CLEARANCE OF AMYLOID-BETA IN ALZHEIMER'S DISEASE

To understand the pathogenesis and develop potential therapies in animal models

A THESIS SUBMITTED IN TOTAL FULFILMENT
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Yan-Jiang Wang
Doctor of Medicine (Neurology)
Master of Medicine (Preventive Medicine)
Bachelor of Medicine (Clinical Medicine)

Department of Human Physiology,
Centre for Neuroscience,
School of Medicine, Flinders University,
Adelaide, South Australia

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THESIS SUMMARY

Alzheimer's disease (AD) is the most common cause of dementia. No strong disease-modifying treatments are currently available. Amyloid-beta peptide (Aβ) appears to play a pivotal role in the pathogenesis of AD. We focused our interest on revealing the pathogenesis of the disease and developing novel therapeutic modalities. The thesis consists of three projects:

1. Prevention of AD by intramuscular delivery of an anti-Aβ single chain antibody (scFv) gene

Immunotherapy is effective in removing brain Aβ, but was associated with detrimental effects. In the present study, the gene of an anti-Aβ scFv was delivered in the hind leg muscles of APPSwe/PS1dE9 mice with adeno-associated virus at three months of age. Six months later, we found that brain Aβ accumulation, AD-type pathologies and cognitive impairment were significantly attenuated in scFv-treated mice relative to enhanced green fluorescence protein (EGFP)-treated mice. Intramuscular delivery of scFv gene was well tolerated by the animals. These findings suggest that peripheral application of scFv is effective and safe in preventing the development of AD, and would be a promising non-inflammatory immunological modality for prevention and treatment of AD.

2. Prevention of AD with grape seed derived polyphenols

Polyphenols extracted from grape seeds are able to inhibit Aβ aggregation, reduce Aβ production and protect against Aβ neurotoxicity *in vitro*. We investigated the therapeutic effects of a polyphenol-rich grape seed extract (GSE) in vivo.

APPSwe/PS1dE9 transgenic mice were fed with normal AIN-93G diet (control diet), AIN-93G diet with 0.07% curcumin, or diet with 2% GSE beginning at 3 months of age for 9 months. Total phenolic content of GSE was 592.5 mg/g dry weight, including gallic acid, catechin, epicatechin and proanthocyanidins. Long-term feeding of GSE diet was well tolerated. The Aβ levels in the brain and serum of the mice fed with GSE were reduced by 33% and 44% respectively compared with the mice fed with the control diet. Amyloid plaques and microgliosis in the brain of mice fed with GSE were also reduced by 49% and 70% respectively. In conclusion, polyphenol-rich GSE is promising to be a safe and effective drug to prevent the development of AD.

3. Roles of p75NTR in the development of AD

P75NTR has been suggested to mediate Aβ induced neurotoxicity. However, its role in the development of AD is undetermined. APPSwe/PS1dE9 transgenic mice were crossed with p75NTR knockout mice to generate APPSwe/PS1dE9 mice with p75NTR gene deleted. P75NTR mainly expressed in the basal forebrain neurons and degenerative neurites in neocortex and hippocampus. Genetic deletion of p75NTR gene in APPSwe/PS1dE9 mice reduced soluble Aβ levels, but increased the insoluble Aβ accumulation and Aβ plaque formation in the brain. P75NTR deletion decreased Aβ production of cortical neurons *in vitro*. Recombinant extracellular domain of p75NTR attenuated the oligomerization and fibrillation of synthetic Aβ42 peptide *in vitro*, and reduced local Aβ plaques after hippocampus injection *in vivo*. Our data suggest that p75NTR plays an important role in AD development and may be a valid therapeutic target for the treatment of AD.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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2. **Wang YJ**, Gao CY, Yang M, Pollard A, Valadares D, Liu XH, Dong XY, Wu XB, Zhong JH, Zhou HD, Zhou XF (2010). Intramuscular delivery of a single chain antibody gene prevents brain Abeta deposition and cognitive impairment in a mouse model of Alzheimer's disease. Brain Behav Immun (in revision).
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CHAPTER 1

Literature review

Clearance of Amyloid-beta in Alzheimer's Disease:

Mechanisms and Therapeutic Strategies

Introduction

AD (AD) is a debilitating neurodegenerative disease and is the most common cause of dementia and the leading cause of death amongst the elderly (World Health Organization, 2003; Ferri et al., 2005). With the world's population ageing, it is estimated that by 2050, 106.8 million will be living with AD (Brookmeyera et al., 2007). Unfortunately, there are no strong disease-modifying treatments available at this time (Vellas et al., 2008). Understanding of the pathogenesis of AD is crucial to provide a strong rationale for development of a potential cure or treatment.

AD is characterized by deposit of amyloid plaques, neuron loss, neuritic degeneration, accumulation of fibrillary tangle in neurons, and a progressive deterioration of cognition. Amyloid-beta peptide (Aβ) appears to play a pivotal role in the pathogenesis of AD according to the amyloid hypothesis, in which accumulation of Aβ in the brain is the primary factor driving AD pathogenesis, and the rest of the disease process, including hyperphosphorylation of tau protein, formation of neurofibrillary tangles and inflammation, is secondary to Aβ accumulation in the brain (Hardy and Selkoe, 2002).

Aβ is generated from sequential cleavage of the amyloid precursor protein (APP). APP is a transmembrane glycoprotein, whose gene is mapped to chromosome 21 (Korenberg et al., 1989). Due to the alternative splicing of the nascent transcript, there are several different isoforms of APP (Preece et al., 2004). The predominant isoforms are APP695, APP751 and APP770. APP695 is the major neuronal isoforms. APP is processed in two distinct pathways: the amyloidogenic and non-amyloidogenic processing (Gandy and Greengard, 1994). Aβ is generated from the amyloidogenic processing, which is initiated with the cleavage of APP by β-

Figure 1.1

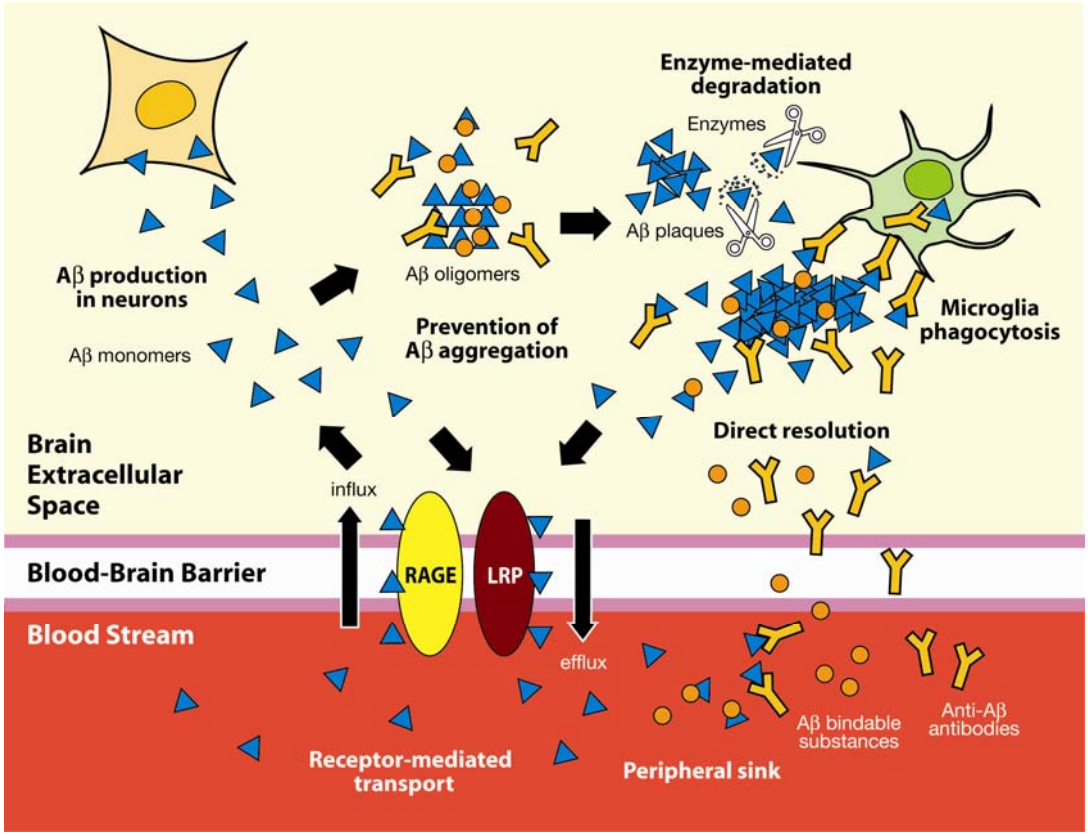

Figure 1.1 Mechanisms of Aβ clearance. The steady state level of Aβ depends on a balance between production, clearance and influx. Transport of Aβ across BBB is mainly mediated by receptors (i.e. RAGE and LRP). Aβ in extra- and intra-cellular space can be degraded by enzymes (i.e. NEP and IDE). Peripheral anti-Aβ antibodies and Aβ-bindable substances are able to enter the brain at low levels, where they bind to Aβ, prevent Aβ aggregation, directly resolve Aβ fibrils, and might also exert an effect of peripheral sink by binding to peripheral Aβ, thereby disrupting the equilibrium, resulting in the sequestration of Aβ away from the brain. These mechanisms are the potential target for the development of AD treatment.

secretase in the lumenal domain proximal to the transmembrane segment and generates the N-terminus of Aβ, the large soluble ectodomain (APPsβ) and a membrane-tethered C-terminal fragment (β-CTF). The subsequent cleavage by γ-secretase cuts the β-CTF within the transmembrain domain and gengerates the C-terminal of Aβ (Gandy and Greengard, 1994). The variation of cleavage by γ-secretase generates variable length (39-42 amino acid long) Aβ. The longer forms of Aβ are prone to rapid aggregation. Aβ peptides form fibrils and deposit outside neurons in dense formations known as senile plaques (Tiraboschi et al., 2004). In non-amyloidogenic pathway, APP is cut by α-secretases within the lumenal domain between β- and γ-secretase cleavage sites, generating an ectodomain of APP (APPsα) and C-terminal fragment (α-CTF)(Gandy and Greengard, 1994). Because α-secretase cuts within the middle of Aβ, non-amyloidogenic processing pathway does not generate Aβ.

Regarding brain Aβ accumulation, only a relatively small number (<5%) of AD patients (familial cases) have increased Aβ production in the central nervous system (CNS) due to inherited mutations in the APP (amyloid protein precursor) gene nearby the Aβ coding region (i.e., Swedish mutation) or presenilins 1 or 2 genes (Hardy and Selkoe, 2002), while the majority of patients with so-called sporadic or late-onset AD (LOAD) do not have an increased Aβ production or APP over-expression in the CNS. These findings implicate that the dysfunction in Aβ clearance plays important roles in the accumulation of Aβ in the brain of AD patients. Emerging evidence suggests that the steady levels of Aβ are determined by the balance between its production and clearance (Figure 1.1). Here, we review the recent progress in the physiological and therapeutic Aβ clearance, and discuss the potential strategies towards a potential prevention and treatment for AD.

Receptor-mediated Aβ transport across blood-brain barrier (BBB)

Soluble Aβ can be removed slowly, *via* interstitial fluid (ISF) bulk flow, into the bloodstream (Shibata et al., 2000; Silverberg et al., 2003; Crossgrove et al., 2005). Ageing, an important risk factor of AD, significantly affects the choroid plexus-CSF circulatory system and the exchange of a variety of components with the blood. However, this is responsible for the clearance of only 10-15% of the total Aβ in the brain and circulating Aβ can also influx into the brain from plasma. Receptor-mediated transport of Aβ is principally responsible for the transport of Aβ across the BBB (Table 1).

Efflux of Aβ from CNS to plasma

LRP-mediated Aβ efflux

Low-density lipoprotein receptor-related protein (LRP), a member of the LDL receptor family, is a multifunctional scavenger and signaling receptor (Herz and Marschang, 2003). LRP can interact with a broad range of secreted proteins and resident cell surface molecules, including AD-related molecules APP, ApoE and alpha2M, tissue plasminogen activator, plasminogen activator inhibitor-1, factor VIII, and lactoferrin, mediate endocytosis and activate signaling pathways through multiple cytosolic adaptor and scaffold proteins (Herz and Marschang, 2003).

LRP mediates the efflux of Aβ from the brain into blood. The interaction between Aβ and LRP mediates Aβ brain capillary binding, endocytosis and transcytosis across the BBB into blood (Shibata et al., 2000; Herz, 2003). Dysfunction of LRP leads to reduced efflux of Aβ from the brain thus increased Aβ deposition in the mouse brain (Kang et al., 2000; Shibata et al., 2000; Van Uden et al., 2002).

LRP has been shown to be genetically linked to AD in epidemiological studies (Lambert et al., 1998a) (Kang et al., 2000). In AD reduced expression of brain endothelial LRP is associated with positive Aβ staining of vessels (Shibata et al., 2000).

The expression of LRP can be regulated by Aβ levels. A possible mechanism is that Aβ can promote proteasome-dependent degradation of LRP (Deane et al., 2004b). This may explain the observations of decreased LRP activity or expression in brain microvessels in AD patients and mouse models (Shibata et al., 2000; Deane et al., 2004b; Donahue et al., 2006).

Recent studies found that soluble LRP (sLRP), a product from cleavage of LRP N-terminal extracellular domain (von Arnim et al., 2005) and normally circulates in human plasma (Quinn et al., 1997), binds 70% to 90 % of plasma Aβ (Sagare et al., 2007). The binding of Aβ to sLRP prevents its access to the brain. In AD individuals, the levels of LRP at the BBB are reduced, as are levels of Aβ binding to sLRP in plasma (Sagare et al., 2007). This, in turn, may increase Aβ brain levels through a decreased efflux of brain Aβ at the BBB and/or reduced sequestration of plasma Aβ associated with re-entry of free Aβ into the brain. Recombinant LRP cluster IV (LRP-IV), a major binding domain of LRP, binds Aβ with high affinity in plasm of mouse model and AD patients, and effectively reduces Aβ-related pathology and dysfunction in a AD mouse model, suggesting that LRP-IV can effectively replace native sLRP, which is comprised in AD patients, and clear Aβ, without direct central action and discernible side effects (Sagare et al., 2007). Thus, therapies which increase LRP expression at the BBB and/or enhance the peripheral Aβ "sink" activity

of sLRP, hold potential to control brain Aβ accumulations, neuroinflammation and cerebral blood flow reductions in AD.

P-glycoprotein mediated Aβ efflux

ATP-binding cassette transporter p-glycoprotein (P-gp), known as multi-drug resistance transporter (MDR1), is highly expressed on the luminal surface of brain capillary endothelial cells, wherein P-gp functionally constitutes a major component of the BBB by limiting CNS penetration of various substances (Cordon-Cardo et al., 1989). Increased levels of Aβ40 and Aβ42 in the temporal lobe of the brain of non-demented elderly people were inversely correlated with P-gp expression levels in cerebral vessels (Vogelgesang et al., 2002). Pharmacological blockade of P-gp rapidly decreases extracellular levels of Aβ secretion. Aβ is transported across the plasma membranes of P-gp enriched vesicles in both ATP- and P-gp-dependent manners (Lam et al., 2001). In P-gp-null mice Aβ clearance in the brain is compromised, while in APP-transgenic mice P-gp administration significantly increases Aβ levels within the brain ISF. Furthermore, APP-transgenic, P-gp-null mice had increased levels of brain Aβ and enhanced Aβ deposition (Cirrito et al., 2005). These findings suggest that P-gp takes part in Aβ clearance as an Aβ efflux pump at the BBB. P-gp activity at the BBB could affect risk for developing AD, and it could be a potential diagnostic and therapeutic candidate.

Influx of Aβ from plasma to CNS

A substantial amount of Aβ is presented in the blood, and is markedly elevated in AD patients. The observation that Aβ can be reversely transported into the brain via

the BBB suggests the possible participation of blood Aβ in the development of amyloid deposition in AD (Kuo et al., 1999; Pluta et al., 2000; Deane et al., 2004a).

RAGE-mediated Aβ influx

Receptor for advanced glycation endproducts (RAGE), a member of the immunoglobulin superfamily, is a multi-ligand, cell surface receptor expressed by neurons, microglia, astrocytes, cerebral endothelial cells, pericytes, and smooth muscle cells, with up-regulated expression in diverse pathologies from atherosclerosis to AD (Schmidt et al., 1999; Alarcon et al., 2005). It binds a repertoire of broad ligands including products of nonenzymatic glycoxidation (AGE), Aβ, the S100/calgranulin family of proinflammatory cytokine-like mediators, and the high mobility group 1 DNA binding protein, amphoterin.

In contrast to LRP, RAGE is considered to be the major player to mediate Aβ influx into the brain from the bloodstream (Mackic et al., 1998). RAGE binds soluble Aβ in the nanomolar range (Yan et al., 2000), and mediates transport of pathophysiologically relevant concentrations of plasma Aβ across the BBB (Deane et al., 2003). Down-regulation of RAGE is able to inhibit the influx of Aβ from the periphery into the CNS (Deane et al., 2003).

In addition to the transcytosis of circulating Aβ into the brain, RAGE plays multiple adverse roles in the pathogenesis of AD, including perturbation of neuronal properties and functions (Arancio et al., 2004), amplification of glial inflammatory responses (Stern et al., 2002; Deane et al., 2003), elevation of oxidative stress and amyloidosis (Yan et al., 1996), triggering autoimmune responses in the periphery

(Mruthinti et al., 2004), and Aβ-induced cell death (Yan et al., 1996; Hadding et al., 2004; Sturchler et al., 2008).

A feature of RAGE that is particularly worth noting is its unusual sustained juxtaposition with its ligand in tissues. In contrast to suppression of receptors observed with LRP in an Aβ rich environment (Shibata et al., 2000), RAGE expression is up-regulated and sustained at an elevated level by excess amounts of Aβ in cerebral microvascular endothelia of AD brain with a positive feedback mechanism (Yan et al., 1996; Mackic et al., 1998; Shibata et al., 2000; Miller et al., 2008). Deposition of Aβ in the brain of AD or transgenic models of AD triggers the expression of RAGE in affected cerebral vessels. Given that the efflux of Aβ from the brain appears compromised during ageing and in AD (Shibata et al., 2000), this mechanism may exacerbate cellular dysfunction due to RAGE/Aβ interaction, as increasing expression of the receptor allows for more profound RAGE-mediated influx of Aβ.

gp330/megalin-mediated Aβ influx

Beside RAGE, gp330/megalin has also been reported to transport circulating plasma Aβ in a complex with ApoJ back into the brain across the BBB (Zlokovic et al., 1996). However, gp330/megalin is normally saturated by high levels of plasma ApoJ which precludes significant influx of Aβ into the CNS under physiological

TABLE 1 Receptors which mediate Aβ transport across BBB

Receptor	Function	Evidence for involvement in *in vitro* or animal models	Evidence for involvement in human AD
LRP	Transport of Aβ from brain into blood	(Kang et al., 2000; Shibata et al., 2000; Van Uden et al., 2002)	Low level of LRP is associated with positive staining of vessels for Aβ (Shibata et al., 2000); Linkage studies (Lambert et al., 1998a).
P-glycoprotein	Transport of Aβ from brain into blood	(Lam et al., 2001; Cirrito et al., 2005)	Levels in cerebral vessels reduced with increased Aβ level in AD brain (Vogelgesang et al., 2002).
RAGE	Transport of Aβ from blood into brain	(Yan et al., 2000; Deane et al., 2003)	Not established
gp330/megalin	Transport of Aβ from blood into brain	(Zlokovic et al., 1996)	Not established

conditions, so RAGE is the most likely receptor responsible for the transport of Aβ back into the brain (Zlokovic, 2004).

Enzyme-mediated Aβ degradation

Aβ is degraded by a number of peptidases, principally two zinc metalloendopeptidases referred to as neprilysin and insulin degrading enzyme (IDE) (Table 2).

Neprilysin

Neprilysin is a type II membrane protein localized in the cell surface, its substrates include the insulin B chain, a variety of neuropeptides, the atrial natriuretic peptide (ANP), endothelins and Aβ. The catalytic site of neprilysin is exposed extracellularly, making it a prime candidate for peptide degradation at extracellular sites of diffuse Aβ deposits and neuritic plaques.

Neprilysin is a rate-limiting Aβ-degrading enzyme in the brain (Kanemitsu et al., 2003). The catalytic site of neprilysin is exposed extracellularly, making it a prime candidate for peptide degradation at extracellular sites of Aβ deposits. Intracerebral human neprilysin gene transfer leads to a remarkable decrease in amyloid deposits in an AD mouse brain (Marr et al., 2003), while inhibition of neprilysin protein or disruption of the neprilysin gene results in a defect in Aβ degradation (Iwata et al., 2001; Newell et al., 2003). In AD brain, the level and activity of neprilysin decrease in the cortex and hippocampus but not in other brain areas or peripheral organs (Yasojima et al., 2001; Caccamo et al., 2005; Maruyama et al., 2005). Neprilysin levels were inversely associated with Aβ peptide levels in the vasculature, brain and CSF of AD patients (Carpentier et al., 2002; Maruyama et al., 2005; Hellstrom-

Lindahl et al., 2008). Mutations in either NEP gene or its promoter have also been suggested to be associated with sporadic AD in different populations (Helisalmi et al., 2004; Sakai et al., 2004; Shi et al., 2005; Wood et al., 2007). These findings suggest that the deficient degradation of Aβ caused by low levels of neprilysin might contribute to AD pathogenesis and elevating neprilysin activity in the brain may have a therapeutic significance.

Insulin degrading enzyme (IDE)

IDE is a zinc metalloendopeptidase that hydrolyzes several regulatory peptides, including insulin, glucagon, atrial natriuretic factor, transforming growth factor, β-endorphin, amylin, Aβ, and the AICD. In contrast to neprilysin, IDE is predominantly localized to the cytoplasm, while only a small fraction resides in the plasma membrane.

IDE is another major enzyme for Aβ degradation in the brain. The levels of IDE in the brain decrease during aging. It has a distinct distribution in the AD brain, with lower levels and being more oxidized in the cortex and hippocampus than in the cerebellum (Caccamo et al., 2005). In animal models, deficits in IDE function lead to the impairment of Aβ degradation in the brain (Farris et al., 2003; Miller et al., 2003; Farris et al., 2004), whereas over-expression of IDE reduces Aβ levels, and retards or completely prevents amyloid plaque formation in the brain (Leissring et al., 2003). IDE activity selectively decreases in the hippocampus of patients at high risk to develop AD (Zhao et al., 2007). Defect in Aβ proteolysis by IDE also contributes to Aβ accumulation in the cortical microvasculature of AD cases with cerebral amyloid angiopathy (Morelli et al., 2004).

Epidemiological studies suggest that chromosome 10q encompassing the gene encoding IDE has genetic linkage for both late-onset AD (LOAD) (Bertram et al., 2000; Ait-Ghezala et al., 2002) and type 2 diabetes mellitus (DM2) (Wiltshire et al., 2001). Within the region, the gene for IDE represents a strong positional and biological candidate for LOAD, DM2, and for the epidemiological relationships among hyperinsulinemia, DM2, and AD (Biessels et al., 2006). In chromosome 10-linked AD families the catalytic activity of the insulin-degrading enzyme is reduced (Kim et al., 2007a). In this regard, sequence variants of IDE have recently been shown to be associated with LOAD and extent of Aβ deposition in the AD brain in some studies (Bian et al., 2004; Blomqvist et al., 2005; Bjork et al., 2007; Vepsalainen et al., 2007).

Other enzymes associated with Aβ degradation

Other metalloendopeptidase candidates, such as endothelin converting enzyme (ECE) and angiotensin-converting enzyme (ACE), also degrade Aβ. ECE-1 and a closely related enzyme, ECE-2, can hydrolyze Aβ in the brain (Eckman et al., 2001; Eckman et al., 2003). ECE is associated with susceptibility to sporadic LOAD in a population study (Scacchi et al., 2008). ACE has been recently found to be capable of degrading Aβ (Hemming and Selkoe, 2005; Zhang et al., 2005). The relationship between ACE and AD was revealed in epidemiological studies (Elkins et al., 2004; Kolsch et al., 2005; Sleegers et al., 2005; Meng et al., 2006; Wang et al., 2006a).

TABLE 2 Enzymes which degrades Aβ

Enzyme	Function	Evidence for involvement in in vitro and animal models	Evidence for involvement in human AD
Neprilysin	Degrades Aβ	(Iwata et al., 2001; Marr et al., 2003; Newell et al., 2003)	Levels and activity decreased in aging and AD brains (Yasojima et al., 2001; Carpentier et al., 2002; Caccamo et al., 2005; Maruyama et al., 2005; Hellstrom-Lindahl et al., 2008) Mutations in NEP gene are associated with sporadic AD (Helisalmi et al., 2004; Shi et al., 2005; Wood et al., 2007)
Insulin degrading enzyme	Degrades Aβ	(Farris et al., 2003; Leissring et al., 2003; Farris et al., 2004)	Levels reduced in AD cases with cerebral amyloid angiopathy (Morelli et al., 2004); some linkage studies (Bertram et al., 2000; Ait-Ghezala et al., 2002)

Natural anti-Aβ autoantibodies

Natural autoantibodies against Aβ have been found in AD patients and healthy individuals (Mruthinti et al., 2004; Brettschneider et al., 2005; Geylis et al., 2005). The anti-Aβ autoantibodies are present in both the IgG and IgM repertoire (Szabo et al., 2008), and recognize multiple linear epitopes in the Aβ monomer and conformation-specific epitopes of aggregated Aβ peptide, including structures from oligomers to fibrils, with an average avidity higher than that of the monoclonal antibody 4G8 (Relkin et al., 2007; Szabo et al., 2008).

The pathophysiological significance of these autoantibodies is undetermined. These autoantibodies exist in very low levels, tend to be reduced in AD patients, and appear to be harmless. In AD patients the autoantibodies against the neurotoxic oligomeric Aβ species decrease in plasma and are correlated with age at onset for AD (Moir et al., 2005). Anti-Aβ autoantibodies isolated from immunoglobulin preparations also strongly blocked Aβ fibril formation, disrupted formation of fibrillar structures and almost completely prevented Aβ neurotoxicity (Du et al., 2003). Additionally, some naturally occurring proteolytic antibodies have also been found to cleave Aβ (Liu et al., 2004a; Paul et al., 2005; Taguchi et al., 2008). In the phase I trials treatment of intravenous immunoglobulins (IVIgG), which contains autoantibodies against Aβ, significantly lowered CSF levels of total Aβ and improved the cognitive performance in AD patients, suggesting that IVIgG can mobilize Aβ from brain to blood (Dodel et al., 2004; Relkin et al., 2008).

These findings make it tempting to speculate that naturally occurring autoantibodies against Aβ might be beneficial to Aβ clearance. Although levels of these

autoantibodies are normally very low in serum, their long-term persistence might be sufficient to protect against AD (Schenk et al., 2004).

Therapeutic clearance of Aβ

Immunotherapy-mediated Aβ clearance

Active immunotherapy

A milestone study of the therapeutic Aβ clearance is the immunological approach which was creatively done by Schenk et al in 1999, in which aggregated Aβ42 immunization of the young PDAPP transgenic mouse over-expressing Aβ in the brain, essentially prevents the development of Aβ deposits, neuritic dystrophy and astrogliosis related to Aβ accumulation in the brain, while immunization of old animals, presumed as a treatment, also led to a marked reduction in the extent and progression of these AD-type pathologies (Schenk et al., 1999). This study opens a new era of anti-Aβ immunotherapy towards Aβ clearance and AD treatment.

After that, lots of studies have verified that Aβ vaccination is effective in reducing Aβ load in the brain, thus leads to a concomitant improvement in neuritic dystrophy and cognitive deficits in animal models (Janus et al., 2000; Morgan et al., 2000; Dodart et al., 2002; Bard et al., 2003; Wilcock et al., 2004a; Billings et al., 2005; Maier et al., 2006). The success in the animal studies pushed the Aβ immunization forward into the clinical trials without any delay. The phase I and phase II clinical trials also suggest that the active immunization with Aβ peptide is therapeutically effective, as demonstrated by eliciting amyloid plaque clearance, attenuating plaque-associated pathology, decreasing CSF tau level and slowing patients' cognitive decline (Nicoll et al., 2003; Ferrer et al., 2004; Gilman et al., 2005; Masliah et al.,

2005). Recently the six-year prospective follow-up study reports that active Aβ immunization in the first phase I clinical trial is not able to alleviate the progression of AD such as cognitive decline or survival outcomes, although the generation of anti-Aβ antibodies are elicited and cortical Aβ is subsequently removed in an antibody titer dependent manner after immunization with Aβ (Holmes et al., 2008). However, the small cohort of patients and intervention time in the middle to late course of the disease in the studies make it premature to draw a conclusion regarding the inefficacy of the active Aβ immunization (Holtzman 2008; St George-Hyslop and Morris 2008). Further clinical trials are called for with power to detect even small changes in progression rates, and differentiate the curative and preventive efficacy.

Passive immunotherapy

Instead of active immunotherapy by immunization with Aβ42, direct administration of anti-Aβ antibodies peripherally or intracranially, called passive immunization, is also effective in reducing Aβ burden and attenuates cognitive deterioration of the animals (Bard et al., 2000; DeMattos et al., 2001; Chauhan and Siegel, 2004; Wilcock et al., 2004c; Lee et al., 2006).

Because of the success in the animal studies and the expectation that administration of purified antibodies would be safer and more adjustable than active immunization protocols, the clinical trial of passive immunotherapy is currently undertaken, in which AD patients are treated with humanized monoclonal antibodies against Aβ, named Bapineuzumab. It is expected that administered antibodies should clear Aβ with the potential added benefit of a better safety and tolerability profile. Recently the phase II clinical trials aiming to examine the safety of the passive approach are reported with an overall negative result balanced against a positive effects on a

subgroup of patients who do not carry the AD risk allele ApoE4, and favouring the improvement of cognition in total treated patients (Salloway et al., 2009). Phase III clinical trials are currently being undertaken to examine the efficacy in a larger cohort of AD patients of ApoE4 carrier or non-carrier (Kaufer and Gandy, 2009).

Mechanisms of Aβ clearance by immunotherapy

The antibodies with specificity to Aβ are the effectors of the Aβ clearance in both active and passive immunotherapy. Three basic hypotheses have been proposed regarding the mechanism of Aβ clearance by immunotherapy. The first mechanism is the antibody mediated opsonization of Aβ. The anti-Aβ antibodies in the serum can cross the BBB, and bind to Aβ in the brain, forming the immune complex then initiating the phagocytosis of the complex by microglia activated through Fc ligation . The second mechanism is the peripheral "sink" hypothesis. Anti-Aβ antibodies existing in the blood can bind and sequester plasma Aβ in the periphery and then prevent Aβ from influxing back into the brain, thus disrupting the free Aβ equilibrium between the BBB and favoring a net transport of Aβ out of the brain (Bard et al., 2000; Brazil et al., 2000; Wilcock et al., 2001; Wilcock et al., 2004a; Wilcock et al., 2004c). The third hypothesis is the disruption of the Aβ aggregation by anti-Aβ antibodies. Antibodies raised against the N terminus of Aβ can inhibit Aβ aggregation and even bind to pre-existing Aβ fibrils, resulting in disaggregation and protection from their neurotoxic effects (Solomon, Koppel et al. 1996; Solomon, Koppel et al. 1997). This effect can prevent the formation of Aβ deposition in the brain and thus favors the efflux of Aβ from the brain to blood. It is noteworthy that these mechanisms are not mutually exclusive and can be simultaneously active to remove amyloid plaques (DeMattos et al., 2001; DeMattos et al., 2002; Lemere et al.,

2003). Additionally, given the presence of natural anti-Aβ autoantibodies in human plasma, these hypotheses also raise the possibility that the immune system may operate naturally to facilitate clearance of Aβ in normal subjects (Bacskai et al., 2002; Das et al., 2003).

Adverse effect of immunotherapy

The Aβ targeting immunotherapy, including both active and passive immunotherapy, forms a promising picture of Aβ clearance towards the effective prevention and treatment of AD. However, despite the results favoring Aβ clearance and cognition improvement, some serious side effects are associated with the immunotherapy.

In the clinical trials of active immunotherapy, a significant number of patients (accounted for 6%) developed autoimmune meningoencephalitis, caused by the infiltration of autoreactive T lymphocytes into the brain in response to active immunization (Moir et al., 2005). Further study suggests that the T lymphocytes activation is provoked by the peptide domains mapped to the Aβ15-42, called T cell epitopes, which is segregated from the dominant B cell epitopes identified in Aβ1-15 (Nicoll et al., 2003; Ferrer et al., 2004).

The passive approach can avoid the activation of autoimmune T lymphocyte seen in active immunization with Aβ42, however, another potential risk is cerebral microhemorrhage, which is firstly observed in the animal models (Pfeifer, Boncristiano et al. 2002). Subsequent studies show that the microhemorrhages occur in animals and patients receiving both active and passive immunotherapies (Monsonego et al., 2003) (Wilcock, Jantzen et al. 2007), suggesting that the microhemorrhage is a common side effect caused in anti-Aβ immunotherapy. The underlying mechanism is unclear yet. It is proposed that the occurrence of the

microhemorrhage requires the presence of CAA and antibody recognition of deposited forms of Aβ (Ferrer et al., 2004). A substantial change in the passive immunotherapy is the shift of Aβ from the parenchymal regions to the blood vessels, which is associated with the microhemorrhages (Racke et al., 2005). In an animal model of passive immunotherapy associated microhemorrhage, no overt structural changes in the vessel wall are observed, suggesting that the microhemorrhage is not a result of obvious vessel structure damage by passive immunotherapy (Wilcock et al., 2004b). Despite the uncertainty of the mechanism, a recent study suggests that both parenchymal and vascular Aβ deposit can be removed while the microhemorrhage can be avoided by modulating antibody dose in the passive immunization (Burbach et al., 2007).

Another significant adverse effect related to passive immunotherapy is the vasogenic edema which shows up on MRI and resolves later, most of them causes few clinical symptoms in the phase II clinical trials of passive immunotherapy (Schroeter et al., 2008). It is unclear at present time how troublesome the edema could be, and whether it is acceptable eventually in balance with treating a deadly disease. The underlying mechanism of the edema remains unclear, a possible explanation might be the increase of protein concentrations and osmotic pressure in the interstitial space in capillary beds resulted from release of Aβ peptide from plaques by the anti-Aβ antibodies (Salloway et al., 2009).

Despite the significant adverse effects, immunotherapy against Aβ still holds the promise to become the first strong disease modifying modality against AD. Currently many approaches are under exploration to avoid the adverse effects of the immunotherapy.

Improvement of vaccine

It has been identified that the autoimmune T lymphocyte activated by T cell epitope was mapped to the Aβ15-42, while the dominant B cell epitopes was mapped to the Aβ1-15 (Gelinas et al., 2004). To avoid the activation of T lymphocyte which occurred in the clinical trials, new vaccines composed of B cell epitopes only are under development. Recent studies suggest that immunization with B cell epitopes (Aβ1–15) is effective in generating anti-Aβ antibodies without provoking T-cell response against full-length Aβ, reducing brain Aβ deposition and attenuating cognitive deficits in AD animal models (Monsonego et al., 2003). Importantly, antibodies generated against N-terminus of Aβ, the B cell epitope, are able to inhibit Aβ aggregation, neutralize Aβ neurotoxicity, disaggregate pre-existing Aβ fibrils, and are most effective in reducing brain amyloid burden (Agadjanyan et al., 2005; Maier et al., 2006). The predominant antibodies generated after immunization with Aβ42 (AN1792) in the clinical phase IIa study are primarily B cell epitope (N-terminal 1-8) specific, which are independent of meningoencephalitis seen in a subset of immunized patients (McLaurin et al., 2002; Bard et al., 2003; Bussiere et al., 2004; Horikoshi et al., 2004). These preclinical and clinical data provide the basis for the future studies to improve the Aβ vaccines by including B cell epitopes and excluding T cell epitopes, to elicit the beneficial immune response and avoid a potentially deleterious cellular immune response.

DNA vaccination provides an attractive approach for better control of the immune response towards a humoral response to Aβ. Compared with conventional peptide/protein-based immunization, DNA vaccination has several significant advantages: easy production, the capability of modifying genes coding for desired

antigens, the stability of episomal DNA, and more importantly, the ability to induce the desired type of immune response using the appropriate immunostimulatory and immunomodulatory sequences. Active vaccination with DNA vaccine encoding full length Aβ peptide can effectively induce anti-Aβ antibody and reduce brain Aβ burden, with long term effects and safety (Lee et al., 2005). Furthermore, the strategies are taken to induce the beneficial Th2-type immune response by using the B cell epitope sequence only, or by including Th2 response-favouring immunostimulatory or immunomodulatory sequences in a DNA vaccine. DNA vaccines encoding B cell epitope sequence of Aβ (i.e. 11 tandem repeats of Aβ1-6 or Aβ1-21) alone are able to induce an anti-inflammatory Th2-type immune response, and does not cause obvious inflammation-related pathology in the brain of mice immunized (Hara et al., 2004; Okura et al., 2006; DaSilva et al., 2009). The sequence of immunostimulatory or immunomodulatory, such as interleukin-4, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage-derived chemokine (MDC/CCL22) and cholera toxin B subunit, are used as a molecular adjuvents in combination with Aβ DNA vaccine to promote a strong anti-inflammatory Th2-type immune response and generate Aβ reduction and cognitive improvement without obvious adverse effects (Hara et al., 2004; Kim et al., 2005). In addition, gene-gun delivery of Aβ DNA vaccines has been suggested to be highly effective in breaking self-tolerance and inducing Th2-type immune responses (Ghochikyan et al., 2003; Zhang et al., 2003a; He et al., 2005; Movsesyan et al., 2008). Thus, DNA vaccine is promising to be a safe and effective Aβ vaccine.

Mucosal immunization via oral or nasal routes is a desirable strategy due to its convenience and high tolerability (Qu et al., 2004). By combining above novel Aβ immunogens targeting the B cell epitope with appropriate immune response–directed

adjuvants and routes of administration, it is promising to develop a safer and effective Aβ vaccine (Lemere et al., 2006).

Modification of anti-Aβ antibody

The autoimmune CNS inflammation and microhemorrhage are observed in anti-Aβ immunotherapy, and are presumably induced by T-cell-mediated and/or Fc-mediated immune responses (Qu et al., 2004). Although Fc receptor mediated phagocytosis of antibody-Aβ immune complexes by microglia plays an important role in reducing brain Aβ (Pfeifer et al., 2002; Wilcock et al., 2004b; Racke et al., 2005), Fc portion of the anti-Aβ antibody is not necessary for Aβ clearance as Aβ immunization is also able to effectively reduce Aβ deposition in Fc receptor knockout mice (Bard et al., 2000). In this regard, removal of Fc portion may minimize the risk of triggering inflammatory side reactions. It is reported that administration of $F(ab)_2$ fragment of a monoclonal anti-Aβ antibody significantly reduces Aβ deposition, and phagocytic infiltration in the brain (Bacskai et al., 2002). Alternatively, deglycosylation of the anti-Aβ antibodies, which results in defective binding to Fc receptor, retains the Aβ-reducing property of the parental antibodies, while attenuates inflammation and microhemorrhage observed with unmodified antibodies (Tamura et al., 2005).

Anti-Aβ single chain antibody (scFv)

Besides the modified antibodies, scFv provides an alternative potentially noninflammatory approach to facilitate Aβ clearance. Up to now some Aβ specific scFvs have been developed with various functions, including interfering with Aβ aggregation (Wilcock et al., 2006; Takata et al., 2007), preventing toxic effects of Aβ (Frenkel et al., 2000; Liu et al., 2004b; Zameer et al., 2006; Habicht et al., 2007;

Robert et al., 2008; Lafaye et al., 2009), being specific to oligomers conformation (Frenkel et al., 2000; Robert et al., 2008; Lafaye et al., 2009), inhibiting β-secretase cleavage (Meli et al., 2009), α-secretase-like activity (Paganetti et al., 2005) and Aβ-hydrolyzing activity (Rangan et al., 2003). ScFv offers an advantage for developing a gene therapy-based approach of Aβ clearance. Intracranial delivery of a scFv gene is able to reduce and prevent the formation of histological Aβ plaques in the AD mouse model (Taguchi et al., 2008). We have demonstrated that intramuscular delivery of scFv gene is as effective as intracranial delivery in reducing cerebral Aβ (Fukuchi et al., 2006; Levites et al., 2006). Notably, intracranial use of scFv, but not intramuscular delivery, significantly increased the soluble form of Aβ, which is likely due to the direct dissolution of aggregated Aβ in the brain (Wang et al., 2009b). This phenomenon raises a concern that the transformation of Aβ from aggregated form to soluble form may increase the toxic oligomers which then cause further damage to the neurons. Since single chain antibodies inherit the Aβ-reducing properties of their parental antibodies, it could be of interest to develop an alternative non-inflammatory approach by mimicking the current immunotherapy without evoking the detrimental T cell response and Fc-mediated inflammation.

Targeting Aβ oligomers

Aβ oligomers, a soluble species of Aβ, has been suggested to be the primary neurotoxin and may be a major therapeutic target of AD (Wang et al., 2009b). It is noteworthy that anti-Aβ immunotherapy reduces the insoluble Aβ, but does not reduce, or even increases the soluble form of Aβ in the brain of transgenic AD mice (Dahlgren et al., 2002; Walsh et al., 2002; Lesne et al., 2006; Lacor et al., 2007; Shankar et al., 2008) and AD patients (Janus et al., 2000; DeMattos et al., 2001;

Petrushina et al., 2007; Wang et al., 2009b). The increased Aβ level might be resulted from mobilization of insoluble Aβ by antibodies (Solomon, Koppel et al. 1996; Solomon, Koppel et al. 1997). These data suggest that immunotherapy could fail to remove the more toxic soluble Aβ, or even produces conditions favoring the formation of Aβ oligomers during the process of Aβ plaque removal (Masliah et al., 2005; Nicoll et al., 2006; Patton et al., 2006; Holmes et al., 2008). This might be a potential reason that the plaque removal is not enough to significantly improve the cognition and to halt the progression of AD in the clinical trials (Petrushina et al., 2007; Holmes et al., 2008). In this regard, Aβ oligomers should be a primary therapeutic target. The key step targeting Aβ oligomers is to develop the confirmation-sensitive antibodies. Up to now a series of oligomer conformation specific antibodies and antibody fragments such as scFv, Fab and antibody domain have been developed, they are able to inhibit Aβ fibrillation, prevent Aβ neurotoxicity in vitro (Gilman et al., 2005; Holmes et al., 2008). The functional efficacy of these antibodies and fragments in term of Aβ reduction and cognition improvement need to be tested. In a recent study, administration of an oligomer specific monoclonal antibody for six months improves the cognitive function, but is not able to clear Aβ pathology (Habicht et al., 2007; Robert et al., 2008; Zameer et al., 2008; Lafaye et al., 2009; Meli et al., 2009; Wang et al., 2009a). The ideal immunotherapy should be able to reduce both the soluble and insoluble Aβ simultaneously. Recently a virosomes-based active vaccination is established to reduce both soluble and insoluble Aβ species (Lee et al., 2006).

Aβ binding substance-mediated Aβ clearance

According to the peripheral sink hypothesis, Aβ binding substances sequester plasma Aβ, leading to clearance of Aβ by promoting a net efflux of a rapidly mobilized soluble pool of Aβ (Fig. 1). Peripheral treatment with gelsolin or GM1, an agent that has high affinity for Aβ, reduced the level of Aβ in the brain, most likely because of a peripheral action (Zurbriggen et al., 2005).

Penetration of Aβ binding substances into the brain provides a chance for them to inhibit the aggregation of soluble Aβ and/or resolubilization of Aβ fibrils, then shift brain equilibrium between soluble and aggregated Aβ species towards soluble ones and finally facilitate Aβ clearance. The phenolic, yellow pigment, curcumin, found naturally in turmeric, a spice used extensively in Indian food, directly binds small Aβ species to block the formation of oligomer and fibril as well as to disaggregate Aβ aggregates *in vitro* and *in vivo*. When administered peripherally, curcumin can cross the BBB, bind plaques, and reduce amyloid levels and plaque burden in aged transgenic AD mice (Matsuoka et al., 2003). Another Aβ binding substance, enoxaparin (a low molecular weight heparin), when administered peripherally, significantly lowered the number of, and the area occupied by, cortical Aβ deposits and the total Aβ40 cortical concentration, possibly by either impeding the structural changes in Aβ necessary for fibril formation in the brain, or by sequestering the plasma Aβ peripherally (Yang et al., 2005). Grape seed derived polyphenols have been also suggested to be able to inhibit the aggregation of Aβ in vitro, and chronic consumption is associated with Aβ reduction and cognitive improvement (Bergamaschini et al., 2004). Besides these natural polyphenols, an antihypertensive drug, valsartan, is capable of prevent Aβ peptide from forming high-molecular

weight (HMW) oligomers. Valsartan administration reduces the AD-type neuropathology and extracellular HMW oligomeric Aβ content in the brain, and attenuate the development of Aβ-mediated cognitive deterioration (Wang et al., 2008; Wang et al., 2009c). These studies suggest that a search for high affinity Aβ binding compounds from Aβ receptors, functional foods or herbs could be a very attractive and promising strategy to shift the Aβ transport equilibrium towards plasma. Another important pool of drug candidates for Aβ clearance is the endogenous soluble receptors which directly interact with Aβ and are able to remove Aβ from the brain, such as sLRP (Wang et al., 2007) and extracellular domain of p75NTR as studied in the present study chapter 4.

Conclusions

The steady state level of Aβ depends on a balance between production, clearance and influx. Recently, pathologic, genetic, and transgenic evidence has suggested that physiological receptor-mediated BBB transport and enzyme-mediated degradation of Aβ are impaired in AD. Immunotherapy is effective in reducing the Aβ load, attenuating AD-like pathology and improving cognitive deficits. Although clinical trials were halted due to the adverse effects, immunotherapy still holds promise to be a disease-modifying treatment for AD. Several Aβ binding substances have been shown to be able to remove Aβ from the brain. Promoting receptor-mediated Aβ efflux from the brain, suppressing the Aβ influx across the BBB, up-regulating the enzyme-mediated degradation, modifying the immunotherapy to overcome its adverse effects, and searching for new high affinity Aβ binding agents are some of the many promising approaches for future treatments for AD.

CHAPTER 2

Intramuscular Delivery of a Single Chain Antibody Gene Prevents Brain Aβ Deposition and Cognitive Impairment in a Mouse Model of Alzheimer's Disease

Summary

Anti-β-amyloid (Aβ) immunotherapy is effective in removing brain Aβ, but was associated with detrimental effects. We have demonstrated that AAV-mediated intramuscular delivery of an anti-Aβ single chain antibody (scFv) gene was as effective as intracranial delivery in clearing brain Aβ of old APPSwe/PS1dE9 transgenic mice. In the present study, we tested the efficacy and safety of intramuscular delivery of scFv gene in preventing brain Aβ deposition. ScFv gene was intramuscularly delivered at three months of age when the brain Aβ deposition was not formed. Six months later, we found that the transgenes were expressed in a stable form in the delivered sites, with small amount of ectopic expression in the liver and olfactory bulb. Brain Aβ plaque formation, Aβ accumulation, AD-type pathologies and cognitive impairment were significantly attenuated in scFv-treated APPSwe/PS1dE9 transgenic mice relative to enhanced green fluorescence protein (EGFP)-treated mice. Intramuscular delivery of scFv gene was well tolerated by the animals, did not cause inflammation and microhemorrhage in the gene expression site and brain, and did not induce neutralizing antibodies in the animals. These findings suggest that peripheral application of scFv is effective and safe in preventing the development of AD (AD), and would be a promising non-inflammatory immunological modality for prevention and treatment of AD.

Key words: AD; amyloid-beta; single chain antibody; adeno-associated virus; intramuscular delivery; immunotherapy

Introduction

AD (AD) is characterized pathologically by the deposition of amyloid-β peptides (Aβ), neurofibrillary tangles, distinctive neuronal loss, and neurite dystrophy.

Aβ has been suggested to play an etiological role in the pathogenesis of AD (Sagare et al., 2007). Clearance of Aβ represents an important therapeutic strategy for AD (Hardy and Selkoe, 2002). Anti-Aβ immunotherapy has been well documented to be effective in removing Aβ from the brain in both experimental and clinical settings (Wang et al., 2006c). However, anti-Aβ immunotherapy is currently associated with detrimental events including brain inflammation and microhemorrhage which are presumably induced by T-cell and/or Fc-mediated immune responses (Schenk et al., 1999; Janus et al., 2000; Hock et al., 2003; Gilman et al., 2005). We have demonstrated previously that intramuscular delivery of a gene encoding for an anti-Aβ single chain antibody (scFv) was as effective as intracranial delivery of the gene in removing Aβ from the brain of 9 months old APPSwe/PS1dE9 mice without eliciting brain inflammation and microhemorrhage, representing a noninflammatory peripheral treatment modality (Pfeifer et al., 2002; Wilcock et al., 2004b; Racke et al., 2005).

Recently, a six-year prospective follow-up study of the first phase I clinical trial of active Aβ immunization reported that the generation of anti-Aβ antibodies was elicited and cortical Aβ plaques were subsequently removed in an antibody titer dependent manner after immunization with Aβ (Wang et al., 2009b). Despite this, the immunization was unable to halt/alleviate the progression of AD such as cognitive decline or survival outcomes. This negative outcome suggests that early prevention of Aβ accumulation may be more practical than the treatment of AD

progression (Holmes et al., 2008). Thus, in the present study we aimed to investigate the preventive efficacy of the intramuscular anti-Aβ scFv gene delivery in the APPSwe/PS1dE9 mouse model.

Materials and methods

Animals

The APPSwe/PS1dE9 transgenic mice bearing a chimeric mouse/human (Mo/Hu) APP695 with mutations linked to familial AD (KM 593/594 NL) and human PS1 carrying the exon-9-deleted variant associated with familial AD (PS1dE9) were obtained from Jackson Laboratory (Holtzman, 2008; St George-Hyslop and Morris, 2008) and bred in the Flinders University Animal House. Genotyping of mice was performed by PCR following the supplier's instructions. Mice were maintained on *ad libitum* food and water with a 12-hour light/dark cycle. All animal husbandry procedures performed were approved by the Flinders University Animal Welfare Committee in accordance with NHMRC guidelines.

rAAV vector construction and production

The scFv used in the present study was obtained from a semi-synthetic human single chain Fv antibody library. The binding epitope of the scFv is located between amino acids 1 and 16 of Aβ (Jankowsky et al., 2001). rAAV2/1-scFv was constructed and produced following methods we reported previously (Cai et al., 2003). Briefly, scFv cDNA was obtained from the scFv-expressing plasmid and inserted into the AAV vector plasmid pSNAV1 under the control of the CMV promoter followed by the SV40 polyadenylation signal. rAAV2/1-scFv and rAAV2/1-EGFP were produced and purified using a preciously reported method (Wang et al., 2009b). The vector

preparations were of high purity as judged by silver stained sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis, and free of rHSV1/repcap contamination (Yan et al., 2005). The viral titer was determined by dot blot DNA analysis with purified plasmid DNA as standard. The rAAV2/1-scFv preparation was adjusted to the concentration of 3×10^{12} particles /ml in PBS and stored at -80°C until use.

Muscle injection

Aβ plaque formation begins around three to four months of age in the brain of APPSwe/PS1dE9 transgenic mice (Wang et al., 2009b). In the present study the scFv gene was delivered at three months of age. On the day of surgery, the mice were anesthetized with halothane and AAV-scFv was injected into the muscles of left and right hind leg (1.5×10^{10} particles/5 μl for each side, n=10). The same amount of AAV-EGFP was injected in the same approach as the control (n=10). After surgery, animals were housed one animal per cage with free access to food and water for six months.

Behavioral test

Six months later after gene delivery when the mice were nine months old, the animals were subjected to a memory behavioral test. This task represents a classic version of the Morris Water Maze task and was performed following the protocols reported previously with minor modifications (Wang et al., 2006d). In brief, the test was conducted in a pool (200 cm diameter) which was filled with water (24±2°C), made opaque with white non-toxic dye and surrounded by a set of spatial cues (Yau et al., 2007). The tank was imaginarily subdivided into four quadrants, and four start positions were located at the intersections of the quadrants (Markowska et al., 1993). Each daily session consisted of four platform trials in which a round escape platform

(8 cm diameter) was submerged 1 cm under water surface in a quadrant. The mouse navigated in the pool to locate the platform and was then able to escape. If the mouse failed to locate the platform within 120 seconds, it was directed to the platform. Once the mouse escaped onto the platform, it remained on the platform for 10 s. After four days of training, all mice were given a single probe trial, in order to assess the final strength of memory traces, where the platform was withdrawn, at two hours after the last platform trial. The duration for probe trial was 40 seconds. Performance in all tasks was recorded and analyzed by a computer-based video tracking system and image analyzing software (ANY-maze, Stoelting).

In platform trials, distance (path from the start location to the platform, cm), latency (the time to reach the platform from the start location, s), and swim speed (average speed during a trial, cm/s) were measured. In probe trials quadrant time (percentages of time spent in the platform quadrant) and platform crossings (the number of times that the mice crossed the exact location of the platform) were measured. For latency and swim distance in platform trials, lower numbers indicated a better performance. For quadrant time and platform crossings in probe trials, higher numbers indicated a better performance (i.e. more time spent in the correct quadrant and more crossings over the platform location) (Frick and Fernandez, 2003).

Tissue sampling

Six months after gene delivery, animals were overdosed with Lethabarb (0.1 ml, ip) and perfused intracardially with 100 ml of 0.1% $NaNO_2$ in phosphate buffer. For histological analysis, left hemibrain, olfactory bulb, cerebellum, spinal cord, muscle, liver, spleen, kidney and intestine were fixed in 4% paraformaldehyde (pH7.4) for 24 hr and subsequently incubated for 24 hr in 30% sucrose for cryoprotection. Coronal sections of the brain and cross-sections of muscles at 35 μm thickness were collected

with a cryosectioning microtome and stored at 4°C in PBS until use. For biochemical analysis, right hemibrain and other tissues mentioned above were sampled, snap frozen in liquid nitrogen and stored at -80°C until processing. Blood was sampled before perfusion and plasma was separated for the detection of Aβ levels, inflammatory cytokines and antibodies against scFv.

Expression of transgenes

The expression of the EGFP gene was examined directly under fluorescence microscope (Olympus BX50). The mRNA expression of scFv gene was detected with RT-PCR. Briefly, total RNA from different tissues was isolated with a TRI REAGENT kit (Sigma, USA). 5 μg of total RNA was used to synthesize cDNA with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The cDNAs encoding scFv or G3PDH were amplified using HotStarTaq DNA Polymerase (Qiagen, USA). The primers used were: scFv 5'AAG GCT TGA GTG GAT GGG ATG GAT 3'(forward), 5'CCT GAG GGC CGG TTG TTT TTA C 3'(reverse); G3PDH 5'ACC ACA GTC CAT GCC ATC AC3' (forward), 5'TCC ACC ACC CTG TTG CTG TA3' (reverse). The cycling parameters were an initial DNA denaturation and polymerase activation at 96 °C for 15 min, followed with 38 cycles of 94°C for 30 sec, 65°C for 45 sec and 72°C for 45 sec, and ended with a DNA extension at 72°C for 10 min. The products were resolved on a 1.5% agarose gel and visualized with ethidium bromide under UV light.

Histology and image quantification

The staining for Aβ plaques, microgliosis, astrogliosis and microhemorrhage in the brain was processed as described previously (Frick and Fernandez, 2003). Briefly, a series of five equally spaced tissue sections (~1 mm apart) spanning the entire brain

were randomly selected and stained using free-floating immunohistochemistry for total Aβ (Biotin-conjugated mouse anti-Aβ antibody 6E10, Serotec, USA; 1:1000 dilution), activated microglia (rat monoclonal anti-CD45, Chemicon, USA; 1:2000 dilution) and astrocyte (rabbit polyclonal anti-glial fibrillary acidic protein, Dako, Denmark; 1:1000 dilution), respectively. Sections were incubated overnight with primary antibodies at 4ºC, further developed with biotinylated secondary antibodies and the ABC kit (Vector Lab, Burlingame, CA) using diaminobenzidine and glucose oxidase as substrates.

Fibrillar Aβ plaques were stained with Congo red. In brief, a series of five equally spaced tissue sections spanning the entire brain were mounted on slides. Sections were treated with working sodium chloride solution (sodium chloride-saturated 80% alcohol containing 0.01% sodium hydroxide) for 20 min at room temperature, then stained with Congo red saturated working sodium chloride solution for 1 hr, and finally dehydrated in absolute alcohol.

Images were collected under light microscope (Olympus BX50) using constant bulb temperature and exposure, with all images acquired in the same session. The area of neocortex and hippocampus was selected for automatic quantification of Aβ plaque, microglia and astrocyte immunostaining, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors.

For microhemorrhage staining, a series of five equally spaced tissue sections spanning the entire brain was mounted and stained for hemosiderin using 2% potassium ferrocyanide in 2% hydrochloric acid for 15 min, followed by a counterstain in a 1% neutral red solution for 10 min at room temperature.

Microhemorrhage events in the form of the number of Prussian blue-positive profiles were counted in the brains of each mouse on all sections, and the average number of hemosiderin deposits was calculated per each brain hemisphere.

To investigate the scFv-related pathological changes, sections of brain, olfactory bulb, liver, and muscle were stained with HE, and examined under light microscope.

All image analyses were processed in a blinded manner.

Quantification of Aβ peptide levels in the brain and plasma by ELISA

Aβ was extracted from each brain in a two-step procedure described previously (Wang et al., 2009b). Briefly, frozen brain was homogenized and sonicated in TBS containing protease inhibitors (Boehringer Mannheim, Germany), then centrifuged at 100,000 $\times g$ for 1 hr at 4°C. Following centrifugation, the resultant supernatant was collected, representing the TBS-soluble fraction (Aβ-TBS). The resultant pellet was then solubilized in 5 M guanidine HCl (GuHCl), centrifuged at 100,000 $\times g$ at 4°C for 1 hr, and the resultant supernatant was collected, representing the GuHCl fraction (Aβ-GuHCl). Levels of total Aβ40 and of Aβ42 in brain extract and plasma were quantitatively measured by sandwich ELISA (Covance, USA) as per the product instruction.

Quantification of inflammatory cytokines in the brain and plasma by ELISA

TNFα, IL-1β and IFN-γ in the TBS fraction of brain homogenates and plasma were measured by sandwich ELISA as per manufacturer's instructions (eBioscience, USA).

ELISA assay for antibodies against scFv in mice after intramuscular delivery of rAAV-scFv.

To investigate whether animals developed antibodies against scFv six months after intramuscular gene delivery, we measured the levels of anti-scFv antibodies in plasma. The antibodies to scFv developed in animals may neutralize the therapeutic effect of the gene therapy. Recombinant scFv at 2 μg/ml was coated on ELISA plates and the wells were incubated overnight with the mouse plasma sampled from the mice with gene delivery at different dilutions, followed by the HRP-conjugated secondary antibody to mouse IgG. The antibody titers were detected with TMB method.

Statistical analysis

Unless otherwise stated, the data in the text and figures are expressed as mean ± SEM. Statistical comparisons between groups were assayed using *t* test and one-way ANOVA for testing the significance of values. *P* values less than 0.05 were considered significant. All these analyses were performed using SPSS for Windows version 13.0 (SPSS Inc.)

Results

Expression of scFv gene after intracranial and intramuscular delivery

The aim of the present study was to examine whether peripheral delivery of scFv gene can prevent the Aβ deposition in the brain. The APPSwe/PS1dE9 transgenic mice, a mouse model for AD overproducing Aβ42 and Aβ40, begin to develop Aβ

Figure 2.1

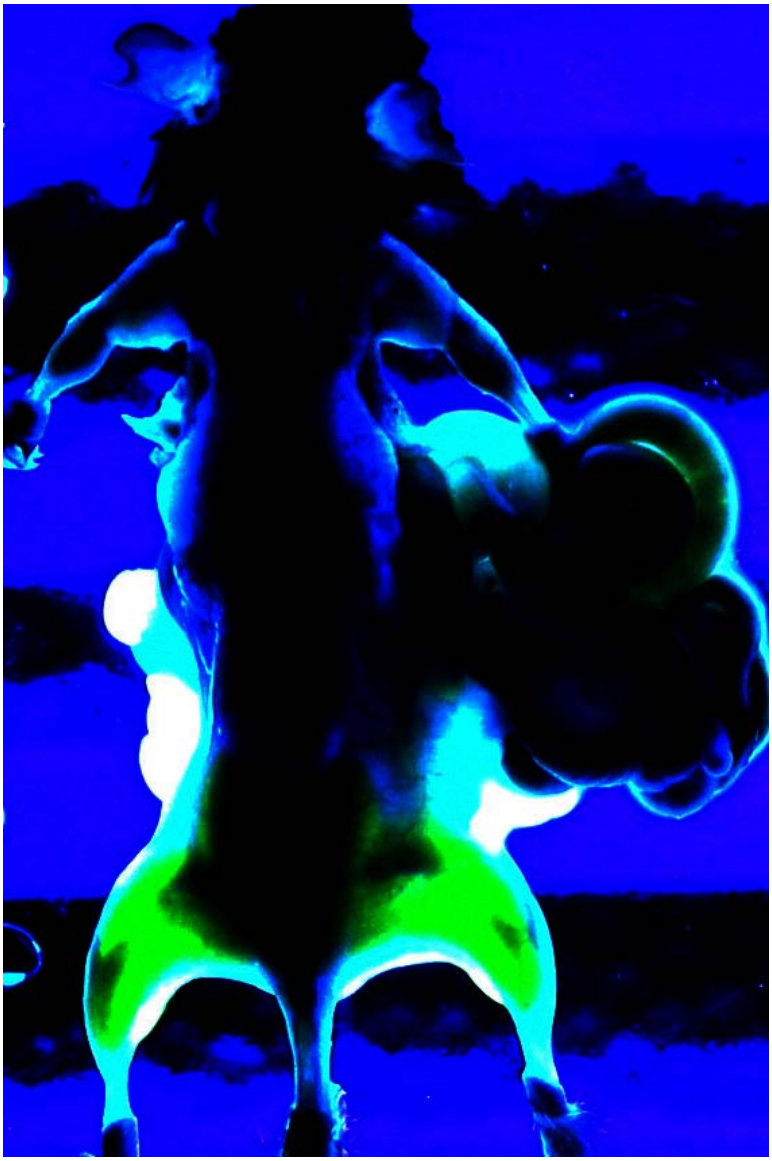

Figure 2.1 Expression of EGFP gene six months after intramuscular injection of AAV-EGFP. When APPSwe/PS1dE9 transgenic mice were three months old, AAV-scFv was injected into the muscles of their left and right hind legs at dose of 1.5×10^{10} particles/5 μl for each side. Six months later, animals were sacrificed and skin was taken off. Obvious expression of EGFP gene was illustrated in green colour in the muscles of both hind legs under ultraviolet illumination.

Figure 2.2

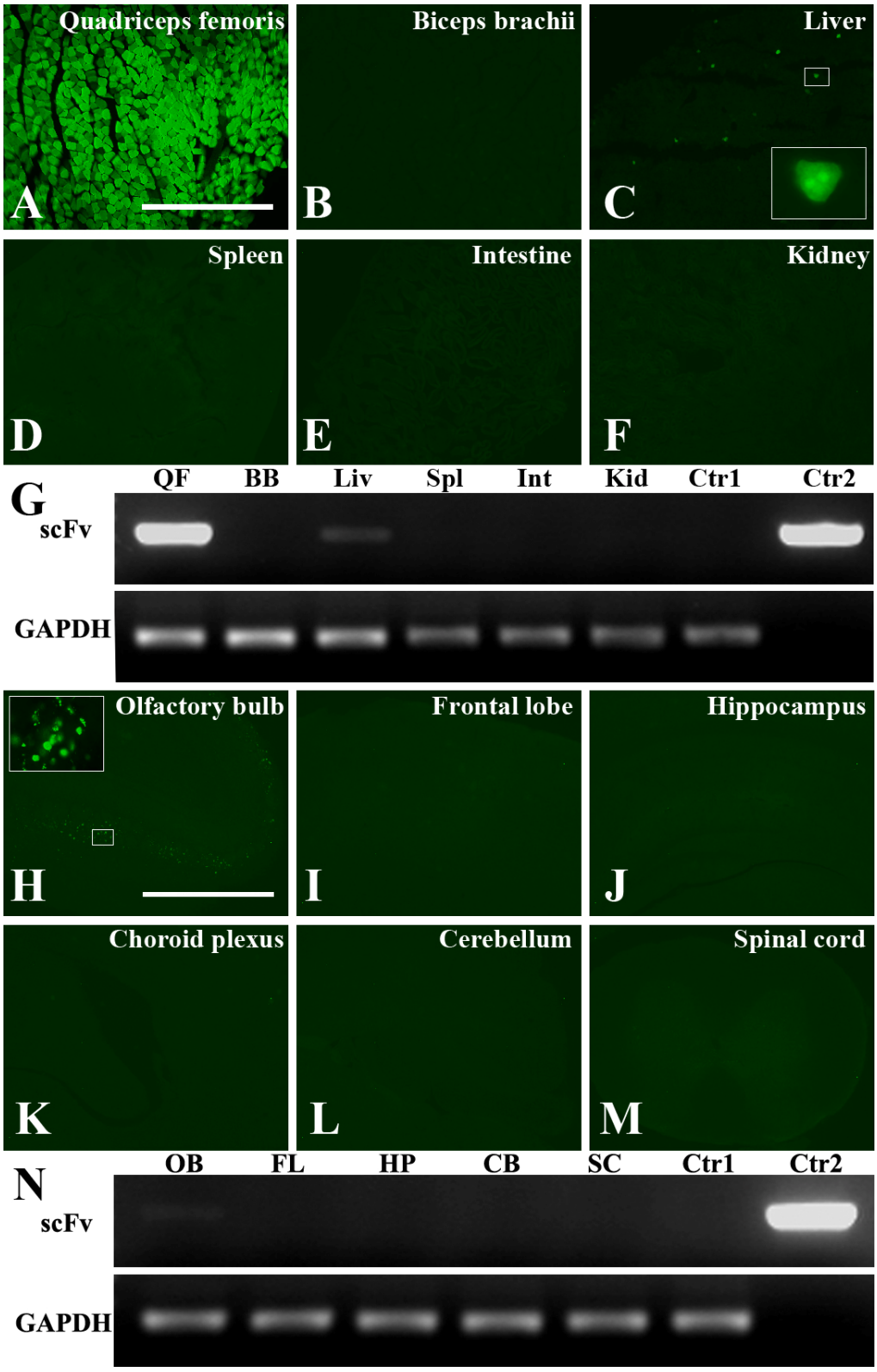
Quadriceps femoris
A
Biceps brachii
B
Liver
C
Spleen
D
Intestine
E
Kidney
F
QF
BB
Liv
Spl
Int
Kid
Ctr1
Ctr2
G
scFv
GAPDH
Olfactory bulb
H
Frontal lobe
I
Hippocampus
J
Choroid plexus
K
Cerebellum
L
Spinal cord
M
OB
FL
HP
CB
SC
Ctr1
Ctr2
N
scFv
GAPDH

Figure 2.2 Profiles of the transgene (EGFP and scFv) expression six months after intramuscular delivery. A-F. Expression of EGFP gene in muscles and peripheral organs. **G.** Expression of scFv gene in muscles and peripheral organs detected by RT-PCR. QF: quadriceps femoris, BB: biceps brachii, Liv: liver, Spl: spleen, Int: intestine, Kid: kidney, Ctr1: RT-PCR products from muscle RNA of non-injected mouse as negative control, Ctr2: PCR products from scFv plasmid as positive control. EGFP and scFv genes were expressed mainly in the injected muscle (quadriceps femoris), and discernable expression was observed in liver cells. **H-M.** Expression of EGFP gene in CNS. **N.** Expression of scFv gene in CNS detected by RT-PCR. OB: olfactory bulb, FL: frontal lobe, HP: hippocampus, CB: cerebellum, SC: spinal cord, Ctr1: RT-PCR products from muscle RNA of non-injected mouse as negative control, Ctr2: PCR products from scFv plasmid as positive control. Discernable expression of EGFP and scFv gene was detected in Olfactory bulb, but not in frontal lobe, hippocampus, choroid plexus, cerebellum and spinal cord. Bar=1 mm.

deposits at three months of age, and develop significant Aβ plaques by the age of nine months. ScFv gene was injected into the left and right hind leg muscles of APPSwe/PS1dE9 transgenic mice at three months of age. EGFP gene was delivered using the same approach as the control. Animals were examined six months after gene delivery. The equivalent dosage of administrated AAV vectors was 3.5×10^{12} vector genomes in a 60 Kg human, as derived using FDA criteria for converting drug equivalent dosages across species, based on body surface area [human equivalent dose in mg/kg = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$] (George et al., 2004). This dosage is in the safe dose range of intramuscular injection of AAV as tested in a clinical trial (Food and Drug Administration, 2003).

We first examined the expression of EGFP directly under ultraviolet light. As shown in Figure 2.1, strong expression of EGFP was observed in the muscles of both hind legs. All animals injected with the AAV-EGPF vector showed green colour in their hind-limbs.

We next considered the expression in different tissues. The expression of EGFP protein was examined by observation directly under fluorescence microscope and the mRNA expression of scFv was examined using RT-PCR (we could not generate scFv specific antibodies due to the low immunogenicity of the scFv, for details, please refer to the following data). As shown in Figure 2.2, six months after intramuscular delivery, the EGFP protein was expressed at a high level in quadriceps femoris at the site of gene delivery (Figure 2.2A). Amongst the peripheral tissues we studied, we also observed a low level of EGFP expression in the liver only (Figure 2.2C). The EGFP expressing cells in the liver appeared to be binucleate cells of hepatocyte morphology (Figure 2.2C inset). We did not see any discernible EGFP expression in other peripheral tissues including biceps brachii (Figure 2.2B), spleen

Figure 2.3

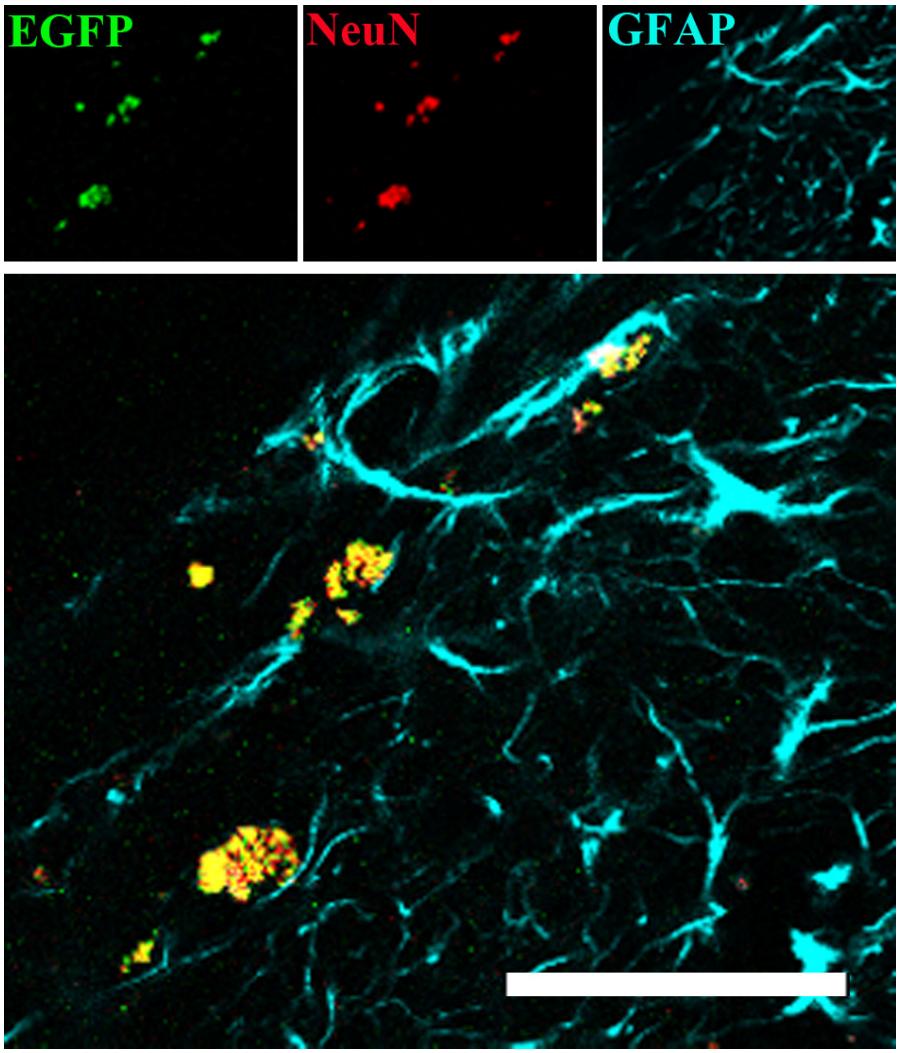

Figure 2.3 Expression of EGFP in olfactory bulb. EGFP expression (green) was detected in the glomerular layer. It was colocalized with NeuN (red, marker of neuron), but not GFAP (blue, marker of astrocyte), suggesting the EGFP was expressed in neurons of glomerular layer. Bar=50 μm.

Figure 2.4

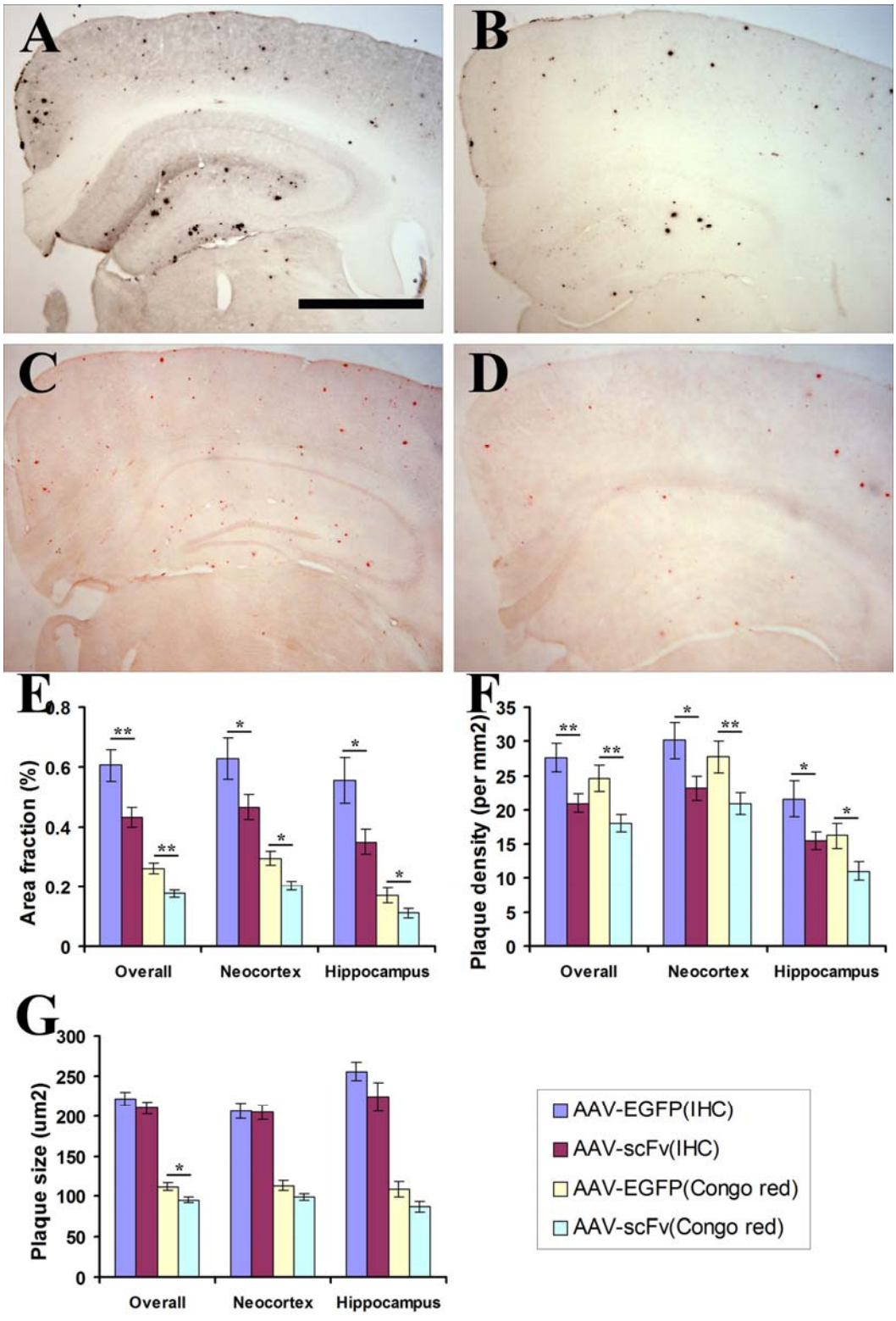
A
B
C
D
E
Area fraction (%)
0
0.2
0.4
0.6
0.8
Overall
Neocortex
Hippocampus
F
Plaque density (per mm2)
0
5
10
15
20
25
30
35
Overall
Neocortex
Hippocampus
G
Plaque size (um2)
0
50
100
150
200
250
300
Overall
Neocortex
Hippocampus
AAV-EGFP(IHC)
AAV-scFv(IHC)
AAV-EGFP(Congo red)
AAV-scFv(Congo red)

Figure 2.4 Aβ plaque burden in neocortex and hippocampus six months after intramuscular scFv gene delivery. A. Immunohistochemical (IHC) positive Aβ deposits in neocortex and hippocampus of mice injected with AAV-EGFP. **B.** IHC positive Aβ deposits in neocortex and hippocampus of mice injected with AAV-scFv. **C.** Congo red positive Aβ deposits in neocortex and hippocampus of mice injected with AAV-EGFP. **D.** Congo red positive Aβ deposits in neocortex and hippocampus of mice injected with AAV-scFv. **E.** Comparison of Aβ plaque area fraction between mice injected with AAV-EGFP and AAV-scFv. **F.** Comparison of Aβ plaque density between mice injected with AAV-EGFP and AAV-scFv. **G.** Comparison of Aβ plaque size between mice injected with AAV-EGFP and AAV-scFv. The results are means ± SEM. * compared with control, $p<0.05$, ** compared with control, $p<0.01$. Bar=1 mm.

(Figure 2.2D), intestine (Figure 2.2E) and kidney (Figure 2.2F). Consistently, the expression of scFv mRNA showed a similar expression pattern to EGFP (Figure 2.2G).

We then examined the expression of transgenes in the central nervous system (CNS), and observed the low level of EGFP expression in the glomerular layer of the olfactory bulb (Figure 2.2H). The EGFP expressing cells were NeuN positive but GFAP negative, indicating a neuronal cell type (Figure 2.3). No discernible expression of EGFP was observed in other parts of CNS including frontal lobe, hippocampus, choroid plexus, cerebellum and spinal cord (Figure 2.2I, J, K, L and M, respectively). Consistently, the scFv mRNA expression was also detected in olfactory bulb, but not in other CNS tissues (Figure 2.2N).

These data suggest that the transgenes are expressed in a stable form in the injected tissues six months after gene delivery, with small amount of ectopic transgene expression in olfactory bulb and liver.

Intramuscular delivery of scFv gene prevents Aβ deposition in the brain

Six months after gene delivery, we examined the amyloid plaque burden in the brain. Aβ plaques exist in two forms, fibrillar and diffuse. Firstly we evaluated the Aβ deposition in the brains by staining the Aβ plaques with anti-Aβ antibody 6E10 to label both diffuse and fibrillar plaques (IHC plaques) and Congo red to detect the fibrillar Aβ plaques (Congo red plaques) (Brantly et al., 2006). Analysis of Aβ plaques generated three parameters: area fraction (representing the percentage of area occupied by the plaques against the total area analysed), plaque density (representing the average number of plaques per area unit), and plaque size (representing the average size of plaques). Representative images are shown in Figure 2.4. Compared

Figure 2.5

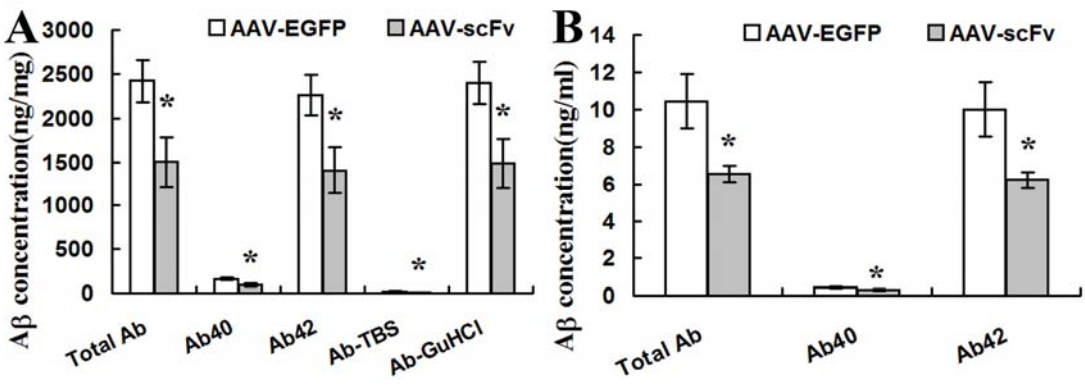

Figure 2.5 Aβ peptide levels six months after intramuscular delivery of scFv gene. A. Comparison of total Aβ, Aβ40, Aβ42, TBS-soluble and GuHCl-soluble Aβ in the brains between mice injected with AAV-EGFP and AAV-scFV. B. Comparison of total Aβ, Aβ40 and Aβ42 in serum between mice injected with AAV-EGFP and AAV-scFv. The results are means ± SEM. * compared with control, $p<0.05$, ** compared with control, $p<0.01$.

with EGFP-treated mice, scFv-treated mice had significantly lower area fraction and plaque density of both IHC and Congo red plaques overall, neocortex and hippocampus. ScFv-treated mice also had smaller size of IHC and Congo red plaques than EGFP-treated mice. However, statistical significance was only achieved for the overall measurement of Congo red plaques.

We next examined the accumulation of Aβ, which exists in soluble and insoluble forms, in the brain. Soluble (TBS fraction) and insoluble (GuHCl fraction) Aβ40 and Aβ42 were extracted sequentially from the brain and quantified by sandwich ELISA with antibodies specific to Aβ40 and Aβ42. For the comparison of Aβ levels between scFv- and EGFP-treated mice, the total Aβ level from individual animals was calculated by the sum of TBS- and GuHCl-Aβ40 and Aβ42. Compared with EGFP-treated mice, scFv-treated mice had significantly lower levels of total Aβ (1500±281 vs 2421±237 ng/mg, F=1.112, p=0.023), Aβ40 (95.1±19.9 vs 161.2±12.3 ng/mg, F=1.185, p=0.013), Aβ42 (1405±263 vs 2259±229 ng/mg, F=0.863, p=0.025), soluble Aβ (11.4±2.2 vs 21.6±3.9 ng/mg, F=3.479, p=0.033), and insoluble Aβ (1489±280 vs 2399±236, F=1.096, p=0.023) (Figure 2.5A). We further examined the Aβ levels in the serum. Consistently, scFv-treated mice had lower concentrations of total Aβ (6.55±0.44 vs 10.46±1.44 ng/ml, F=11.403, p=0.014), Aβ40 (0.305±0.04 vs 0.439±0.03 ng/ml, F=0.198, p=0.016) and Aβ42 (6.25±0.43 vs 10.02±1.43 ng/ml, F=12.203, p=0.016) in plasma than EGFP-treated mice (Figure 2.5B).

Figure 2.6

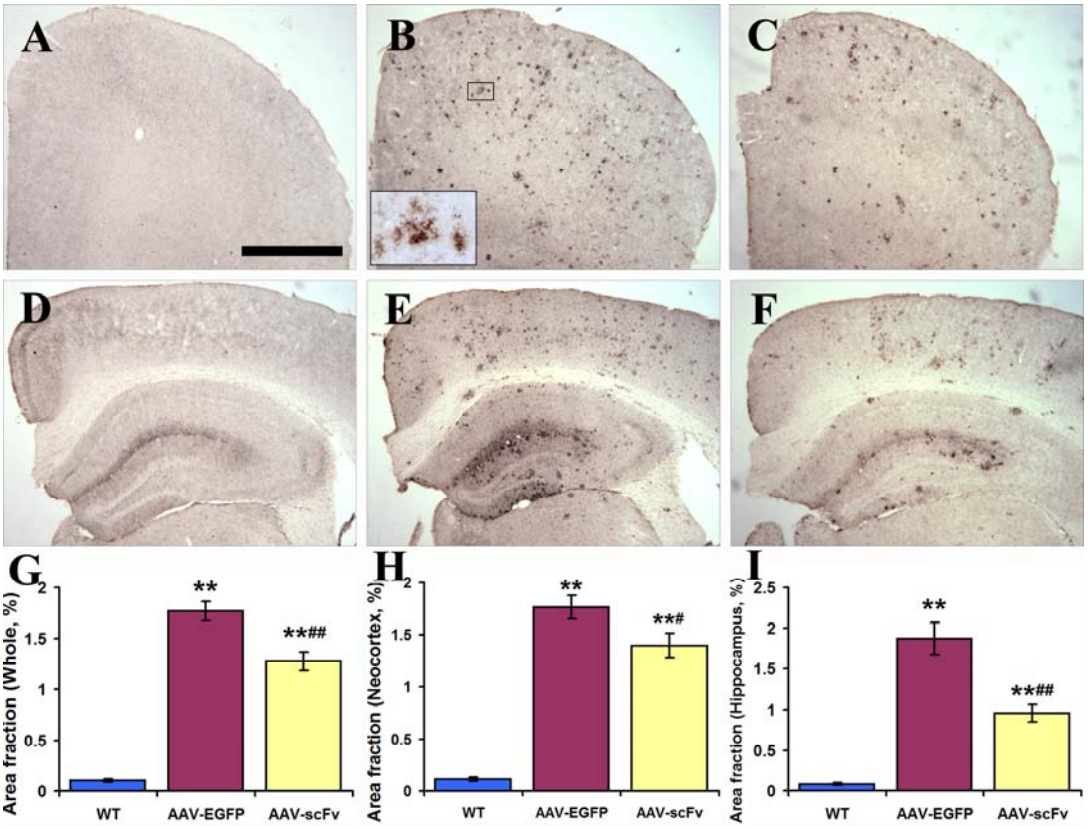

Figure 2.6 Microgliosis in the brain six months after intramuscular delivery of scFv gene. A. No obvious microgliosis was observed in the frontal lobe of wild type littermates. B. Microgliosis in the frontal lobe of APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP. C. Microgliosis in frontal lobe of APPSwe/PS1dE9 transgenic mice injected with AAV-scFv. D. No obvious microgliosis was observed in the hippocampus of wild type littermates. E. Microgliosis in the hippocampus of APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP. F. Microgliosis in the hippocampus of APPSwe/PS1dE9 transgenic mice injected with AAV-scFv. G-I. Comparison of microgliosis area fraction in the entire brain (G), cortex (H) and hippocampus (I) among wild type (WT), mice injected with AAV-EGFP and AAV-scFv. The results are means ± SEM. ** denote $p<0.01$ versus wild type littermate, $^{\#}$ and $^{\#\#}$ denote $p<0.05$ or $p<0.01$ versus APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP. Scale bar=1 mm.

Figure 2.7

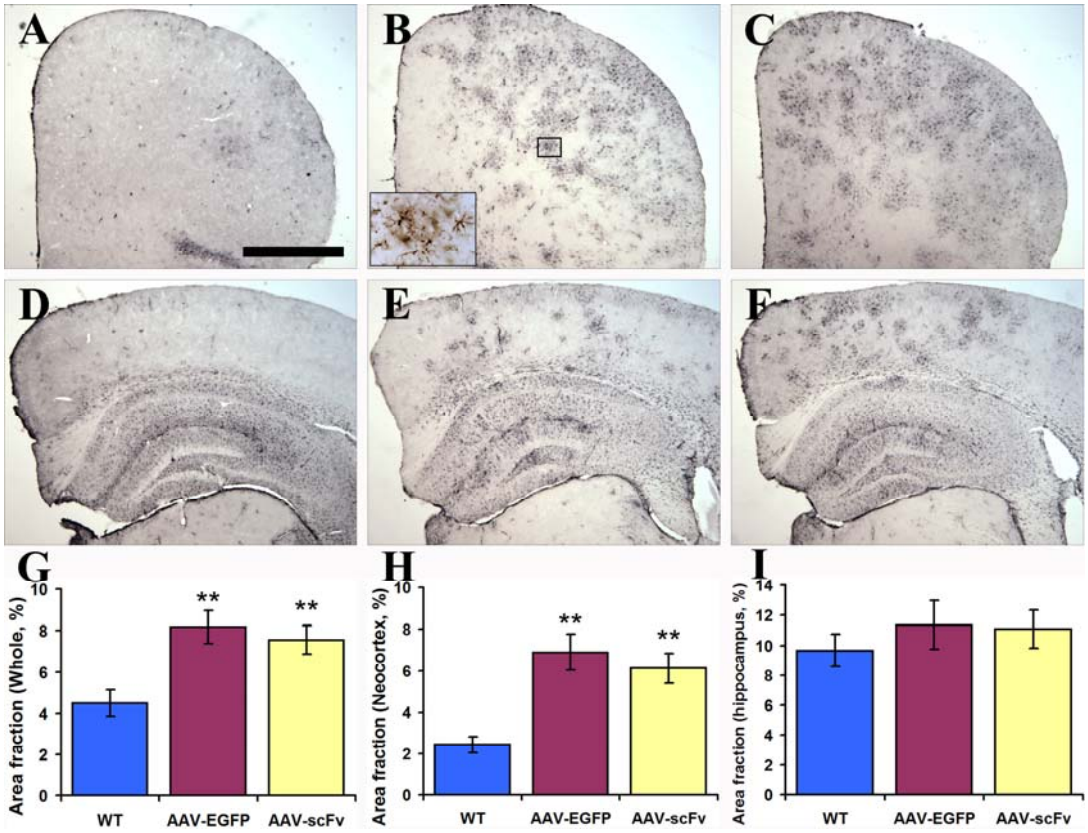

Figure 2.7 Astrogliosis in the brain six months after intramuscular delivery of scFv gene. A-C. Astrogliosis in frontal lobe of wild type littermates (A), APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP (B) and AAV-scFv (C). D-F. Astrogliosis in the hippocampus of wild type littermates (D), APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP (E) and AAV-scFv (F). G-I. Comparison of GFAP area fraction in the entire brain (G), cortex (H) and hippocampus (I) among wild type littermate (WT), mice injected with AAV-EGFP and AAV-scFv. The results are means ± SEM. ** denote $p<0.01$ versus wild type littermate, $^{\#}$ and $^{\#\#}$ denote $p<0.05$ or $p<0.01$ versus APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP. Scale bar=1 mm.

These histological and biochemical data indicate that intramuscular delivery of scFv gene effectively prevents Aβ accumulation and deposition in the brain.

Intramuscular delivery of scFv gene attenuates AD-type pathologies

We further investigated whether delivery of scFv gene can attenuate microgliosis and astrogliosis in the brain. Activated microglia in the regions of neocortex and hippocampus were stained with monoclonal anti-CD45 antibody. As shown in Figure 2.6, there was no obvious activation of microglia in the brain of wild type littermates (Figure 2.6A and D), while significant microgliosis developed in the brain of APPSwe/PS1dE9 transgenic mice (Figure 2.6B and E). Compared with EGFP-treated mice (Figure 2.6B and E), scFv-treated mice (Figure 2.6C and F) had significantly lower area fraction of overall brain regions (Figure 2.6G, 1.27±0.09% vs. 1.77±0.10%, One way ANOVA $F=42.676$, $p<0.001$), in neocortex (Figure 2.6H, 1.40±0.12% vs 1.76±0.11%, ANOVA $F=27.970$, $p<0.001$) and in the hippocampus (Figure 2.6I, 0.96±0.10 vs 1.78±0.19%, ANOVA $F=17.667$, $p<0.001$) with respect to microgliosis.

We used the GFAP antibody to illustrate astrocytes (Figure 2.7). Unlike microgliosis, we saw obvious astrocytes in both wild type and APPSwe/PS1dE9 transgenic mice. In general, APPSwe/PS1dE9 transgenic mice had higher extent of astrogliosis in neocortex but not hippocampus than wild type mice. The scFv-treated mice had lower extent of overall astrogliosis and astrogliosis in the neocortex and hippocampus, however, the difference did not reach statistical significance.

Figure 2.8

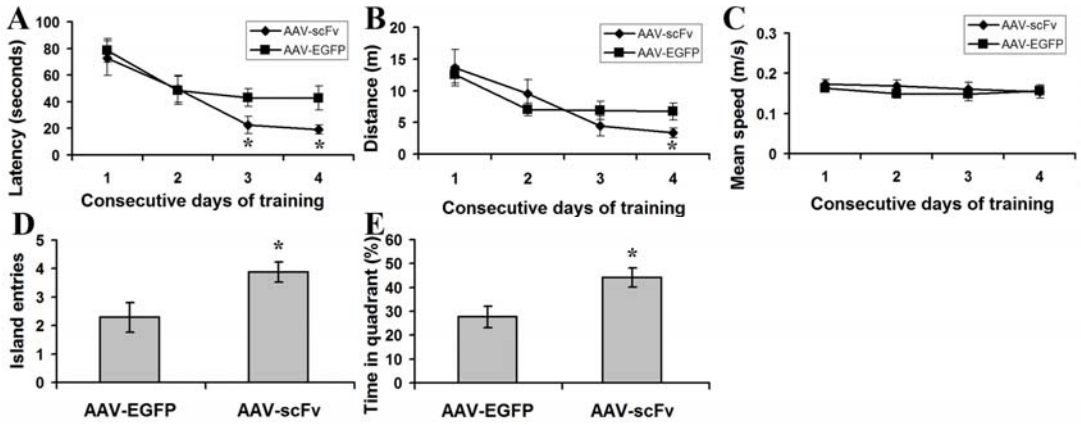

Figure 2.8 AAV-scFv injection prevented cognitive impairment of APPSwe/PS1dE9 transgenic mice. Six months after intramuscular delivery of scFv gene, all the animals were subjected to Morris Water Maze test for a consecutive four days. A. Latency taken to escape from the water in the platform trials. B. Distance taken to escape from the water in the platform trials. C. Motor function of scFv- and EGFP-treated mice as reflected by their swimming speed during the consecutive days of training. D. The number of crosses over the exact location of the hidden platform in the probe trial. E. Percentage of time spent in the target quadrant area relative to the total time spent in the the pool in the probe trial. The results are means ± SEM. * compared with control (AAV-EGFP), $p<0.05$.

Intramuscular delivery of scFv gene prevents amyloid-associated cognitive impairments

As scFv gene delivery reduced the Aβ accumulation and deposition, and attenuated the microgliosis and astrogliosis in the brain (Wang et al, 2009a), we hypothesized that scFv treatment should functionally prevent the amyloid-associated cognitive deterioration. The cognitive function of the animals was tested using classic Morris Water Maze. Consistently, the scFv-treated mice performed significantly better than EGFP-treated mice, as reflected by significant reductions in escape latency time and distance taken to escape onto the platform with progressive learning trials (Figure 2.8A and B). ScFv-induced attenuation of cognitive impairment was confirmed in the probe trial showing that scFv-treated mice entered the platform area more times (3.88±0.35 vs 2.89±0.52, p=0.023) and spent more time in the target quadrant area (44.11±4.07% vs 27.70±4.49%, p=0.018) relative to EGFP-treated control mice (Figure 2.8D and E). This effect was not attributable to the presence of motor deficits, because the two groups of mice exhibited similar swimming speeds (Figure 2.8C).

Intramuscular delivery of scFv gene is well tolerated and does not cause inflammation and microhemorrhage in the injection sites and brain

It is important to understand the safety of the scFv gene treatment. During the period of the present study, no animal death occurred and no behavioural abnormality was observed. Furthermore, the gene delivery did not significantly influence animal body weight (data not shown).

Autoimmune CNS inflammation and bleeding of small vessels are the major adverse effects of active and passive immunotherapies. We investigated whether scFv treatment caused inflammation and microhemorrhage. Microhemorrhage was

Figure 2.9

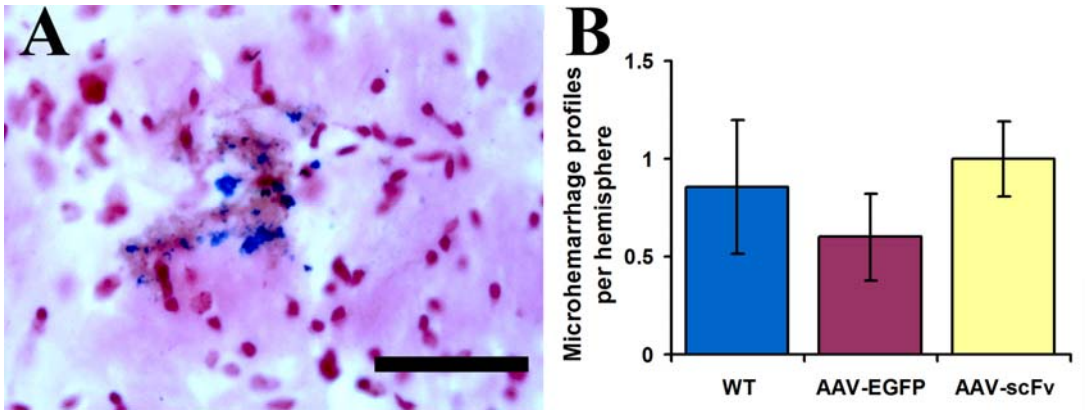

Figure 2.9 Microhemorrhage profiles in the brain six months after intramuscular delivery of scFv gene. A. An example of microhemorrhage profile observed in hippocampus. B. Comparison of microhemorrhage profiles per each brain hemisphere among wild type littermates (WT), APPSwe/PS1dE9 transgenic mice injected with AAV-EGFP and AAV-scFv. Scale bar=50 μm.

Figure 2.10

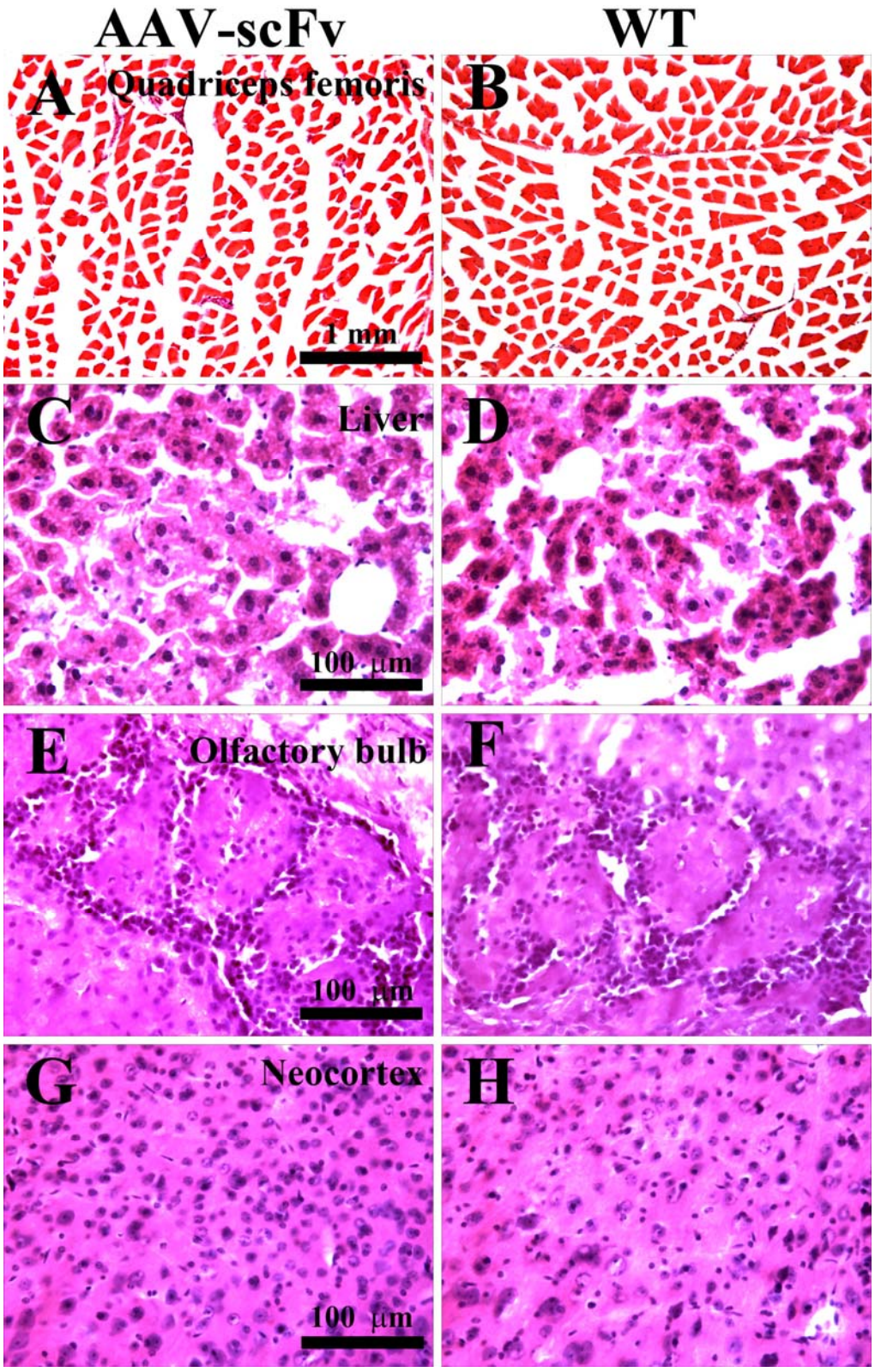
AAV-scFv
WT
A
Quadriceps femoris
1 mm
B
C
Liver
100 μm
D
E
Olfactory bulb
100 μm
F
G
Neocortex
100 μm
H

Figure 2.10 ScFv gene delivery does not cause pathological changes in the scFv expressing tissues and brain. A-B. HE staining of the quadriceps femoris of APPSwe/PS1dE9 mice injected with AAV-scFv (A) and of wild type littermates without AAV injection (B). **C-D.** HE staining of the livers of APPSwe/PS1dE9 mice injected with AAV-scFv (C) and of wild type littermates without AAV injection (D). **E-F.** HE staining of the glomerular layer of the olfactory bulb of APPSwe/PS1dE9 mice injected with AAV-scFv (E) and of wild type littermates without AAV injection (F). **G-H.** HE staining of the cortex of APPSwe/PS1dE9 mice injected with AAV-scFv (G) and of wild type littermates without AAV injection (H). There were no discernable pathological changes in quadriceps femoris, liver, olfactory bulb and neocortex of APPSwe/PS1dE9 mice injected with AAV-scFv.

Figure 2.11

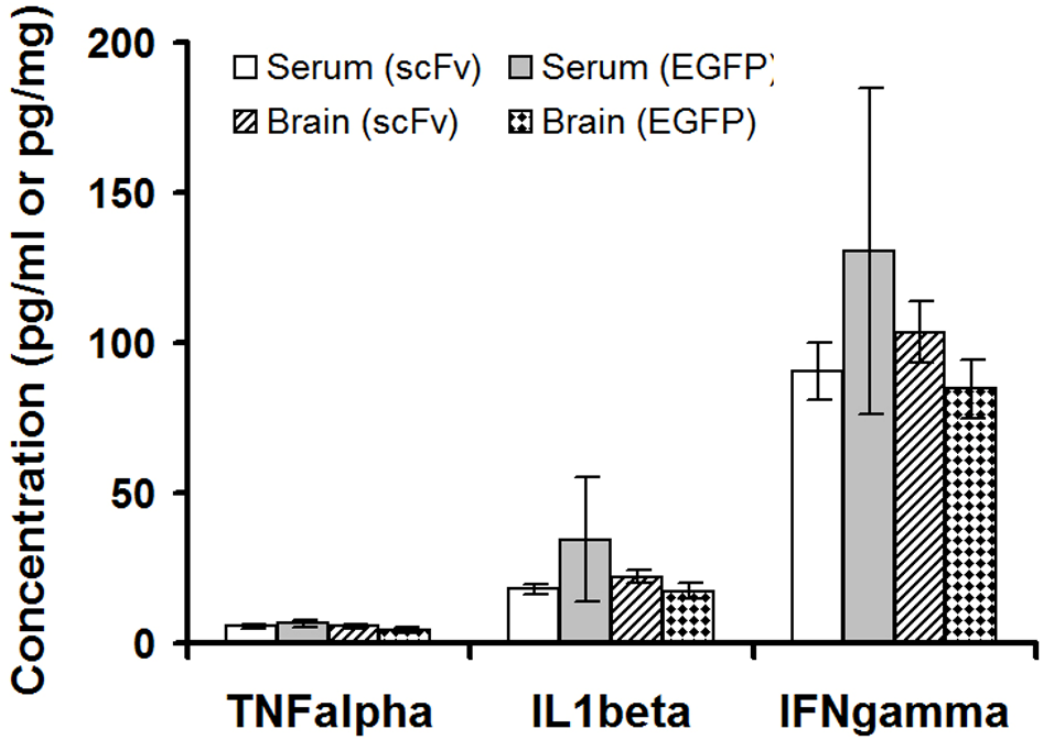

Figure 2.11 Levels of TNFα, IL-1β and IFN-γ in serum and brain of APPSwe/PS1dE9 mice injected with AAV-scFv and AAV-EGFP. The cytokine concentration unit is pg/ml for serum and pg/mg for brain samples. Results are mean±SEM.

illustrated with potassium ferrocyanide. We did not observe any significant difference in microhemorrhage profiles among the wild type, EGFP- and scFv-treated mice (One way ANOVA F=1.879, p=0.174) (Figure 2.9). We next stained the sections of tissues expressing scFv gene, including quadriceps femoris, liver, olfactory bulb, and the brain with HE, and did not see any discernible inflammatory responses or obvious pathological abnormalities in these tissues when compared with tissues from wild type controls (Figure 2.10).

We further measured the levels of the key inflammatory cytokines TNFα, IL-1β and IFNγ in serum and brain homogenates, and consistently there were no significant differences in the serum and brain levels of these cytokines between the scFv- and EGFP-treated mice (Figure 2.11).

These data indicate that long-term expression of scFv gene in the muscle is well tolerated by the APPSwe/PS1dE9 mice, and scFv treatment did not cause microhemorrhages and inflammatory responses in AAV-injected tissues, transgene expressing tissues and the brain, indicating the safety of long-term use of scFv treatment.

ScFv has low immunogenicity and does not induce antibodies

A major concern of long-term use of antibodies as a treatment is the generation of neutralizing antibodies, which will compromise the therapeutic effect. We investigated whether long-term expression of scFv gene in the body would generate neutralizing antibodies. Six months after intramuscular delivery of scFv gene, despite the stable expression of scFv gene, there were no detectable neutralizing antibodies against scFv in all mice injected with the AAV-scFv (data not shown). We also did not detect the scFv specific antibodies in the sheep and mice immunized with either

recombinant scFv protein or chemically synthesized scFv peptide fragments, with intention to generate anti-scFv antibodies (data not shown). The results suggest that the scFv has low antigenicity and did not evoke a significant immune response from the host in the present study.

Discussion

In the present study, we have demonstrated that intramuscular delivery of the gene encoding for a single chain antibody against Aβ isolated from a human single chain antibody library (Frid et al., 2007) is capable of preventing the Aβ accumulation and deposition in the brain, attenuating AD type pathologies, and improving the cognitive functions of the APPSwe/PS1dE9 transgenic mice. Importantly, long-term treatment with scFv was well tolerated by the animals, did not cause abnormal behaviors, microhemorrhages, and cerebral and systematic inflammatory responses, and had low antigenicity without inducing the secondary neutralizing antibodies.

Regardless of Aβ-reducing effects of both active Aβ immunization and passive immunization with antibodies against Aβ in animal experiments and clinical trials (Cai et al., 2003), (Schenk et al., 1999; Dodart et al., 2002; Bard et al., 2003; Wilcock et al., 2004a; Billings et al., 2005; Brendza et al., 2005; Maier et al., 2006), several major adverse effects arose, including autoimmune meningoencephalitis by evoking autoreactive T lymphocytes (Nicoll et al., 2003; Ferrer et al., 2004; Gilman et al., 2005; Masliah et al., 2005), and multiple cortical haemorrhages possibly due to Fc-mediated inflammation in amyloid-laden vessels (Nicoll et al., 2003; Ferrer et al., 2004). Other concerns include those of anti-Aβ antibodies binding to epitopes on CNS neurons that can also induce autoimmune reactions (Pfeifer et al., 2002; Ferrer

et al., 2004; Wilcock et al., 2004b; Racke et al., 2005), and Fc-mediated microglia activation and phagocytosis of insoluble aggregates which may also result in inflammatory responses (Rohn et al., 2000). These adverse effects preclude the clinical use of immunotherapy.

However, the immunotherapy against Aβ still has the potential of becoming the first strong disease-modifying modality against AD. Currently several approaches are under exploration to avoid the adverse effects of the immunotherapy, including vaccine refinement (Rogers and Shen, 2000), novel immunization approach (Lemere, 2009) and modified antibodies (Lemere et al., 2006). ScFv is in a format where the VH and VL domains are joined, and usually retains the specific, monovalent, antigen-binding affinity of the parent IgG (Rebe and Solomon, 2005; Tamura et al., 2005). Due to the lack of Fc fragment, scFv may not cause Fc-related cerebral microhemorrhage and inflammation. We have demonstrated that intramuscular delivery of the scFv gene is effective in reducing the brain Aβ burden in 9 months old APPSwe/PS1dE9 transgenic mice without eliciting cerebral microhemorrhages, activating microglia and lymphocyte infiltration in the brain (Holliger and Hudson, 2005). In the present study, we further demonstrated the efficacy of intramuscular delivery of scFv gene in preventing the accumulation and deposition of Aβ in the brain and functionally attenuating Aβ-related cognitive decline without eliciting microgliosis, microhemorrhage, cerebral and systematic inflammatory responses. Our studies suggest that the systemic use of scFv is potentially an effective and safe therapeutic strategy.

Intracranial delivery of a scFv gene is able to reduce and prevent the formation of histological Aβ plaques in the AD mouse model (Wang et al., 2009b). We have demonstrated that intramuscular delivery of scFv gene is as effective as intracranial

delivery in reducing cerebral Aβ (Fukuchi et al., 2006; Levites et al., 2006). Notably, intracranial use of scFv, but not intramuscular delivery, significantly increased the soluble form of Aβ, which is likely due to the direct dissolution of aggregated Aβ in the brain (Wang et al., 2009b). This phenomenon raises a major concern that the transformation of Aβ from aggregated form to soluble form may increase the toxic oligomers which then cause further damage to the neurons, if the scFv is not able to neutralize the Aβ neurotoxicity. In the present study, soluble Aβ in TBS in the brain is decreased after intramuscular delivery of scFv gene. Taken together, our studies suggest that intramuscular delivery of scFv gene provides a less invasive and maybe a safer therapy than intracranial delivery.

Based on our studies, scFv represents a potentially noninflammatory approach to facilitate Aβ clearance. Up to now some single chain antibodies specific against Aβ have been developed with different functions, including interfering with Aβ aggregation (Wang et al., 2009b), preventing toxic effects of Aβ (Frenkel et al., 2000; Liu et al., 2004b; Zameer et al., 2006; Habicht et al., 2007; Robert et al., 2008; Lafaye et al., 2009), being specific to oligomers conformation (Frenkel et al., 2000; Robert et al., 2008; Lafaye et al., 2009), inhibiting β-secretase cleavage (Meli et al., 2009), α-secretase-like activity (Paganetti et al., 2005) and Aβ-hydrolyzing activity (Rangan et al., 2003). It is of significant interest to develop alternative immunological approaches with these types of scFv to target Aβ production and Aβ clearance.

AAV has been suggested to be a safe vector of gene delivery (Taguchi et al., 2008). In the present study, the dosage of rAAV2/1 vector injected (equivalent human dose 3.5×10^{12} vector genomes) is low, within the safe dosage range (2.1×10^{12} - 6.9×10^{13}

vector genomes) as tested in a phase I clinical trial of intramuscular injection of the similar rAAV2 vector (Flotte, 2004). We did not see discernable pathological changes in the muscles where rAAV2 vectors were injected. Injection of rAAV2 into the blood is known to widely transform different cells (Brantly et al., 2006). In our previous study, ectopic expression of transgenes were obvious in olfactory bulb, choroid plexus, liver and intestine besides the muscle at three months after intramuscular delivery of the genes. The widespread existence of AAV in different tissues after intramuscular injection may not pose a significant set-back, since AAV is the safe vector available for the gene therapy (Mori et al., 2006). In the present study, the ectopic expression of transgenes was further limited to olfactory bulb and liver six months after gene delivery, suggesting that the ectopic expression in intestine and choroid plexus is transient. This limited expression reduces the concerns of any potential side effects arising from ectopic transgene expression. Our findings are consistent with recent clinical trials where intramuscular AAV injection did not cause serious vector-related adverse events, and detectable vector DNA in the blood which may lead to ectopic expression (Flotte, 2004).

It is important to note that the scFv in the present study has very low antigenicity. No antibodies against the scFv were generated after gene delivery and even after vaccination with routine immunization protocols. This would be a potential advantage in the future human use where neutralizing antibodies against the biopharmaceuticals will compromise the therapeutic effect (Brantly et al., 2006; Jiang et al., 2006).

Conclusions

In summary, our studies suggested that intramuscular delivery of a scFv gene is effective in preventing brain Aβ accumulation and cognitive impairment, without

eliciting cerebral microhemorrhage, inflammatory responses, and neutralizing antibodies in a prevention modality. Recombinant AAV2/1-mediated scFv gene delivery is well tolerated by animals. This study further extends our understanding of the therapeutic effect and safety of the intramuscular delivery of the scFv gene. Together with the safety of human AAV application, our studies suggest that AAV-mediated intramuscular delivery of scFv is a promising tool for both prevention and treatment of AD. Our studies also present a "proof of principle" for a novel peripheral immunological modality that may also be of relevance for other misfolding diseases such as Parkinson disease, Huntington disease and prion diseases (Chirino et al., 2004).

CHAPTER 3

Grape Seed Derived Polyphenols Attenuate Amyloid-beta Neuropathology in the Brain of Alzheimer's Disease Mice

Summary

Polyphenols extracted from grape seeds are able to inhibit amyloid-beta (Aβ) aggregation, reduce Aβ production and protect against Aβ neurotoxicity *in vitro*. We aimed to investigate the therapeutic effects of a polyphenol-rich grape seed extract (GSE) in AD mice. APPSwe/PS1dE9 transgenic mice were fed with normal AIN-93G diet (control diet), AIN-93G diet with 0.07% curcumin, or diet with 2% GSE beginning at 3 months of age for 9 months. Total phenolic content of GSE was 592.5 mg/g dry weight, including gallic acid (49mg/g), catechin (41mg/g), epicatechin (66mg/g) and proanthocyanidins (436.6mg catechin equivalents/g). Long-term feeding of GSE diet was well tolerated without fatality, behavioural abnormality, changes in food consumption, body weight or liver function. The Aβ levels in the brain and serum of the mice fed with GSE were reduced by 33% and 44% respectively compared with the Alzheimer's mice fed with the control diet. Amyloid plaques and microgliosis in the brain of Alzheimer's mice fed with GSE were also reduced by 49% and 70% respectively. Curcumin also significantly reduced brain Aβ burden and microglia activation. With the dosage used in the present study, GSE appeared more effective in suppression of brain Aβ burden and microglia activation than curcumin. In conclusion, polyphenol-rich GSE is well-tolerated and prevents development of AD in a transgenic mouse model.

Keywords: AD; Polyphenols; Grape seed extract; Amyloid-beta; Microglia

Introduction

AD (AD) is the most common form of senile dementia occurring in later life and is a major cause of disability and death in the elderly (Cardinale and Biocca, 2008). With the world population ageing, it is estimated that the number of people affected with AD will double every 20 years from today's estimate of 26.6 million to 106.8 million by 2050 (World Health Organization, 2003). However, no strong disease-modifying treatment or preventative measures are currently available (Brookmeyera et al., 2007).

AD is characterized neuropathologically by deposits of amyloid-beta peptides (Aβ), neurofibrillary tangles, reactive microgliosis and astrogliosis, cerebral amyloid angiopathy and neuronal loss that result in the progressive deterioration of cognition and memory (see review in (Citron, 2004)). According to the amyloid hypothesis, the accumulation of Aβ in the brain is the primary factor driving AD pathogenesis (Thomas and Fenech, 2007). It has been suggested that neuroinflammation may significantly contribute to disease progression and chronicity of AD (Hardy and Selkoe, 2002). Therefore, clearance of Aβ from the brain and anti-inflammation represent potential important strategies that may be used to prevent and treat the disease (Heneka and O'Banion, 2007).

Epidemiological studies have shown that consumption of diets rich in anti-inflammatory agents, such as those found in fruits and vegetables, or anti-inflammation drugs, may lower the risk of developing age-related neurodegenerative diseases such as Parkinson's disease and AD (Citron, 2004; Wang et al., 2006c). Polyphenols from grape seeds extract (GSE) have been suggested to be able to inhibit Aβ aggregation, reduce Aβ production, protect against Aβ neurotoxicity, and

attenuate oxidative stress *in vitro* (Lau et al., 2005; Barberger-Gateau et al., 2007; McGeer and McGeer, 2007). GSE has been widely used as food additive in order to benefit health and chronic illness (for review see(Bastianetto et al., 2000; Jang and Surh, 2003; Ono et al., 2003; Savaskan et al., 2003; Li et al., 2004; Marambaud et al., 2005; Ono et al., 2005; Mancuso et al., 2007; Riviere et al., 2007)). However, whether polyphenols from grape seeds can prevent or slow down the pathogenesis or reduce brain pathology and improve cognitive functions in AD patients or animal models is not clear. It is known that polyphenols such as curcumin found in tumeric which is an active component of curry (Shi et al., 2003) or epigallocatechin-3-gallate (EGCG) from green tea (Yang et al., 2005) can prevent the Aβ deposit in the brain and reduce neuropathology in AD mice. In the present study, we investigated the effects of polyphenol-rich GSE on cognitive protection, Aβ deposition and AD-type pathology in a transgenic AD mouse model which over expresses Aβ in the brain.

Material and Methods

Transgenic mouse model

Approval for this study was obtained from CSIRO Human Nutrition and Flinders University Animal Ethics committees. APPSwe/PS1dE9 transgenic mice were provided by Jackson Laboratory, USA. These mice were constructed on a C57BL/6 background, and bear a chimeric mouse/human (Mo/Hu) APP695 with mutations linked to familial AD (KM 593/594 NL) and human PS1 carrying the exon-9-deleted variant associated with familial AD (PS1dE9) in one locus under control of a brain- and neuron-specific murine Thy-1 promoter element (Rezai-Zadeh et al., 2005).

Genotyping of mice was performed by PCR following the supplier's instructions. Mice were maintained on *ad libitum* food and water with a 12-hour light/dark cycle.

The 3 months old APPSwe/PS1dE9 transgenic mice were randomly assigned to a polyphenol group (N=12, fed with polyphenol diet), curcumin group (N=12, fed with curcumin diet) and normal diet group (N=12, fed with normal diet). The age and sex matched wild type littermates were used as a normal control (N=20, fed with normal diet). All animal husbandry procedures performed were approved by the Flinders University and CSIRO Human Nutrition Animal Welfare Committees in accordance with NHMRC Australian guidelines in Australia.

HPLC analysis of polyphenols in GSE

GSE (Vinlife N05010) was purchased from Tarac Technologies P.L. and was characterised by high performance liquid chromatography (HPLC) analysis without any further extraction process. GSE was dissolved in 80% methanol acidified with 0.1% HCl to obtain final concentration of 2.0 mg/mL. 10 µL was injected into the HPLC column for the analysis of polyphenolic compounds. HPLC analysis was carried out according to methods described previously (Jankowsky et al., 2001). Analytical HPLC was run at 25°C and monitored at 280nm (hydroxybenzoic acids and flavanols), 320nm (hydroxycinnamic acids, stilbenes) and 370 nm (flavonols).

Diets

Before experiments, all animals were fed with commercial standard diet pellets (Gordon's Specialty Stock Feeds Pty Ltd , NSW, Australia). All experimental diets were prepared by Specialty Feeds, Glen Forrest, Western Australia. The **Control diet** was the standard AIN-93G rodent diet (Kammerer et al., 2004) comprising of 39.7% corn starch, 20% casein (vitamin free), 13.2% dextrin, 10% sucrose, 7%

soybean oil, 5% powdered cellulose, 3.5% AIN-93G mineral mix, 1% AIN-93G vitamin mix, 0.3% L-cysteine, 0.25% Choline bitartrate, 0.001% t-Butylhydroquinone and 5% maize starch. **Curcumin diet** consisted of the AIN-93G diet with the exception that it contained 4.93% maize starch and 0.07% Curcumin (Sigma, Cat No: C1386, USA). **GSE diet** consisted of the AIN-93G diet with the exception that it contained 3% maize starch and **2% GSE**. The dosage of curcumin and GSE was based on previous reports indicating highest possible doses with lack of toxicity and in the case of curcumin preventive effects against AD in a mouse model (Reeves, 1997). Sufficient diet was prepared at the beginning of the study for the duration of the study and housed at the Flinders Medical Centre animal holding facility at 4 °C. The animals were fed with above diets for 9 months starting from when they were 3 months old at which time no Aβ deposition was formed in the brain of the animal. Food consumption and animal body weight were monitored every three months throughout the study.

Tissue sampling

Animals were sacrificed by overdosing with chloral hydrate (1.5 g/kg). Blood was sampled from the right atrium of the heart, followed by intracardial perfusion with 100 ml of 0.1% $NaNO_2$ in phosphate buffer. Brains were sampled and weighed on a digital electronic balance with a readability of 1mg (BX-420H, Shimadzu Scientific Instruments, USA). Left brain hemisphere for histological analysis was fixed in 4% paraformaldehyde (pH 7.4) for 24 h and incubated for 24 h in 30% sucrose for subsequent cryoprotection. Coronal sections of the brain at 35 μm thickness were collected with a cryosectioning microtome and stored at 4 ºC in PBS containing 0.1% sodium azide until use. Right brain hemisphere was snap frozen in liquid nitrogen and stored at -80 ºC for future biochemical analysis.

AD-type pathology and quantitative image analysis

The staining for brain total Aβ, microgliosis, astrogliosis and microhemorrhage was processed as described previously (Yamakoshi et al., 2002; Deshane et al., 2004; Yang et al., 2005). Briefly, three series of six equally spaced tissue sections (~200 μm apart) spanning the hippocampus were randomly selected and stained using free-floating immunohistochemistry for total Aβ (Biotin-conjugated mouse anti-Aβ antibody 6E10, Serotec, USA; 1:1000 dilution), activated microglia (rat monoclonal anti-CD45, Chemicon, USA; 1:2000 dilution) and astrocyte (rabbit polyclonal anti-glial fibrillary acidic protein, Dako, Denmark; 1:1000 dilution), respectively. Sections were incubated overnight with primary antibodies at 4ºC, further developed with biotinylated secondary antibodies and the ABC kit (Vector Lab, Burlingame, CA) using diaminobenzidine and glucose oxidase as substrates. Quantification of total Aβ deposit, microgliosis and astrogliosis were performed on images acquired with a digital camera and analysed with NIH Image J (http://rsbweb.nih.gov/ij/).

Images were collected at 4× magnification using constant bulb temperature and exposure, with all images acquired in the same session. The area of cortex and hippocampus was selected for automatic quantification of Aβ, microglia and astrocyte immunostaining, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors.

A series of six equally spaced tissue sections (~ 1 mm apart) spanning the entire brain was mounted and stained for hemosiderin using 2% potassium ferrocyanide in 2% hydrochloric acid for 15 min, followed by a counterstain in a 1% Neutral Red solution for 10 min at room temperature. Microhemorrhage events in the form of the

number of Prussian blue-positive profiles were counted in the brains of each mouse on all sections, and the average number of hemosiderin deposits was calculated per each brain hemisphere. All image analyses were processed in a blinded manner.

Quantification of Aβ peptide levels in the mouse brain and plasma by ELISA

ELISA analysis of the brain Aβ was processed as described previously (Wang et al., 2009b). Briefly, frozen brain was homogenized and sonicated in water containing 2% Sodium dodecyl sulphate (SDS) and protease inhibitors (Boehringer Mannheim, Germany). Homogenates were centrifuged at 100,000×*g* for 1 h at 4 ºC, and the resultant supernatant was collected, representing the SDS-soluble fraction (Aβ-SDS). The resultant pellet was then extracted in 70% formic acid, centrifuged, and the resultant supernatant was collected, representing the SDS-insoluble fraction (Aβ-FA). Before ELISA assay, SDS extracts were diluted 1:50 and formic acid extracts were neutralized by 1:20 dilution into 1 M Tris phosphate buffer, pH 11, and then diluted 1:20 in sample buffer. Concentration of Aβ40 and of Aβ42 in brain extract and plasma were quantitatively measured by ELISA (catalogue nos. 8940 and 8942; Signet Laboratories, Dedham, MA) according to manufacturer's instructions. Using the wet weight of brain tissue in the original homogenate, the final values of Aβ were expressed as picomoles per gram wet weight of cortex.

Western blot for APP and BACE expression

The expression of APP and BACE1 was analyzed using Western blot. Brain homogenates were subjected to SDS-PAGE (8-12% acrylamide). The blots were probed with the following antibodies: 6E10 (directed to Aβ and recognize full length APP), anti-BACE1 monoclonal antibody (MAB931, R&D Systems), and anti-β actin

monoclonal antibody (Sigma-Aldrich). The band density of the APP and BACE1 was normalized with β-actin.

Quantification of TNFα, IL-1β and IFN-γ in the mouse plasma by ELISA

TNFα, IL-1β and IFN-γ in the plasma of mice were measured using ELISA kits (Cat No. 88-7342, 88-7913, 88-7914, eBioscience, USA) as per manufacturer's instructions.

Assessment of toxicity of polyphenol from grape seeds

Total bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in serum were analyzed using commercial enzyme assays according to the manufacturer's instructions (Roche Diagnostics, GmbH, d-68298 Mannheim).

Statistical analysis

The data in the text and figures are expressed as mean±S.E.M, unless otherwise stated. Inter-group comparisons were assayed using one-way ANOVA and *post hoc* tests to determine the significance values. Spearman correlation coefficient was used to analyze the correlation of brain weight with Aβ level, microglia activation and astrocytosis in the brain, and the correlation between brain Aβ level and serum Aβ level. *p* values less than 0.05 were considered to be statistically significant. All the analyses were performed using SPSS for Windows version 13.0 (SPSS Inc.)

Figure 3.1

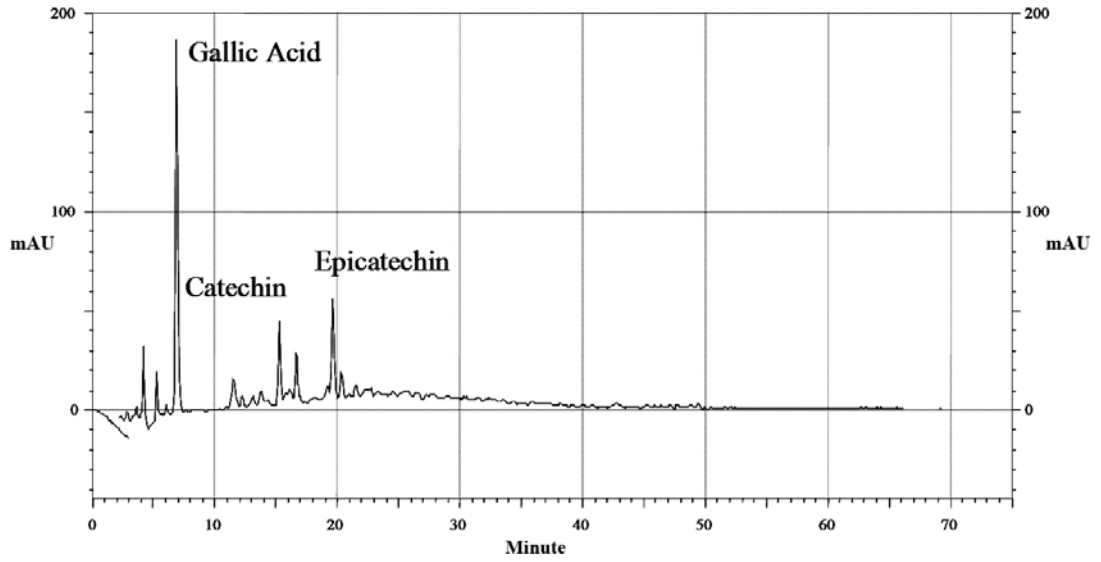

Figure 3.1 HPLC profile of phenolic compounds in GSE. Analytical HPLC was run at 25°C and monitored at 280nm (hydroxybenzoic acids and flavanols), 320 nm (hydroxycinnamic acids, stilbenes) and 370 nm (flavonols). Compounds were detected at 280 nm including phenolic acids (gallic acid) and flavanols (catechins). The level of proanthocyanidins was calculated as a difference between the total peak area at 280 nm and the area of individual peaks that represent the monomers. The mobile phase consisted of 2% acetic acid in water (solvent A) and 1.0 % acetic acid in water and acetonitrile (50:50 v/v, solvent B). The flow rate was 1 mL min^{-1}. The following gradient program was used: from 10 to 24% solvent B (20 min), from 24 to 30% B (20 min), from 30 to 55% B (20 min), from 55 to 100% B (15 min), 100% B isocratic (8 min), from 100 to 10% B (2 min). The total run time was 85 min.

Results

Chemical analysis of polyphenols in GSE

HPLC analysis detected compounds in GSE only at 280 nm (Figure 3.1). At this wavelength phenolic acids (gallic acid) and flavanols (catechins) were detected. The level of proanthocyanidins was calculated as a difference between the total peak area at 280 nm and the area of individual peaks that represent the monomers.

Through spectral characteristics and comparison with standards we detected three main compounds which were gallic acid (49 mg/g DW), catechin (41 mg/g DW), and epicatechin (66 mg/g DW). The concentration of proanthocyanidins was 436.6 mg Catechin Eq./g DW. The total polyphenolic content was 592.5 mg/g DW. A similar total phenolic content was obtained when analysed by the Folin-Ciocalteu method (data not shown).

GSE is well tolerated in APPSwe/PS1dE9 transgenic mice

The overall goal of the present study was to test the hypothesis that polyphenols from grape seeds may prevent AD-type Aβ associated pathology and behaviour change. The feeding of the GSE polyphenols and curcumin diet was from 3 months of age when Aβ deposition was not formed yet in the brain, to 12 months of age. In APPSwe/PS1dE9 transgenic mice, Aβ deposition begins at 6 months of age and become obvious at 9 months of age (Wang et al., 2009b).

The mean daily food consumption of the mice was 0.11-0.14g per gram body weight, the corresponding daily polyphenol consumption was 1.2-1.7 mg per gram body weight, and daily curcumin consumption was 77-98 μg per gram body weight. The equivalent consumption in a 60 Kg human is about 5.9 g per day for GSE

Figure 3.2

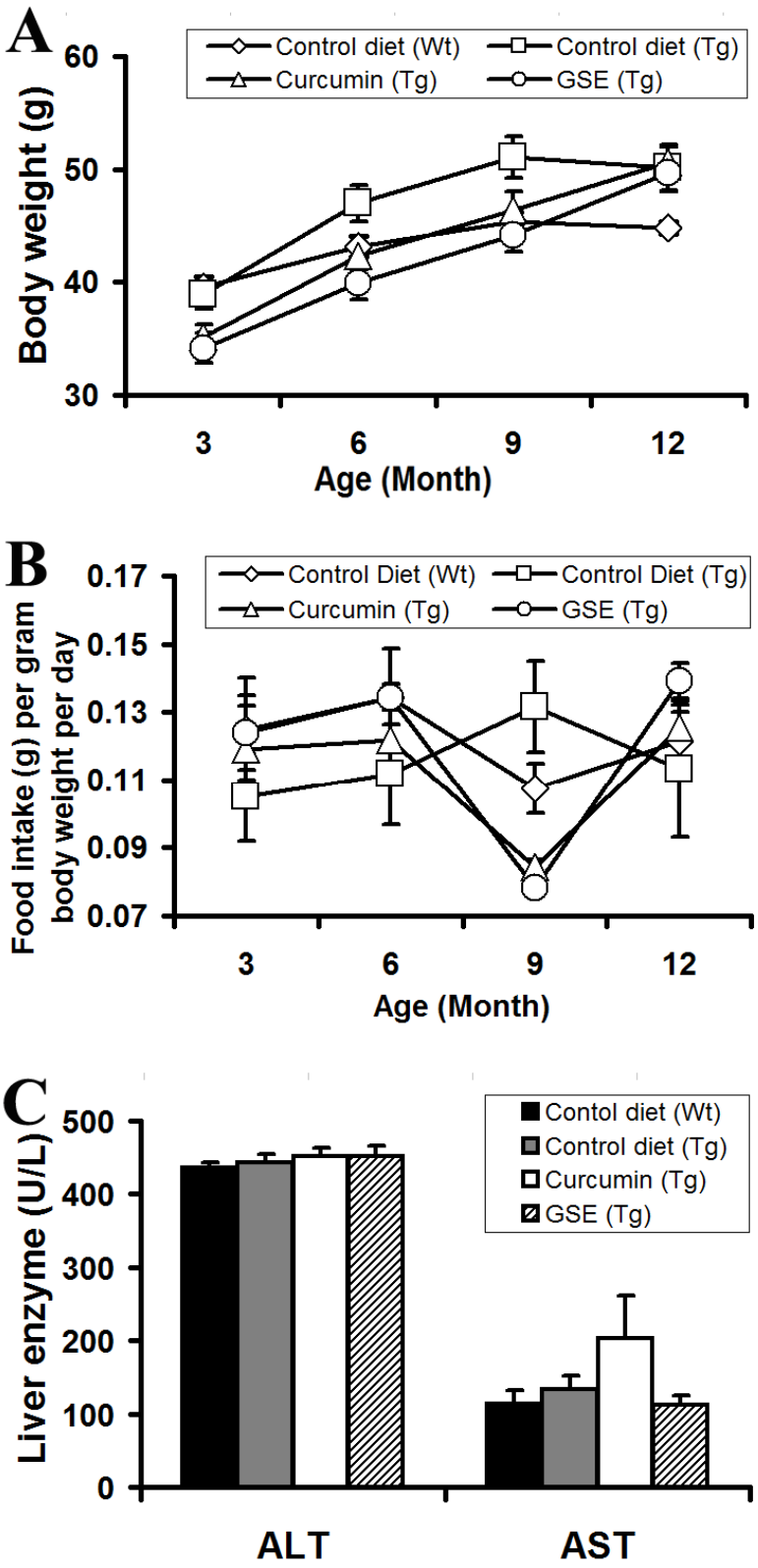
A
Control diet (Wt)
Control diet (Tg)
Curcumin (Tg)
GSE (Tg)
Body weight (g)
60
50
40
30
3
6
9
12
Age (Month)
B
Control Diet (Wt)
Control Diet (Tg)
Curcumin (Tg)
GSE (Tg)
Food intake (g) per gram body weight per day
0.17
0.15
0.13
0.11
0.09
0.07
3
6
9
12
Age (Month)
C
Contol diet (Wt)
Control diet (Tg)
Curcumin (Tg)
GSE (Tg)
Liver enzyme (U/L)
500
400
300
200
100
0
ALT
AST

Figure 3.2 Polyphenols from grape seeds are well tolerated in APPSwe/PS1dE9 transgenic mice. APPSwe/PS1dE9 transgenic mice were fed Control, Curcumin or GSE diets for 9 months from 3 months of age. In parallel control studies, gender- and age-matched wild type littermates were fed with Control diet. **A.** Body weight was measured at 3, 6, 9 and 12 months of age. **B.** Food intake was monitored at 3, 6, 9 and 12 months of age, and was calculated as food intake (gram) per gram body weight per day. **C.** Serum indices of liver functional status such as AST and ALT. Points and bar graphs represent group mean (+/- SEM).

polyphenols and 0.35 g per day for curcumin, as derived using FDA criteria for converting drug equivalent dosages across species, based on body surface area [human equivalent dose in mg/kg = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$] (Wang et al., 2006d).

During the period of the study, no animal death occurred and no behaviour abnormality was observed. We found that the long term daily consumption for 12 months in APPSwe/PS1dE9 transgenic mice, delivered in the food, did not significantly influence animal body weight (Figure 3.2A) and daily food consumption (Figure 3.2B).

It is important to note that the chronic consumption of polyphenols and curcumin did not cause liver function damage, as reflected by the normal serum levels of bilirubin, aminotransferase (AST) and alanine aminotransferase aspartate (ALT) (Figure 3.2C) and bilirubin which was detected at a very low level (< 1 μmol/L) in serum of all animals (data not shown).

GSE reduces brain and serum Aβ levels and prevents Aβ deposition in APPSwe/PS1dE9 transgenic mice

After 9 months feeding on different diets, Aβ in SDS fraction (Aβ-SDS) and in formic acid fraction (Aβ-FA) were quantified utilising a sandwich ELISA. Aβ-SDS represents the soluble forms of Aβ, while Aβ-FA represents the insoluble forms of Aβ. The total Aβ level from individual animals was calculated by the sum of total SDS-soluble Aβ (SDS-soluble Aβ42 and Aβ40) and total FA soluble Aβ (FA soluble Aβ42 and Aβ40).

Figure 3.3

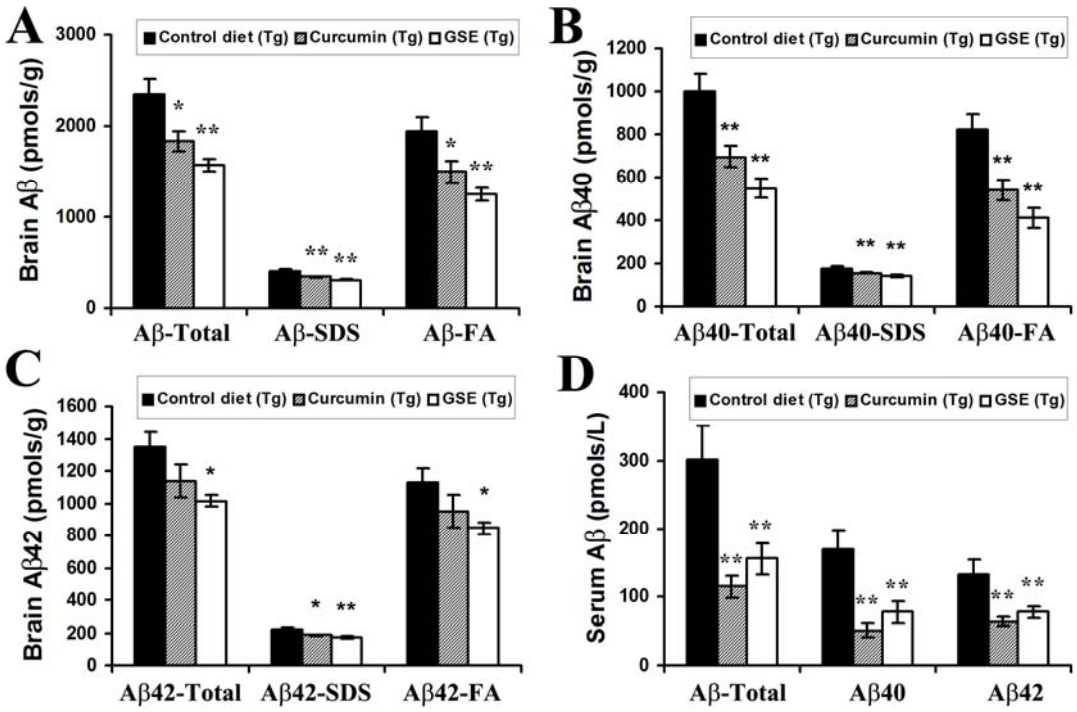

Figure 3.3 Effects of polyphenol consumption on Aβ levels in the brain and serum of APPSwe/PS1dE9 transgenic mice. Aβ peptide concentration in the brain and serum was measured using ELISA. A. Comparison of total Aβ, Aβ in SDS fraction (Aβ-SDS) and Aβ in formic acid fraction (Aβ-FA) among groups. B. Comparison of total Aβ40, Aβ40-SDS and Aβ40-FA. C. Comparison of total Aβ42, Aβ42-SDS and Aβ42-FA. D. Comparison of total Aβ, Aβ40 and Aβ42 in serum. * and ** denote $p<0.05$ or $p<0.01$ versus APPSwe/PS1dE9 transgenic mice fed with Control diet.

Figure 3.4

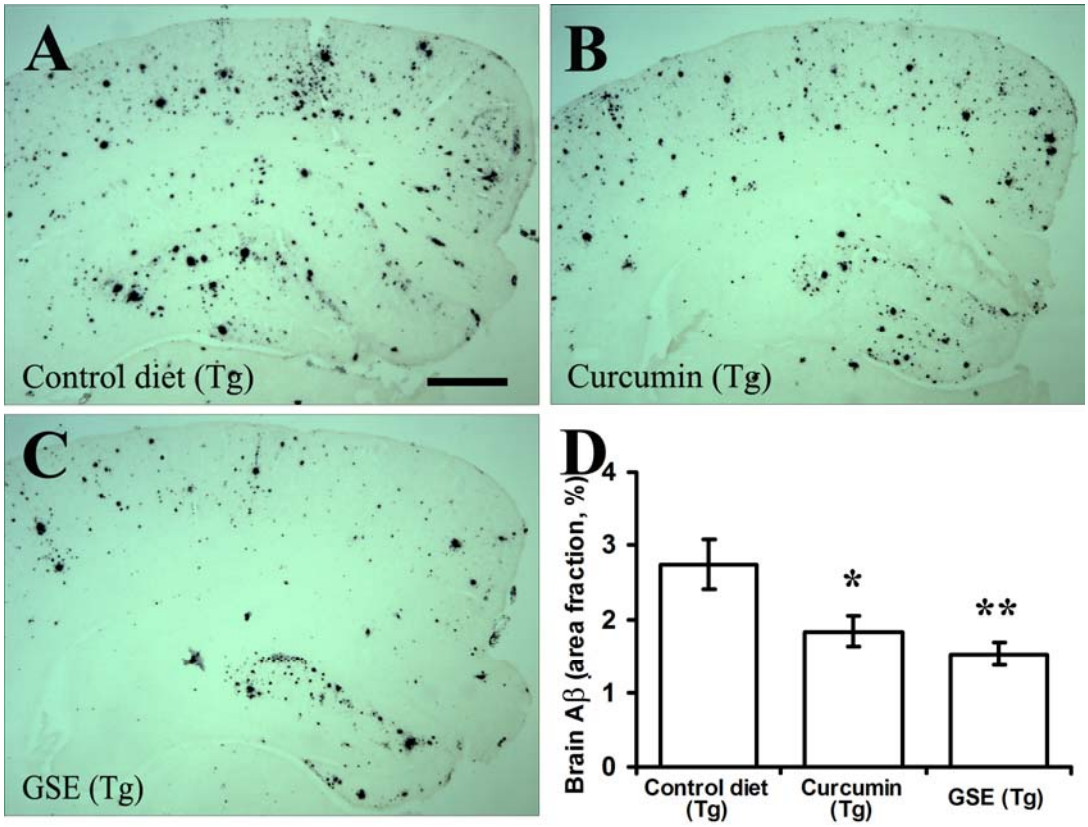

Figure 3.4 Effects of polyphenol consumption on Aβ plaque burden in the brain of APPSwe/PS1dE9 transgenic mice. A series of six equally spaced tissue sections (~200 μm apart) spanning the hippocampus were stained using free-floating immunohistochemistry for Aβ plaque (anti-Aβ antibody 6E10, Serotec) and developed with DAB. The area of cortex and hippocampus was selected for automatic quantification of Aβ plaque immunostaining with ImageJ, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors. A-C. Aβ plaques in hippocampus and cortex of APPSwe/PS1dE9 transgenic mice fed with Control, Curcumin or GSE diets. D. Comparison of Aβ plaque area fraction in cortex and hippocampus among groups. * and ** denote $p<0.05$ or $p<0.01$ versus APPSwe/PS1dE9 transgenic mice fed with Control diet, as determined by Student *t* test. Scale bar=0.5mm. Original magnification, ×4.

Compared with transgenic mice consuming Control diet, there was a significant reduction in the total brain Aβ burden in mice consuming a GSE diet ($p<0.001$) or Curcumin diet ($p=0.01$) (Figure 3.3A). GSE diet consumption led to a 33% reduction in brain Aβ burden, while Curcumin diet consumption resulted in a 22% reduction. Consistently, inter-group comparisons of SDS-Aβ and FA-Aβ, Aβ40 and Aβ42 are essentially the same as those of total Aβ (Figure 3.3B and C).

GSE or Curcumin diet consumption also reduced the Aβ concentrations in the serum (Figure 3.3D). The total Aβ concentration in serum correlated significantly and positively with brain total Aβ burden (Pearson $r=0.348$, $p=0.021$), suggesting the potential value of serum Aβ concentration monitoring to reflect brain Aβ burden during the course of Alzheimer's dietary prevention studies with polyphenols and curcumin.

Aβ plaques were observed primarily in the neocortical and hippocampal areas of the brain. Quantitative histology analysis also generated similar results. Compared with Control diet consumption (Figure 3.4A), consumption of GSE ($p=0.002$) or Curcumin ($p=0.015$) diets reduced the Aβ deposition in the neocortex and hippocampus (Figure 3.4B and C). Similar to the brain Aβ levels determined by Aβ ELISA, the total Aβ plaque burden determined by immunohistochemistry was reduced by 45% in the GSE diet group and 33% in the Curcumin diet group. These data suggest polyphenols from grape seeds are effective in reducing Aβ burden and preventing Aβ deposition in the brain.

Figure 3.5

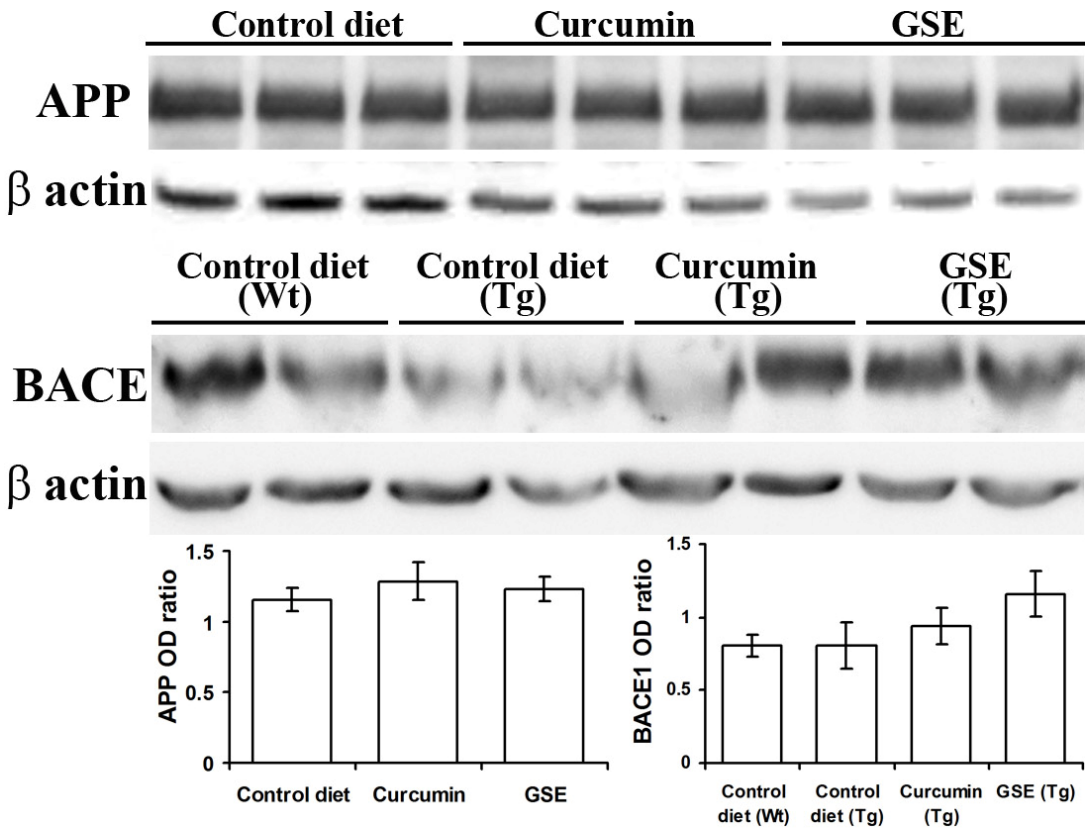

Figure 3.5 Effects of GSE consumption on the expression of APP and BACE1 in the brain of APPSwe/PS1dE9 transgenic mice. Brain homogenates from APPSwe/PS1dE9 transgenic mice fed with Control, Curcumin or GSE, and wild type littermates fed with Control diet were subjected to Western blot to detect the level of APP and BACE1 by 6E10, anti-BACE1 monoclonal antibodies, and anti-β actin monoclonal antibody. The band density of the APP and BACE1 was normalized with β-actin.

Figure 3.6

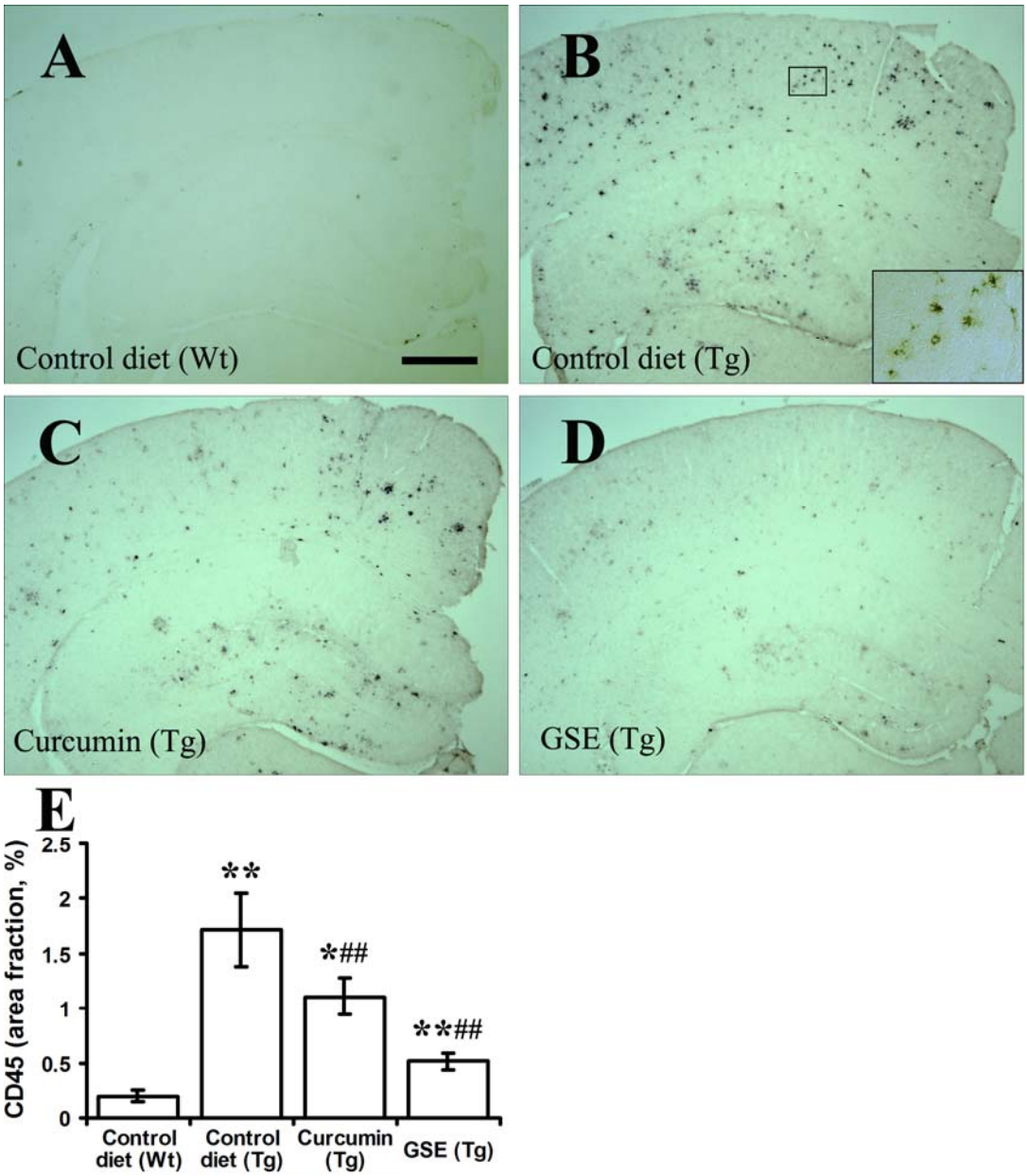
A
Control diet (Wt)
B
Control diet (Tg)
C
Curcumin (Tg)
D
GSE (Tg)
E
CD45 (area fraction, %)
2.5
2
1.5
1
0.5
0
**
*##
**##
Control diet (Wt)
Control diet (Tg)
Curcumin (Tg)
GSE (Tg)

Figure 3.6 Effects of polyphenol consumption on microgliosis in the brain. A series of six equally spaced tissue sections (~200 μm apart) spanning the hippocampus were stained using free-floating immunohistochemistry for activated microglia (rat monoclonal anti-CD45, Millipore) and developed with DAB. The area of cortex and hippocampus was selected for automatic quantification of activated microglia immunostaining with ImageJ, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors. **A.** No obvious microgliosis was observed in the brain of wild type littermates fed with Control diet. **B-D.** Microgliosis in hippocampus and cortex of APPSwe/PS1dE9 transgenic mice fed with Control, Curcumin or GSE diets. **E**. Comparison of CD45 area fraction in cortex and hippocampus among groups. ***** and ****** denote $p<0.05$ or $p<0.01$ versus wild type littermate fed with normal diet, $^{\#}$ and $^{\#\#}$ denote $p<0.05$ or $p<0.01$ versus APPSwe/PS1dE9 transgenic mice fed with Control diet, as determined by Student *t* test. Scale bar=0.5mm. Original magnification, ×4.

Figure 3.7

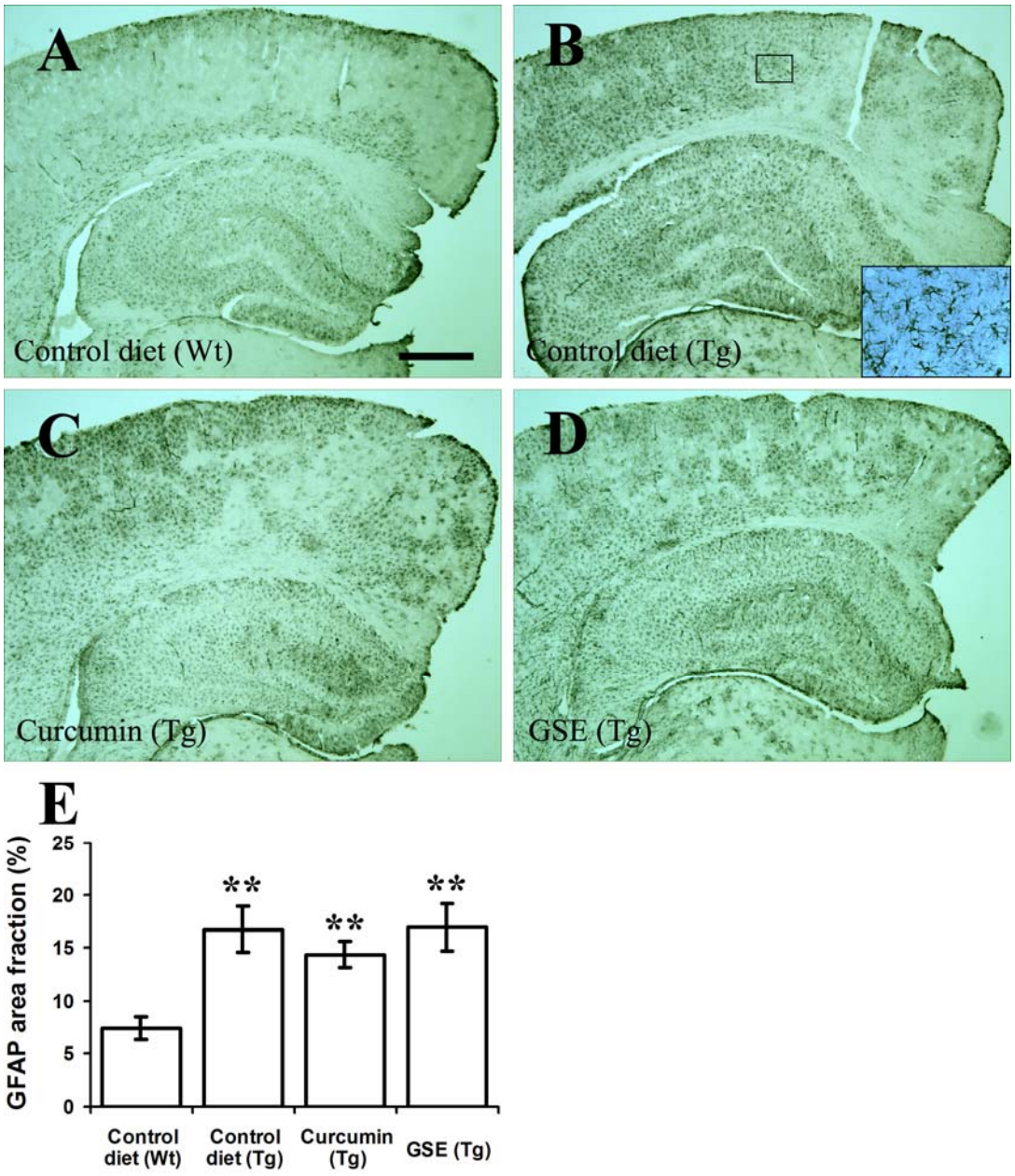
A
Control diet (Wt)
B
Control diet (Tg)
C
Curcumin (Tg)
D
GSE (Tg)
E
GFAP area fraction (%)
25
20
15
10
5
0
**
**
**
Control diet (Wt)
Control diet (Tg)
Curcumin (Tg)
GSE (Tg)

Figure 3.7 Effects of polyphenol consumption on astrogliosis in the brain. A series of six equally spaced tissue sections (~200 μm apart) spanning the hippocampus were stained using free-floating immunohistochemistry for astrocyte (rabbit polyclonal anti-glial fibrillary acidic protein, Dako) and developed with DAB. The area of cortex and hippocampus was selected for automatic quantification of astrogliosis immunostaining with ImageJ, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors. **A.** astrogliosis in hippocampus and cortex of wild type littermates fed with Control diet. **B-D.** astrogliosis in hippocampus and cortex of APPSwe/PS1dE9 transgenic mice fed with Control, Curcumin or GSE diets. **E.** Comparison of GFAP area fraction in cortex and hippocampus among groups. * and ** denote $p<0.05$ or $p<0.01$ versus wild type littermate fed with Control diet, as determined by Student *t* test. Scale bar=0.5mm. Original magnification, ×4.

GSE does not change the expression of APP and BACE1 in the brain of APPSwe/PS1dE9 transgenic mice

To further understand the mechanisms underlying the brain Aβ reduction after GSE and curcumin consumption, we examined the expression of APP and BACE1 in the brain homogenates. As shown in Figure 3.5, there were no significant differences in the levels of APP, the precursor of Aβ, and BACE1, the key enzyme to generate Aβ by cleaving APP. These data suggest that the reduction of brain Aβ after GSE and Curcumin consumption is not due to the changes in APP expression and β-cleavage of APP by BACE1.

GSE prevents AD-type neuropathology in APPSwe/PS1dE9 transgenic mice

Microgliosis and astrogliosis were observed primarily in the neocortical and hippocampal areas of the brain. We examined the area fraction of CD45+ microglia and GFAP+ astrocytes in neocortical and hippocampal regions.

No obvious microgliosis was observed in brains of wild type littermates (Figure 3.6A), while APPSwe/PS1dE9 transgenic mice showed obvious microgliosis (Figure 3.6B). Compared with transgenic mice fed with Control diet, the mice fed GSE had a significantly lower level of microgliosis (0.51±0.08 vs. 1.72±0.34, $p<0.001$), and such a reduction was also observed in the mice fed curcumin (1.11±0.16 vs. 1.72±0.34, $p=0.032$) (Figure 3.6 C, D and E).

Wild type littermate fed with control diet had obvious astrogliosis (Figure 3.7A), which, however, was significantly lower than that in APPSwe/PS1dE9 transgenic mice (Figure 3.7B). There was no significant difference in astrogliosis among the groups of transgenic mice fed with Control, Curcumin and GSE diets (Figure 3.7B to

Figure 3.8

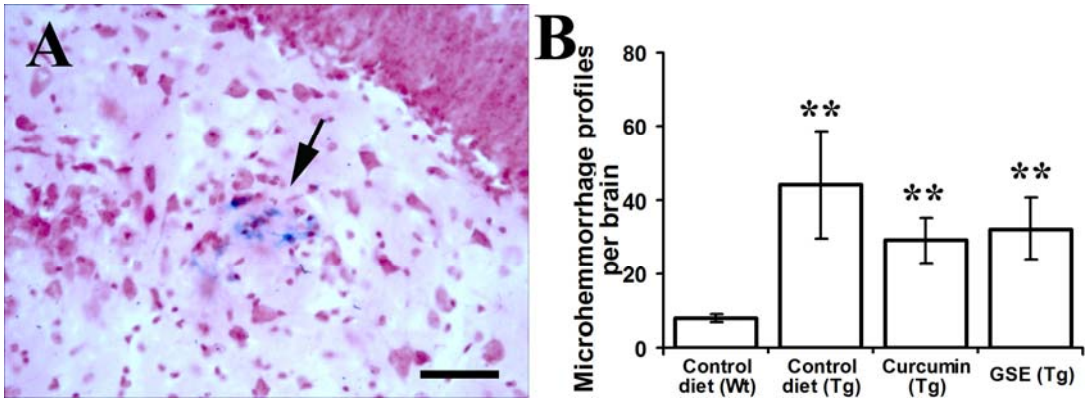

Figure 3.8 Effects of GSE and curcumin consumption on microhemorrhage profiles in the brain. A series of six equally spaced tissue sections (~ 1 mm apart) spanning the entire brain was mounted and stained for hemosiderin using 2% potassium ferrocyanide in 2% hydrochloric acid, followed by a counterstain in a 1% Neutral Red solution. Microhemorrhage events in the form of the number of Prussian blue-positive profiles were counted, and the average number and standard error of hemosiderin deposits was calculated per each brain hemisphere. A. An example of microhemorrhage profile (solid arrow) observed in hippocampus. B. Comparison of microhemorrhage profiles per each brain hemisphere among groups. * and ** denote $p<0.05$ or $p<0.01$ versus wild type littermate fed with Control diet, as determined by Student *t* test. Scale bar=50μm. Original magnification, ×40.

Figure 3.9

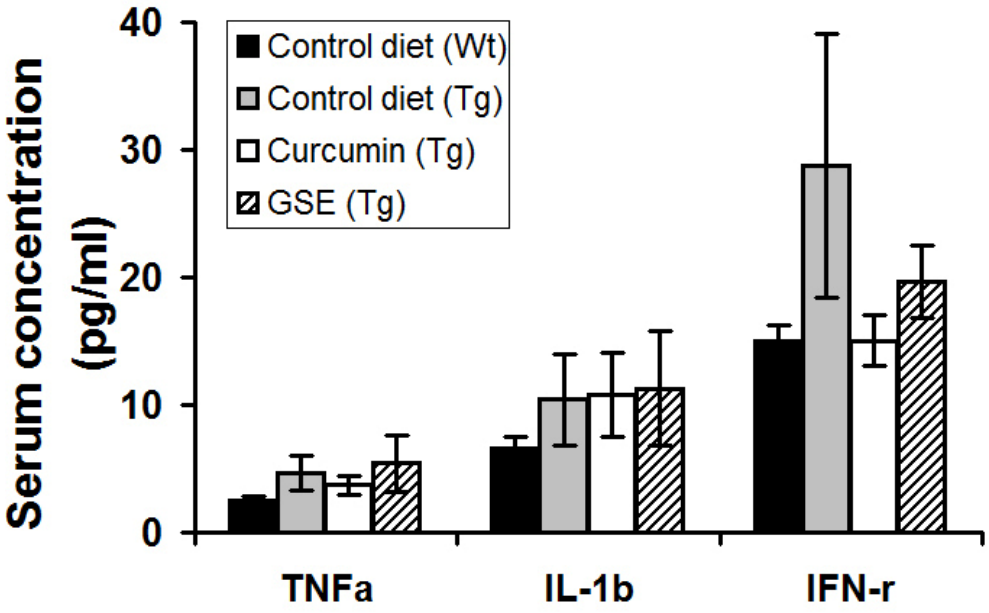

Figure 3.9 Effects of curcumin and GSE consumption on plasma levels of TNFα, IL-1β and IFN-γ. Plasma levels of TNFα, IL-1β and IFN-γ were measured using ELISA kits (eBioscience). ** denotes $p<0.01$ versus wild type littermate fed with normal diet, # denotes $p<0.05$ versus APPSwe/PS1dE9 transgenic mice fed with Control diet, as determined by Student *t* test.

E). Characteristic blue hemosiderin-positive profiles were observed primarily in the neocortical, leptomeningeal, hippocampal and thalamic areas of the brain (Figure 3.8A). The microhemorrhage was detected at a rate of 44.0±14.6 per hemibrain in transgenic mice fed with normal diet, which was higher than in wild type littermate (7.76±1.07 per hemibrain, p=0.006). Non-significant lower rates of microhemorrhage were observed in mice fed with GSE (29.0±6.2 per hemibrain, p=0.270) or Curcumin (32.1±8.4 per hemibrain, p=0.383) diets when compared with transgenic mice fed with Control diet (Figure 3.8B).

Plasma and brain levels of inflammatory cytokines after GSE consumption

Activated microglia and microphages secrete cytokines such as IL-1β, TNF-α and IFN-γ. This is a major pathologic event in the progression of inflammatory cascades within the AD brain. We measured the levels of IL-1β, TNF-α and IFN-γ in the brain homogenates and plasma. In general, cytokines were detected but at very low levels which were at the lower range of the sensitivity of the ELISA kit used (8-15 pg/ml). Levels of IL-1β and TNF-α tended to be higher in transgenic mice than in wild type littermates, but the difference between groups did not reach statistical significance (Figure 3.9). The level of IFN-γ was higher in transgenic AD mice fed with Control diet (28.7±10.3 pg/ml) than in their wild type littermates (14.9±1.2 pg/ml, p=0.008) on the same diet. Compared with Control diet consumption, GSE (19.6±2.0 pg/ml vs. 28.7±10.3 pg/ml, p=0.336) and Curcumin (15.1±2.0 pg/ml vs. 28.7±10.3 pg/ml, p=0.021) diet consumption decreased the IFN-γ level in the plasma of APPSwe/PS1dE9 transgenic mice.

Discussions

Both genetics and environment determine the development of many chronic diseases including AD. The genetic mutations of APP, presenilin and other genes only account for a relatively small number (<5%) of the total number of AD patients (familial cases) whereas the majority of AD patients are likely due to environmental factors and other genetic factors affecting Aβ clearance (Food and Drug Administration, 2003). Major environmental factors are likely to include excess or deficiency of dietary constituents consumed on a regular basis which have bioactivity in relevant pathways. Our understanding of how food and drink can potentially influence the development of AD will help to develop and implement practical and inexpensive treatments that may aid in combating this devastating disease.

In the present study, we report that polyphenols-rich GSE fed for 9 months as a food additive dramatically could prevent the AD development in a genetic mouse model. We found that the chronic consumption of polyphenols extracted from grape seeds was well tolerated, effectively reduced the Aβ burden in the brain and blood, prevented the Aβ deposition, and attenuated the microgliosis.

Aβ accumulation and deposition in the brain is one of the histopathological hallmarks of AD. Our study clearly demonstrated that the polyphenols in GSE when fed to a transgenic mouse model for 9 months reduced the total brain amyloid burden by 33% to 45%, depending on the analysis methods used. Aβ40 and Aβ42 are the major forms of amyloid-beta peptides in the brain. Aβ42 is much more prone to aggregation and more toxic to neurons than Aβ40 (Hardy and Selkoe, 2002). In the present study, it appears that GSE reduced Aβ40 slightly more than Aβ42, which is consistent with a recent study (Jarrett et al., 1993; El-Agnaf et al., 2000). However,

the mechanism on this differential effect is not known. We used curcumin as a positive control at a dietary concentration known to reduce the brain amyloid burden (Wang et al., 2008). Our data indicate that the polyphenols-rich GSE are comparable to curcumin in the reduction of brain and plasma amyloid burden although they were added at a higher concentration in the diet. As there are a number of polyphenols in GSE, it is not known which polyphenol plays a major role in these events. However, we assume that the combination of all polyphenols could be important in achieving these desirable effects. The role these polyphenols play in the reduction of the amyloid burden is not known. Recently, other known polyphenols have been shown to be able to influence Aβ metabolism and protect against Aβ neurotoxicity. Curcumin, a phenolic yellow pigment found in turmeric and a spice used extensively in Asian Indian food, can directly bind small Aβ species to block the formation of oligomer and fibril as well as to disaggregate Aβ aggregates *in vitro,* and can reduce amyloid levels and plaque burden in aged transgenic AD mice when administered peripherally (Yang et al., 2005). The consumption of the green tea polyphenol epigallocatechin-3-gallate has also been shown to reduce the overproduction of Aβ *in vitro* (Lim et al., 2001; Yang et al., 2005) and in the transgenic AD mouse brain (Levites et al., 2003) by promoting the nonamyloidogenic alpha-secretase proteolytic pathway, and reduce the generation of holo-APP and Aβ presumably via iron chelating effects of the polyphenol *in vitro* (Rezai-Zadeh et al., 2005). In the present study, holo-APP and BACE1 expression did not change after chronic consumption of GSE or curcumin, suggesting that GSE and curcumin probably do not exert their protective effects by modifying expression of holo-APP or its processing by BACE1. Wine is rich in polyphenols and wine consumption is related to a lower risk for AD in epidemiological studies (Reznichenko et al., 2006). This has been confirmed in an animal study showing that wine consumption is effective in preventing AD, also by

increasing the nonamyloidogenic alpha-secretase activity (Orgogozo et al., 1997; Lindsay et al., 2002; Letenneur, 2004). It is known that grape-derived polyphenols inhibit Aβ aggregation, reduce Aβ production, degrade intracellular Aβ, protect Aβ neurotoxicity, and attenuate oxidative stress in vitro (Wang et al., 2006b). It is likely that the polyphenols from GSE act on similar multiple pathways to reduce the brain amyloid burden. A reducing effect was observed preferentially on the insoluble fraction of both Aβ40 and Aβ42 in our study, as well as others, suggesting that GSE and curcumin probably affect the assembly or disruption of preformed fibrils and Aβ aggregation (Bastianetto et al., 2000; Jang and Surh, 2003; Ono et al., 2003; Savaskan et al., 2003; Li et al., 2004; Marambaud et al., 2005; Ono et al., 2005; Mancuso et al., 2007; Riviere et al., 2007). However, chronic consumption of GSE derived polyphenols did not change the activities of Aβ generating and degrading enzymes (Wang et al., 2008). Thus, mechanisms of GSE in modulating Aβ metabolism need further investigation and novel pathways need to be explored.

Inflammation is another hallmark of AD. There is mounting evidence that chronic inflammatory processes play a fundamental role in the progression of neuropathological changes in the AD brain (Wang et al., 2008). The major players involved in the inflammatory process in AD are thought to be microglia. Cytokine production by activated microglia, such as IL-1β, TNF-α and IFN-γ, is a key pathologic event in the progression of inflammatory cascades (Akiyama et al., 2000; von Bernhardi, 2007). In the present study, chronic polyphenol consumption effectively alleviated microgliosis. The serum levels of IL-1β, TNF-α and IFN-γ in transgenic mice were higher than in wild type littermates. This is consistent with the findings from AD patients that IL-1β, TNF-α and IFN-γ are up-regulated (Jekabsone et al., 2006; Meme et al., 2006; Yamamoto et al., 2007). Polyphenol and curcumin

consumption tend to decrease plasma levels of IFN-γ. However, we did not observe a difference in serum levels of IL-1β and TNF-α among transgenic mice fed with Control, Curcumin or GSE diets. It should be noted that these cytokines exist at very low levels in the serum which is within the lower range of sensitivity of the ELISA kits used (8-15 pg/ml) which might not be sensitive enough to reflect the difference between groups. Another possible explanation is that the cytokines in the serum might not have a good correlation to the cytokines in the brain. The IL-1β, TNF-α and IFN-γ levels in the transgenic mice have not been well characterized. So far limited studies have examined these cytokines in the APP transgenic mouse model and generated controversial results, primarily as a result of the low levels of these cytokines (Fillit et al., 1991; Blum-Degen et al., 1995; Solerte et al., 2000)(see review in (Mehlhorn et al., 2000; Sly et al., 2001; Abbas et al., 2002; Yamamoto et al., 2007)). However, our quantitative data on the microglia activation by immunostaining demonstrated that the microglia activation was suppressed by over 70% in animals on the GSE diet. The inflammation suppression by the GSE diet indicates that the polyphenols from GSE are also powerful ingredients which inhibit the inflammation in the brain of AD. It is well known that GSE has a property of anti-inflammation in other inflammation models such as chemically-induced dermatitis (Heneka and O'Banion, 2007), ultraviolet B (UVB) induced oxidative stress models (Li et al., 2001; Bralley et al., 2007), atherosclerosis model (Sharma et al., 2007) or systemic sclerosis in patients (Vinson et al., 2002). How the GSE polyphenols in the present study affect the inflammation is not clear. It is known that the green tea polyphenol EGCG or GSE suppresses NF-kappaB activation and phosphorylation of p38 MAPK and JNK in human astrocytoma U373MG cells (Kalin et al., 2002) or a in UVB-induced oxidative stress mouse model (Kim et al., 2007b). As NF-kappaB, p38 MAPK and JNK are major signal pathways involving

inflammation, it is likely that the polyphenols from GSE suppress inflammation by inhibiting these signalling pathways. In a human umbilical vein endothelial cell culture model, GSE significantly inhibited the expression of adhesion molecule VCAM-1 and activated peroxisome proliferators-activated receptor gamma (PPAR gamma) and reduced the content of Von Willebrand factor, indicating that GSE may suppress inflammation by inhibiting the cell inflammatory factor expression and activating PPAR gamma (Sharma et al., 2007). Other mechanisms such as oxygen free radical scavenging, anti-lipid peroxidation, and inhibition of the formation of inflammatory cytokines may also be involved (Ma et al., 2007). Meanwhile, decrease of Aβ accumulation in the brain may also contribute to the reduced microgliosis observed in the present study.

In our present study, consumption of GSE or curcumin did not significantly reduce the GFAP positive astrogliosis, although Aβ pathology and microgliosis in the brain were significantly attenuated. Significant astrogliosis were also observed in the brain of wild type control mice. Our findings are consistent with a recent study showing that area reactivity of GFAP did not correlate with Aβ immunoreactivity in AD patient's brains, which suggests other factors such as age-associated events may also contribute to the astrocyte pathology in AD brain (Li et al., 2001).

Conclusions

Moderate wine consumption has been recommended for the prevention of AD (Simpson et al., 2008). Considering that the disease primarily affects the old generation most of whom are contraindicated to alcohol, polyphenol extracts from grape seeds might be a better alternative to wine for the prevention of AD. In a parallel study, we have shown that the consumption of the polyphenols from GSE

significantly suppressed the genomic instability events associated with DNA damage in those animals with a high polyphenol or curcumin diet (Hampl et al., 2002) which are in agreement with other studies from our group showing that wine polyphenols protect against DNA damage induced by oxidative stress in vitro and ex vivo (Thomas et al., 2009). In the present study, we have clearly demonstrated the benefit of GSE as a food additive in the prevention of AD. Our studies showed that 9 months of continuous consumption of GSE did not cause any damage to the liver as the bilirubin level and amino acid transferase activities are normal. The food intake and body weight in the experimental animals are well maintained and comparable to those of animals fed with the Control diet. In addition, all animals fed with GSE were found to be normal with no tumour development or unexpected death. Our study indicates that GSE is a safe food additive in mice and therefore it is possible that it may be safely consumed in a long-term manner in humans to prevent the development of AD, although this has yet to be tested. As this food additive has a strong anti-inflammatory effect shown in the previous studies and our current studies, the consumption of GSE may be beneficial for chronic inflammatory diseases. Given the safety of GSE in long-term use and that no strong disease-modifying therapeutic and preventive measures are currently available in clinical settings, consuming GSE would be promising in developing practical preventive and therapeutic measures for AD.

CHAPTER 4

Deletion of P75NTR Reduces Aβ Production But Exacerbates Aβ Pathology by Increasing Aβ Deposition in an Alzheimer's Disease Mouse Model

Summary

Accumulation of toxic amyloid-beta (Aβ) in cerebral cortex and hippocampus is a major pathological feature of Alzheimer's disease (AD). The neurotrophin receptor, p75NTR, has been proposed to mediate Aβ- induced neurotoxicity; however its role in the development of AD remains to be clarified. Study of p75NTR expression in the AD model APPSwe/PS1dE9 transgenic mice, indicates localization to the basal forebrain neurons and degenerative neurites in amyloid plaques of neocortex, increased expression with aging, and further activation by Aβ accumulation. Deletion of p75NTR gene by crossing APPSwe/PS1dE9 mice with p75NTR knockout mice reduced soluble Aβ levels in the brain and serum, but increased the accumulation of insoluble Aβ and Aβ plaque formation. This caused no change in either the expression of APP and its proteolytic derivatives, or α, β and γ secretase activities, or in levels of BACE1, neprilysin (NEP) and insulin degrading enzyme (IDE) proteins. Recombinant extracellular domain of p75NTR attenuated the oligomerization and fibrillation of synthetic Aβ42 peptide *in vitro*, and reduced local Aβ plaques after hippocampus injection *in vivo*. In addition, deletion of p75NTR attenuated microgliosis but increased the microhemorrhage profiles in the brain. The deletion of p75NTR did not significantly change the cognitive function of the mice up to the age of 9 months. Our data suggest that p75NTR plays a critical role in regulating Aβ levels by both increasing Aβ production and attenuating its aggregation and they caution that a therapeutic intervention simply reducing p75NTR may exacerbate AD pathology.

Key words: p75NTR, deletion, amyloid-beta, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by accumulation of amyloidogenic plaques and neurofibrillary tangles in the brain, loss of cholinergic neurons and a progressive loss of cognitive functions. Soluble Aβ peptide plays a central role in the neurotoxicity and the development of AD (Greenrod and Fenech, 2003; Greenrod et al., 2005). The oligomers are the most toxic species of Aβ, causing neuronal death, neuritic degeneration and dysfunction in synaptic transmission in AD (Ma et al., 2006; Lacor et al., 2007). The level of Aβ in the brain is maintained by the dynamic balance of Aβ production from the cleavage of amyloid precursor protein (APP) and the clearance of Aβ by its enzymatic degradation and transport (Lambert et al., 1998b; Hardy and Selkoe, 2002; Malaplate-Armand et al., 2006; Lacor et al., 2007). Mutations in genes directly involved in the cleavage of APP or in clearance of Aβ can break this balance and cause accumulation of Aβ in patients or in mutant mouse models (Hardy and Selkoe, 2002; Wang et al., 2006c). Understanding the pathways involving Aβ production, transport and clearance will allow developing therapies to hold the progression of the disease.

Neurotrophin signaling pathways are known to affect production of Aβ and development of AD (Citron et al., 1992; Borchelt et al., 1996; Irizarry et al., 1997). Transgenic animals expressing the antibody against nerve growth factor (NGF) develop AD-associated pathology that can be alleviated by the delivery of NGF (Capsoni et al., 2000; Capsoni et al., 2002). NGF signaling via TrkA reduces Aβ production whereas it facilitates Aβ production via p75 neurotrophin receptor (p75NTR) (Capsoni et al., 2002). p75NTR is highly expressed in cholinergic neurons

in the basal forebrain which are most vulnerable in AD. Over-expression or delivery of NGF in primates and in human can ameliorate the degeneration of cholinergic neurons (Costantini et al., 2005a). However, the significance of p75NTR in AD pathogenesis is not clear and conflicting data have been reported. Recent studies suggest that binding of Aβ to p75NTR mediates neuronal death *in vitro* and *in vivo* (Koliatsos et al., 1991; Tuszynski et al., 1996; Tuszynski et al., 1998) and neuritic degeneration in AD mice (Kuner et al., 1998; Costantini et al., 2005b; Sotthibundhu et al., 2008). However, the activation of p75NTR by Aβ has also been shown to promote survival of human neurons (Knowles et al., 2009). Also, NGF signaling via p75NTR increases the production of Aβ during normal aging (Zhang et al., 2003b).

p75NTR is a type I receptor membrane protein which is proteolytically processed by a two-step mechanism similar to the processing of APP and involves α-secretase-like and γ-secretase mediated cleavages (Costantini et al., 2005a). APP directly interacts with p75NTR and this interaction mediates neuronal death of basal forebrain neurons but whether this mediates the production Aβ and AD pathology is not clear (Jung et al., 2003; Gowrishankar et al., 2004; Zampieri et al., 2005). Presenilin 1 M146V mutation increases γ-secretase cutting of p75NTR (Fombonne et al., 2009). The cleavage of p75NTR by extracellular metalloproteases sheds p75 extracellular domain and generates a membrane-associated C-terminal fragment (Hatchett et al., 2007). Further processing of the C-terminal fragment by γ-secretase releases the p75-ICD cytosolic fragment that mediates signaling (Sotthibundhu et al., 2008).

The complexity of signals mediated by p75NTR raises the question of the functional roles of p75NTR in the development of AD *in vivo* (Jung et al., 2003; Bronfman,

2007). Although a large body of evidence shows p75NTR plays a critical role in the death of neurons mediated by Aβ and proneurotrophins, the role of p75NTR in Aβ metabolism in AD has not been explored so far *in vivo*. This critical experiment is essential to reveal the functional role of p75NTR in the development of AD pathology. In the present study we used a genetic approach to examine the roles of p75NTR in regard to Aβ burden and AD-like pathology. We found that p75NTR plays different roles in Aβ metabolism.

Materials and methods

Generation of APPSwe/PS1dE9 transgenic mice with deletion of p75NTR gene

p75NTR knockout mice (p75NTR/ExonIII-/- mice) and APPSwe/PS1dE9 transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). p75NTR/ExonIII-/- mice were constructed on a 129/Sv and have a targeted deletion of exon III of the p75NTR locus (Coulson, 2006). These mice produce a shorter isoform of p75NTR lacking the extracellular domain of the receptor responsible for neurotrophin binding (Lee et al., 1992). APPSwe/PS1dE9 transgenic mice were on a C57BL/6 background, and bear a chimeric mouse/human (Mo/Hu) APP695 with mutations linked to familial AD (KM 593/594 NL) and human PS1 carrying the exon-9-deleted variant associated with familial AD (PS1dE9) in one loci under control of prion promoter element (von Schack et al., 2001). APPSwe/PS1dE9 transgenic mice were APPSwe/PS1dE9+/- and begin to develop senile plaques in the cerebral cortex and hippocampus at three to four months of age.

APPSwe/PS1dE9 and p75NTR/ExonIII-/- mice were crossed to generate APPSwe/PS1dE9/p75NTR+/- (APP+/-p75+/-) mice. APP+/-p75+/- mice were backcrossed with the p75NTR/ExonIII-/- mice for ten generations to produce APP+/-

p75-/- mice with nearly pure 129/Sv background. An APP+/-p75-/- mouse was crossed with a 129/Sv mouse to obtain a male APP+/-p75+/- mouse, which was next crossed with female 129/Sv mice and p75NTR knockout mice to breed six kinds of genotypes, including APP+/-p75-/-, APP+/-p75+/-, APP+/-p75+/+, APP-/-p75+/+, APP-/-p75+/- and APP-/-p75-/-. The genotyping of p75NTR and APPSwe were performed with PCR as per the product instructions (Jackson Laboratory, Bar Harbor, ME, U.S.A.). Mice were maintained on *ad libitum* food and water with a 12-hour light/dark cycle. All animal cross-breeding and husbandry procedures performed were approved by the Flinders University Animal Welfare Committee and Flinders University Biosafety Committee.

Behavioural test

This task represents a classic version of the Morris Water Maze task and was conducted following the protocols reported previously with minor modification (Jankowsky et al., 2001). In brief, the test was conducted in a pool (diameter 200 cm) which was filled with opaque water containing white non-toxic dye (24±2°C) and surrounded by a set of spatial cues (Yau et al., 2007). The tank was imaginarily divided into four quadrants, and four start positions were located at the intersections of the quadrants (Markowska et al., 1993). Each daily session consisted of 4 platform trials during which a round platform (diameter 8 cm) was submerged 1 cm under water surface in a quadrant. The mouse explored in the pool and escaped on the platform. If the mouse failed to locate the platform in 120 seconds, it was directed to the platform. Once the mouse escaped on the platform, it remained on the platform for 10 seconds. After four days of training, all mice were given a single probe trial, where platform was withdrawn, at two hours after the last platform trial in order to assess the final strength of memory traces. In platform trials, distance (path from the

start location to the platform, cm), latency (the time taken to reach the platform from the start location, s), and swim speed (average speed during a trial, cm/s) were measured, while in probe trials quadrant time (percentages of time spent in the platform quadrant) and platform crossings (the number of times that the mice crossed the exact location of the platform) were measured. For latency and swim distance in platform trials, lower numbers indicate better performance. For quadrant time and platform crossings in probe trials, higher numbers indicate better performance (more time spent in the correct quadrant and more crossings over the platform location) (Frick and Fernandez, 2003).

Tissue sampling

Animals were sacrificed by overdosing with pentobarbitol (0.08 g/kg). Blood was sampled from the right atrium of the heart, followed by intracardial perfusion with 100 ml of 0.1% $NaNO_2$ in phosphate buffer. Brains were sampled and weighed on a digital electronic balance with a readability of 1 mg (BX-420H, Shimadzu Scientific Instruments, USA). Left brain hemisphere for histological analysis was fixed in 4% paraformaldehyde (pH 7.4) for 24 h and incubated for 24 h in 30% sucrose for subsequent cryoprotection. Coronal sections of the brain at 35 μm thickness with a cryosectioning microtome and stored at 4ºC in PBS containing 0.1% sodium azide until use. Right brain hemisphere was snap frozen in liquid nitrogen and stored at -80ºC for future biochemical analysis.

AD-type pathology and quantitative image analysis

To examine the expression of p75NTR in the brain, brain sections of 9 months old APPSwe/PS1dE9 mice were stained using rabbit polyclonal anti-human p75NTR antibody (Ab 9650, a gift from Dr. M. Chao, Skirball Institute, New York University,

New York, NY) following the free-floating immunohistochemistry protocol as described previously (Frick and Fernandez, 2003). Immunofluorescence was used to examine the colocalization between p75NTR positive fibers and amyloid plaques, with Ab 9650 for p75NTR, antibody N52 (Sigma-Aldrich) for neurofilament 200 (NF200) and Thioflavine S for fibrillar plaque. Sections were observed with confocal fluorescence microscope (Radiance 2000MP, Bio-Rad).

The staining for brain total Aβ, microgliosis, astrogliosis and microhemorrhage was processed as described previously (Wang et al., 2009b). Briefly, a series of five equally spaced tissue sections (~1.3mm apart) spanning the entire brain were randomly selected and stained using free-floating immunohistochemistry for total Aβ (Biotin-conjugated mouse anti-Aβ antibody 6E10, Serotec, USA; 1:1000 dilution), activated microglia (rat monoclonal anti-CD45, Chemicon, USA; 1:2000 dilution) and astrocyte (rabbit polyclonal anti-glial fibrillary acidic protein, Dako, Denmark; 1:1000 dilution), respectively. Sections were incubated overnight with primary antibodies at 4ºC, further developed with biotinylated secondary antibodies and the ABC kit (Vector Lab, Burlingame, CA) using diaminobenzidine and glucose oxidase as substrates.

For the compact Aβ plaque staining, a series of sections was mounted and stained with Congo red. In brief, the sections were treated with working sodium chloride solution (containing sodium chloride saturated in 80% alcohol and 0.01% sodium hydroxide) at room temperature for 20 minutes, then placed directly into working Congo red solution (containing saturated Congo red in working sodium chloride solution) for 1 hour, and dehydrate rapidly in absolute alcohol.

Images were collected at 4× magnification using constant bulb temperature and exposure, with all images acquired in the same session. The area of neocortex and hippocampus was selected for automatic quantification by ImageJ of Aβ, microglia, astrocyte immunostaining and Congo red positive Aβ plaque, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors.

Microhemorrhage staining and quantification were performed following the method described before (Wang et al., 2009b). In brief, a series of five sections were mounted on the slides and stained for hemosiderin using 2% potassium ferrocyanide in 2% hydrochloric acid for 15 min, followed by a counterstain in a 1% Neutral Red solution for 10 min at room temperature. Microhemorrhage events in the form of the number of prussian blue-positive profiles were counted in the brains of each mouse on all sections under microscope, and the average number of hemosiderin deposits was calculated per each brain hemisphere.

All image analyses were processed in a blinded manner.

Quantification of Aβ peptide levels by ELISA

ELISA analysis of the brain Aβ was processed as described previously (Wang et al., 2009c). Briefly, frozen brain was homogenized and sonicated in TBS containing protease inhibitors (Boehringer Mannheim, Germany). Homogenates were centrifuged at 100,000×*g* for 1 h at 4 ºC, and the resultant supernatant was collected, representing the TBS-soluble fraction (Aβ-TBS). The resultant pellet was suspended and sonicated in water containing 2% Sodium dodecyl sulphate (SDS) and protease inhibitors. The SDS solubilized homogenates were centrifuged at 100,000×*g* for 1 h

at 4 ºC, and the resultant supernatant was collected, representing the SDS-soluble fraction (Aβ-SDS). The resultant pellet was then extracted in 70% formic acid, centrifuged, and the resultant supernatant was collected, representing the SDS-insoluble fraction (Aβ-FA). Before ELISA assay, formic acid extracts were neutralized by 1:20 dilution into 1 M Tris phosphate buffer (pH 11) and then diluted in sample buffer. Concentrations of Aβ40 and of Aβ42 in brain extract and plasma were quantitatively measured by ELISA according to manufacturer's instructions (Covance, USA). Using the wet weight of brain tissue in the original homogenate, the final values of brain Aβ were expressed as picomoles per gram wet weight of brain.

APP proteolytic processing and α, β, γ-secretase activities

APP derivatives were analyzed by Western blot analyses as performed according to our procedures described previously (Wang et al., 2009b). Briefly, brain proteins (20 μg) from brain homogenates were boiled in Laemmli sample buffer containing 8% β-mercaptoethanol, loaded onto 4-12% Bis-Tris gradient gels (Invitrogen), and electrophoresed. Separated proteins were transferred from the gels onto pure nitrocellulose (Bio-Rad), and the membranes were boiled in PBS for 5 min. Membranes were then blocked in casein-blocking buffer [0.5% (w/v) in PBS. The blots were probed with the following antibodies: WO2 (mouse monoclonal directed to residue 5-8 of Aβ and which recognizes APPfl, APPα and CTFα) (George et al., 2004), 369 (rabbit polyclonal directed to C-terminal region of APP, and which recognizes APPfl , CTFα and CTFβ) (Ida et al., 1996) and anti-APPβ antibody (Signet). After incubation with the primary antibody and then the secondary HRP-conjugated antibody, the blots were developed using an enhanced chemiluminescence (ECL) kit (Lumigen™ TMA-6, Amersham-Pharmacia Biotech)

as per the manufacturer's instructions. The GeneGnome chemiluminescence imager (Syngene, Cambridge, UK) was used to capture the signals and quantitation was performed using GeneTools analysis software (Syngene). The membranes were stripped and re-probed for β-actin with polyclonal antibody directed to β-actin (Sigma). The band density of the APP derivatives was normalized to the density of β-actin signal.

α, β and γ secretase activities were measured according to the manufacturer's protocols (R&D). In brief, the fresh brain tissues were pulverized in liquid nitrogen, and aliquoted for secretase assays. Secretases were extracted in the extraction buffer provided in the product kits, and their activities were measured by adding a secretase-specific peptide conjugated to the reporter molecules EDANS and DABCYL. Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal, which is proportional to the level of secretase enzymatic activity. The fluorescent signal was read at an excitation wavelength of 355 nm and emission at 510 nm with a 495 nm cutoff.

Western blot analysis

The expression of p75NTR and the molecules involving Aβ production and degradation were analyzed using Western blotting. Total brain homogenates were subjected to SDS-PAGE (8-12% acrylamide). The blots were probed with the following antibodies: rabbit anti-p75NTR polyclonal antibody (G3231, Promega), anti-BACE1 monoclonal antibody (MAB931, R&D Systems), anti-IDE polyclonal antibody (25970, Abcam), anti-NEP polyclonal antibody (NEP11-A, Alpha Diagnostic), anti-LRP monoclonal antibody (5A6, Abcam), anti-RAGE antibody (H-

300, Santa Cruz), and anti-β actin monoclonal antibody (Sigma-Aldrich). The band density of the APP derivatives was normalized to that of β-actin.

Primary cortical neuron culture

Primary neuron culture was carried out according to our methods described previously (Buxbaum et al., 1990). In brief, one month old female APP+/-p75-/- and APP+/-p75-/- mice were sacrificed by Lethabarb. The cortex was sliced into 0.5mm thickness in PBS supplemented with HibemateA/B27, and shaken in a tube containing papain for 30 min in a 30°C water bath. The cell suspension was applied to Optiprep density gradient medium (Sigma-Aldrich) and centrifuged at 800×g. Fractions containing neurons were collected and washed briefly in HibernateA/B27 with 0.5mM glutamine by centrifuging at 200×g for two times. The cell pellets were resuspended in Neurobasal A/B27 with 0.5mM glutamine, 5 ng/ml bFGF, 10,000 u/ml penicillin and1 mg/ml streptomycin. Cell density was counted and adjusted accordingly to 2.5×10^5/ml with culture medium. The cells were plated in triplicate onto poly-D-lysine (100 mg/ml) coated 24 multi-well plates and were maintained in a humidified atmosphere in 37 °C, 5% CO_2. Both culture medium and cell lysate prepared in RIPA buffer were collected after culture for 3 and 5 days for Aβ ELISA assay.

Effects of p75NTR extracellular domain on Aβ oligomerization and fibrillation

Aβ preparation. Synthetic Aβ42 was purchased from American Peptide (Sunnyvale, CA) and prepared following the protocols described elsewhere (Wang et al., 2010). In brief, the Aβ42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma) at 1 mg/ml, and was aliquoted in Eppendorf tubes. The HFIP was allowed to

evaporate in the fume hood, and the resulting clear peptide film was dried under vacuum overnight.

Inhibition of Aβ oligomerization. 2.5 μg Aβ42 was dissolved and incubated at a final concentration of 20 μm with recombinant extracellular domain of p75NTR, which is fused with Fc fragment of human IgG (p75/Fc), at various concentrations (0.2, 2 and 10 μm) in DMEM at 37°C for 24 hours according to the protocol describe elsewhere (Dahlgren et al., 2002). The same amount of Aβ42 without incubation or incubated with human IgG at a molar ratio of 1:0.5 were used as controls. Samples were run on Tris-Tricine gel and Aβ was visualized by Western blot analysis with biotin-conjugated 6E10 antibody (Signet).

Inhibition of Aβ fibrillation and disaggregation of preformed Aβ fibril. The effects of p75NTR extracellular domain on Aβ fibrillation and Aβ fibril disaggregation were examined with our methods described previously (Wang et al., 2008). For the inhibition of Aβ fibrillation, 25 μm Aβ42 (30 μg) was incubated with 12.5 μm p75/Fc or HuIgG at 37 ºC for 4 days. For disaggregation of pre-formed Aβ fibril, 30 μg Aβ42 was dissolved in DMEM at a concentration of 25 μm and incubated at 37 ºC for 4 days to generate fibrils. Pre-formed fibrils were then incubated with 12.5 μm p75/Fc or HuIgG for an additional 3 days at 37 ºC. For both incubations, Aβ42 and HuIgG were incubated alone at the same conditions, along with the experiment as controls. At the end of incubations, the samples were measured by adding the reaction solution to 3 ml of 5 μM Thioflavine T (ThT) solution (50 mM phosphate buffer, pH 6.0, Sigma, USA). Fluorescence emission of ThT is shifted when it binds to β-sheet aggregate structures of amyloid fibrils (Wang et al., 2009b). Fluorescence intensity was monitored at an excitation wavelength of 450 nm and an emission

wavelength of 482 nm by a Luminescence spectrometer PE-LS50B (Perkin-Elmer, USA) with both excitation and emission bandwidths of 5 nm. Readings were the results of the average of three values determined by a time scan after subtracting out the fluorescence contribution from free ThT. Each experiment was performed in triplicate.

Hippocampus injection of p75/Fc and Aβ plaque analysis

Nine months old APPSwe/PS1dE9 mice, which contained numerous amyloid deposits in the brain, were anesthetized with halothane, and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). A mid-sagittal incision was made to expose the cranium, and a burr hole was drilled with a dental drill over the left hippocampi to the following coordinates: anteroposterior, -2.3 mm; lateral, 1.8 mm, ventral, 2.0 mm, all taken from bregma. 3 μg in 3 μl p75/Fc was injected into the left hippocampus of mice (n=4). 6 μg in 3 μl of HuIgG (the equivalent molar to p75/Fc) was injected in the same approach as a control. One week after injection, brains were fixed and sectioned. Five sections around the injection site were selected per animal. Aβ plaques were stained using biotin-conjugated 6E10 antibody. The area fraction of Aβ plaque in hippocampus was quantified with ImageJ under the same settings in a blinded manner. The area fraction of Aβ plaque in hippocampus of the injection side was normalized with the control side.

Statistical analysis

The data in the text and figures are expressed as mean±S.E.M, unless otherwise stated. Inter-group comparisons were assayed using *t* test, one-way ANOVA and *post hoc* for testing the significance of values. *p* values less than 0.05 were considered as

Figure 4.1

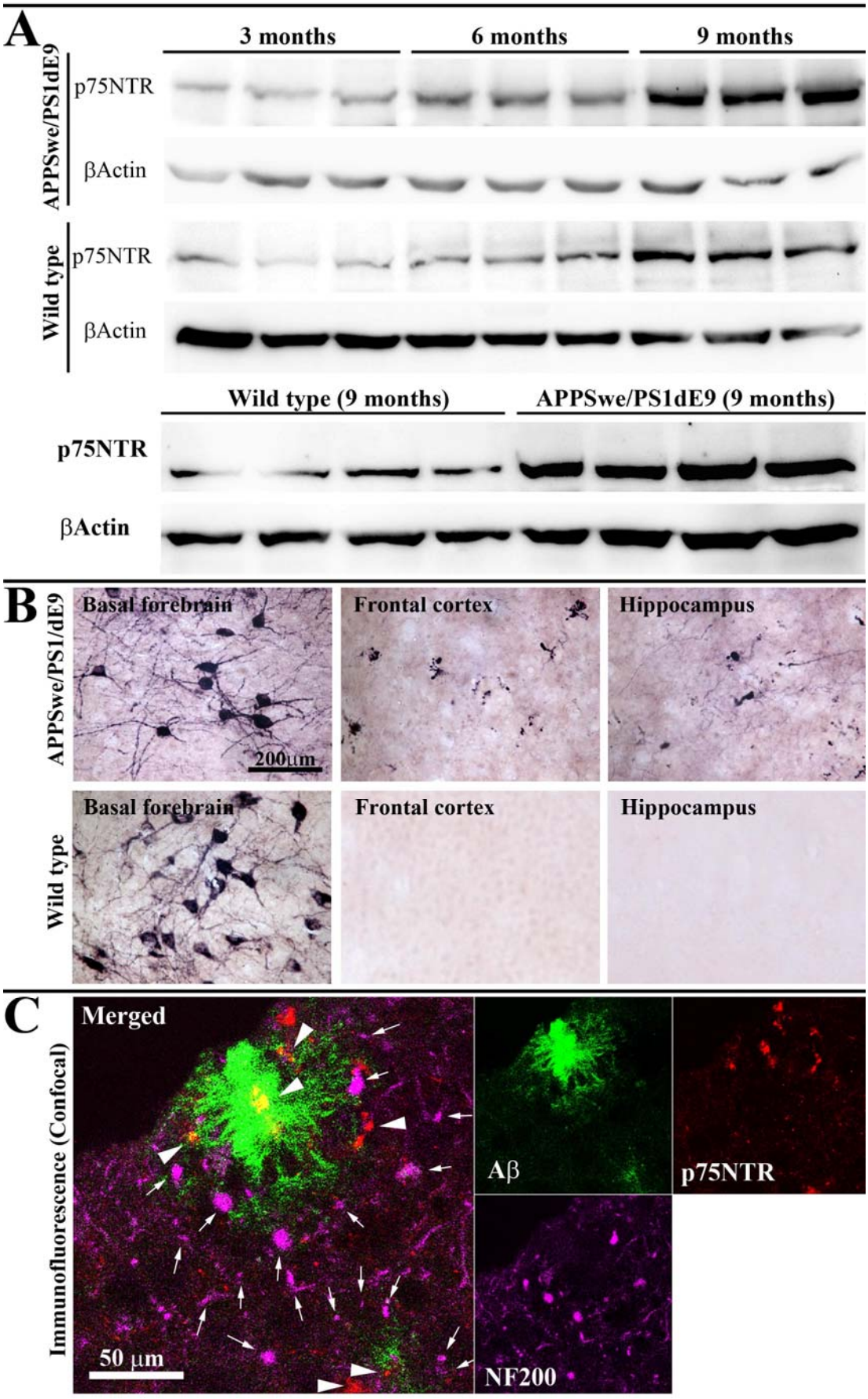
A
3 months
6 months
9 months
APPSwe/PS1dE9
p75NTR
βActin
Wild type
p75NTR
βActin
Wild type (9 months)
APPSwe/PS1dE9 (9 months)
p75NTR
βActin
B
APPSwe/PS1/dE9
Basal forebrain
Frontal cortex
Hippocampus
200μm
Wild type
Basal forebrain
Frontal cortex
Hippocampus
C
Immunofluorescence (Confocal)
Merged
Aβ
p75NTR
50 μm
NF200

Figure 4.1 Expression of p75NTR in the brain. P75NTR expression in the brain was measured by Western blot and immunohistochemistry. **A.** Brain homogenates of APPSwe/PS1dE9 mice and their wild type littermates at three, six and nine months of age were subjected to Western blot analysis probed with rabbit anti-p75NTR polyclonal antibody (Ab 9650) and monoclonal antibody to β-actin. **B.** Sections of basal forebrain, frontal lobe and hippocampus were stained using free-floating immunohistochemistry for p75NTR with rabbit anti-p75NTR polyclonal antibody. C. Representative confocal images for colocalization of p75NTR positive fibers and fibrillar plaques, with Ab 9650 for p75NTR, N52 for neurofilament 200 (NF200) and Thioflavine S for fibrillar plaque.

statistically different. All the analyses were performed using SPSS for Windows version 13.0 (SPSS Inc.)

Results

Expression pattern of p75NTR in the brain of APPSwe/PS1dE9 transgenic mice

To understand the roles of p75NTR in the development of AD, we examined the expression of p75NTR in the brain. Firstly, we measured p75NTR expression levels in brain homogenates at various ages, using western blotting. As shown in Figure 4.1A, p75NTR expression level increased with age in both the transgenic mice ($F=14.762$, $p<0.001$) and their wild type littermates ($F=8.057$, $p=0.002$). p75NTR level at 9 months of age was significantly higher in the AD transgenic mice than in the wild type mice ($t=4.184$, $p=0.001$). We next examined the sites of expression of p75NTR in the brain by immunohistochemistry and found that p75NTR was expressed in the cholinergic neurons of basal forebrain of both APPSwe/PS1dE9 transgenic mice and their littermates (Figure 4.1B). There was no obvious difference in the expression of p75NTR in the basal forebrain cholinergic neurons over time in either transgenic or wild type mice. However, in the brain of APPSwe/PS1dE9 mice, we observed p75NTR positive fibers in the neocortex and hippocampus at three months of age when Aβ begins to deposit (data not shown), and observed the p75NTR positive fibers and degenerative neurites at six months of age in the neocortex and hippocampus of APPSwe/PS1dE9 transgenic mice. The number of degenerative neurites increased at nine months of age. We also found that amyloid plaques in the neocortex of APPSwe/PS1dE9 transgenic mice were almost always associated with p75NTR positive degenerative neurites located in their cores (Figure 4.1C). However, we did not observe the p75NTR positive fibers and degenerative

Figure 4.2

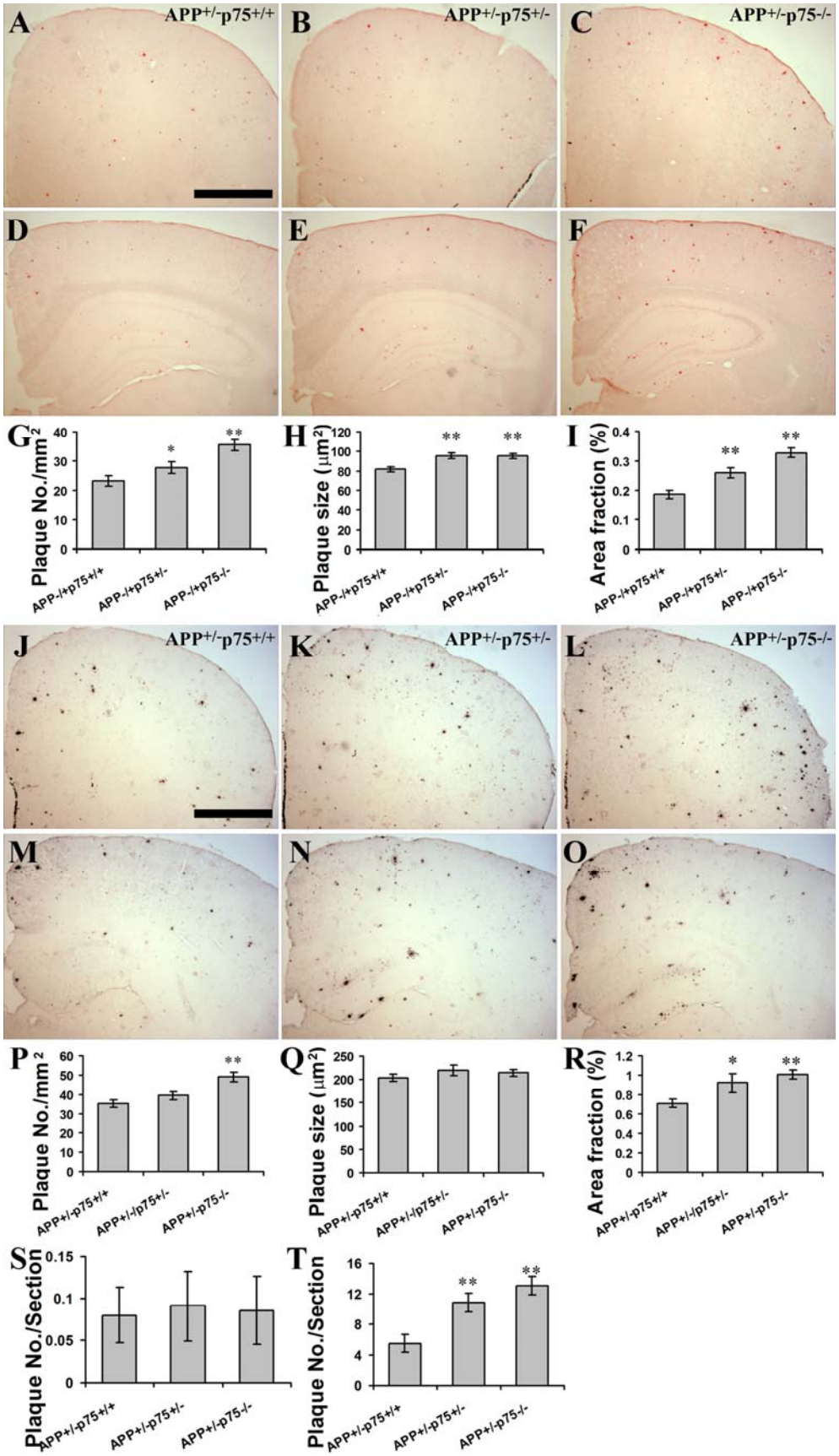
A
APP+/-p75+/+
B
APP+/-p75+/-
C
APP+/-p75-/-
D
E
F
G
Plaque No./mm2
H
Plaque size (μm2)
I
Area fraction (%)
APP-/+p75+/+
APP-/+p75+/-
APP-/+p75-/-
J
APP+/-p75+/+
K
APP+/-p75+/-
L
APP+/-p75-/-
M
N
O
P
Plaque No./mm2
Q
Plaque size (μm2)
R
Area fraction (%)
APP+/-p75+/+
APP+/-p75+/-
APP+/-p75-/-
S
Plaque No./Section
T
Plaque No./Section

Figure 4.2 Aβ plaque burden in the brain of mice with different genotypes. A series of five equally spaced tissue sections (~1.3 mm apart) spanning the whole brain were stained using free-floating immunohistochemistry for total Aβ plaque, or using Congo red staining for compact Aβ plaque. The area of neocortex and hippocampus was selected for automatic quantification of Aβ plaque staining with ImageJ, yielding the area fraction of the total positive staining against the area of tissue analysed. The average of the individual measurements was used to calculate group means and standard errors. **A-C.** Congo red positive Aβ plaques in frontal lobe of nine months old APP+/-p75+/+, APP+/-p75+/- and APP+/-p75-/-. **D-F.** Congo red positive Aβ plaques in hippocampus of nine months old APP+/-p75+/+, APP+/-p75+/- and APP+/-p75-/-. **G-H.** Comparison of Congo red positive Aβ plaque density (**G**), average size (**H**) and area fraction (**I**) in neocortex and hippocampus of nine months old animals. **J-L.** IHC positive Aβ plaques in frontal lobe of nine months old APP+/-p75+/+, APP+/-p75+/- and APP+/-p75-/-. **M-Q.** IHC positive Aβ plaques in hippocampus of nine months old APP+/-p75+/+, APP+/-p75+/- and APP+/-p75-/-. **P-R.** Comparison of IHC positive Aβ plaque density (**P**), average size (**Q**) and area fraction (**R**) in neocortex and hippocampus of nine months old animals. **S-T**. Comparison of Congo red positive Aβ plaque density in the brains of three (**S**) and six (**T**) months old animals. * and ** denote $p<0.05$ or $p<0.01$ versus APP+/-p75+/+. Scale bar=1mm.

neurites at all time points in the brain of wild type mice. We did not see any obvious p75NTR expression in the thalamus at all time points (data not shown). These data indicate that the expression of p75NTR increases with the age and is related to Aβ load in the brain.

Deletion of p75NTR exacerbates Aβ-related pathologies

To investigate if p75NTR plays a role in Aβ deposition, APP+/-p75-/- mice were generated by crossing APPSwePS1dE9 mice with mice lacking the p75NTR gene. Firstly we evaluated Aβ deposition in the brains by staining the Aβ plaques with Congo red, a histological dye which binds to fibril proteins enriched in β-sheet conformation at high affinity, and is commonly used to detect the fibrillar Aβ plaques (LeVine, 1993). Interestingly, we found a clear trend of a p75NTR gene dosage effect on Aβ deposition in the brain. At the age of nine months, mice with p75NTR gene deleted (APP+/-p75-/-) had significantly higher plaque density (number of plaques per mm^2 of area, 35.6±1.99 vs. 23.1±1.74, $p<0.001$), average plaque size (95.7±2.7 vs. 81.8±2.6 μm^2, $p<0.001$) and area fraction (percentage of area occupied by Aβ plaques, 0.33±0.02% vs. 0.19±0.01%, $p<0.001$) in their brains than mice with two copies of p75NTR gene (APP+/-p75+/+), while mice with one copy of p75NTR gene (APP+/-/p75+/-) showed plaque density (27.8±2.1) and area fraction (0.26±0.02%) in the middle between APP+/-p75+/+ and APP+/-p75-/- mice (Figure 4.2A-I).

As Congo red stains the fibrillar plaques only, we further stained the brain with anti-Aβ antibody 6E10 to illustrate the diffuse and fibrillar Aβ deposition. While we found that the 6E10-stained pan Aβ plaques had higher density, bigger size and area fraction than Congo red stained fibrillar Aβ plaques, comparative results of 6E10-

stained Aβ plaque among mice with various copies of p75NTR gene were similar to those of Congo red stained fibrillar plaque, with APP+/-p75-/- mice having higher plaque density (49.1±2.5 vs. 35.3±2.0, $p<0.001$) and area fraction (1.01±0.05% vs. 0.71±0.04%, $p=0.004$) than APP+/-/p75+/+ mice (Figure 4.2J-R).

To examine the time course effect of p75NTR deletion on Aβ deposition, we next stained the Aβ plaque in the brains at the age of three and six months. We counted the plaque numbers in the brain under the microscope in a blind manner, and found that the young animals had a few plaques in their brains. APP+/-p75-/- mice had more plaques in their brains than APP+/-p75-/+ (10.1±1.2 vs. 13.0±1.2, $p=0.185$) and APP+/-p75+/+ (5.5±1.2 vs.13.0±1.2, $p<0.001$) at six months of age (Figure 4.2T), when Aβ accumulation in the brain begins to accelerate, but not at three months of age (Figure 4.2S), when Aβ plaque just begins to form (Frid et al., 2007).

These results clearly indicate that deletion of p75NTR exacerbates the Aβ plaque formation in a gene dosage dependent manner.

Deletion of p75NTR increases insoluble Aβ but decreases soluble Aβ levels in the brain

To further understand the effect of p75NTR deletion on Aβ deposition, we measured the levels of the different aggregation states of Aβ in the brain by ELISA. Aβ was sequentially extracted in TBS, 2% SDS and 70% formic acid. In general, Aβ in the TBS fraction represents the soluble form, Aβ in the SDS fraction represents the diffuse Aβ plaques, whereas Aβ in the formic acid fraction represents the fibrillar Aβ plaques. Aβ levels were determined by both, Aβ40- and Aβ42-specific sandwich ELISA. Total Aβ level was generated from the sum of Aβ40 and Aβ42 of the

Figure 4.3

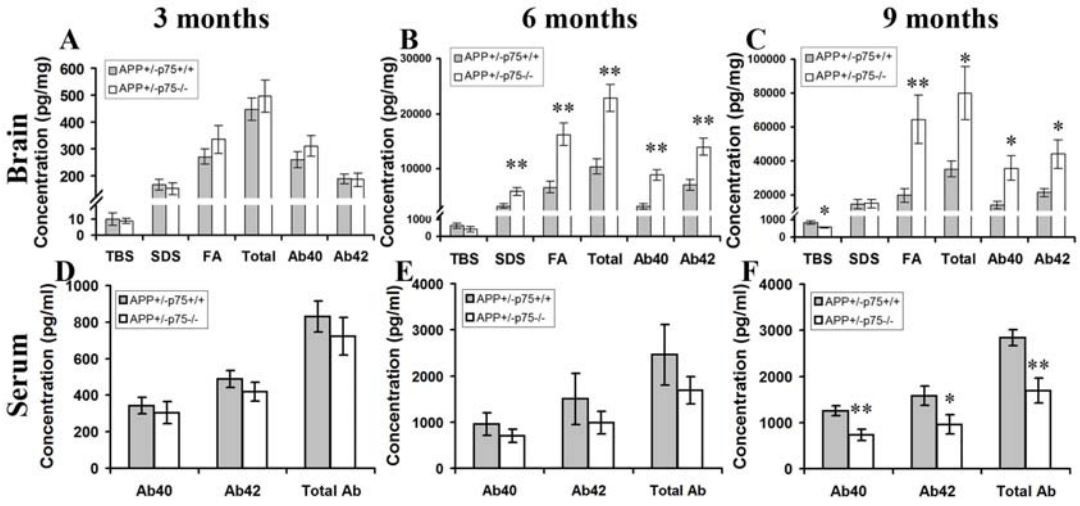

Figure 4.3 Aβ levels in the brain and serum. Aβ in the brain was extracted sequentially in TBS, 2% Sodium dodecyl sulphate (SDS) and 70% formic acid (FA) water solution. Aβ peptide concentrations in the brain and serum of animals were measured by ELISA. **A-C.** Comparison of Aβ in TBS, SDS and FA, total Aβ, Aβ40 and Aβ42 among groups at three, six and nine months of age. **D-F.** Comparison of total Aβ, Aβ40 and Aβ42 in serum at three, six and nine months of age. * and ** denote $p<0.05$ or $p<0.01$ versus APP+/-p75+/+ mice.

different fractions. Consistent with the Aβ pathology results, the APP+/-p75-/- mice had more total Aβ, Aβ40 and Aβ42 in their brain than APP+/-p75+/+ mice at three months (total Aβ: 496±60 vs. 448±41 pg/mg, p=0.502; Aβ40: 311±39 vs. 259±30 pg/mg, p=0.306; Aβ42: 185±24 vs. 188±18 pg/mg, p=0.914), six months (total Aβ: 22832±2413 vs. 10417±1394 pg/mg, p<0.001; Aβ40: 8860±994 vs. 3315±493 pg/mg, p<0.001; Aβ42: 13972±1526 vs. 7101±944 pg/mg, p=0.001) and nine months of age (total Aβ: 79884±15605 vs. 35389±4734 pg/mg, p=0.014; Aβ40: 35758±7357 vs. 14011±2221 pg/mg, p=0.011; Aβ42: 44126±8354 vs. 21378±2616 pg/mg, p=0.018) (Figure 4.3A-C). Further analysis of distinct Aβ pools showed a difference between APP+/-p75-/- mice and APP+/-p75+/+ mice. Compared with APP+/-p75+/+ mice at all time points, APP+/-p75-/- mice had less TBS soluble Aβ (APP+/-p75-/- vs APP+/-p75+/+, three months: 8.81±1.54 vs. 9.86±3.83 pg/mg, p=0.823; six months: 426±157 vs. 634±150 pg/mg, p=0.354; nine months: 568±42 vs. 857±111 pg/mg, p=0.025), comparable SDS soluble Aβ (three months:153±22 vs. 167±19 pg/mg, p=0.635; six months: 5934±733 vs. 3310±374 pg/mg, p=0.005; nine months: 14907±2250 vs. 14777±2672 pg/mg, p=0.971) and more SDS insoluble Aβ (formic acid fraction, three months: 335±52 vs. 271±27, p=0.262; six months: 16264±2012 vs. 6681±1084 pg/mg, p<0.001; nine months: 79885±15606 vs. 35390±4735 pg/mg, p=0.007) (Figure 4.3A-C).

We further measured the serum Aβ. Consistent with the brain TBS soluble Aβ levels, APP+/-p75-/- mice had lower serum levels of total Aβ (three months: 722±102 vs. 831±85 pg/ml, p=0.423; six months: 1691±293 vs. 2458±659 pg/ml, p=0.252; nine months: 1693±271 vs. 2839±171 pg/ml, p=0.002), Aβ40 (three months: 304±60 vs. 343±45 pg/ml, p=0.604; six months: 704±144 vs. 955±212 pg/ml, p=0.356; nine months: 732±120 vs. 1257±104 pg/ml, p=0.004) and Aβ42 (three months: 419±52

Figure 4.4

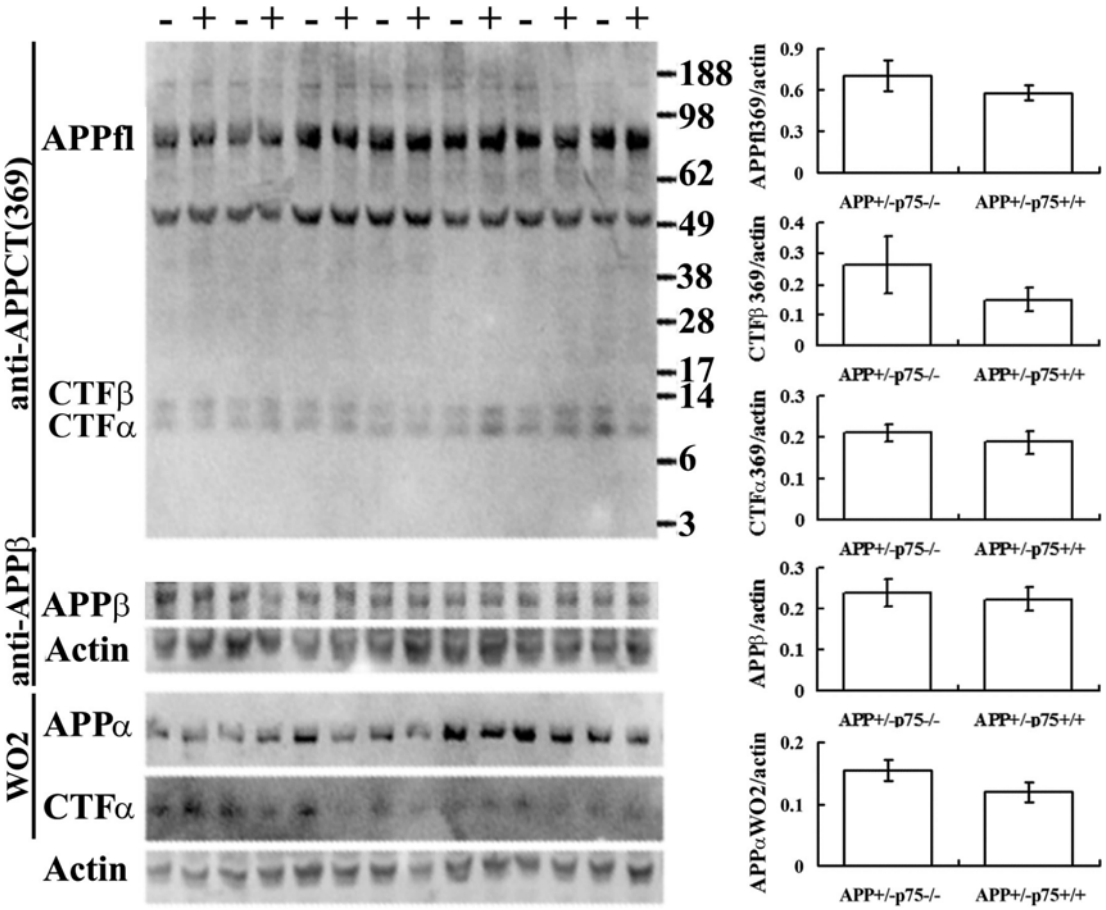

Figure 4.4 APP expression and proteolytic processing. Western blot analyses were performed to detect the APP expression and APP proteolytic derivates in the brain homogenates of nine months old animals using antibodies directed to APPfl, APPα, APPβ, CTFα and CTFβ. "-" donates APP+/-p75-/- mice, "+" donates APP+/-/p75+/+ mice.

vs. 488±47 pg/ml, p=0.340; six months: 987±241 vs. 1504±553 pg/ml, p=0.353; nine months: 962±209 vs. 1583±206 pg/ml, p=0.045) than APP+/-p75+/+ mice at three, six and nine months of age.

The above data indicate that deletion of p75NTR increases the total Aβ levels in the brain, with increased fibrillar Aβ levels but decreased soluble Aβ levels. Alternative possibilities may underlie these changes: p75NTR deletion would either influence Aβ production or/and Aβ deposition.

Deletion of p75NTR does not affect APP proteolytic processing

Aβ is generated from sequential APP cleavages by β and γ secretases on the cell membrane. APP is mainly cleaved by three proteolytic enzymes: α, β and γ secretases. Sequential cleavage by β and γ secretases generates Aβ (amyloidogenic processing of APP), while sequential cleavage by α and γ secretases precludes generation of Aβ (non-amyloidogenic processing of APP) (Ωανγ ετ αλ., 2006δ). To investigate whether exacerbated Aβ pathology by p75NTR deletion resulted from increased amyloidogenic processing of APP, APP proteolytic products present in the brain homogenates were analysed by western blotting using three selective antibodies. As shown in Figure 4.4, the expression of full length APP (APPfl) and its derivates APPα, APPβ, CTFα and CTFβ. did not significantly differ between the APP+/-p75+/+ and APP+/-p75-/- mice.

We next measured the activities of the α, β, γ-secretase, which are key enzymes of the APP proteolytic processing, by adding secretase-specific peptides conjugated to the reporter molecules. Consistently, the activities of these enzymes are similar between APP+/-p75-/- and APP+/-p75+/+ mice at three, six and nine months of age

Figure 4.5

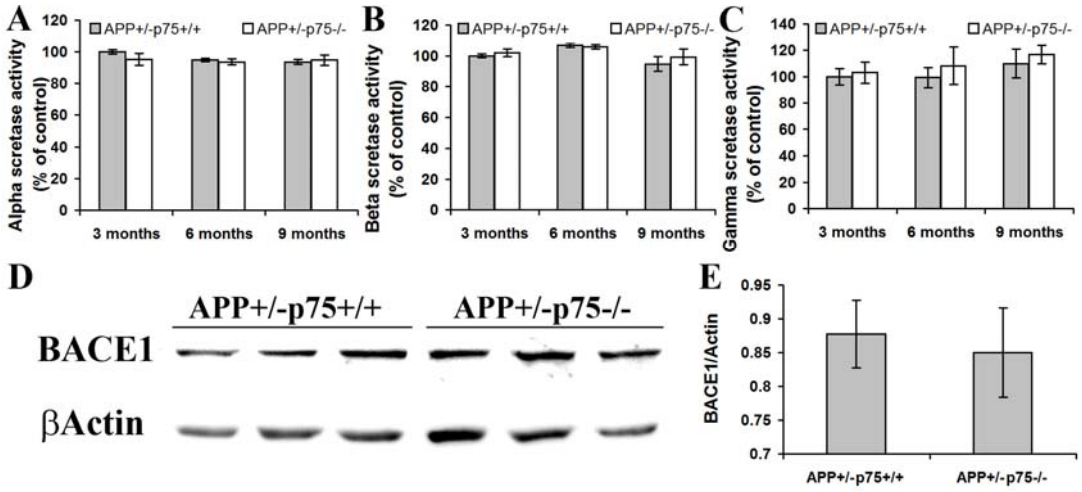

Figure 4.5 Secretase activities and BACE1 expression. A-C. α, β and γ secretase activities in the brain of nine months old APP+/-p75+/+ and APP+/-p75-/- mice, which were measured with secretase-specific peptides conjugated to the reporter molecules EDANS and DABCYL. Cleavage of the peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal, which is proportional to the level of secretase enzymatic activity. **D.** The protein levels of BACE1 in the brain of APP+/-p75+/+ and APP+/-p75-/- mice were measured by Western blot analysis probed with anti-BACE1 monoclonal antibody.

Figure 4.6

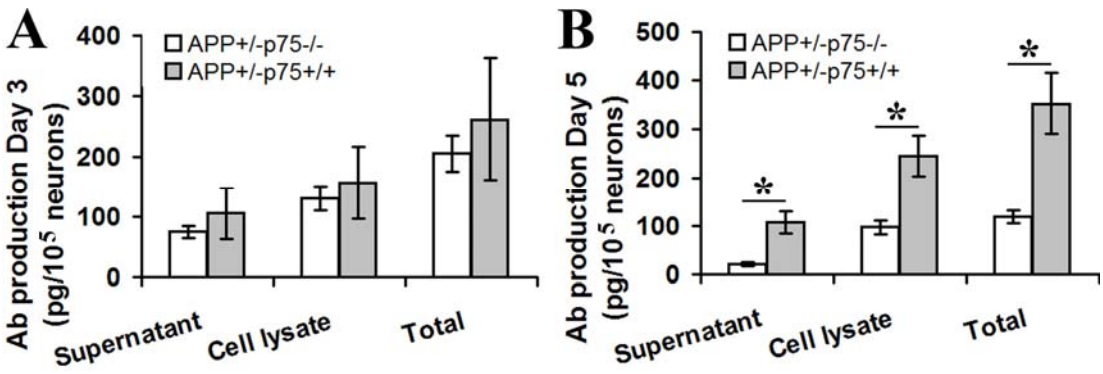

Figure 4.6 Total Aβ production of cortical neurons *in vitro*. The cortex of one month old female APP+/-p75-/- and APP+/-p75-/- mice was isolated and cultured at 2.5 × 10^5/ml in Neurobasal A/B27 with 0.5mM glutamine, 5 ng/ml bFGF, 10,000 u/ml penicillin and1 mg/ml streptomycin in triplicate. Both culture medium and cell lysate prepared in RIPA buffer were collected after culture for three and five days and measured for Aβ with sandwich ELISA. * donates $p<0.05$.

(Figure 4.5A-C). We further examined the expression of BACE1, the limiting enzyme of Aβ production, with Western blot and consistently the BACE1 expression in APP+/-p75-/- mice did not differ from that in APP+/-p75+/+ at nine months of age (Figure 4.5D). These data suggest that p75NTR deletion does not affect the cleavage of APP.

Deletion of p75NTR decreases Aβ production *in vitro*

We further investigated whether p75NTR deletion poses significant impact on the Aβ production by determining the Aβ production of cortical neurons in vitro. Cortical neurons were isolated from brains of one month old APP+/-p75-/- and APP+/-p75+/+ mice, and cultured for three or five days. The supernatant and cell lysate of cultured neurons were collected and measured for Aβ concentration by a sandwich ELISA. As shown in Figure 4.6, neurons from APP+/-p75-/- mice produced less total Aβ in both supernatant and cell lysate fractions relative to neurons from APP+/-p75+/+ mice at 3 days and 5 days, with significant difference at 5 days. These data are consistent with the levels of brain and serum soluble Aβ which were lower in APP+/-p75-/- mice than in APP+/-p75+/+ mice. Taken together, these data suggest that deletion of p75NTR gene decreases Aβ production, however this is not through the changes in the APP expression and proteolytic processing.

Deletion of p75NTR does not affect Aβ degrading enzymes in the brain

We determined the expression of neprilysin (NEP) and insulin degrading enzyme (IDE), the two key Aβ degrading enzymes, in the brain homogenate of nine months old mice by western blot. As shown in Figure 4.7, there is no significant difference in brain protein levels of NEP and IDE between APP+/-p75-/- and APP+/-p75+/+ mice.

Figure 4.7

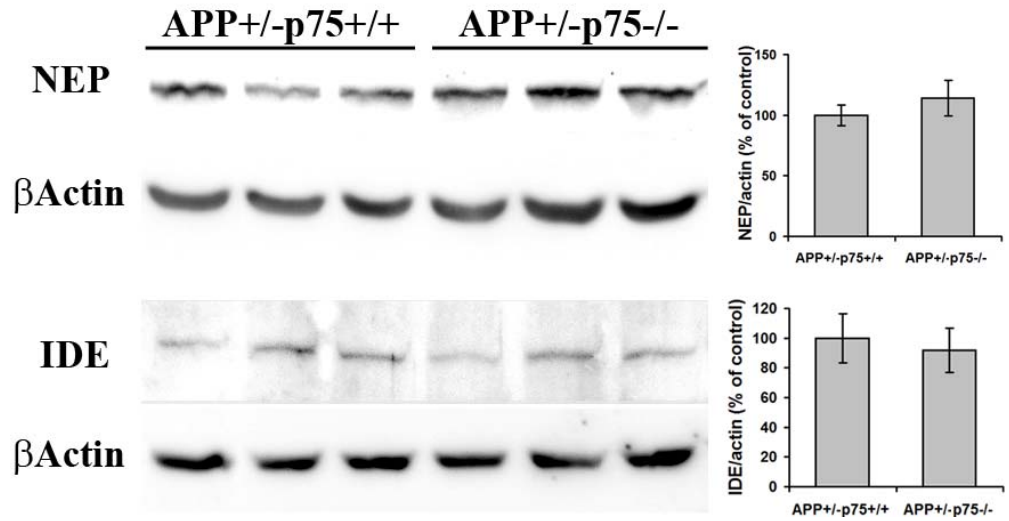

Figure 4.7 Levels of NEP and IDE in the brain. The protein levels of NEP and IDE in the brain homogenates of nine months old APP+/-p75+/+ and APP+/-p75-/- mice were measured by Western blot analysis with antibodies directed to NEP and IDE.

These results suggest that deletion of p75NTR has no significant influence on Aβ degradation.

Recombinant extracellular domain of p75NTR inhibits the formation of Aβ fibrils

As p75NTR binds Aβ (Thinakaran and Koo, 2008) and can be extracellularly cleaved by metalloproteases to generate soluble p75NTR (Yaar et al., 1997; Yaar et al., 2002), we hypothesized that the p75NTR extracellular domain may exert a protective role against Aβ deposition and aggregation. Firstly we examined if p75NTR could interfere with the aggregation of Aβ. Aβ monomers were incubated with recombinant extracellular domain of p75NTR (the domain of p75NTR which directly interacts with Aβ) fused with Fc fragment of human IgG (p75/Fc), at various concentrations, with human IgG as a control. Interestingly, we found that p75/Fc, but not human IgG, inhibited Aβ oligomerization in a dose-dependent manner (Figure 4.8A).

We next determined whether p75/Fc interferes with Aβ fibrillation, Aβ42 was incubated alone, or with p75/Fc or human IgG (HuIgG), and the formed fibrils were measured by adding Thioflavine T, a dye with high affinity for β-sheet conformation. The fluorescence intensity of Aβ incubated with p75/Fc was lower than that of Aβ incubated alone ($p<0.001$) or with HuIgG ($p<0.001$) (Figure 4.8B). The fluorescence intensity did not differ between Aβ incubated alone and Aβ incubated with HuIgG ($p=0.329$), indicating that p75/Fc inhibited Aβ fibrillation.

To determine whether p75/Fc promotes the disaggregation of pre-formed Aβ fibrils, the pre-formed Aβ fibrils were incubated with p75/Fc. As shown in Figure 8C, the

Figure 4.8

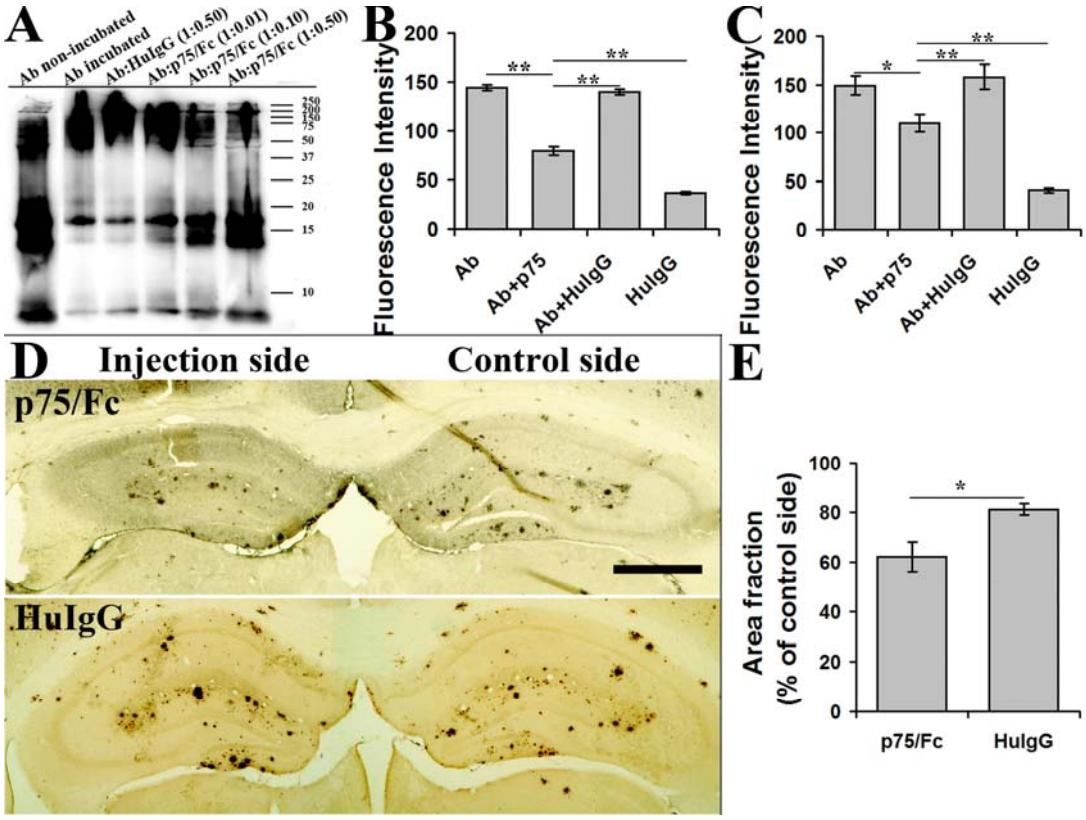
A
Ab non-incubated
Ab incubated
Ab:HuIgG (1:0.50)
Ab:p75/Fc (1:0.01)
Ab:p75/Fc (1:0.10)
Ab:p75/Fc (1:0.50)
250
150
100
75
50
37
25
20
15
10
B
Fluorescence Intensity
200
150
100
50
0
**
**
**
Ab
Ab+p75
Ab+HuIgG
HuIgG
C
Fluorescence Intensity
200
150
100
50
0
*
**
**
Ab
Ab+p75
Ab+HuIgG
HuIgG
D
Injection side
Control side
p75/Fc
HuIgG
E
Area fraction
(% of control side)
100
80
60
40
20
0
*
p75/Fc
HuIgG

Figure 4.8 Extracellular domain of p75NTR attenuates Aβ aggregation. A. Dose-dependent inhibition by p75/Fc of synthetic Aβ42 peptide aggregation into soluble oligomeric forms in vitro. Aβ42 (final concentration 20 μm) was incubated with p75/Fc at various molar ratio (1:0.01, 1:0.1, 1:0.5) or human IgG (molar ratio, 1:0.5). "Aβ non-incubated" is the control that Aβ42 was not incubated at 37°C for 24 h. "Aβ incubated" is the control that Aβ42 peptide (final concentration 20 μm) was incubated alone at 37°C for 24 h. Bands were visualized by Western blot analysis probed with biotin-conjugated 6E10 antibody. **B.** Inhibition of Aβ aggregation by p75/Fc. 25 μm Aβ42 peptide (30 μg) was incubated with 12.5 μm p75/Fc or HuIgG at 37 ºC for 4 days. The same amount of Aβ42 or human IgG was incubated alone at the same condition as controls. The Aβ fibrils were measured by ThT assay. **C.** Disaggregation of pre-formed Aβ fibrils by p75/Fc. 25 μm Aβ42 (30 μg) was incubated at a concentration of at 37 ºC for 4 days to generate fibrils. Pre-formed fibrils were then incubated with the 12.5 μm p75/Fc or HuIgG for an additional 3 days at 37 ºC. Aβ42 and HuIgG were incubated alone at the same conditions, along with the experiment as controls. The Aβ fibrils were measured by ThT assay. **D**. Representative images of hippocampus Aβ plaque staining 7 days after injection of p75/Fc or HuIgG into the hippocampus of nine months old APPSwe/PS1 mice. * and ** donate $p<0.05$ or $p<0.01$.

fluorescence intensity of pre-formed Aβ fibrils incubated with p75/Fc was lower than that of pre-formed Aβ fibrils incubated alone ($p=0.023$) or with HuIgG ($p=0.008$). No difference in fluorescence intensity was observed between pre-formed Aβ fibrils incubated alone or with HuIgG ($p=0.546$). These data indicate that p75/Fc has the ability to promote the disaggregation of pre-formed Aβ fibrils, and also imply the potential of the p75/Fc in Aβ clearance.

We further investigated the effect of p75/Fc on Aβ plaques *in vivo*. p75/Fc was injected stereologically into the hippocampus of nine month old APPSwe/PS1dE9. One week later we analyzed Aβ plaques in the injected site. Interestingly we found that the local plaque number was reduced at the p75/Fc injection site in comparison with the control side (Figure 4.8.D and E). This effect may not be due to the local injury which could remove the local plaque by an inflammatory mechanism (Sotthibundhu et al., 2008), as the HuIgG injection did not induce a significant reduction in plaque number. These findings further confirm the effect of the p75NTR extracellular domain on Aβ aggregation in vivo, and importantly, suggest the physiological roles of the p75NTR shedding event in the development of AD.

Deletion of p75NTR exacerbates other AD type pathologies

We also examined microgliosis in the mouse brains. Obvious microgliosis was observed in the brains of APP+/-p75-/- and APP+/-p75+/+ mice, but not APP-/-p75+/+ and APP-/-p75-/- mice (Figure 9A-D). Consistent with Aβ plaque burden in the brains, APP+/-p75-/- mice had a higher overall area fraction of microgliosis (2.49 ± 0.46 vs. 0.92 ± 0.28, $p<0.001$), as well as in both neocortex (2.23 ± 0.36 vs. 0.85 ± 0.24, $p<0.001$) and hippocampus (2.43 ± 0.49 vs. 0.92 ± 0.20, $p<0.001$) than APP+/-/p75+/+ mice (Figure 9E-G).

Figure 4.9

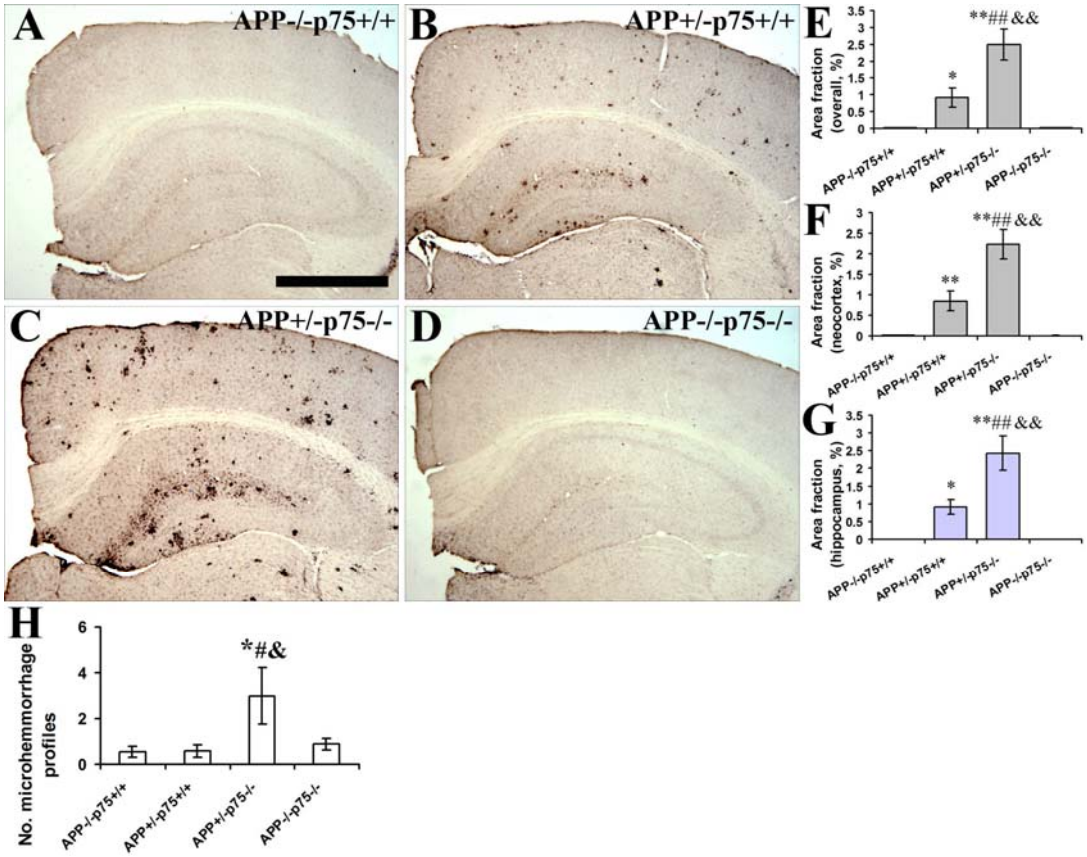
A
APP-/-p75+/+
B
APP+/-p75+/+
C
APP+/-p75-/-
D
APP-/-p75-/-
E
Area fraction (overall, %)
*
**##&&
F
Area fraction (neocortex, %)
**
**##&&
G
Area fraction (hippocampus, %)
*
**##&&
APP-/-p75+/+
APP+/-p75+/+
APP+/-p75-/-
APP-/-p75-/-
H
No. microhemmorrhage profiles
*#&

Figure 4.9 Microgliosis and microhemorrhage in the brain of animals with different genotypes. A series of five equally spaced tissue sections spanning the brain were stained using free-floating immunohistochemistry for activated microglia. The area of neocortex and hippocampus was selected for automatic quantification of activated microglia immunostaining with ImageJ, yielding the area fraction of the total positive staining against the area of tissue analysed. No obvious microgliosis was observed in the brain of APP-/-p75+/+ (**A**) and APP-/-p75-/- (**D**) animals. **B-C.** Microgliosis in hippocampus and neocortex of APP+/-p75+/+ and APP+/-p75-/- mice. **E.** Comparison of CD45 area fraction in neocortex and hippocampus among groups. **F.** Comparison of CD45 area fraction in neocortex. **G.** Comparison of CD45 area fraction in hippocampus. * and ** denote $p<0.05$ or $p<0.01$ versus APP-/-p75+/+ mice, $^{\#}$ and $^{\#\#}$ denote $p<0.05$ or $p<0.01$ versus APP+/-p75+/+ mice, $^{\&}$ and $^{\&\&}$ denote $p<0.05$ or $p<0.01$ versus and APP-/-p75-/- mice, as determined by one-way ANOVA. Scale bar=1mm.

We examined the microhemorrhage in the brain. There were low levels of microhemorrhage profiles in APP-/-p75+/+, APP+/-p75+/+ and APP-/-p75-/- mice, and a significant higher levels of microhemorrhage profiles in APP+/-p75-/- mice (Figure 9 H).

Deletion of p75NTR does not lessen memory deficits till nine months of age

To investigate the influence of p75NTR deletion on cognitive function, mice with different genotypes (APP+/-p75-/-, APP+/-p75+/+, APP-/-p75+/+ and APP-/-p75-/-) were tested in the Morris Water Maze at three, six and nine months of age. We did not observe any significant difference in performance of either platform trials or probe trials between the different genotypes (data not shown), suggesting that deletion of p75NTR has no significant impact on the cognition in the animal till nine months of age.

Discussion

Although genetic studies clearly demonstrate that the aberrant production of Aβ plays a central role in the development of early onset familial AD, the etiology of sporadic late onset AD (LOAD), which accounts for 95% of total AD patients, remains debated (Nakagawa et al., 2000). Multiple genes and signaling pathways involved in the production and elimination of Aβ and phosphorylation of tau are implicated in the development of LOAD (Small, 2008; Small and Duff, 2008). This study focuses on the role of p75NTR in the production of Aβ and amyloid plaque formation using a genetic approach in APPSwe/PS1dE9 transgenic AD mice. We found that p75NTR may play differential roles in Aβ metabolism. The deletion of

p75NTR resulted in an increased deposition of *insoluble* Aβ in the brain but decreased *soluble* Aβ in the brain and blood, and decreased Aβ production by cortical neurons *in vitro*. Consequently, the deletion of p75NTR exacerbates AD pathologies such as increased amyloid plaques and microgliosis. We further found that the extracellular domain of p75NTR suppresses Aβ aggregation *in vitro* and *in vivo*. Our studies provide further evidence that p75NTR may play a critical role in the development of AD.

It is well known that cholinergic neurons in the basal forebrain express p75NTR, TrkA, TrkB and TrkC (Small, 2008; Small and Duff, 2008). Strikingly, neurons in other brain regions normally express little or no p75NTR. However, the reports on the levels of p75NTR expression in AD patients and during aging remain controversial, being either increased (Salehi et al., 2000), reduced (Mufson and Kordower, 1992; Hu et al., 2002) or unchanged (Arendt et al., 1997; Salehi et al., 2000). In the present study, we showed that p75NTR expression increases with age in both wild type and APPSwe/PS1dE9 mice, and APPSwe/PS1dE9 mice had a higher p75NTR expression level relative to their wild type littermates. Consistent with previous findings, we found that p75NTR is mainly localized in basal forebrain neurons. In APPSwe/PS1dE9 mice we found that some degenerative fibers and neurites also express p75NTR, suggesting that p75NTR may play a role in neurite degeneration. Indeed a recent study has demonstrated that p75NTR mediates Aβ-induced neurite atrophy via the c-Jun pathways (Goedert et al., 1989; Treanor et al., 1991; Ginsberg et al., 2006). Accumulating evidence indicate that p75NTR also plays a critical role in degeneration of cholinergic neurons in response to Aβ (Κνοωλεσ ετ αλ., 2009) and to proneurotrophins (Sotthibundhu et al., 2008). Several studies have focused on developing small molecules to block p75NTR

receptor signals and protect cholinergic neurons as a therapeutic strategy for the treatment of AD (Friedman, 2000; Podlesniy et al., 2006; Volosin et al., 2008). However, how p75NTR may regulate Aβ production, deposition and clearance has been neglected.

In the present study we found that the knockout of p75NTR decreases soluble Aβ in the brain, as reflected by TBS extracted fraction, and in the blood. The reduction of soluble Aβ in the p75NTR knockout AD mice suggests that the production of Aβ in these mice may be reduced. Indeed, our *in vitro* studies, where Aβ clearance is not complicated, showed that Aβ production by cortical neurons of AD mice was significantly reduced in the absence of p75NTR, suggesting p75NTR mediates Aβ production by neurons. Our data are consistent with *in vitro* data suggesting that p75NTR signaling may play a role in the production of Aβ (Massa et al., 2006; Yang et al., 2008). The levels of soluble Aβ in the brain may reflect a steady-state production of Aβ (Costantini et al., 2005b; Costantini et al., 2005a). However, α-, β- and γ-secretase assays did not show any significant changes in enzymatic activities in p75NTR knockout AD mice. Western blot analysis on APP fragments also did not show any significant differences in APP expression and processing. These data suggest that the likely reduction in soluble Aβ in these animals was not due to a change in APP processing enzymes. How p75NTR regulates Aβ production is not clear. It is known that neurotrophin signaling plays an important role in AD development (Cirrito et al., 2008). Blocking endogenous NGF with antibodies in the mouse promotes the development of AD with amyloid deposition and cholinergic neuron degeneration (Coulson et al., 2009) and supplement of NGF can ameliorate the AD pathology (Capsoni et al., 2000). *In vitro* studies showed that NGF suppresses Aβ production via TrkA signal pathway whereas increases Aβ via the

p75NTR signaling pathway (Capsoni et al., 2002). Multiple ligands bind to p75NTR which may mediate AD development (Costantini et al., 2006). In addition to neurotrophins and proneurotrophins which are prototypic ligands for p75NTR, Aβ is also a ligand of p75NTR (Dechant and Barde, 2002). Neurotoxic Aβ oligomers can directly interact with p75NTR extracellular domain as determined by FRET (Yaar et al., 1997; Yaar et al., 2002). Aβ can activate p75NTR and causes degeneration of basal forebrain neurons via increasing the generation C-terminal fragments (Knowles et al., 2009) and activation of p38, JNK and NF-kappaB signaling pathways (Sotthibundhu et al., 2008). Whether the Aβ/p75NTR signaling causes a positive feedback in Aβ production is not known but the NGF/p75NTR signaling pathway increases the production of Aβ by activation of ceramide signals (Costantini et al., 2005b) and p38, JNK and NF-kappaB signaling pathways (Costantini et al., 2005a).

Although the deletion of p75NTR gene reduced soluble Aβ, the total Aβ levels, in particular, the insoluble fractions as reflected by formic acid extraction were significantly increased in p75NTR knockout AD mice. The increase in total Aβ after p75NTR deletion suggests that p75NTR plays a protective role against amyloid plaque formation and Aβ deposition in AD. Our studies showed that the extracellular domain of p75NTR inhibited the aggregation of Aβ and increased dissociation of preformed fibrous Aβ as reflected by measurement of Thioflavin-T fluorescence *in vitro*. In addition, we found that p75NTR inhibited the oligomerization of Aβ as demonstrated by Western blot showing p75NTR increased the level of monomer of Aβ and reduced the levels of high molecular weight oligomers. Furthermore, after injection of the extracellular domain-Fc into the hippocampus, the number and the area of amyloid plaques in the injected hippocampus were reduced. The mechanism by which the p75NTR extracellular domain reduced amyloid plaques may be similar

to that of anti-Aβ antibodies (Costantini et al., 2005b). It may inhibit the aggregation *in vivo* and enhance transport and removal from the extracellular space. Accumulating evidence show that p75NTR can directly bind Aβ and Aβ oligomers (Klyubin et al., 2005), and mediate neurotoxic signals of Aβ (Yaar et al., 1997; Yaar et al., 2002). Thus it is not surprising that the extracellular domain of p75NTR may act as a sequestering molecule for Aβ to clear Aβ *in vivo*.

The effects of the extracellular domain of p75NTR may have physiological significance, as p75NTR, like APP and other type I transmembrane receptors, may be cleaved extracellularly by metalloproteases (Perini et al., 2002; Costantini et al., 2005b; Sotthibundhu et al., 2008; Knowles et al., 2009). The cleavage of p75NTR by α-secretase is the first step towards the receptor mediated intramembrane proteolysis (RIP) by γ-secretase which releases the intracellular domain, causing apoptosis of neurons (Bronfman, 2007; Sotthibundhu et al., 2008). In the physiological condition, the extracellular domain of p75NTR released by the proteolysis may suppress the deposition of Aβ and promote its clearance. The loss of p75NTR by gene deletion caused significant increase in Aβ deposition and insoluble Aβ, while steady-state production of Aβ by neurons *in vitro* and *in vivo* is significantly reduced in the p75NTR knockout mice. The evidence from our current study strongly suggests that the extracellular domain of p75NTR may play a role in reducing the aggregation and deposition of Aβ.

The increase in amyloid plaques in the brain of p75NTR knockout AD mice can be due to the reduction in Aβ degradation by enzymes. IDE and neprilysin are two main enzymes which degrade Aβ (Jung et al., 2003; Zampieri et al., 2005; Bronfman, 2007; Sotthibundhu et al., 2008). To see whether the absence of p75NTR affects

degradation of Aβ, we analysed the level of IDE and neprilysin. Western blot analysis showed that there was no significant change in the level of IDE and neprilysin in p75NTR knockout AD mice. The data suggest that the absence of p75NTR may not significantly affect the enzymatic degradation of Aβ.

Conclusions

Collectively, our data suggest that p75NTR may have double faced effects on Aβ metabolism. On one hand, p75NTR signaling may increase Aβ production and enhance steady state levels of Aβ, which may increase AD pathology. On the other hand, the extracellular domain of p75NTR after shedding from the membrane may bind and sequester Aβ, thus suppressing its aggregation and β-sheet formation and reducing Aβ deposition in the brain. Our data, together with previous studies, which showed the roles of p75NTR-mediated neurotoxicity in response to Aβ and proneurotrophins, strongly suggest that p75NTR represents a valid therapeutic target for the treatment of Alzheimer's disease. Our data caution that simply reducing expression of p75NTR may exacerbate AD pathology. However, the use of the extracellular domain may not only attenuate neurotoxic signals by blocking Aβ- and proneurotrophin-p75NTR interactions on cell membrane, but also may suppress Aβ aggregation and deposition and enhance Aβ clearance from the brain.

CHAPTER 5

Future Directions

With the world's population ageing, more than 100 million will be living with AD by the middle of this century (Mukherjee and Hersh, 2002). However, no strong disease-modifying treatments are available at the present time (Brookmeyera et al., 2007). An important task to deal with this challenge is to develop effective therapies. Understanding of the pathogenesis of AD is crucial to provide a strong rationale for development of a potential cure or treatment. In this regard, we focused our interest on revealing the pathogenesis of the disease and developing novel therapeutic modalities. We have done three studies: (1) to develop a safe and effective modified immunotherapy based on a single chain antibody directed to Aβ; (2) to examine the efficacy of the polyphenols derived from grape seeds in preventing the development of AD; and (3) to elucidate the roles of p75NTR, a low-affinity neurotrophin receptor, in the development of AD. In these studies, we have obtained some novel findings, which extend our understanding of the AD pathogenesis, and pave the way to develop novel safe and effective therapies for AD. Despite these findings, there are still many important issues need to be addressed in the future. Here I will give some perspectives for our future study.

1. Intramuscular delivery of a single chain antibody gene prevents brain Aβ deposition and cognitive impairment in a mouse model of AD

Anti-β-amyloid (Aβ) immunotherapy is effective in removing brain Aβ, but is associated with detrimental effects, which are assumed to be related to the activation of autoimmune T lymphocytes and inflammatory responses mediated by Fc fragment of antibodies. To avoid these adverse effects, we developed a novel approach by delivering the scFv gene into the muscles to prevent the Aβ deposition in brain, and found that the scFv gene expressed in a stable form in the delivered sites, with limited ectopic expression. Intramuscular delivery of scFv gene was well tolerated by

the animals, and effectively attenuated the Aβ deposition, Aβ related pathologies and cognitive impairment. Despite these findings, the following issues will be addressed in the future.

(1) What is the mechanism underlying the Aβ reducing effect of the scFv?

Currently three basic hypotheses have been proposed regarding the mechanism of Aβ clearance by immunotherapy, including the antibody mediated opsonization of Aβ (Vellas et al., 2008), sequestering plasma Aβ (Bard et al., 2000; Brazil et al., 2000; Wilcock et al., 2001; Wilcock et al., 2004a; Wilcock et al., 2004c) and disruption of the Aβ aggregation by anti-Aβ antibodies (Solomon, Koppel et al. 1996; Solomon, Koppel et al. 1997). At the present time the mechanism underlying the scFv mediated Aβ reduction is unknown. We have demonstrated that the scFv of our study is able to inhibit the aggregation of Aβ monomers and promote the Aβ fibril disaggregation in vitro (DeMattos et al., 2001; DeMattos et al., 2002; Lemere et al., 2003). In addition, it is also unclear if the scFv can interact with cellular APP. A recent study found that intracellular expression of a scFv specific to β-site of APP was able to prevent the cleavage of APP by β-secretase Aβ and Aβ production (Wang et al., 2009b). The scFv can not mediate the opsonization of Aβ in the brain due to lack of Fc fragment, but it is possible that the scFv can sequester the plasma Aβ and enter the brain to inhibit the Aβ aggregation and disaggregate the Aβ fibrils. These hypotheses need to be tested in the future.

(2) How to improve the therapeutic effect of the scFv?

In our studies, we found that intramuscular delivery of scFv gene is able to reduce around 30% of the total Aβ deposition in the brain. While in some other studies,

active immunotherapy can attenuate majority of the Aβ deposition (Paganetti et al., 2005). The extent of the Aβ reduction by scFv in our study may be able to slow the disease progress, but not enough to halt the disease progress. It is necessary to improve the Aβ-reducing efficacy of the scFv. An important factor limiting the efficacy may be the short half-life of the scFv. ScFv is a small size molecule. Although we did not measure the pharmacokinetics of the scFv, other study demonstrated that scFvs usually have a much shorter half-life (2 hours) in the blood than full antibodies (1-2 weeks) (Schenk et al., 1999; Bard et al., 2000). Another key factor would be that the antigen affinity of the scFvs is lower than that of their parent antibodies. The full antibody is bi-valent, while scFv is mono-valent. Thus, we hypothesize that to increase the half-life and the valence of the scFv would be effective to improve the therapeutic efficacy. To achieve these goals, we plan to connect multiple tandems of scFvs together, to increase the molecular size then increase the half-life time, and to generate the multiple valences.

(3) Whether the scFv is able to inhibit Aβ oligomerization and block the neurotoxicity of Aβ.

Aβ oligomers, a soluble species of Aβ, has been suggested to be the primary neurotoxin and may be a major therapeutic target of AD (Holliger and Hudson, 2005). Aβ oligomers should be a primary therapeutic target. The ideal immunotherapy should be able to reduce both the soluble and insoluble Aβ simultaneously. The scFv of our study has been demonstrated to be able to inhibit the aggregation of Aβ monomers and promote the Aβ fibril disaggregation (Dahlgren et al., 2002; Walsh et al., 2002; Lesne et al., 2006; Lacor et al., 2007; Shankar et al.,

2008). Thus it is interesting to investigate if the scFv is also able to inhibit the Aβ oligomerization, and block the Aβ neurotoxicity.

2. Grape seed derived polyphenols attenuate amyloid-beta neuropathology in the brain of AD mice

Polyphenols extracted from grape seeds have been suggested to inhibit Aβ aggregation, reduce Aβ production and protect against Aβ neurotoxicity in vitro. However, the in vivo evidence is lacking. Thus we investigated the therapeutic effects of a polyphenol-rich grape seed extract (GSE) in AD mice, and found that chronic consumption of grape seed derived polyphenols was well tolerated and effective in preventing the brain Aβ deposition and attenuating the AD pathologies in APPSwe/PS1dE9 mouse model. In the future, several important issues need to be addressed:

(1) What are the mechanisms of the Aβ reducing effect of grape seed derived polyphenols?

It is known that grape-derived polyphenols inhibit Aβ aggregation, reduce Aβ production, degrade intracellular Aβ, protect Aβ neurotoxicity, and attenuate oxidative stress in vitro (Cai et al., 2003; Wang et al., 2009b). But whether these in vitro effects also take into action in vivo is unknown. A reducing effect of grape seed polyphenols was observed preferentially on the insoluble fraction of both Aβ40 and Aβ42 in our study, as well as others, suggesting that grape seed derived polyphenols probably affect the assembly or disruption of preformed fibrils and Aβ aggregation in vivo (Bastianetto et al., 2000; Jang and Surh, 2003; Ono et al., 2003; Savaskan et al., 2003; Li et al., 2004; Marambaud et al., 2005; Ono et al., 2005; Mancuso et al., 2007; Riviere et al., 2007). In the present study, we did not find any effects of grape

seed derived polyphenols on the APP expression and BACE1 expression. In a similar study, chronic consumption of GSE derived polyphenols did not change the activities of Aβ generating and degrading enzymes (Ono et al., 2008; Wang et al., 2008). These data suggest that it is unlikely that GSE reduces the Aβ production and regulates the Aβ degradation. Thus, mechanisms of GSE in modulating Aβ metabolism need further investigation and novel pathways need to be explored.

(2) Which components of GSE are active in reducing Aβ in the brain?

In the present study, the grape seed derived polyphenols consist of several major components, including gallic acid, catechin, epicatechin and proanthocyanidins. Which components are active and contribute to the Aβ reduction is unclear. We assume that the combination of all polyphenols could be important in achieving these desirable effects, because several individual polyphenols have been recently shown to be able to reduce brain Aβ in vivo, such as curcumin (Wang et al., 2008), green tea polyphenol epigallocatechin-3-gallate (EGCG) (Lim et al., 2001; Yang et al., 2005) and citrus bioflavonoid luteolin (Rezai-Zadeh et al., 2005). To address this issue, the individual components of GSE need to be examined in the future in terms of Aβ reduction and relevant mechanisms.

(3) Are grape seed derived polyphenols also able to treat other protein misfolding diseases?

Besides AD, other protein misfolding diseases such as Parkinson disease, Huntington disease and prion diseases also share the similar pathogenesis in which cellular proteins misfold from their native conformation to a pathological isoform and form intracellular or/and extracellular aggregates (Rezai-Zadeh et al., 2008). As the grape seed derived polyphenols are able to attenuate the aggregation of Aβ, it is tempting

to speculate that grape seed derived polyphenols are also able to inhibit the misfolding of proteins, thus generate therapeutic effects.

3. Deletion of p75NTR reduces Aβ production but exacerbates Aβ pathology by increasing Aβ deposition in an AD mouse model

Although a large body of evidence shows p75NTR plays a critical role in the death of neurons mediated by Aβ and proneurotrophins, little work has been done on the role of p75NTR in Aβ metabolism in vivo. In the present study we examined the roles of p75NTR in Aβ production and deposition in APPSwe/PS1dE9 mice with p75NTR gene deleted. Our data suggest that p75NTR have double faced effects on Aβ metabolism. On one hand, p75NTR signaling may increase the Aβ production and enhances steady state levels of Aβ which may enhance the AD pathology. On the other hand, the extracellular domain of p75NTR after shedding from the membrane may bind and sequester Aβ, thus suppressing the aggregation and β-sheet formation and reducing Aβ deposition and accumulation in the brain. Despite these novel findings, several important issues remain to be addressed.

(1) What is the mechanism for promotion of Aβ production by p75NTR?

In our study we found that deletion of p75NTR decreased the steady state levels of soluble Aβ in vivo and in vitro, suggesting that p75NTR signal promote the Aβ production. However, the relevant mechanism is unknown. Recent studies suggest that p75NTR signaling may play a role in the production of Aβ (Chiti and Dobson, 2006). However, we did not see any significant changes in APP expression and processing, secretase activities, and Aβ degrading enzymes in p75NTR knockout AD mice relative to AD mice. Thus it is necessary to investigate the novel pathways by

which p75NTR regulates the Aβ production, There are a number of ligands which bind to p75NTR (Costantini et al., 2005b; Costantini et al., 2005a). Which ligand that regulates Aβ production via p75NTR remains to be investigated. In the future studies, effects of the p75NTR ligands such as neurotrophins, proneurotrophins, Aβ, Nogo receptor ligands on the Aβ production should be investigated using p75NTR wild type and mutant cells. Further studies should be also on their down-streaming signal pathways such as ceramide, p38, JNK or NF-kappaB which may be involved in Aβ production regulation. It is also necessary to examine whether the interaction between p75NTR and APP influences the Aβ production, and whether p75NTR regulates the Aβ endocytosis and transportation.

(2) What are the roles of p75NTR in the neuronal degeneration in AD?

Accumulating evidence indicate that p75NTR also plays a critical role in degeneration of cholinergic neurons in response to Aβ (Dechant and Barde, 2002) and to proneurotrophins (Coulson et al., 2009). However, the evidence is controversial because p75NTR also attenuates the Aβ neurotoxicity (Friedman, 2000; Podlesniy et al., 2006; Volosin et al., 2008). Most of these studies provide in vitro evidence. It is critical to use the animal models to investigate the exact roles of p75NTR in Aβ neurotoxicity in AD. The AD mouse model with deletion of p75NTR gene generated in our study represents an ideal animal model to investigate the roles of p75NTR. In the future, we plan to examine the changes in p75NTR-deleted AD mice of cholinergic neuron loss and the neurite degeneration, by examining the number and volume of cholinergic neurons in the basal forebrain, cholinergic fibre length and fibre density using stereological assessment techniques, and ChAT and AChE enzyme activity and expression using biochemical and histological methods.

(3) Development of novel therapies targeting p75NTR

Our data, together with previous studies which showed the roles of p75NTR mediated neurotoxicity in response to Aβ and proneurotrophins, strongly suggest that p75NTR may be a valid therapeutic target for the treatment of AD. P75NTR pathway can be blocked by targeting the binding of ligands such as Aβ and neurotrophins to p75NTR, and signaling pathways such as calpain, GSK3b and c-Jun, which mediate Aβ toxicity. Several studies have focused to develop small molecules blocking p75NTR receptor signals to protect cholinergic neurons for the treatment of AD (Massa et al., 2006; Yang et al., 2008). In our study, we found that recombinant extracellular domain of p75NTR inhibited the Aβ aggregation and disaggregated the Aβ fibrils in vitro. Importantly, we also provided preliminary data showing that recombinant extracellular domain was also able to reduce the local Aβ plaques of injection site. Based on these findings, we hypothesize that the use of extracellular domain may not only attenuate neurotoxic signals such as Aβ- and proneurotrophin-mediated neurotoxicity, but also may neutralize Aβ, suppress Aβ deposition and enhance Aβ clearance from the brain. Thus we plan to explore the efficacy of p75NTR extracellular domain in the prevention and treatment of AD, by delivering the p75NTR extracellular domain gene into the brain or muscle with AAV.

Summary

AD has become an urgent public health issue which is calling for effective prevention and treatment. The current available medications approved for AD treatment, such as cholinesterase inhibitors, antidepressants and antipsychotics, are primarily to relieve the symptoms, and but are not able to cure the disease (see review in (Zhang et al., 2003b)). Some available medications designed for other

diseases, such as estrogen (Rosenberg, 2005) and its analogs (Shumaker et al., 2003), statins (Yaffe et al., 2005), non-steroidal anti-inflammatory agents (Sparks et al., 2005) and vitamin E (Rogers et al., 1993), have been implied for AD treatment, but their efficacy in AD treatment need further clinical investigation. Based on the latest understanding of the AD pathogenesis, lots of efforts are made to develop novel drugs to target the disease cascade, such as β or γ secretase inhibitors to reduce Aβ production (Petersen et al., 2005), agents to remove phosphorylated tau protein (Henley et al., 2009; Huang et al., 2009) and antioxidants to relieve oxidative stress in the brain (Hanger et al., 2009). Our present studies show the potential of the anti-Aβ scFv, grape seed derived polyphenols and recombinant p75NTR extracellular domain in prevention and treatment of AD, and further efforts should be made to translate these novel candidates into disease-modifying drugs against AD.

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