CHAPTER II

Review Literatures

2.1. Iron

Iron is vital for all living organisms because it is essential for a wide variety of biological process such as DNA synthesis, oxygen binding and electron transport. Within the brain, iron is essential for a number of enzymes involved in neurotransmitter synthesis. For example, iron as a cofactor of the tyrosine hydroxylase which catalyzes the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA), the precursor of catecholamine neurotransmitters such as dopamine, adrenaline and noradrenaline (Erikson *et al.*, 2001). Additionally, tryptophan hydroxylase is also iron containing enzyme that catalyzes the first step of the conversion of tryptophan to serotonin (Ichiyama *et al.*, 1974). Iron is also involved in the enzymatic degradation of neurotransmitters since it is a cofactor of monoamine oxidase (MAO), the enzyme responsible of oxidative deamination of dopamine, adrenaline and noradrenaline (Kumar & Andersen, 2004). Moreover, iron is also required for normal myelin synthesis because several iron dependent emzymes are involved in the synthesis of myelin lipids such as HMG-CoA reductase, which catalyzes the NADPH-dependent reduction of HMG-CoA to mevalonate.

Although iron is extremely important for a wide variety of biological process, excessive free iron is dangerous owing to its ability to increase oxidative stress through the conversion of H_2O_2, generated during normal cellular metabolic processes or under pathological condition, to more cytotoxic hydroxyl radical via the Fenton reaction (McCord, 1998; Andrews, 1999). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules which causing lipid peroxidation, DNA strand breaks and degradation of biomolecules. In healthy people, the total body iron content is approximately 3-5 g while brain iron content is less than 2% of the total body iron content. It has been reported that brain iron

increases with age. Exhibit a dramatic increase in brain iron accumulation has also been reported in many neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Connor *et al.*, 1992a; Connor *et al.*, 1992b; House *et al.*, 2006; Wallis *et al.*, 2008). Although it has not yet known that this iron accumulation is an initial event that causes neurodegeneration in these diseases or a consequence of the disease process, recent evidences have been reported that mutation in the gene associated with iron absorption, HFE gene, altered brain iron levels and also associated AD (Sampietro et al. 2001; Pulliam et al. 2003; Berlin et al. 2004; Connor and Lee 2006; Bartzokis et al. 2010). These contents have opened the possibility that increased brain iron concentration may be a one factor that contribute to neurodegeneration in AD. Therefore, identification of novel molecular mechanisms or target proteins associated with iron-mediated neuropathology in AD could encourage the development of a drug which may help prevent or slow diseases progression. However, why does iron increase abnormally in some regions of the AD brain has not yet completely answer.

The mechanism by which iron transported across blood-brain barrier

The mechanisms how iron moves through the brain and accumulates within regions in AD still need to be elucidated. However, to enter the brain, iron must cross the blood-brain barrier (BBB), a tight structure composed of endothelial cells, pericytes and astrocytic foot processes that restrict blood-to-brain trafficking. To date, the mechanisms of iron transport across the BBB have not been completely clarified. The accumulated evidence suggests that the transferrin (Tf) and transferrin receptor (TfR)-mediated endocytosis may be the major route of iron transport across the luminal membrane of the capillary endothelium, and that iron crosses the abluminal membrane and enters into the brain (Bradbury, 1997; Moos & Morgan, 2000). The evidence shows that the uptake of Tf-bound iron by TfR-mediated endocytosis from the blood into the cerebral endothelial cells is no different in nature from the uptake

into other cell types (Bradbury, 1997). As shown in Figure 2.1, when Tf-iron (Fe^{3+}) complexes attach to TfR on the cell surface, the TfR-Tf-iron complex is internalized into clathrin-coated endosomes. Here an ATP-dependent proton pump acidifies the endosome until the pH is less than 6 (about 5.5) (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). At this pH, iron is released from the complex and reduced to its Fe^{2+} form by endosomal oxidoreductase. Fe^{2+} then transported out the endosomal membrane though the action of divalent methyl transporter (DMT) 1 while Tf and TfR both recycle to the cell surface where Tf releases back into the circulation. In the cytosol Fe^{2+}enter the labile iron pool (LIP; Fe^{2+}> Fe^{3+}) from which it is available for intracellular use or for storage (in ferritin).

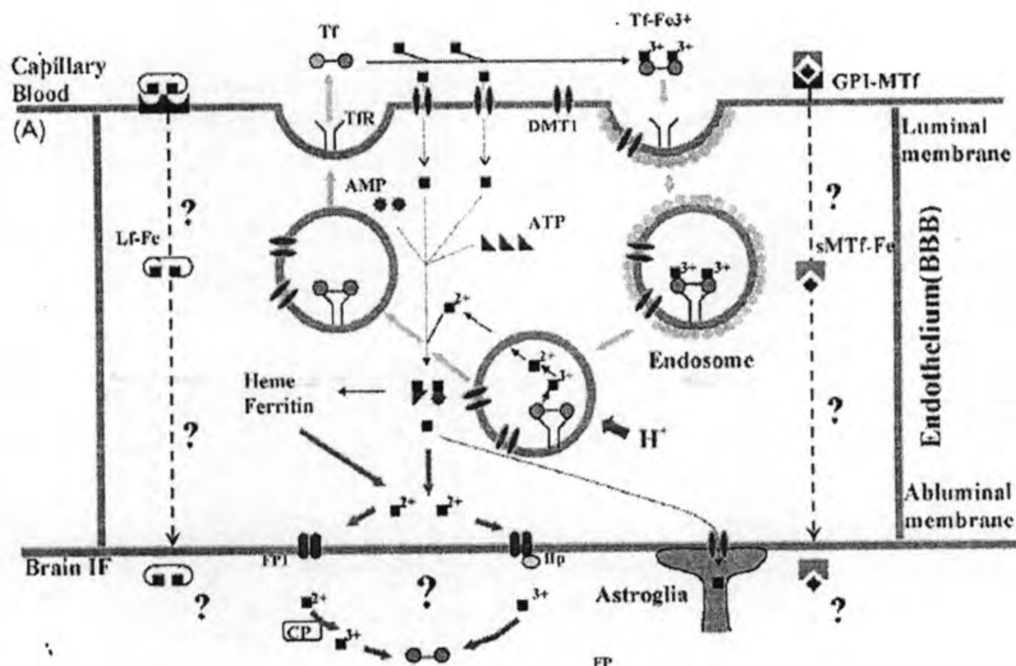

Figure 2.1 A hypothetical scheme for the possible mechanism by which iron transport across BBB. Tf-Fe uptake by endothelial cells is similar in nature to the uptake into other cell types. DMT1 might play a role in translocation of iron from endosome to cytosol. Iron (Fe^{2+}) probably crosses the abluminal membrane via ferroportin 1 (FP1) – hephaestin (Hp) and/or Hp-independent export systems (light-blue arrows). LfR–Lf a nd GPI-anchoredp97–secreted-p97 pathways might also be

involved in iron transport across the BBB (dark-blue arrows). The presence of DMT1 on astrocytic end feet suggests that astrocytes may take up iron from endothelial cells through their end feet processes on the capillary endothelia vai a DMT1-mediated process (Ke & Qian, 2007).

LIP is potentially toxic. Therefore, the maintenance of cellular iron homeostasis is necessary to achieve an optimal balance of iron import and export that ensures rerelease of the required amounts of iron to sustain biochemical activities while preventing the harmful effects of excessive iron in cells. Sensitive control mechanisms that monitor iron levels in LIP and prevent the expansion of this pool to combat the Fenton reaction, while still making iron available for iron-dependent proteins and enzymes, have been found at the level of post transcription regulation of TfR and ferritin mRNAs. The mRNA for each of these proteins contains at least one stem–loop structure known as an iron responsive element (IRE) in their untranslated regions (UTR) that can be bound by iron responsive element binding proteins (IRPs) (Rouault *et al.*, 1990; Hentze & Kuhn, 1996). The IRE–IRP mechanism provides for posttranscriptional iron regulation in response to intracellular iron concentrations (LIP). The ferritin mRNA contains RE in their 5' UTR whereas the TfR mRNA contains IRE in the 3' UTR (Hentze *et al.*, 1987; Rouault *et al.*, 1988; Koeller *et al.*, 1989; Gunshin *et al.*, 2001). When cellular iron levels are depleted, in the case of the TfR, the IRP bind to the 3' IREs, the mRNA is stabilized and translation enabled, resulting in increased iron uptake from the circulation. In the case of ferritin mRNAs, IRPs binds to the 5' IRE prevent translation initiation by blocking the ability of the 40S ribosomal subunit to scan for the AUG start codon where it initially binds (Walden *et al.*, 1989; DeRusso *et al.*, 1995). Without additional ferritin molecules the iron imported into the cytosol cannot be stored, thereby increasing the cellular iron concentration. In contrast, when cellular iron levels are elevated, the IRP dose not binds to the IRE which allows for TfR degradation and ferritin subunit translation,

decreasing iron uptake and increasing iron storage, respectively (Chen *et al.*, 1998). The mechanisms of IRE–IRP regulate iron concentration is shown in Figure 2.2.

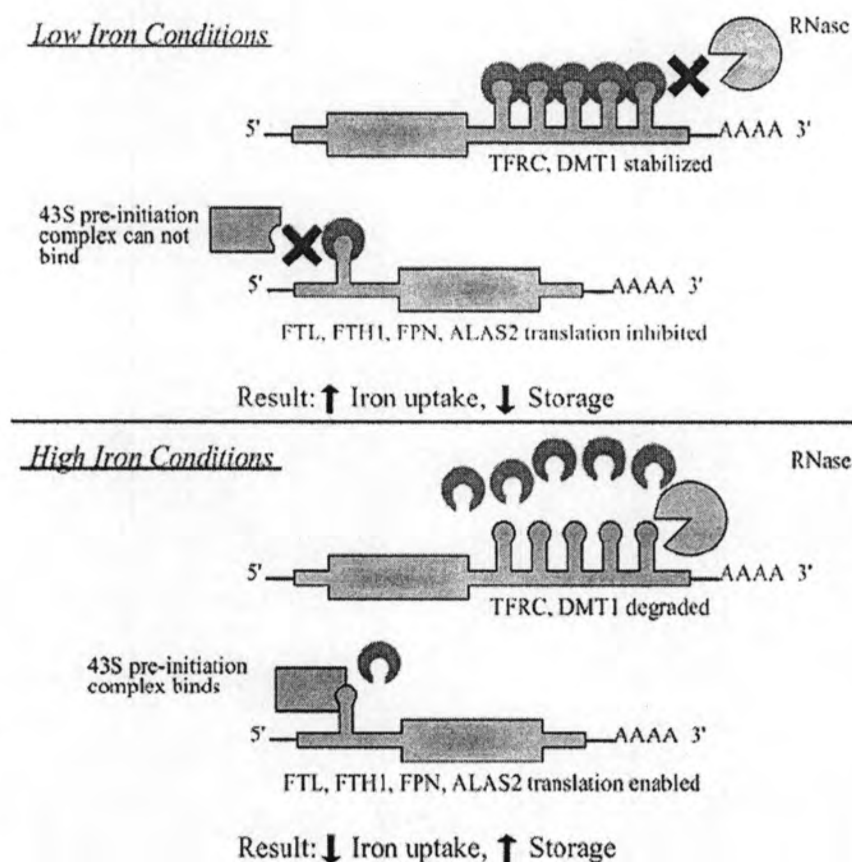

Figure 2.2 IRE–IRP regulations of iron concentrations. The IRE–IRP mechanism provides for posttranscriptional iron regulation in response to intracellular iron concentrations (Rhodes & Ritz, 2008).

In addition to Tf/TfR pathway, it has been suggested that the lactoferrin receptor/ lactoferrin and glycosylphosphatidylinositol (GPI) anchored p97/secreted p97 pathways might play a role in iron transport across the BBB (Faucheux *et al.*, 1995; Rothenberger *et al.*, 1996; Malecki *et al.*, 1999; Moroo *et al.*, 2003; Ji *et al.*, 2006). It is also possible that a small amount of iron might cross the BBB in the form of intact Tf-Fe complex by receptor-mediated transcytosis (Moos & Morgan, 2000;

Morgan & Moos, 2002). However, the mechanism by which iron crosses the abluminal membrane of the BBB and then enters the interstitial fluid of the brain is unclear. Since it has been reported that FP-1 and hephaestin are found the brain, it is possible that FP/hephaestin or FP/çeruloplasmin system might play a role in iron transport across abluminal membrane of the BBB. Because the form of iron transport across this membrane might be Fe^{2+}, therefore, a ferroxidase such as hephaestin (or ceruloplasmin) might be necessary for Fe^{2+} to be oxidized to Fe^{3+}. After crossing the basolateral membrane of the BBB cells, Fe^{3+} could be carried away by Tf. Another proposed mechanism involved in Fe^{2+} iron transport across abluminal membrane is the role of astrocytes. The astrocytes probably have the ability to take up Fe^{2+} from endothelial cells through their end feet processes on the capillary endothelia (Malecki *et al.*, 1999; Oshiro *et al.*, 2000).

After the iron is transported across the BBB to the interstitial fluid of the brain, it is likely to bind quickly to the Tf (secreted from the oligodendrocytes) or other transporters and then transported to where iron is needed (Espinosa de los Monteros *et al.*, 1990; Moos & Morgan, 1998). Within interstitial fluid (IF) of the brain, there are probably two transport forms of iron, Tf bound iron (Tf-Fe) and non Tf bound iron (NTBI) (Bradbury, 1997; Moos & Morgan, 1998). The latter probably includes citrate-Fe^{3+} or Fe^{2+}, ascorbate-Fe^{2+} and also Lf-Fe^{3+} and sMTf-Fe^{3+}. However, the affinity of Tf with iron is the highest, compared with other iron transporters (the equilibrium constant for formation of diferric Tf is more than 10^{10} times that of ferric citrate). So Fe^{3+} in IF will bind to Tf first. The excess iron will bind to other transporters. The Tf-Fe or probably Lf-Fe and sMTf-Fe will be taken up by brain cells via TfR or LfR and GPI-anchored MTf-mediated processes, respectively (Malecki *et al.*, 1999; Qian & Shen, 2001), however, the relevant mechanisms have not yet been detailed. It has been reported the major mechanism that neurons uptake iron is TfR-mediated endocytosis. However, neuron can also take up iron by a Tf-indipendent pathway. TfR-mediated endocytosis has also been anticipated to be involved in Fe^{3+} uptake in astrocyte and

oligodendocytes, a myelin-forming cell type in the brain which has high iron need for their normal function (Roskams & Connor, 1992; Giometto *et al.*, 1999). In addition, based on the recent identification of a ferritin receptor (FtR) predominantly on oligodendrocytes, this has been suggested that these cells may obtain iron via ferritin/FtR pathway (Hulet *et al.*, 2000). The major pathway that microglia uptake iron has been report that to be GPI-anchored MTf-mediated processes. The mechanism by which iron uptake by the brain cells is shown as Figure 2.3.

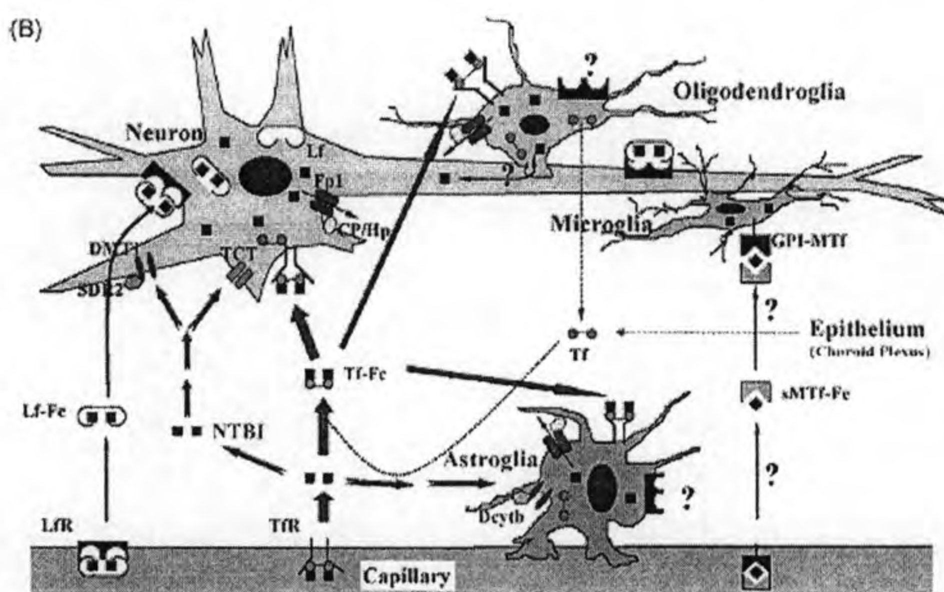

Figure 2.3 A hypothetical scheme for the possible mechanism by which iron uptake by the brain cells. There are two transport forms of iron in the brain: Tf-Fe and non transferring boud iron (Ke & Qian, 2007).

In the brain, the most common cell type to stain for iron under normal conditions is the oligodendrocyte (Zecca *et al.*, 2001). In contrast, microglial cells under physiological conditions are only rarely stainable with iron. However, microglia, express ferritin, indicating that all of this cell type has the capacity to store iron. In contrast, very little ferritin expression is seen in astrocytes, indicating that these cells provide little iron storage. It is generally accepted that iron accumulation in the brain

progresses with age (Aquino *et al.*, 2009). When older individuals (60–90 years of age), are compared with younger subjects (28–49 years), more iron staining is observed in the microglia and astrocytes of the cortex, cerebellum, hippocampus, basal ganglia and amygdala, and ferritin immunoreactivity is also stronger in these cells (Connor *et al.*, 1990). Oligodendrocytes contain the largest amount of iron, ferritin and Tf, but this content remains constant during ageing. Significant elevations of iron have also been reported in several brain regions of individuals with AD, including frontal, temporal, parietal neocortex, hippocampus and amygdale, when compared with age-matched control. Postmortem analysises of AD brain tissue reveal that amyloid plaques are stained robustly for iron, as shown in table 1. Elevated levels of brain iron may have a direct effect on amyloid precursor protein (APP) processing, because of the presence of IRE within the 5' URT of the APP transcript. In response to cellular iron influx by a regulatory mechanism similar to iron control of ferrin translation, APP levels was increased upon iron treatment where as decreased upon treatment with an iron chelator. Iron may enhance Aβ production by decreasing α-secretase cleavage rate. Iron is believed to play a role in the plaque forming process by directly binding to Aβ peptides; therefore facilitating Aβ aggregation. Iron was also shown to enhance neurotoxicity of Aβ, providing a direct link between excessive iron accumulation and the known cause of cell death in AD brain. Moreover, it has also been reported that binding of iron to the tau protein precedes the aggregation of hyperphosphorylated tau and the subsequent formation of NFTs. Therefore, iron may implicate in the formation of the NFTs. The iron-possitive cells recognized as activated microglia are also associated with amyloid cores of the senile plaques and have been proposed to contribute to conversion of diffuse plaques to dense core plaques.

Table 1 Iron levels in patients with Alzheimer's disease compared with healthy individuals

Location	Iron μg/g (mM)[a]
Plaque rim	52.4 (938)[b]
Plaque core	53.1 (951)[b]
Total senile plaque	52.5 (940)[b]
Alzheimer's neuropil	38.8 (695)
Control neuropil	18.9 (338)

[a]Numbers in brackets represent molar concentrations, which were converted with the assumption of a sample density equivalent to 1 g cm^{-3}. [b]$P < 0.05$ (plaque values compared with neuropils from patients with Alzheimer's disease). [c]$P < 0.05$ (neuropils from patients with Alzheimer's disease compared with neuropils from control individuals)(Lovell *et al.*, 1998).

Recent evidences have been reported that mutation in the gene associated with iron absorption, HFE gene, increased brain iron accumulation and also increased a risk factor for AD (Moalem *et al.*, 2000; Sampietro *et al.*, 2001; Pulliam *et al.*, 2003; Berlin *et al.*, 2004; Connor & Lee, 2006). This gene is located on the short arm of chromosome 6. As it encodes a 343-amino acid glycoprotein located at the plasma membrane which exhibits significant similarity to the HLA class I molecules, it was designed HLA-H or HFE. As shown in Figure 2.4, the structure of HFE protein is composed of three extracellular loops (α1, α2, and α3), the transmembrane portion, and intracytoplasmic portion. Like other class I molecules, HFE contains disulfide bridges that stabilize its tertiary structure (Feder *et al.*, 1996; Lebron *et al.*, 1998). One of these S-S bonds at α3 loop is required to allow its interaction with β_2 microglobulin (β_2MG), which in turn is important for the trafficking of HLA class I proteins to the

cell surface. The biological basis of the role of HFE in the control of iron absorption derives from studies showing that this protein is able to interact with TfR receptor at a gap between loops α1 and α2 (Bennett *et al.*, 2000). This interaction affects the binding between TfR and iron bound Tf leading to limit iron uptake. The mutant HFE protein does not have this effect, resulting in increased iron uptake in cells. HFE is highly expressed in the duodenum, it was hypothesized that HFE modulates the uptake of Fe-Tf from plasma by crypt cells and participates in the mechanism through which the crypt enterocytes sense the levels of body iron stores. HFE mutation could impair this function and provide a false signal to crypt cells resulting in an abnormal programmer of increased iron absorption from the lumen leading to excessive iron accumulation in tissues and resultant organ damage which is associated with hemochromatosis (HH), an autosomal recessive condition in which intestinal iron absorption is greatly elevated (Brittenham *et al.*, 2000). HH is one of the most common diseases among individuals of Northern European (1/200 to 1/400 individuals have HH, with estimated carrier frequency ranging from 1/8 to 1/10). About one million individuals in the United State are affected by this disease (Levy *et al.*, 1999). The clinical features of the disease arise as result of decades of progressive accumulation of iron in parenchymal cells of the liver, pancreas, and heart. The most common clinical consequences of iron accumulation include liver cirrhosis, hepatocellular cancer, diabetes, cardiac failure, and arthritis. The clinical disease of HH is usually caused by a homozygous autosomal recessive mutation in the HFE gene.

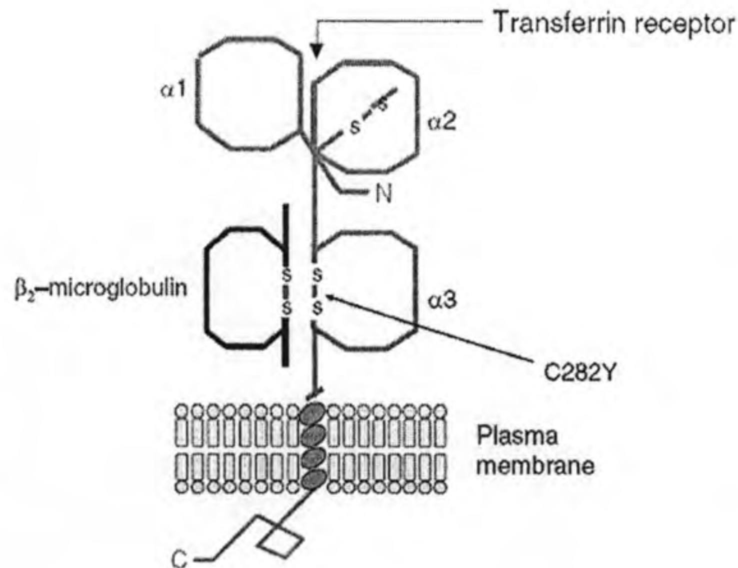

Figure 2.4 Structure of HFE, the candidate protein for HH. HFE is a major histocompatibility complex class I-like protein (HLA-H) that associated with β_2MG. (Redraw with modification from Griffiths W.J.H. Aliment Pharmacol Ther., 26: 331-342, 2007)

The two most common mutations of HFE are the C282Y and the H63D. The former mutation is caused by a guanine to adenine substitution at nucleotide 845 (845G⟶A), which results in cysteine to tyrosine substitution at amino acid position 282 (C282Y) in the HFE protein. This mutation occurs at highly conserved residue and disrupts a disulphide bridge in the α3 loop. This loss impedes the interaction of HFE with β_2MG and prevents the expression of the protein on cell surface, with the protein instead being retained within the golgi complex, with the result being unregulated TfR-mediated iron uptake in the intestine (Feder *et al.*, 1997; Waheed *et al.*, 1997; Lebron *et al.*, 1998). This mutation is approximately 60% to 90% of HH patients. In addition to the mutation of C282Y, a second mutation has been found at position 187, where a cystein is replaced by guanine (187C⟶G), which results in histidine to aspartic acid substitution at amino acid position 63 (H63D) in the HFE protein. This residue lies

within the loop of the $\alpha 1$ domain, where it forms a salt bridge with Asp95. This mutation appears to lack the normal high degree of inhibitory effect on the TfR (Feder *et al.*, 1996). The H63D mutation is even more common than C282Y and is found in 15% to 40% of white individuals where as mutation of C282Y is approximately 10% to 15% of white individuals (Steinberg *et al.*, 2001). Homozygosity for H63D slightly increases body iron status (Tf saturation, serum ferritin) but does not result in clinically significant iron overload (Gochee *et al.*, 2002). However, compound heterozygosity for the H63D mutation with C282Y is found with an increased frequency in patients with iron overload than predicted for the general population (Bacon, 2001). The risk for iron loading in the C282Y/H63D compound heterozygote is increased but is estimated to be nearly 200 fold lower than in the C282Y homozygote (Risch, 1997)

A number of recent studies suggest that carrying an H63D HFE mutation is a risk factor or genetic modifier for AD. These include a study of HFE mutations in patients with familial AD and found that HFE mutations were overrepresented in males, but underrepresented in females (Moalem *et al.*, 2000). Furthermore, Pulliam et al studied conventional AD patients and non-demented patients with AD-like pathology changes and compared them to non-demented controls without pathology and showed higher rates of HFE mutations in both cases compared to non-demented controls without pathology (Pulliam *et al.*, 2003). While, analysis of patients with sporadic late onset AD suggested that the frequency of the H63D mutation was highest in the patients younger than 70 years at the time of disease onset compared to patients over 80 (Sampietro *et al.*, 2001). Consistent with these reports, recent evident has been reported that hemochromatosis protein was found on brain capillaries, choroid plexus, and ependymal cells along with TfR. Interestingly, it has also been reported that iron accumulation in the brain does occur in individuals with HH patients. Therefore, it is possible that iron uptake throughout the brain of HH patients could be influenced by the HFE mutation. However, the mechanisms by which mutation in HFE gene associate with AD has not yet know. Since the elevation of iron in AD brain has been

observed in many pathogenesis of AD such as NFTs, amyloid plaques and neuroinflammation, the elevation of iron may be involved in the initiation of these lesions leading to enhance neuronal cells death in AD.

2. 2 Alzheimer's disease

Alzheimer's disease (AD) is the neurodegenerative disorder characterized by progressive death of neurons in the brain areas associated with learning and memory, particularly in the hippocampus and cerebral cortex. AD was described in 1906 by the German physician Alois Alzheimer in a patient with strange behavioral symptoms, short term memory loss, and a progressive decline of cognitive and motor function. Since the first report describing AD, it has become the most common cause of dementia in Western societies. There are currently 5.5 million people in the United States and more than 35 million worldwide afflicted with the disease. Every one in eight people over the age of 65 and every nearly half of those over 85 have AD. With its aging population, the prevalence of AD patients will approach 13.2 to 16.0 million cases in the United States. In Thailand, there are approximately 670,000 people afflicted with AD. There are two basic types of AD: early-onset (also known as familial AD) and late-onset. The former is defined by the development of the disease before the age of 65 years. The statistical incidence of early onset AD is less than 5% of all AD cases. The majority of early onset AD appears to be linked with genetic defects on chromosome 21, 1 and 14 which include the genes encoded for beta amyloid (Aβ) protein precursor (APP), presenilin 2 (PS2) and presenilin 1 (PS1) respectively. Unlike early-onset, late-onset is the most common form of AD, accounting for about 95% of AD cases and usually occurring after age 65. The exactly causes of late-onset AD has not yet known. It is believed that a combination of several factors may be involved in late-onset AD. The major pathological hallmarks in AD brain are composed of intracellular neurofibrillary tangles (NFTs), extracellular deposition of amyloid plaques. The other pathological features that appear in the brain

parenchyma of AD patients include mitochondrial damage, oxidative stress, reactive gliosis with neuroinflammation and neuronal apoptosis.

NFTs are intraneuronal bundles of paired helical filaments. The main structure component of these tangles is an abnormally hyperphosphorylated and aggregated form of tau. Tau, a phosphoprotein encoded by the gene located on chromosome 17, is a major microtubules associated protein of a normal mature neuron. A major known function of tau is its interaction with tubulin, the major protein subunit of microtubules. Tau promotes assembly of tubulin into microtubules and helps stabilize the structure of microtubules (Weingarten *et al.*, 1975). These functions of tau are regulated by its degree of phosphorylation. Normal brain, tau has two to three moles phosphase/mole of protein, which appears to be optimal for its biological activity. However, in AD brain, tau accumulates in the affected neurons as abnormally hyperphosphorylated protein, which is at least three to four-fold more phosphorylated than in normal adult brain (Kopke *et al.*, 1993). Hyperphosphorylated tau lacks affinity for microtubules, and self-associates into paired helical filament structures. Generally, microtubules are a structure of cytoskeleton which is essential for maintaining cell structure and intracellular trafficking of proteins and organelles, including transport along axons. Thus, disturbances of microtubules are likely to impair axonal transport and thereby compromise the functions of synaptic inputs and, eventually, the viability of neurons. In AD abnormally hyperphosphorylated tau are phosphorylated over 30 sites, mostly are serine/threonines kinases such as glycogen synthase kinase 3 beta (GSK-3β), cyclin dependent kinase and MAP kinase (Hanger *et al.*, 1992; Morishima-Kawashima *et al.*, 1995). Among these kinases, GSK-3β has been reported to be a major kinase that phosphorylates many tau epitopes *in vitro* which are the same sites as seen in AD (Mandelkow *et al.*, 1992; Ishiguro *et al.*, 1993; Yang *et al.*, 1993; Singh *et al.*, 1995a; Singh *et al.*, 1995b). Moreover, it has been reported that activated forms of GSK-3β are concentrated in neuron with pretangle and tangle pathology. These evidences indicate that GSK-3β may be a principal kinase that

phosphorylates tau in AD. Interestingly, it was neither tau mutation nor GSK-3β mutation occur in AD. Figure 2.5 shown hyperphosphorylation of tau protein.

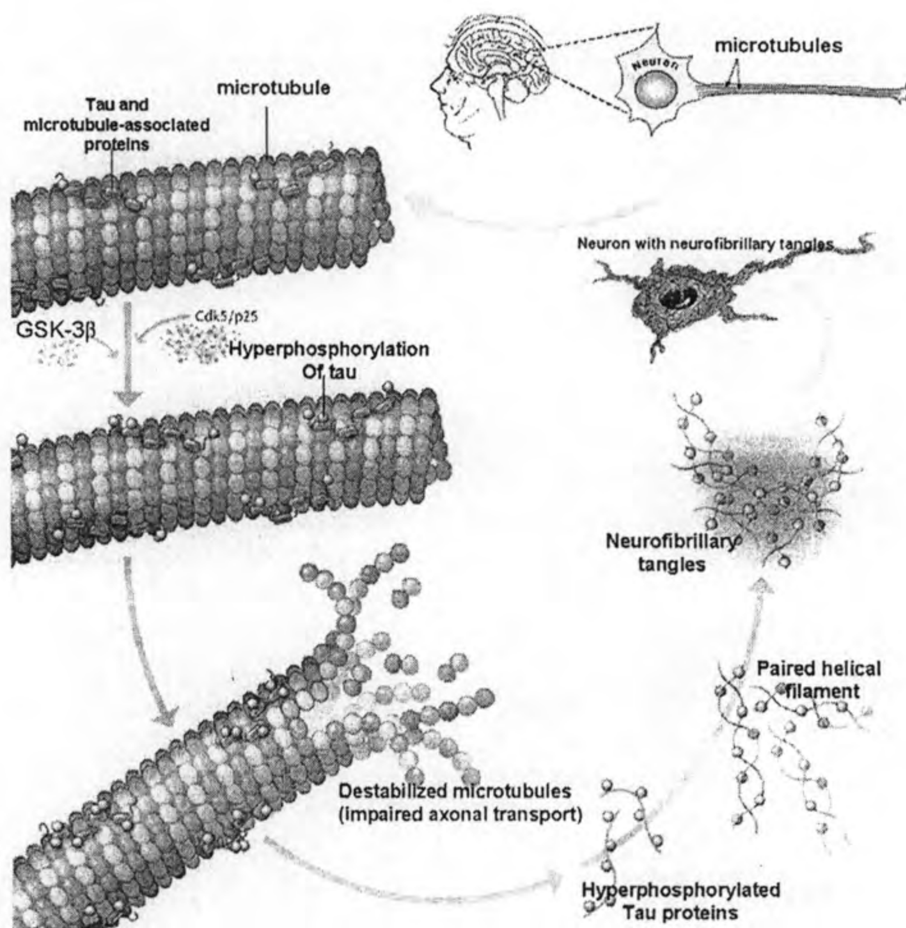

Figure 2.5 Neurofibrillary tangles. Biochemical analysis has shown that neurofibrillary tangles are composed mainly of abnormally-phosphorylated tau protein (Querfurth & LaFerla).

In addition to NFTs, the brain regions affected by AD also contain "amyloid plaques" in which extracellular deposits of beta amyloid (Aβ) peptide of about 50-100 μm in diameter (Selkoe, 1991; Kosik, 1994; LeBlanc *et al.*, 1996). The name amyloid comes from the early mistaken identification of the substance as starch (amylum in Latin), based on crude iodine-staining techniques. The later period, the scientific was finally resolved that it was a deposition of proteinaceous mass. The histopathological

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definition of amyloid is an extracellular, proteinaceous deposit exhibiting beta sheet structure, therefore, its name beta amyloid plaques. Amyloid plaques are classified into two major types: the classical (neuritic) plaques and the diffuse (pre-amyloid) plaques. The classical plaque is a complex lesion of the cortical neuropil containing several abnormal elements with a central deposit "the core" composed of extracellular amyloid fibrils or Aβ (Glenner & Wong, 1984; Masters *et al.*, 1985) surrounded by dystrophic neurites (both dendrites and axonal terminals), activated microglia and reactive astrocytes (Selkoe, 1991). The diffuse plaques contain nonfibrillar Aβ and are devoid of amyloid core with very few activated microglia. The principal constituent of amyloid is a 4 kDa peptide called Aβ which is cleaved from amyloid precursor protein (APP) (Haass *et al.*, 1992), a single membrane spanning protein which composed of a large hydrophilic amino-terminal extracellular domain, a single hydrophobic transmembrane domain consisting of 23 residues and small carboxyterminal cytoplasmic domain (Turner *et al.*, 2003). The pre-mRNA of APP is spliced to produce three major isoforms; APP_{770}, APP_{751} and APP_{695} with the APP_{695} isoform expressed at high levels in neurons (the ratio of APP_{770},:APP_{751} :APP_{695} is 1:10:20). During APP transit from the endoplasmic recticulum to the plasma membrane through the constitutive secretory pathway, APP undergoes post-translational modification by N- and O-glycosylation in the Golgi apparatus, and delivered to the cell surface as an integral membrane protein. However, it has been reported that only 10% of APP molecules are successfully delivered to the cell membrane. APP can be processed along two main pathways, non-amyloidogenic and amyloidogenic pathway involving three secretases (α, β and γ) as shown in Figure 2.6 (Selkoe, 2001a). Generally, APP is mainly cleaved by α-secretase (non-amyloidogenic pathway). With this pathway, α-secretase cleaves APP within the Aβ domain, releasing the large soluble APP fragment (α-sAPP). The remaining C-terminal fragment (CTF) is cleaved by the γ-secretase complex releasing the short p3 peptide (3 kDa). The remaining APP intracellular domain (AICD) is metabolised in the cytoplasm (Haass *et al.*, 1993). Since APP

cleavage by α-secretase is within the Aβ domain this precludes Aβ generation and aggregation. In amyloidogenic (β-secretase pathway), β-secretase cleaves APP just before the Aβ domain, releasing soluble βsAPP (Vassar *et al.*, 1999). The remaining CTF is cleaved by the γ-secretase releasing the free 40 or 42 amino acid Aβ peptide. The remaining AICD is metabolised in the cytoplasm (Cook *et al.*, 1997). Protein subunits of the γ-secretase consists of four components; presenilin 1, presinilin 2, nicastrin (Yu *et al.*, 2000; Esler *et al.*, 2002), PEN-2 (Francis *et al.*, 2002), and APH-1 (Francis *et al.*, 2002; Goutte *et al.*, 2002). It has been reported that a pair of conserved aspartate residues within the predicted transmembrane domains 6 and 7 of PS1 and PS2 is crucial for γ-secretase activity. APH-1 and PEN2 are thought to stabilize the γ–secretase complex and nicastrin to mediate the recruitment of APP CTF to the catalytic site of the γ-secretase. The major sites of γ-secretase cleavage correspond to positions 40 and 42 of Aβ. The mutations in presenilin 1 and presenilin 2 influence γ-secretase cleavage and promote the overproduction of Aβ1-42. It has been reported that Aβ production is promoted by GSK-3β and reduced by GSK-3 inhibitors (Phiel *et al.*, 2003). However, the mechanism whereby GSK-3β promotes Aβ production remains to be clarified. Because GSK-3β can phosphorylate APP and presenilin-1 (a catalytic domain of γ-secretase), this mechanism may be involved in Aβ production.

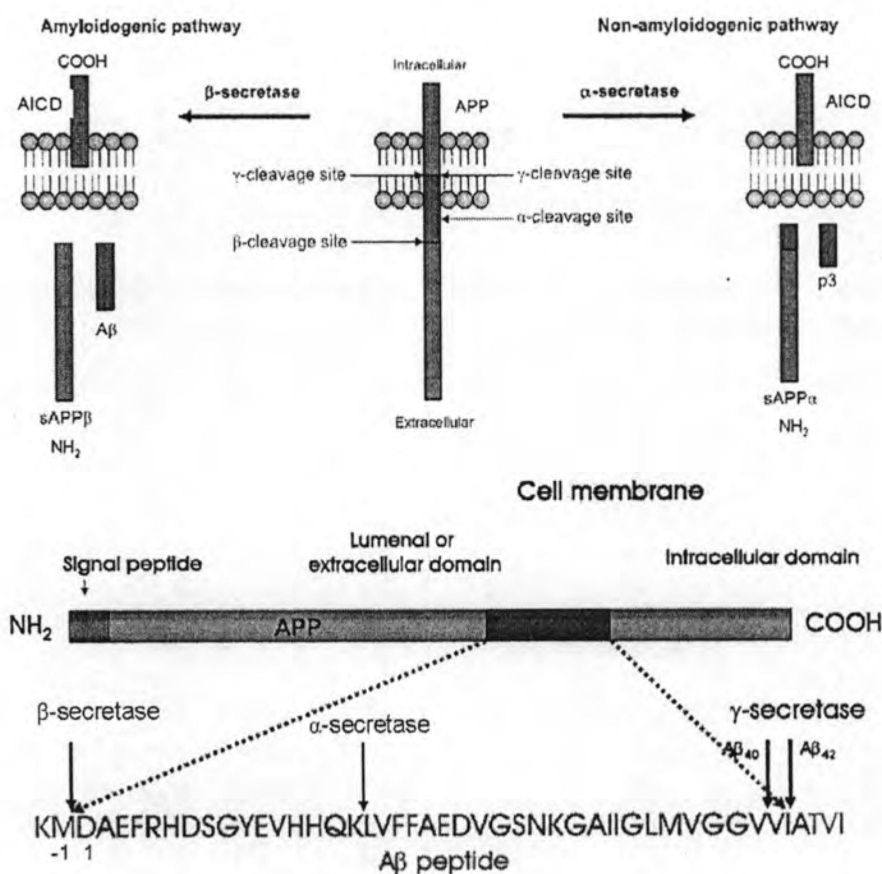

Figure 2.6 Proteolytic processing of APP by α-, β-, and γ-secretase. α -secretase cleaves within the Aβ region, resulting in the secretion of the extracellular domain of APP; hence, the cleavage does not produce the Aβ peptide. In contrast, the β-secretase and γ-secretase cleavages do result in production of the peptide (Hunt & Turner, 2009).

Under normal conditions, brain Aβ clearance occurs through at least three pathways: extracellular proteolysis by degrading enzymes (Selkoe, 2001b), transport across the BBB, and receptor-mediated endocytosis (Zlokovic, 2004). Several proteolytic enzymes have been involved in the degradation of Aβ such as insulin-degrading enzyme (IDE), endothelin-converting enzyme (ECE) 1 and 2, plasmin and neprilysin. Microglia and astrocytes has also been reported to take up Aβ through receptor mediated mechanisms. Generally, local catabolism or clearance mechanisms efficiently prevent accumulation of these amyloidogenic peptides in the brain. A loss of such clearance mechanisms may be responsible for the accumulation of Aβ in AD.

It has been reported that Aβ is neurotoxic both *in vitro* and *in vivo*. Aβ itself has been shown to damage neurons directly by causing plasma membrane lipid peroxidation, impairment of glutamate uptake and induce neuronal apoptosis. Although the extracellular exposure of cells to Aβ peptides is traditionally considered the primary pathway for generating cellular toxicity, a recent study has observed the Aβ and APP in mitochondria in brains from APP transgenic mice and AD patients which may promote mitochondrial stress (Anandatheerthavarada *et al.*, 2003; Devi *et al.*, 2006). The mechanisms by which Aβ and APP induce mitochondrial damage has not yet known. However, Aβ and APP are known to localize to mitochondrial membranes, block the transport of nuclear-encoded mitochondrial proteins to mitochondria, interact with mitochondrial proteins, alcohol dehydrogenase (ABAD), disrupt the electron-transport chain, increase reactive oxygen species production. These mechanisms may cause mitochondrial damage. Mitochondrial damage such as reduced mitochondrial membrane potential and ATP levels have been detected in the brains AD brain. However, the mechanism by which Aβ and APP transport to mitochondria has not yet known. Moreover, it has also been reported that Aβ can activate microglial cells to release a wide range of glial-derived neurotoxic molecules such as nitric oxide, proinflammatory cytokines and chemokines, creating apparent sites of intensive chronic inflammation. Importantly, it has been reported that activation of microglia is a relatively early pathogenic event that precedes the process of neuropil destruction in AD. According to this view, local recruitment of activated microglia may be a key step that enhanced neuronal cells death in AD.

2.3 Microglia

Microglial cells were first distinguished as a specific type of glial cells which are different from other glial cell population in the brain by Del Rio-Horteca. They are small glial cells (diameter of their somsta usually dose not exceed 5-10 μl) which are mesodermal in origin. During embryonic development, microglia progenitor, derived

from mesenchymal myeloid lineage progenitor cells, invade the developing brain regions at the time when the vascular supply of the brain is being elaborated. Until the early postnatal period, they migrate into essentially all brain areas and distribute throughout all parts of the CNS, where are more in gray than in white matter and the cerebral cortex and hippocampus have more microglia than do regions in brainstem and cerebellum. Due to their origin, microglia share the phenotypic markers of monocytes and macrophages, many of which are surface antigens with significant functional properties. Microglia represent the brain resident tissue macrophages and function as the scavenger cells in brain. In mature brain, microglia constitute from 5-20% of the total glial cells population (Lawson *et al.*, 1990) and constitute from 10-20% of the total cells population in the brain. Microglia are classified according their morphology into three types: resting ramified, activated, and amoeboid phagocytic, as shown in Figure 2.7. In mature brains, resting microglia exhibit a characteristic ramified morphology and are responsible for immune surveillance. Resting microglia become activated in response to brain injury or to degenerative process. Under such conditions the microglial cells undergo rapid proliferation and dramatic morphologic alterations, changing from resting ramified microglia into activated amoeboid microglia. These activated microglia migrate toward the site of the injury, where they try to remove the dying cells and cellular debris by releasing a variety of inflammatory mediator including cytokines and chemokines (as shown in table 2) that promote the acute inflammatory response. They also release trophic factors and anti-inflammatory factors that promote neuronal survival. If the dying cells and cellular debris can be removed and the neuronal injury can be repaired, activated microglia will return to resting state. Therefore, it is believed that an acute inflammatory response is generally beneficial to the CNS, since it tends to minimize further injury and contributes to repair of damage tissue. In contrast, if the dying cells and cellular debris can not be removed and the neuronal injury can not be repaired, the inflammatory mediators will be sustained release resulting in promote the chronic inflammation. Owing to the

chronic inflammation, there is often compromise of the BBB which increases infiltration of peripheral macrophages into the brain parenchyma to further perpetuate the inflammation. Rather than serving a protective role as does acute neuroinflammation, chronic inflammation is most often detrimental and damaging to nervous tissue. Thus whether neuroinflammation has beneficial or harmful outcomes in the brain may depend critically on duration of the inflammatory response. CNS degenerative diseases, including AD are associated with chronic inflammation and elevated levels of several cytokines.

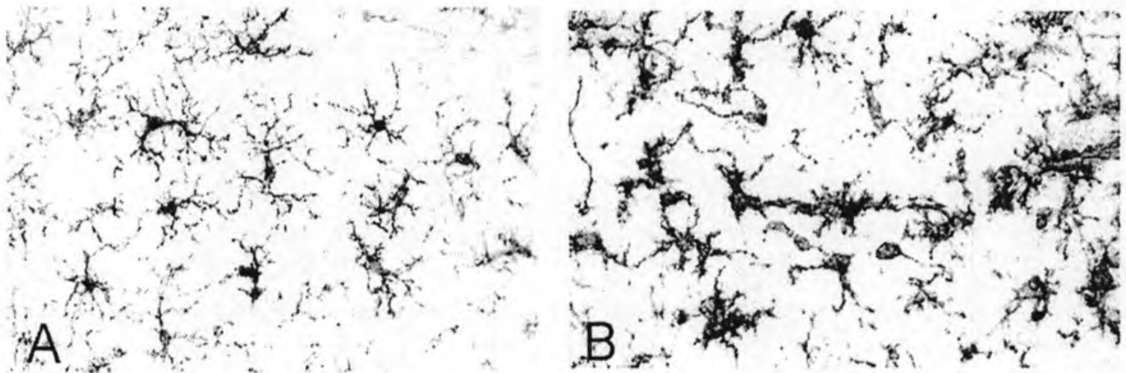

Figure 2.7 Morphology of microglial cells. (A) In the healthy brain, microglia are in a resting state. With their large cell surface pleated into humerous delicate cell branches, they survey the surrounding tissue. (B) Activated microglia, they change in cell shape (they start retracting many of the dilicate cell branches.) At this stage microglia begin to produce numerous important molecules, e.g. for cell-cell signalling.

Table 2 Pro-inflammatory cytokines, chemokines and neurotrophic factors with microglial synthesis (Block & Hong, 2005).

Abbrevation	Full length name	Function/effect
MCP-1	Monocyte chemoattractant protein 1	Chemotaxis
IL-8	Interleukin-8	Chemotaxis
MDC	Macrophage-derived chemokine/CCL22	Chemotaxis
MIP-1α	Macrophage inflammatory protein 1	Chemotaxis
MIP-1β	Macrophage inflammatory protein 1β	Chemotaxis
MIP-2	Macrophage inflammatory protein 2	Chemotaxis
MIP-3β	Macrophage inflammatory protein 3β	Chemotaxis
IL-3	Interleukin-3	Proliferation
M-CSF	Macrophage colony stimulating factor	Proliferation
IL-2	Interleukin-2	Growth factor/proliferation
IL-12	Interleukin-12	Proliferation/T-cell differentiation
PGE2	Prostaglandin E2	Pro-inflammatory, proliferation
IL-1β	Interleukin-1β	Pro-inflammatory
TNF-α	Tumor necrotic factor α	Pro-inflammatory
TGFβ	Transforming growth factor beta	Anti-inflammatory
IL-13	Interleukin-13	Immunosuppressive
IL-10	Interleukin-10	Immunosuppressive
NGF	Nerve growth factor	Neurotrophic
BDNF	Brain-derived neurotrophic factor	Neurotrophic
NT-3	Neurotrophin-3	Neurotrophic
NT-4	Neurotrophin-4	Neurotrophic
NO	Nitric oxide	Neurotoxic
$O^{\bullet}_2$	Superoxide	Neurotoxic
H_2O_2	Hydrogen peroxide	Neurotoxic
$OH^{\cdot}$	Hydroxyl radical	Neurotoxic
$NOO^{\cdot}$	Peroxynitrite	Neurotoxic
MMP-9	Matrix metalloproteinase 9	Neurotoxic

Clinicopathological studies and neuroimage reveal that neuroinflammation and microglial activation in specific cortical regions precedes neuronal damage in AD. Also, case-control and population-based studies supported a roughly 50% reduction in AD risk after long-term use of anti inflammatory drug. Moreover, it has been reported that inhibition of microglial activation in animal AD models attenuates neurotoxicity. These finding suggest that neuroinflammation and microglial activation is importance in AD. In AD brains, activated microglia are widely distributed throughout the brain parenchyma but are also focally concentrated within amyloid plaques where they generally throught to be actively engaged in clearance of Aβ from the plaque interior via phagocytosis, as shown in Figure 2.8.

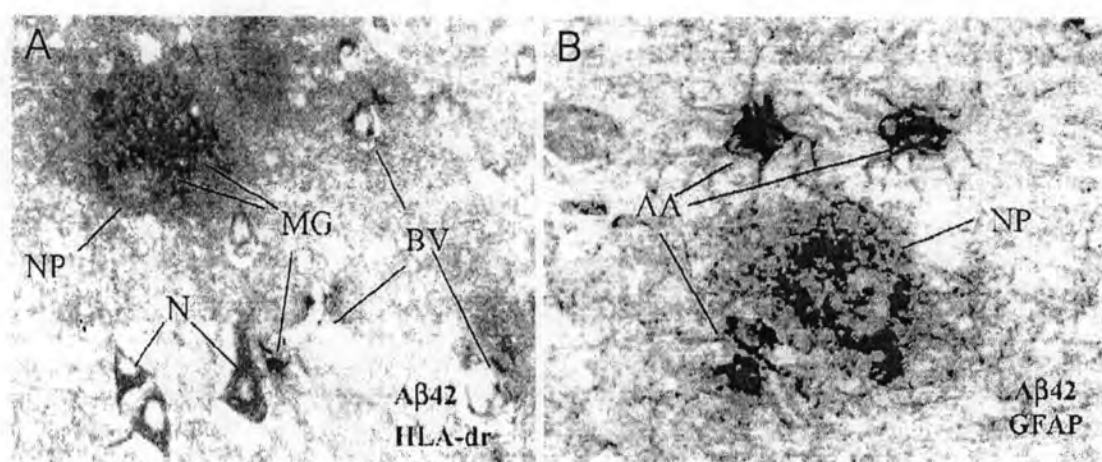

Figure 2.8 Activated microglia and activated astrocytes accumulate Aβ42-positive material. (A) Double immunolabeling with Aβ42 (red) and HLA-dr (blue) showing Aβ42-burdened neurons in the vicinity of a large neuron-derived plaque (NP). Microglia (MG) appear to immigrate into the brain from local blood vessels (BV) and congregate at the core of plaques (NP). (B) Double immunolabeling with GFAP (black)- and Aβ42 (red)-specific antibodies confirms the presence of intracellular Aβ42 in activated astrocytes (AA) positioned just outside of the boundary of plaques (NP) in the pyramidal cell layers (Nagele *et al.*, 2004).

It has been reported Aβ can induce microglial activation result in the release of neurotoxic factors from microglia, such as NO, TNF-α and superoxide which contribute substantially to tissue damage as a pathogenic factor of AD. The transcription factor that plays a significant role in the inflammatory response of activated microglia includes an inducible nuclear factor-kappa B (NF-κB), which requires the activation of IκB kinase that phosphorylates inhibitor alpha of NF-κB leading to ubiquitination and degradation and resulting in the nuclear translocation of NF-κB. Aβ has also been reported to interacts with CD14, the LPS receptor, which induce nuclear translocation of NF-κB in activated microglia. However, because microglia are phagocytic cells, many studies indicate that the phagocytosis of Aβ is neuroprotective, as microglia may take up and degrade Aβ. In vitro studies have clearly demonstrated that microglia can actively degrade Aβ isolated from AD brain (Shaffer *et al.*, 1995; Ard *et al.*, 1996). However, this condition dose not occurs at all in AD brain. If microglial cells become unable to clear Aβ in AD brain, Aβ will sustain microglial activation subsequence over activation of microglia, which become detrimental, inducing the release of potentially toxic mediators. However, it has not yet know that why microglia appear unable to remove plaque deposition in AD brain.

In fact that microglia activation in AD brain are progressive and generally accompanied by intracellular accumulation of iron (Grundke-Iqbal *et al.*, 1990; Connor *et al.*, 1992a; Connor *et al.*, 1992b). These activated microglia increased cellular iron uptake by specifically expressed iron binding protein p97. Serum and CSF levels of p97 are also elevated in AD patients. These data lead us to hypothesize that iron loading in activated microglia could impair microglial phagocytic activity, therefore promoting extracellular deposits of tissue debris and protein fragment. Subsequently, Aβ-activated microglia within the plaques release multiple inflammation-related factors, featuring a site of intensive chronic inflammation. However, it is not known what role of iron played in activated microglia on pathogenesis of AD, particularly in the formation of the senile plaques. Since it has been reported that iron progressively

accumulated in brain with age, interestingly, more iron staining has also been observed in the microglia. Therefore, iron accumulation in microglia might affect microglial neuroinflammatory processes that contribute to AD. Recent study has been reported that GSK-3β, a serine/threonine kinase originally identified as a key enzyme regulating glycogen synthesis regulated the transcription activity of NF-κB. Moreover, GSK-3β has also been reported to be involved inflammatory processes in peripheral nervous system. Importantly, GSK-3β activity has been reported to be increased with age (Lee *et al.*, 2006). Similar findings have also been reported for brain iron levels. Furthermore, the activity of GSK-3β has been shown to be elevated in AD brain (Pei *et al.*, 1999), where iron accumulation is pronounced (Connor *et al.*, 1992a; Kasarskis *et al.*, 1995). Accordingly, increased levels of brain iron may be related to elevated levels of GSK-3β activity and may contribute to the neuroinflammatory processes

2.4 Glycogen synthase kinase-3

Glycogen synthase kinase-3 (GSK-3) was characterized in 1980 as a one of protein kinase that able to phosphorylated and deactivated glycogen synthase, a key enzyme involved in the glycogen synthesis (Embi *et al.*, 1980). There are two mammalian isoforms of GSK-3: GSK-3β and GSK-3β. These isoforms are encoded by different genes and have molecular weights of 51 and 47 kDa, respectively. The gene that encodes GSK-3β is located on chromosome 19 while the gene that encodes GSK-3β is located on chromosome 3. These two isoforms of GSK-3 share nearly identical sequences in their kinase domains (98%) but differ within their N- and C-terminal regions. The largest structural difference between the two is amino-terminal, glycine-rich region specific to GSK-3β and this accouts for the 4 kDa difference in the protein masses. In adulthood GSK-3β and GSK-3α are most prominently expressed in the brain with particular abundance in hippocampus, neocortex, and cerebellum, where are found in both neurons and glia. However, in adult hippocampus, GSK-3β expression is

more abundant than GSK-3α expression, and the hippocampal GSK-3β, but not GSK-3 α expression increases with ageing.

The constitutive activity of GSK-3 arises from phosporylation of tyrosines 279 and 216 (for GSK-3α and GSK-3β, respectively) (Hughes *et al.*, 1993). However, little is known about regulation of the processes that modulate tyrosine phosphorylation of GSK-3. In opposition to tyrosine phosphorylation, the most well-defined regulatory mechanism is inhibition of the activity of GSK-3 by phosphorylation of a regulatory serine in either of the two isoforms of GSK-3, serine-9 in GSK-3β or serine-21 in GSK-3α (Woodgett, 1990). This inhibitory phosphorylation can be mediated by several signaling cascades, however, the most well-described signaling pathway is the insulin signaling pathway (as shown in Figure 2.9) (Cross *et al.*, 1995; Grimes & Jope, 2001). The binding of insulin to its receptor activates the intrinsic protein tyrosine kinase activity of the receptor, allowing it to phosphorylate itself at several sites. One phosphotyrosine residue interacts with the phosphotyrosine-binding domain of the insulin receptor substrate proteins (IRS), recruiting them to the plasma membrane, where they undergo phosphorylation by the insulin receptor. As a result, they interact with the Src-homology-2 (SH2) domains of the p85 subunit of phosphatidylinositol 3-kinase (PI3K). This recruits PI3K to the plasma membrane, enabling the p110 catalytic subunit to catalyse the formation of phosphatidylinositol-3,4,5- trisphosphate (PI(3,4,5)P3) from PI(4,5)P2. PI(3,4,5)P3 binds to the domains of both 3-phosphoinositide-dependent protein kinase 1 (PDK1) and PKB/AKT, co-localizing them at the membrane and allowing PDK1 to activate protein kinase B (PKB/Akt). PKB/Akt, in turn, phosphorylates and inhibits GSK-3, resulting in the dephosphorylation of substrates of GSK-3, including glycogen synthase and eukaryotic initiation factor 2B (eIF2B). This contributes to the insulin-induced stimulation of glycogen and protein synthesis. In addition to mediating insulin's effects, PI3K and Akt have distinct properties as mediators of the actions of neurotrophic molecules and this represents the most established pathway by which GSK-3 exerts its neurotrophic/

neuroprotective effects. Neurotrophic factors are necessary for survival and functioning of neurons. Their actions increase cell survival both by providing necessary trophic support for growth and by exerting inhibitory effects on cell death pathways. The primary actions of neurotrophic factors are mediated via intracellular signaling pathways following binding to a Trk receptor. For instance, the most prevalent neurotrophic factor, brain-derived neurotrophic factor (BDNF), exerts its initial actions by interacting with TrkB. TrkB binding of BDNF initiates TrkB dimerization, which results in autophosphorylation of multiple tyrosine residues in its cytoplasmic domain, and thereafter modulation of intracellular signaling pathways including activation of PI3K, of which a primary target is Akt. Akt is then phosphorylates GSK-3. The precise downstream mechanisms that mediate GSK-3's actions in neurotrophic pathways are not fully understood, but are believed to include effectors such as p53, cyclic AMP response element binding protein (CREB), heat–shock factor-1, C-Jun, and Bax. Other kinases that can regulate GSK-3 include protein kinase C (PKC) and protein kinase A (PKA), as shown in Figure 2.9 (Fang *et al.*, 2000; Li *et al.*, 2000; Fang *et al.*, 2002). For regulation by PKC and PKA, activation of G-protein-coupled receptors (GPCRs) linked to heterotrimeric G proteins (α-, β- and γ-subunits) that activate phospholipase-C β (PLCβ) causes the hydrolysis of PI(4,5)P2 to two second messengers, inositol trisphosphate (IP3), which increases intracellular calcium levels, and diacylglycerol (DAG). These messengers induce activation of PKC, which is capable of phosphorylating Ser9 of GSK-3β. Similarly, ligand binding of GPCRs coupled to G proteins that activate adenylyl cyclase (AC) to produce cyclic-AMP, leads to activation of PKA, which can also phosphorylate Ser9 of GSK-3β.

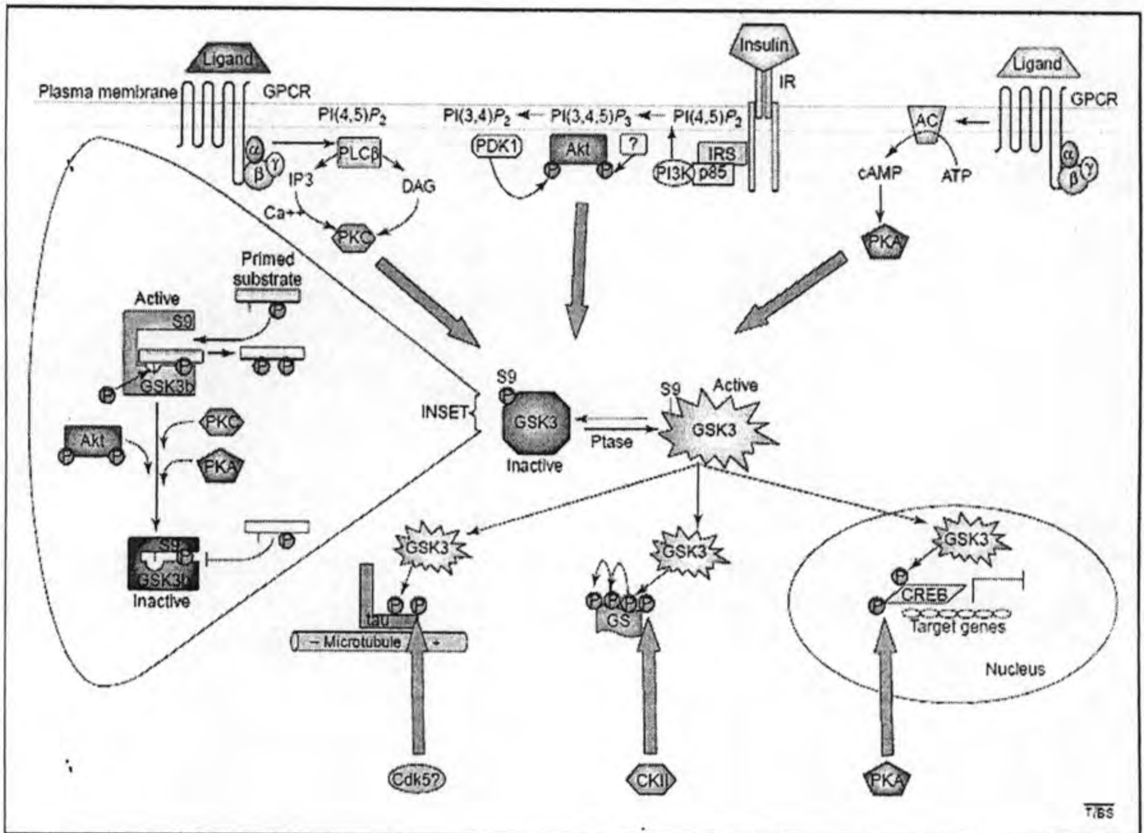

Figure 2.9 Regulation of GSK-3 activity by phosphorylation. Several signaling cascades initiate the phosphorylation of GSK-3 on its regulatory N-terminal serine residue (Jope & Johnson, 2004).

GSK-3 activity is additionally regulated by binding proteins; for example, in the Wnt signaling pathway, a pathway that controls multiple aspects of development, including the proliferation and migration of cells (Ruel *et al.*, 1999; Ding *et al.*, 2000). A proportion of GSK-3 in cells is present in a multiprotein complex together with axin, the adenomatous polyposis coli (APC) protein and β-catenin. In the absence of secreted glycoproteins, called Wnt, the GSK-3 in this complex is active and phosphorylates axin, APC and β-catenin. Axin is stabilized by phosphorylation, but phosphorylation of β-catenin targets it for ubiquitylation and subsequent proteolytic destruction. The role of APC phosphorylation is less clear, but it seems to enhance its interaction with β- catenin. After the binding of Wnt to their receptors results in the activation of disheveled (dvl), which, in concert with the GSK-3-binding protein Frat

(frequently rearranged in advanced T-cell lymphomas), facilitates disruption of axin (and hence APC and β-catenin). This leads to decrease the phosphorylation of axin, APC and β-catenin by GSK-3. The decreases the phosphorylation of β-catenin by GSK-3 leads to its accumulation in cells and translocation to the nucleus, where it binds to members of the T-cell factor (TCF) family of transcription factors (also called LEF, for lymphoid-enhancer factor), and stimulates the transcription of genes that are required for embryogenesis. This is a classical example of how GSK-3 binding proteins can regulate the action of GSK-3 towards individual substrates (as shown in Figure 2.10).

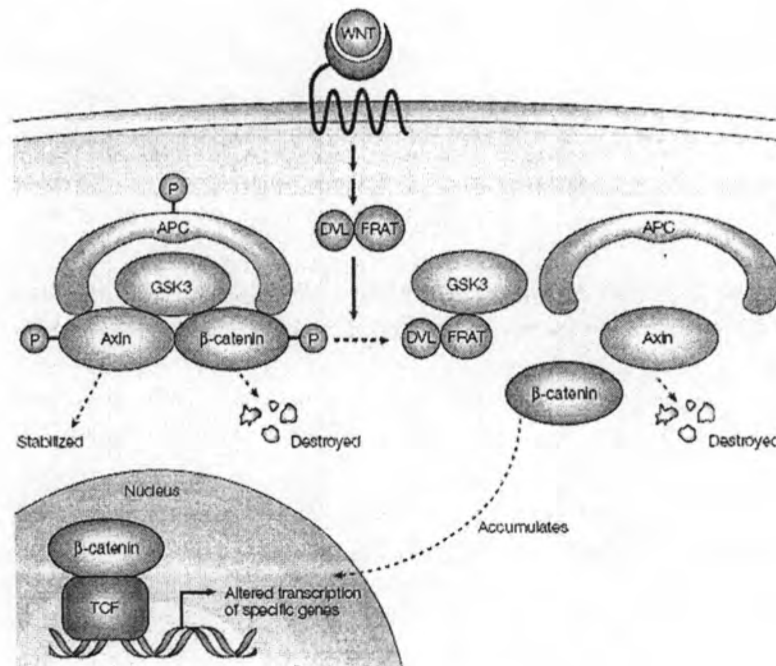

Figure 2.10 The mechanism by which Wnt prevent the GSK3-catalysed phosphorylation of axin, APC and β-catenin (Cohen & Frame, 2001).

The mechanism by which N-terminal serine phosphorylation inhibits GSK-3 activity relates to an unusual property of GSK-3 in phosphorylating its substrates. The minimal recognition motif for phosphorylation by GSK-3 is **S/TXXXS/T**(P), where 'X' is any amino acid, and the last Ser-P/Thr-P is the site of priming phosphorylation.

In order for GSK-3 to efficiently phosphorylate its substrate proteins, it requires that the substrate is previously phosphorylated (by another protein kinase) at a serine or threonine residue located four residues C-terminal (underlined in consensus motif) to the site of GSK-3 phosphorylation (S/T residue in bold). However, upon phosphorylation of serine 9/21, folds back on itself, forming electrostatic interactions with several residues (including R96) that are involved in binding the priming phosphate (Figure 2.11). This conformation occludes the active site, preventing binding of primed substrates. Thus N-terminal serine phosphorylation acts as a 'pseudosubstrate' competitive inhibitor.

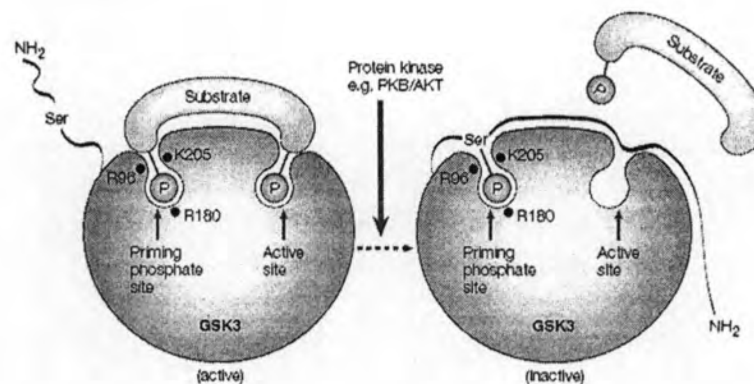

Figure 2.11 The molecular mechanism by which phosphorylation inhibits GSK-3. GSK-3 preferentially phosphorylates substrates that are pre-phosphorylated by a priming kinase. However, the N-termini of GSK-3α and β contain serine residues (S21 and S9 respectively) that, when phosphorylated by an inhibitory kinase, serve as primed pseudosubstrates that can occupy the substrate binding pocket of GSK-3, inhibiting its activity towards true substrates (Cohen & Frame, 2001).

Initially, the functions of GSK-3 were focused on glycogen metabolism; however, later studies discovered that GSK-3 is a multifunctional kinase with a critical role in the regulation of many cellular processes including glycogen metabolism, cell

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division, apoptosis, oncogenesis, and embryonic development. Due to it involved in the regulation of many cellular processes, the dysregulation of which has been implicated in the development of cancer, diabetes as well as in neurodegenerative diseases. In the brain of adulthood, GSK-3β and GSK-3α are most prominently expressed with particular abundance in hippocampus, neocortex, and cerebellum (Yao *et al.*, 2002). However, in adult hippocampus, GSK-3β expression is more abundant than GSK-3α expression, and the hippocampal GSK-3β, but not GSK-3 α expression increases with ageing (Lee *et al.*, 2006; Plattner *et al.*, 2006). Moreover, GSK-3β activity has been reported to be increased in AD (Pei *et al.*, 1999). From this reason GSK-3β has been extensively studied more than GSK-3β in neu-rodegenerative disease.

In AD, increased the levels of active GSK-3β has been found in AD brains compared with non-diseased (Woodgett, 1990; Pei *et al.*, 1999). It was proposed that the increased activity of GSK-3β accounts for tau phosphorylation, increased Aβ production and increased neuronal apoptosis. All of which are features of AD. The role of GSK-3β on tau phosphorylation has been immerged since it has been reported that GSK-3β associated with NFT in AD brains. GSK-3β is now widely acknowledged to be a tau kinase. GSK-3β can phosphorylate many sites on tau in vitro such as Ser199, Thr231, Ser235, Ser396, Ser404, and Ser413 (Ishiguro *et al.*, 1992; Yang *et al.*, 1993). These phosphorylations depress its ability to promote assembly of tubulin in microtubules. Recently, it was found specifically that the phosphorylation of Thr231 in tau by GSK-3β is a critical site in impairing the ability of tau to bind and stabilize microtubules in AD. Furthermore, in animal model, tau phosphorylation is increased in transgenic mice overexpressing GSK-3β (Lucas *et al.*, 2001). In addition to tau hyperphosphorylation, abnormal accumulation of Aβ is also a critical early stage in AD neuropathology, and several studies have shown that Aβ production is promoted by GSK-3β and reduced by GSK-3 inhibitors (Phiel *et al.*, 2003). However, the mechanism whereby GSK-3β promotes Aβ production remains to be clarified.

Because GSK-3β can phosphorylate APP and presenilin-1 (a catalytic domain of γ-secretase), this mechanism may be involved in Aβ production.

Moreover, a growing body of evidence suggests that GSK-3β is an important modulator of apoptosis. GSK-3β was first linked to the promotion of apoptosis by the finding that overexpression of GSK-3β is sufficient to cause apoptosis (Pap & Cooper, 1998) indicating its direct contribution to apoptosis signaling. Moreover, inhibition of GSK-3β has also been reported to protect cells from apoptotic cell death generated by a number of insults, such as DNA damage (Watcharasit *et al.*, 2003), serum deprivation (Linseman *et al.*, 2004), mitochondrial toxins (King *et al.*, 2001) and endoplasmic reticulum stress (Song *et al.*, 2002; Kim *et al.*, 2005). The two most common apoptotic pathways are the 'intrinsic' pathway and the 'extrinsic' pathway. The intrinsic pathway in which cellular stresses disrupt mitochondrial integrity while the extrinsic pathway is initiated by stimulation of 'death receptors' in the plasma membrane (Hengartner, 2000). Both pathways culminate in the activation of intracellular caspases. For intrinsic pathway, pro-apoptotic members of the Bcl-2 family of proteins are key messengers for delivering the apoptotic signal to the mitochondria (Akhtar *et al.*, 2004). "For example, one of these, Bax, undergoes an activating conformational change following cellular insults that causes it to translocate from the cytoplasm to the mitochondria where it can both sequester anti-apoptotic Bcl-2 family proteins and oligomerize within the mitochondrial membrane (Martinou & Green, 2001)". This oligomerization of Bax in the outer mitochondrial membrane contributes to the disruption of the mitochondrial membrane potential and the release of apoptotic proteins such as cytochrome c from the mitochondrial intermembrane space into the cytoplasm (Armstrong, 2006) which can bind to the protein apoptotic protease activating factor-1 (APAF-1), ATP/dATP, and procaspase-9 to form the apoptosome in the cytoplasm. This causes the activation of caspase-9, thereby triggering the activation of the caspase cascade (Armstrong, 2006). GSK-3β has been reported to enhance this pathway by phosphorylation of Bax which lead to increase

Bax translocation to mitochondria. Several studies have demonstrated that dying cells display the characteristics of apoptosis in AD brains and in cultures of neurons exposed to Aβ. Moreover, it has also been reported that Aβ1-42 treatment significantly increased the activity of GSK-3β f ollow by increased neuronal cell death through intrinsic apoptotic pathway while treatment with a GSK-3β inhibitor prevented this mechanism.

Recently, a novel role of GSK-3β in peripheral inflammatory process has been reported. This role of GSK-3β in inflammation was first established by the report that GSK-3β activity is necessary for full stimulation of the production of several pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-1β, and TNF, following stimulation of Toll-like receptors in monocytes and peripheral blood mononuclear cells (Martin *et al.*, 2005). In contrast, GSK-3β reduced the production of the anti-inflammatory cytokine IL-10 (Martin *et al.*, 2005).Moreover, inhibitors of GSK-3β significantly reduced the production of pro-inflammatory cytokines while increased anti-inflammatory cytokine production. This raised the novel possibility that inhibitors of GSK-3β may prove to be beneficial in many conditions involving inflammation. GSK-3β inhibitors were also reported to reduce the systemic inflammatory response, renal dysfunction, and hepatotoxicity associated with endotoxemia in rats (Dugo *et al.*, 2005). Furthermore, GSK-3β inhibitors also diminished inflammatory responses in experimentally induced colitis in rats (Whittle *et al.*, 2006) and in arthritis caused by administration of type II collagen in mice (Cuzzocrea *et al.*, 2006). Arthritis and peritonitis in mice were also reduced by *in vivo* administration of the GSK-3β inhibitor lithium (Xiao *et al.*, 2006). The mechanism by which GSK-3β regulate the production of pro-inflammatory cytokines is not known. One proposes mechanism by which GSK-3β regulates the inflammatory process through regulation of IκBα kinase, known to be required for efficient activation of NF-kB. From this mechanism, it has been demonstrated that GSK-3β involved in IKK activation, IκBα degradation and NF-kB nuclear translocation (Takada *et al.*, 2004; Whittle *et al.*, 2006). Altogether, it is now

evident that GSK-3β is an important component of the peripheral inflammatory response.

These combined evidences strongly suggest that increased GSK-3β activity may contribute not only to the formation of NFT, amyloid plaques and apoptosis but also to inflammatory component of AD neurodegeneration. However, little is known about the function of GSK-3β in the cerebral inflammatory response, which is predominantly mediated by microglia. If, as discuss above, increased GSK-3β activity in AD brain might impact upon the cerebral inflammatory response leading to the sustained secretion of neuro-toxic inflammatory mediators by microglia, in turn causing by-stander damage to neighboring neurons and contributing to neurodegenerative processes. Therefore, if we know the factor that increased GSK-3β activity in AD may be beneficial in the treatment of this disease. Importantly, GSK-3β activity has been reported to be increased with age (Lee *et al.*, 2006). Similar findings have also been reported for brain iron levels. Furthermore, the activity of GSK-3β has been shown to be elevated in AD brain (Pei *et al.*, 1999), where iron accumulation is pronounced (Connor *et al.*, 1992a; Kasarskis *et al.*, 1995). Accordingly, increased GSK-3β activity in AD brain may be related to elevated levels of brain iron and may contribute to the pathogenesis of AD.

Therefore, the first part of this study iron supplemented and lipopolysaccharide (LPS)-activated cultures of BV2 microglia was developed to mimic progressive iron accumulation by activated microglia and used this model to address the functional relationship between iron and microglial activation, which demonstrated to be partially mediated by GSK-3β. The influence of iron on NF-κB-mediated microglial activation in association with GSK-3β activity levels was also investigated. The involvement of GSK-3β in the control of LPS-activated microglial cells was examined using lithium chloride (LiCl), a potent pharmacological inhibitor of GSK-3. The cellular activation of microglial cells induced by LPS was determined by the levels of NF-κB nuclear

translocation, the expressions of pro-inflammatory cytokines, the inflammatory mediators, including IL-1β, TNF-α, NO and MMP-9 and neurotoxicity respectively.

As mentioned earlier that mutation in the HFE gene increased cellular iron uptake and altered brain iron level in HH, a disorder of iron overload (Nielsen *et al.*, 1995; Berg *et al.*, 2000). Furthermore, several epidemiological studies have found an association of HFE variants with AD (Sampietro *et al.*, 2001; Pulliam *et al.*, 2003; Berlin *et al.*, 2004; Connor & Lee, 2006). However, the mechanism has not yet unknown. There is increasing evidence that accumulation of iron in the brain can induce oxidative stress, alter mitochondrial function and neurodegeneration. Increased cellular iron uptake in HFE mutation might increase oxidative stress, alter mitochondrial function which might be a factor of neuronal cell death. Moreover, increased cellular iron uptake in HFE mutation might alter GSK-3β activity leading to increase neuronal Aβ production and apoptosis. All of which are AD features. Thus, the second part, neuroblastoma cell lines expressing a mutation of H63D HFE variant was used to explore the mechanisms behind the associations between cellular iron status, oxidative stress, mitochondrial function, GSK-3β activity, neuronal apoptosis, and Aβ production. GSK-3β activity, Aβ production and neuronal apoptosis. Because it has been reported that Aβ peptide increased GSK-3β activity leading to increase neuronal apoptosis in AD. Therefore, the effect of Aβ peptide on neuronal GSK-3β activity and neuronal apoptosis in H63D HFE variant were also investigated.