity box for 10 min of free exploration, in which two identical objects (object 1) were positioned in two adjacent corners. During the retention session for the short-term memory test, mice were placed back into the same box 1 h later containing one of the previous objects (object 1) and a new object (object 2) for a 5 min testing session. During the retention session for the long-term memory test, animals were subsequently placed back into the same box 23 h after the short-term memory test (24 h after the training session) for 5 min of free exploration, where object 2 was replaced by a novel object. During the retention session, the time spent exploring the novel object (object 1 or novel object) was used to measure memory function.