CHAPTER V

CONCLUSION AND DISCUSSION

In this study, GSK-3β, a major kinase that plays a pivotal role in tauopathies as reported in Alzheimer's disease (AD) and frontotemporal dementia, was introduced as a molecular target involving in microglial activation. The results demonstrated that inhibition of GSK-3β by LiCl attenuated the production of IL-1β, TNF-α, NO, MMP- 9 from activated microglia. These results are in line with an emerging role of GSK-3β in central (Cheng et al. 2009; Huang et al. 2009) and peripheral inflammatory processes (Hoeflich et al. 2000; Martin et al. 2005; Whittle et al. 2006; Zhang et al. 2009). Therefore, agents that inhibit GSK-3β activity may benefit the treatment of neurodegenerative diseases that involve microglial activation and neuronal dysfunction associated with abnormal tau phosphorylation. This view has been supported by recent in vivo findings that the inhibition of GSK-3β reduces microgliosis and protects hippocampal neurons from Aβ-induced neurodegeneration (Hu et al. 2009).

In the brain, GSK-3β is constitutively expressed (Yao et al. 2002) and its activity has been reported to be increased with age (Lee et al. 2006). Similar findings have also been reported for brain iron levels, numbers of activated microglia and iron accumulation by microglia (Zecca et al. 2004). 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. 1992; Kasarskis et al. 1995). Accordingly, increased GSK-3β activity in the normal aged brain and in AD brain might relate to elevated levels of brain iron. Since in most cases, if not all, more iron is observed in reactive phenotype of microglia; I, therefore, examined the relationship between iron, GSK 3β activity and microglial activation. The results clearly demonstrated that elevated levels of extracellular iron increased GSK-3β activity and nuclear translocation of NF-κB in microglia. These results provide evidence that iron accumulation in normal aged brain and AD brain may be one of the primary

events that initiate a cascade of neurodegeneration, at least, by priming the activation of microglia. Although a mechanism by which iron increased GSK-3β activity in microglia reported herein is not known, a recent finding indicates that iron may increase GSK-3β activity by reducing the activation of Akt, an upstream kinase that inhibitory phosphorylates GSK-3β at serine 9 (Dongiovanni et al. 2008). The present study also demonstrated that increased cellular iron status during microglial activation due to the elevated levels of extracellular iron enhanced the release of MMP-9, on the other hand, ameliorated the release of IL-1β and NO. Together with the results obtained from neurotoxicity assay, it is most likely that iron enhanced neurotoxicity of activated microglia may be mediated through MMP-9. Although, our neurotoxicity was performed in neuroblastoma cells line, this preliminary results may be modified to take advantage of both in primary cells and in vivo approaches. Indeed, other studies have demonstrated the neurotoxicity of MMP-9 in different experimental models. For example, the neurotoxicity of TNF-α-stimulated primary microglial cells derived from MMP-9 null mice is substantially reduced, compared to wide type microglia (Kauppinen et al. 2005). Furthermore, the neurotoxic effects associated with rapid microglial activation provoked by kainate-induced excitotoxicity are alleviated by the inhibition of MMP-9 (Jourquin et al. 2003). Specifically, these results also suggest that iron accumulation by activated microglia may contribute to the elevated levels of MMP-9 in the brain and plasma of patients with AD as previously reported (Lorenzl et al. 2003). Although the concentrations of iron (100 and 300 μg/ml) used in this study appears to lower the release of NO and IL-1β, iron at 300 μg/ml tended to increase transcript levels and the production of IL-1β and iNOS/NO, as well as GSK-3β activity and nuclear translocation of NF-κB in activated microglia compared to iron at 100 μg/ml. Therefore, it was speculated that if activated microglia are saturated with iron, their ability to produce cytokines should be enhanced. Supportively, a recent finding demonstrates that if microglia are loaded with lipidsoluble iron compound prior to their activation, the release of IL-1β and TNF-α is significantly increased. In the contrary, if iron in microglia is depleted prior to their activation, the

release of these cytokines is significantly reduced (Zhang et al. 2006). Taken together, it is clear that state of microglial activation is strongly influenced by cellular iron status. The results demonstrating that iron differently regulated the expression of MMP-9, iNOS, IL-1β and TNF-α imply that elevated cellular iron levels may trigger an iron-responsive mechanism capable of modulating pattern of gene expression in activated microglia. Such mechanism may involve other transcription factors in addition to NF-κB. This is consistent with the finding that although inhibition of GSK-3β by LiCl reduced the nuclear levels of NF-κB close to that of control, even in the presence of iron, but it does not completely inhibited the expression of the examined genes. Thus, multiple NF-κB independent pathways may be activated during microglial activation. For example, the binding of NF-κB and transcription activator protein-1 (AP-1) to the MMP-9 promoter has been reported to be essential for the induction and expression of MMP-9 gene in response to the stimuli, including LPS (Yoshizaki et al. 1998). Here, the result was demonstrated that iron promoted NF-κB nuclear translocation. Furthermore, it has been reported that AP-1 DNA binding activity is enhanced by iron (Kaomongkolgit et al. 2008). Therefore, iron-enhanced expression and release of MMP-9 from activated microglia as reported herein may result from a synergistic activation of NF-κB and AP-1 in response to LPS and iron. Although the results shown that iron increased NF-κB nuclear translocation, it has been reported that the key transcription factors that regulate the iNOS expression are NF-κB and NF-IL6 (Dlaska and Weiss 1999; Goldring et al. 1996; Lowenstein et al. 1993; Martin et al. 1994). The former acts as a first inducer of the iNOS transcription whereas the later is importance for maintaining a high transcriptional rate of the iNOS transcription after LPS stimulation (Dlaska and Weiss 1999; Sawada et al. 1997). However, it has been reported that the binding of the NF-IL6 to its promoter was reduced by iron (Dlaska and Weiss 1999). Therefore, the presence of iron in cultured of LPS-activated microglia might reduce the binding of NF-IL6 to the iNOS promoter leading to a decrease in the expression of iNOS when compared to LPS-treated alone. Therefore, although, iron increased NF-κB nuclear translocation, in the same time iron might reduce the binding of

NF-IL6 to iNOS promoter leading to a decrease in iNOS expression. This result was consistent with previous study where iron increased the activation of NF-κB while reduced NO production (Ward et al. 2002). NF-κB and the NF-IL6 have also been reported to be the key transcription factors that regulated the expression of IL-1β (Zhang and Rom 1993). Therefore, the mechanism by which iron reduced IL-1β expression in cultured of LPS-activated microglia might be similar to that iron regulate iNOS expression. Although, NF-IL6 binding site has been reported on the TNF-α promoter, the contribution of NF-IL6 to the transcriptional activation of TNF-α may be minor, because, unlike that of iNOS and IL-1β, iron does not affect the expression TNF-α from activated microglia. This is consistent with the finding that NF-κB is a primary transcription factor that regulates TNF-α expression by acting in synergy with other transcription factors including AP-1 (Collart et al. 1990).

The results in this part suggest that the presence of iron appears to modify microglial activation and its associated neurotoxicity, which were partly due to the regulatory role of iron on GSK-3β activity.

Recent evidences have been reported that mutation in the gene involved in iron absorption such as H63D HFE increased brain iron accumulation and associated with AD (Berlin et al. 2004; Connor and Lee 2006; Pulliam et al. 2003; Sampietro et al. 2001). However, the mechanism has not yet known. Previously, it has been reported that neuroblastoma cells line expressing H63D HFE variant increased cellular iron concentration when compared to cells expressing WT HFE. Therefore this model was took to further investigate the mechanisms of iron on neurodegeneration. Here I demonstrated that neuroblastoma cells line expressing H63D HFE variant increased ROS. There is increasing evidence that accumulation of iron in the brain can induce oxidative stress which may induce neurodegeneration. Free iron induces oxidative stress through its interaction with hydrogen peroxide (Fenton reaction), resulting in increased formation of hydroxyl free radicals. Free radical-related oxidative stress causes molecular damage that can then lead to a critical failure of biological functions and ultimately cell death.

Hydroxyl radicals might also injure mitochondrial proteins or induce lipid peroxidation. Lipid peroxidation may lead to modifications of the mitochondrial membrane functional integrity which lead to increase mitochondrial membrane permeability, cause mitochondria to become further depolarized, meaning that mitochondrial membrane potential is abolished. Loss of mitochondrial membrane potential interferes with the production of adenosine triphosphate (ATP), the cell's main source of energy, because mitochondria must have an electrochemical gradient to provide the driving force for ATP production. The cells expressing H63D HFE variant also showed significantly decreased mitochondrial membrane potential and cytochrome c oxidase activity when compared to the cells expressing WT HFE. Therefore, these finding suggest that increased cellular iron levels in H63D mutation might be a one mechanism that trigger the onset of neurodegeneration at least in part by increasing oxidative stress, alteration of mitochondrial function.

It has been reported that oxidative stress can induce GSK-3β activity. The first part of this study, the result was demonstrated that iron increased GSK-3β in activated microglia. Since over-activity of GSK-3β has also been reported to play a pivotal role in others pathogenesis of AD such as tau hyperphosphorylation, Aβ production and neuronal apoptosis and its activity has been shown to be elevated in AD brain (Pei et al. 1999), where iron accumulation is pronounced (Connor et al. 1992), It was hypothesized that elevated iron concentration in H36D HFE variants might increase GSK-3β activity, neuronal apoptosis and Aβ production. The result showed that cells expressing the H63D allele increased GSK-3β activity and neuronal apoptosis compared to the cells expressing WT HFE. GSK-3β is a critical activator of neuronal apoptosis that can phosphorylate Bax and promote its mitochondrial localization during neuronal apoptosis (Linseman et al. 2004). In this study, increased baseline levels of Bax in the mitochondria in H63D cells compared to WT cells and the levels of Bax in the mitochondria increased even further with Aβ exposure were demonstrated. Therefore, increased mitochondrial Bax in H63D cells is consistent with increased the activity of GSK-3β. It has been reported that Bax can

release cytochrome c by interacting with other family proteins generating pores in the mitochondria membrane that stimulate the release of cytochrome c from the mitochondria (Zong et al. 2001). The subsequent cytosolic cytochrome c then interacts with Apaf-1 and pro-caspase-9 to form a functional apoptosome that ultimately activates downstream executioner caspases like caspase-3 (Zou et al. 1999). Cytochrome c levels, caspase-9 and caspase-3 were also increased in H63D expressing cells compared to cells expressing WT HFE. These effects were further elevated with Aβ peptide exposure. Since the result shown that a great degree of intrinsic apoptotic pathway in H63D HFE variant, however, the levels of apoptosis shown slightly increased compared to the cells transfected with WT HFE. Therefore, active caspase-8 was measured to evaluate the extrinsic apoptotic pathway. Cells expressing H63D HFE displayed a reduction in active caspase-8 compared to WT HFE cells. Aβ treatment also resulted in a decrease of active caspase-8 in the H63D cells compared to cells expressing WT HFE. Importantly, GSK-3β activity has been reported to influence the two major pathways of apoptosis, but in opposite directions; directly enhancing the intrinsic pathway while inhibiting the extrinsic pathway (Beurel and Jope 2006). Collectively, these findings support the idea increased cellular iron concentration in H63D cells elevates GSK-3β activity leading to an up-regulation of the intrinsic apoptotic pathway while inhibition of the extrinsic apoptotic pathway. The mechanism by which H63D mutation decreased extrinsic apoptotic pathway may be compensatory effect to decrease a number of neuronal cell death in H63D HFE mutation. However, these findings implicate that the intrinsic apoptotic pathway might be prominent in cells expressing the H63D HFE variant.

The cells expressing the H63D HFE variant also increased Aβ production in mitochondrial. However, Aβ levels in cell cultured medium did not change. Although the extracellular Aβ production is the major pathological features of AD, it has been reported that in AD patients, intracellular Aβ precedes extracellular Aβ deposition (Gouras et al. 2000). Moreover, in transgenic AD mice has also been reported intracellular Aβ accumulates early in AD progression (Gouras et al. 2000; Murphy et al. 2007; Oddo et al.

2003). Aβ is generated in neurons, wherever APP and β and γ-secretases are present, in particular, in several intracellular sites, including Golgi apparatus, endoplasmic reticulum and endosomal–lysosomal systems. Several studies suggest that Aβ accumulates in cellular compartments, interferes with normal cell function and promotes cellular changes. Aβ in mitochondria has been reported in neurons in a post-mortem analysis of AD patients and in brain neurons from cell models and transgenic mouse models of AD (Devi et al. 2006). Recent studies have shown that mitochondrial Aβ can induces the generation of free radicals, increases oxidative damage lead to impaired oxygen consumption, decreased respiratory chain complex, decreased mitochondrial membrane potential (Anandatheerthavarada et al. 2003; Bishop et al. 2002; Cardoso et al. 2001; Limdi and Crampton 2004; Lustbader et al. 2004). Therefore increased mitochondrial Aβ production in the cells expressing the H63D variant might be a one factor that enhances oxidative stress, alters mitochondrial functions leading to increase neuronal dying by apoptosis. Increased GSK-3β activity has been reported to promote Aβ production, however, the mechanism whereby GSK-3β promotes Aβ production remains to be clarified. Since GSK-3β can phosphorylate presenilin-1, a catalytic domain of γ-secretase, this mechanism may be involved in Aβ production. Therefore, increased cellular iron concentration in the cells expressing the H63D variant leading to increase GSK-3β activity might be a one factor that enhances Aβ production. However, the mechanisms by which Aβ transported thought mitochondria is unclear. It has been reported that mitochondrial import of Aβ may be required the interaction with all major translocates of outer membrane (TOM) protein such as TOM20, TOM70 and channel forming protein TOM40 (Hansson Petersen et al. 2008).

In summary, it has been reported brain iron increases with age and is abnormally elevated early in the disease process in several neurodegenerative diseases including AD. However, it has not yet known that elevated iron in AD brain is an initial event that causes neurodegeneration or a consequence of the disease process. The present study demonstrates that iron increase GSK-3β activity, nuclear levels of NF-κB and neuronal

toxicity mediated by activated microglia. These results provide evidence that iron accumulation in normal aged brain and AD brain may be a one of the primary events that initiate a cascade of neurodegeneration, at least, by priming the activation of microglia. Increased cellular iron levels in the mutation of H63D HFE increase oxidative stress while decrease mitochondrial membrane potential and cytochrome c oxidase activity, markers of mitochondrial damage. The mutation of H63D HFE also increases GSK-3β activity and neuronal apoptosis. All of which might be factors that trigger the onset of neurodegeneration in AD. Therefore, these results provide insight into a novel mechanism of how the dysregulation of brain iron may contribute to a cascade of neurodegeneration in AD.