BRAIN AMYLOIDOPATHY IN A MOUSE MODEL OF ALZHEIMER'S PATHOLOGY

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THESIS

Presented to the Faculty of

The University of Texas at Dallas

In Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN

MOLECULAR AND CELL BIOLOGY

THE UNIVERSITY OF TEXAS AT DALLAS

May 2020

ACKNOWLEDGMENTS

I would like to take this opportunity to express my sincere gratitude to Dr. Heng Du, my supervising professor, for giving me an opportunity to work on this project. I would like to thank Dr. Lan Guo for her support and guidance throughout the course of my thesis. I wish to thank my supervising committee member, Dr. Zhenyu Xuan, for his support and advice. I am also very thankful to my lab colleagues, Dr. Chunxia Yu, Jing Tian and Nazia H. Aminath, for their cordial support. I would like to give special credits to Dr. Chunxia Yu and Jing Tian for helping me with the experiments, for performing immunofluorescence staining experiments and for performing microglia analysis to support my data. Finally, I would like to acknowledge the love and support of my parents, Udhayakumar Ramakrishnan and Sandhiya Udhayakumar, and my sister, Bhuvaneshwari Udhayakumar.

April 2020

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The University of Texas at Dallas, 2020

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Alzheimer's disease (AD) is a chronic neurodegenerative disorder, characterized by progressive cognitive decline. Presence of amyloid plaques and neurofibrillary tangles in the brain are hallmark AD pathology, which mediates synaptic injury and neuronal death, as well as neuroinflammation. Here we outline the various factors associated with brain amyloidosis in an AD mice models. We observed progressive Amyloid beta (Aβ) production and deposition in the brain of the model mice. In addition, activation of microglia accompanies the development of amyloid plaques with age, which is associated with increased neuroinflammation in the mouse model.

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CHAPTER 1

INTRODUCTION

Neurodegeneration occurring in the central nervous system is characterized by chronic progressive loss of the neuronal structure and functions (Huang, Chen, & Zhang, 2017). Most of the diverse neurodegenerative disorders share the common mechanism, characterized by the aggregation and deposition of abnormal proteins which leads to progressive central nervous system diseases. A significant proportion of morbidity and mortality in the developed world is caused by neurodegenerative disorders like Alzheimer's and Parkinson's disease (Skovronsky, Lee & Trojanowski, 2006).

Among the hundreds of different neurodegenerative disorders, attention has been given only to a handful of diseases and Alzheimer's is one of them. Alzheimer's, a common cause of dementia, accounts for 60 to 80 percent of dementia cases worldwide (Przedborski, Vila & Jackson-Lewis, 2003). Symptoms of Alzheimer's vary among people and individuals experience multiple symptoms that change over a period of years. Degree of damage to neurons in different parts of the brain is reflected in these symptoms (2018 Alzheimer's disease facts and figures).

Alzheimer's is a progressive neurologic disease that results in the irreversible loss of neurons, particularly in the cortex and hippocampus. The major pathological hallmarks of AD include extracellular and intracellular deposition of amyloid beta (Aβ), neurofibrillary tangles (NFTs), loss of synaptic transmission and ultimately neuron loss in the cortex and hippocampus area. (Guo, Tian, & Du, 2017) . About 7 percent of early-onset AD cases are familial with an autosomal dominant pattern of inheritance. These familial Alzheimer's disease (FAD) are linked to the presence of mutant genes that encode the amyloid precursor protein (APP) or the presenilins (PS1 or PS2). More than 270 highly penetrant mutations in these genes were reported to cause FAD (http://www.molgen.ua.ac.be/admutations/; http://www.alzforum.org/mutations). These mutant genes result in the dysfunction/death of

vulnerable populations of nerve cells, with the resulting clinical syndrome of progressive dementia (Price & Sisodia, 1998).

The APP and presenilin mutations associated with FAD result in the increased production of Aβ42 which has a main role in the pathogenesis of the disease. Several transgenic mice models of AD have been adopted in the research to gain more insights into the pathogenesis of the disease. 5xFAD mice expressing human APP with the Swedish double (K670N/M671L), Florida (I716V) and London (V717I) mutations, along with the PS1 mutation (M146L/L286V) were generated with robust neuronal loss. These mice models have high Aβ42/Aβ40 and Aβ42 accumulations were rapid at young ages (Schaeffer, Figueiro, & Gattaz, 2011). 5xFAD mice rapidly recapitulate major features of AD amyloid pathology and have been used as models to understand Aβ42 induced neurodegeneration and amyloid plaque formation (Oakley, Cole, Logan, Maus, Shao, et al., 2006).

Aβ is a product of the cleavage of APP protein by β-secretase followed by γ-secretase. APP can also be processed within its Aβ sequence by α-secretase, in a nonamyloidogenic pathway, resulting in sAPPα which is neuroprotective. APP processing has a therapeutic standpoint in AD because modulation/inhibition at different stages can be feasible to direct APP processing towards the nonamyloidogenic pathway (Thordardottir, KinhultStåhlbom, Almkvist, Thonberg, Eriksdotter, Zetterberg, et al. (2017). Mutations in APP alter the endoproteolytic cleavage pattern during APP processing, promoting β- and γ-secretase cleavage activities over α-secretase. Similarly, PS1 mutations alter APP processing in such a way as to enhance the production of Aβ42 (Hardy, and Selkoe, 2002).

Increasing evidence shows that deficient clearance rather than increased production of Aβ contributes to its accumulation in the brain (Baranello, Bharani, Padmaraju, Chopra, Lahiri, Greig, Pappolla, Sambamurti, 2015). Microglia are the primary immune effector cells in the central nervous system. They are found closely associated with the amyloid plaques and have the capability to internalize Aβ plaques (Lee, Landreth, 2010). Microglia can produce Aβ degrading proteases that are involved in the clearance of Aβ. After being phagocytosed by

microglia, Aβ are either degraded or reduced to shorter less toxic forms (Ries & Sastre, 2016). In addition to phagocytosis, microglia secrete proteolytic enzymes that degrade Aβ, indicating its neuroprotective role in AD (Hickman, Allison, El Khoury, 2008).

Tau is a microtubule-associated protein and is involved in stabilizing microtubule by promoting their polymerization and suppressing their dissociation. Neurofibrillary tangles found in the neurons are composed of aggregates of paired helical filaments, which are made up of abnormally phosphorylated tau that lost its microtubule binding capacity (Lee, Perry, Moreira, Garrett, Liu, Zhu, Takeda, Nunomura, Smith, 2005). Increase in tau expression enhances susceptibility to toxic stimuli and/or neurodegeneration and also results in increased production of neurotoxic amyloidogenic peptides (Hanger, Anderton, Noble, 2009). A link between APP processing and tau proteostasis that is regulated by γ- and β-secretase has been found (Moore, Evans, Andersson, Portelius, Smith, et al., 2015). Also, Aβ oligomers have the capacity to cause abnormal tau phosphorylation in brain and this phenomenon is found in numerous APP-Tg mice models (Tomiyama, Matsuyama, Iso, Umeda, Takuma, et al., 2010).

Inflammation is a complex cellular and molecular defense mechanism. Neuroinflammation, characterized by the upregulation in inflammatory molecules and activation of glial cells (microglia and astrocytes) surrounding the plaques is involved in neurodegenerative disease. When microglial cells are heavily activated by high levels of Aβ, there is an increase in the production of proinflammatory molecules which trigger neuronal damage with reduced ability to clear Aβ (Liu, Chan, 2014). The innate immunity mediators in the brain like microglia and astrocytes are always on high alert for pathogens or other inflammatory triggers and are involved in the assembly and activation of inflammasome, ultimately leading to caspase-1 mediated IL-1β cytokine maturation. Inflammasomes have a role in the pathogenesis of AD by regulating the maturation of interleukins which induce the elevation of amyloidogenesis and neurofibrillary tangles (Tan, Yu, Jiang, Zhu, Tan, 2013).

The goals of this article are i) to analyse APP processing in AD mice models, ii) to assess the enzymes involved in Aβ clearance in AD mice models, iii) to examine the phosphorylation of

tau proteins in AD mice and iv) to learn about inflammation and the proteins involved in it. We have used two, four and nine month old 5xFAD mice, that is widely used to understand AD phenotypes, for our study.

CHAPTER 2

LITERATURE REVIEW

2.1 APP mutations and Familial Alzheimer's Disease

Amyloid precursor protein is a transmembrane protein and has been found to have neuromodulatory and neuroprotective function in neuronal cells. When the function of APP in mediating the transduction of extracellular stimuli to the cytoskeleton is compromised, it results in aberrant stress responses and, indirectly more APP, higher inflammation and eventually more Aβ peptide formation (Soldano, Hassan, 2014). Aβ is a proteolytic product of APP and mutations in the genes for APP, PS1, and PS2 are found to increase the production of Aβ42 and cause FAD. Aβ42 is more fibrillogenic and when present in high concentrations, it drives the formation of insoluble fibrils that compose amyloid plaques in the brain (Oakley, Cole, Logan, Maus, Shao, et al., 2006).

Familial Alzheimer's has early onset and is related to a genetic predisposition, including mutations in the APP gene on chromosome 21, presenilin 1 (PS1) gene on chromosome 14 and presenilin 2 (PS2) gene on chromosome 1. Most of the FAD mutations cause aberrant APP processing toward the longer, more amyloidogenic Aβ42 species (Schaeffer, Figueiro, & Gattaz, 2011). Presenilin is a member of γ-secretase that is involved in processing APP into Aβ. Both PS-1 and PS-2 mediate γ-secretase activity, although PS-1 containing complex is more active than that containing PS-1. Takeda et al., demonstrated that mutations in both PS-1 and PS-2 alter APP processing and are linked to early-onset FAD. Transgenic mouse studies have revealed that PS-1 and PS-2 FAD mutations do not inactivate their activity, rather have a gain of function effect, enhancing Aβ42 production. Mice models that co-express mutant APP and PS-1 variants have increased burden of extracellular Aβ peptide, due to altered secretase cleavage pattern (Borchelt, Wong, Sisodia, & Price, 1998).

The finding that the genes that encode for proteins that are deposited in plaques are mutated suggested their role in the disease and led to the generation of transgenic animal

models (Götz, Ittner, 2008). Mutations in APP that cluster near the β- and γ-secretase cleavage sites either elevate the production of total Aβ peptide, as in the case of Swedish mutation (K670N/M671L substitution), or increase the generation of Aβ42 specifically, as occurs with the Florida (I716V) and London (V717I) mutations. The two PS-1 FAD mutations M146L and L286V when introduced together into PS-1, doubled the production of Aβ42 when compared with the individual mutations.

Oakley et al., generated 5xFAD transgenic mice model that co-express the above three APP mutations along with the two PS-1 mutations additively increased Aβ42 generation and acts as a very rapid AD amyloid model with intraneuronal Aβ42 accumulation before plaques form. These transgenic mice develop cerebral amyloid plaques and gliosis at two months of age, achieve massive Aβ42 burdens, and have reduced synaptic markers. The intraneuronal Aβ aggregates have a role in neurodegeneration, neuron loss and amyloid plaques formation, thus making this model very useful to study FAD.

2.2 APP processing in Alzheimer's

APP is a type I transmembrane protein, synthesized in the endoplasmic reticulum and then transported through the Golgi apparatus to the trans-Golgi network (TGN), where it is found in high concentration. From the TGN, APP is transported to the cell surface via vesicles for it to be cleaved by the secretase enzyme. APP processing occurs in two pathways, non-amyloidogenic and amyloidogenic pathway. Three secretases, α, β and γare involved in APP processing. In the non-amyloidogenic pathway, α-secretase cleaves in the middle of the Aβ domain and generates sAPPα and C-terminal fragment C83. The C-terminal fragment is further cleaved by γ-secretase, producing P3 and ACID domains which are mostly degraded (Kuruva, Reddy, 2017). sAPPα has been found to play an important role in neuronal plasticity/survival and is protective against excitotoxicity. It also regulates the neural stem cell proliferation and is important for early CNS development (Zhang, Thompson, Zhang, Xu, 2011).

Action of β secretase on APP is the first step in Aβ generation. BACE1, the major β-secretase is a membrane-bound aspartyl protease with a characteristic type I transmembrane domain near the C-terminus. Activity of BACE1 is a major rate-limiting factor in Aβ generation from APP. β-cleavage results in the release of a soluble APPβ which has been found to mediate axonal pruning and neuronal cell death. The membrane carboxy terminal fragment, βCTF is further cleaved by the γ-secretase to generate Aβ. The γ-secretase cleavage takes place within the transmembrane domain and yields either Aβ40 (majority species) or Aβ42 (amyloidogenic species) along with the intercellular domain of APP (AICD).

Gamma secretase is a high molecular weight complex consisting of four components: presenilin (PS-1or PS-2), Nicastrin, anterior, pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN-2). Presenilins are the transmembrane aspartyl proteases that cleave APP, nicastrin recognises the substrate and APH-1 and PEN-2 are regarded as regulators of the maturation process of PSs. All four are essential to generate an active, stable γ-secretase complex that cleaves APP (Zhang, Li, Xu, & Zhang, 2014).

The five mutations, present in the 5xFAD transgenic mice model are found to divert APP processing to generate more Aβ peptides in the brain. Johnston et al., discovered that in Swedish mutation, proximity of the mutation to the amino terminus of the β-amyloid peptide renders the cleavage site more susceptible to β-secretase activity. Both Florida and London mutations, adjacent to each other are in the carboxy terminus of the β-amyloid peptide and affect the γ-secretase activity (Eckman, Mehta, Crook, Perez-tur, Prihar, et al., 1997, De Jonghe, Esselens, Kumar-Singh, Craessaerts, Serneels, et al., 2001). PSs, being the crucial catalytic components of γ-secretase, mutations in their genes are also the major causative in FADs (Li, Lai, Xu, Huang, DiMuzio-Mower, Sardana, at al., 2000).

2.3 Microglia in AD

Microglia are found in every location within the nervous system and comprise approximately 10-12% of the cells in the brain. Astrocytes found in the CNS interact with

microglia and appear to play an important role in microglial cell biology, and also, both glial cells act together as the intrinsic immune system of the CNS. They are the brain's tissue macrophage and are key players in brain injury and disease (Tremblay, Stevens, Sierra, Wake, Bessis, A., Nimmerjahn, 2011). Microglia have the ability to perform phagocytosis, release inflammatory cytokines and act as antigen presenting cells in the brain (Rock, Gekker, Hu, Sheng, Cheeran, Lokensgard, Peterson, 2004).

Microglia are normally present in quiescent or "resting" states in the healthy adult brain. Morphologically, they are characterized by small soma and long, thin (ramified) processes. Although termed "resting", microglia are not dormant in the adult brain, they continuously span the CNS microenvironment. Even minor alterations in the CNS homeostasis alert microglia, allowing them to provide the first line of defense during infection, injury and disease.

Microglia are rapidly activated in response to a variety of stimuli and undergo morphological transformation characterized by large soma, shorter, stouter (deramified) branches. They also upregulate cell surface markers (MHC I and II), cytokine receptors (IL-1, IL-6, CD68, CD11-b, CD200 etc) and several other markers representative of increased activity. Activated microglia release immune mediators that coordinate the response of both innate and adaptive immunity to control infection, remove cell debris and promote tissue repair. Microglial activation is essential for host defense and neuroprotection, but prolonged activation can have detrimental and neurotoxic effects (Block et al., 2007, Jurgens & Johnson, 2012)

Microglia are found closely associated with the amyloid plaques in the brain of AD patients and AD mice models and exhibit an 'activated' proinflammatory phenotype. As the resident immune cells in the brain, these cells are professional phagocytes and can internalize and degrade fibrillar Aβ. Microglia are able to clear soluble forms of Aβ through different direct and indirect mechanisms such as phagocytosis, micropinocytosis and proteolytic degradation through the production of various Aβ-degrading enzymes, which could be secreted to degrade Aβ extracellularly (Cameron & Landreth, 2010, E. Simon, Obst, Gomez-Nicola, 2019).

Neuroinflammation has been found to be involved in AD pathogenesis. In the brain, high activation of microglia also results in the upregulation of inflammatory signalling and the release

of proinflammatory mediators. Activated microglia are one of the major sources of inflammatory molecules, such as cytokines, chemokines, neurotransmitters, reactive oxygen species, and nitric oxide (NO). Released cytokines are the major effectors of the neuroinflammatory signals. In addition, the secreted inflammatory molecules also recruit other cells such as monocytes and lymphocytes to cross the blood–brain barrier to enhance neuroinflammation in the CNS (Allan, Rothwell, 2003).

2.4 Clearance of Aβ plaques by Microglia

The steady levels of Aβ are determined by the balance between its production and clearance. Dysfunction in Aβ clearance is very crucial for the accumulation of Aβ in AD brains (Wang, Zhou, Zhou, 2006). Two principal mechanisms are involved in the removal of Aβ from the brain: efflux of intact soluble Aβ (sAβ) to the peripheral circulation, mediated by receptors, and proteolytic degradation of both soluble and fibrillar forms of Aβ (fAβ) (Lee & Landreth, 2010). Aβ40 is mainly degraded intracellularly, whereas Aβ42 is degraded outside the cell. So, several enzymes working cooperatively together are involved in clearing intra- and extra-cellular pools of Aβ. Aβ is degraded by several peptidases, principally two zinc metalloendopeptidases, referred to as neprilysin and insulin-degrading enzyme (IDE) are found to play an important role (Grimm, Mett, Stahlmann, Haupenthal, Zimmer, & Hartmann, 2013).

Neprilysin is an integral membrane peptidase found in activated microglia. It localizes to the cell surface where it acts as a rate-limiting Aβ-degrading enzyme. The catalytic site of neprilysin is exposed extracellularly, making it a prime candidate for peptide degradation at extracellular sites of Aβ deposits. Neprilysin degrades soluble Aβ40 and Aβ42, as well as insoluble forms of Aβ (Iwata, Tsubuki, Takaki, Shirotani, Lu, Gerard, Gerard, Hama, Lee, Saido, 2001). Inverse correlation between neprilysin and Aβ peptide levels has been found in AD patients, suggesting that the deficient degradation of Aβ caused by low levels of neprilysin might contribute to AD pathogenesis.

IDE is synthesized by microglia and is released via exosomes to act on extracellular Aβ deposits. IDE displays substrate specificity without requiring a specific amino acid sequence for cleavage and represents the major soluble Aβ-degrading protease in the human brain. IDE is neuroprotective, such that it degrades soluble Aβ and its degradation products do not form oligomers, inhibiting Aβ neurotoxicity (Kurochkin & Goto, 1994). In animal models, impairment in Aβ degradation in brain was observed when there is a deficit in IDE function, whereas overexpression of IDE reduced Aβ levels and retards or completely prevents amyloid plaque formation in the brain.

Cathepsin D is a lysosomal aspartyl protease enzyme and is responsible for intracellular clearance of aggregatable Aβ. It hydrolyses Aβ in the middle of the sequence, by cleaving full-length Aβ between Phe19 and Phe20, to eliminate the amyloidogenic peptide (Hamazaki, 1996). Ries et al., demonstrated that cathepsin B, a lysosomal cysteine protease, can degrade Aβ, once it is phagocytosed by microglia. This enzyme is able to reduce longer toxic forms of Aβ into shorter less toxic species, such as Aβ38.

2.5 Role of Aβ in Tau phosphorylation

Tau is a microtubule-associated protein, participating in the assembly and stability of microtubules which in-turn are important in maintaining neuronal morphology and structural integrity. Tau stabilizes microtubules by promoting their polymerization and suppressing their dissociation. Within neurons, tau is predominantly in axons, where it exists as a highly soluble, phosphorylated protein that stabilizes and promotes the polymerization of microtubules, principally through the microtubule-binding domain. There are two proline rich regions, flanking the microtubule-binding domain in tau, phosphorylation of which affects the ability of tau to bind to microtubules. Hyperphosphorylated tau present in AD shows a low capacity for binding to microtubules (Heutink, H. 2000, Lee, Perry, Moreira, Garrett, Liu, Zhu, Takeda, Nunomura, Smith, 2005).

Appearance of neurofibrillary tangles (NFTs), primarily composed of aggregations of paired helical filaments (PHFs) made of abnormally phosphorylated tau, within specific neuronal populations, is a known neuropathological feature in Alzheimer's known as 'tauopathies'. In AD, in addition to Aβ, tau increasingly seems obligatory to the process leading to neurodegeneration in the tauopathies and lack or a reduced amount of tau has been identified to be associated with neuroprotection and resistance to Aβ toxicity. PHF-tau is assumed to be a neurotoxic agent and several mechanisms have suggested its role in neurodegeneration. First, phosphorylated tau has been shown to cause disassembly of microtubules, it also compromises the stability and function of microtubule, resulting in a loss or decline in axonal or dendritic transport in disease. Furthermore, PHF-tau disrupts intracellular compartments that are essential for normal metabolism. These pathogenic mechanism posed by tau phosphorylation and aggregation is directly involved in neurodegeneration (Jesús Avila, 2006).

It has been proposed that the soluble oligomers of Aβ peptide (also known as ADDLs), that comprise the primary neurotoxic species in AD, instigate formation of tangles, and increased brain levels of soluble Aβ correlate with NFT density in AD patients. Aβ oligomers are found to activate glycogen synthase kinase-3β (GSK-3β) by targeting insulin or wnt signaling pathways. GSK-3β, also known as tau kinase I, appears to be involved in pathological tau hyperphosphorylation (De Felice, Wu, Lambert, Fernandez, Velasco, et al., 2008).

Tau is phosphorylated at nearly 25 different sites and s396 and s404 epitopes appears to be the most highly phosphorylated site by GSK-3β. It is also believed that the kinase phosphorylates the residue in a sequential manner, first s404, followed by s396. Phosphorylation of tau at these sites is involved in microtubule destabilization and tau hyperphosphorylated at these sites is mostly found in Alzheimer's disease (Peel, Sorscher, Kim, Galvan, Chen, Bredesen, 2004).

2.6 Inflammasome

Inflammasomes, inducible high molecular weight complexes, are the platform for proIL-1β (proinflammatory cytokine) processing. There are four different well-characterized inflammasomes and their activators: $NLRP_1$, $NLRP_3$, $NLRC_4$, and AIM_2. $NLRP_3$ is particularly involved in many pathological mechanisms including soluble and aggregated Aβ. Inflammasome is activated by both soluble and fibrillar Aβ. Microglial cells are activated by Aβ fibrils and therefore provide signal via NF-κB transcription of $NLRP_3$ and IL-1β. Intracellular aggregation of soluble Aβ or lysosomal rupture through phagocytosed Aβ fibrils can also provide signals for inflammasome activation. The basic components of inflammasomes include a NOD-like receptor (NLR) that recognizes danger signals or ligands, procaspase-1, central to inflammasome activation and adaptor protein, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), which is involved in the assembly or activity of inflammasomes (Baroja-Mazo, Martín-Sánchez, Gomez, Martínez, Amores-Iniesta, et al., 2014).

Inflammasomes receptor, NLR, that sense the pathogen and damage associated molecules are predominantly cytosolic proteins. In response to danger signals, inflammasomes assemble by self-oligomerizing the NLRs, this recruits and interacts with the adaptor protein ASC, which in turn recruits procaspase-1, the effector protein that is central to the activation of inflammasome. Once procaspase-1 is recruited to assemble the inflammasomes, it cleaves itself to form the active caspase-1. Mature caspase-1 is a protease that cleaves proinflammatory molecules like IL-1β and IL-18 to form their mature form. IL-1β is a very potent pyrogenic cytokine and seems to impact long-term potentiation and synaptic plasticity in hippocampus (Liu & Chan, 2014).

Two steps are necessary for the activation of NLRP3 inflammasome: First step involves the 'priming' of the inflammasome, and is the result of disinhibition and nuclear translocation of NF-κB which leads to the transcription of NLRP3 and pro-IL-1β, both are prerequisites for the actual activation of the inflammasome. Second step is the oligomerization of NLRP3 and the assembly of the inflammasome complex (Gold, & El Khoury, 2015).

2.7 Role of Inflammasome and Inflammation in AD

Inflammation has a potential role in AD pathogenesis and high levels of inflammatory cytokines are expressed during the early stages of AD. It is a complex cellular and molecular defense mechanism, characterized by the activation of glial cells and the release of proinflammatory mediators. In CNS, activated glial cells are the major sources of inflammatory molecules, such as cytokines, chemokines neurotransmitters, ROS and nitric oxide. Released cytokines like IL-1β, TNFα, are the major effectors of the neuroinflammatory signals and are capable of affecting neurophysiologic mechanisms regarding cognition and memory. It has been identified that these cytokines are capable of establishing feedback loop to activate more glial cells and will lead to further generation of inflammatory molecules. They also recruit other cells such as monocytes and lymphocytes to enhance neuroinflammation in the CNS. The neuroinflammatory component of AD is also characterized by a local cytokine-mediated acute-phase response, activation of the complement cascade and induction of inflammatory enzyme systems such as the inducible nitric oxide synthase (iNOS) and the prostanoid generating cyclooxygenase-2 (COX-2). All these factors are capable of contributing to neuronal dysfunction and cell death, either alone or in concert.

Aβ itself is capable of inducing a local inflammatory-type response. Fibrillar Aβ can bind complement factor C1 and activates the classical complement pathway in an antibody-dependent manner. Such activated complement factors are identified to play an important role in the local recruitment and activation of microglial cells expressing the complement receptors, CR3 and CR4 (Rozemuller, Eikelenboom, Stam, Beyreuther, Masters, 1989).

IL-1β, produced mainly by inflammasomes in microglia, mediates a variety of local and systemic immune responses. It is usually present at low levels in health brain, and its secretion could lead to a potent inflammatory response and/or modulate several pathophysiological functions (Tan, Yu, Jiang, Zhu, Tan, 2013). In response to Aβ deposition, it is present in high levels in AD mice models. Suzanne et al., 2008 demonstrated that cytokines, including IL-1β, were found to impair Aβ clearance function of microglial cells by reducing their uptake in APP/PS-1 mice

models. They were also found to promote Aβ generation by stimulating γ-secretase mediated APP cleavage, through a signalling cascade mediated by MEKK1 and JNK (Liao, Wang, Cheng, Kuo, Wolfe, 2004). Thus, NLRP3 activation in microglia has significant contribution in AD pathogenesis via two processes. First, it regulates the production of IL-1β and second, it can reduce Aβ clearance leading to enhanced plaque deposition.

CHAPTER 3

MATERIALS AND METHODS

Mice

Animal studies were approved by The University of Texas at Dallas Institutional Animal Care and Use Committee (IACUC) and were performed according to the National Institutes of Health guidelines for animal care. 5x FAD mice that overexpress mutant human APP with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations along with human PS1 mutations (M146L and L286V) linked to familial AD were used. 5x FAD mice B6SJL-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799Vas/Mmjax of mixed gender were obtained from the Jackson Laboratory. Mice were allocated randomly to experimental groups and two, four and nine month old nonTg and 5x FAD mice of mixed genders were used for the experiments. Genotypes of the mice were confirmed by performing PCR on the tissue samples. The number of mice was determined from the data of previous experiments and power calculation, to ensure that the minimal number of mice as required were used for the experiments.

Genotyping

Tails were cut from the mice once they were 21 days old, after administering anaesthesia. DNA was digested using Sigma Extract-N-Amp tissue PCR kits. 20µl of Extraction buffer and 5µl of tissue preparation buffer was added to the tails. PCR tubes with the reaction mixture was incubated in a thermocycler (Applied Biosystems) for 3 mins at 95°C and then at 25°C for 15 mins. Once the reaction came to an end, 20µlof Neutralization solution was added to neutralize the acidic solution. For 5x FAD genotyping, IMR3610 5’ AGGACTGACCACTCGACCG 3’ (Transgene) and oIMR3611 5’ CGGGGGTCTAGTTCTGCAT 3’ (Transgene) primers were used. For 5x FAD, the samples were run on a 3% agarose gel for 30 mins at 120V, in the presence of 100bp ladder. The bands were confirmed with gel electrophoresis at 377 bp.

Slicing

Two, four and nine month old nonTg and 5xFAD mice were sacrificed and the brain was dissected. Half brain was placed in 4% PFA for slicing and the other half was frozen at -80°C for other experiments. Tissue sectioning vibratome (Leica VT 1200 S) was used to make thin tissue slices with a thickness of 40 µm, which were then stored in PBS for later use in immunofluorescence staining.

Immunofluorescence staining

Tissue slices prepared were blocked with blocking buffer(5% donkey serum, 0.3% Triton-X-100 in PBS) for 1 hr at room temperature in the shaker. Then primary antibody was added and the slices were incubated at room temperature in the shaker, overnight. Dilutions of the antibodies were as follows: anti-Iba1 (Abcam, #ab5706, 1:600), anti- Aβ (CST, 1:500) and 0.1 µg/ml DAPI (Invitrogen, D1306), Slices were washed and incubated with secondary antibodies on the next day for 1hr at room temperature, in shaker. The slices were then washed and mount using mounting medium and imaged on a Nikon confocal microscope.

ELISA

For ELISA, 6mg of tissue samples were collected from the frozen brain tissues of nonTg and 5xFAD mice. The samples were homogenized in PBS, containing protease inhibitor, PMSF, Na_3VO_4 and NaF. After centrifugation, the supernatant were transferred to another tube and the pellets were resuspended in PBS with PI, Na_3VO_4, NaF and PMSF. Supernatant was mixed with the previously collected supernatant and the tubes were centrifuged to remove any solid particles present. The pellet collected was resuspended in guanidine solution and stored overnight at room temperature. Both guanidine and PBS samples were used for analysing the soluble and insoluble Aβ using human Aβ 40 and 42 ELISA kits as per manufacturer's protocol (Thermo Fisher Scientific, catalog# KHB3481 for Aβ40 and catalog# KHB3441 for Aβ42).

For IL-1β, 12mg of brain tissues collected from the mice models were homogenized in RIPA buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA, 0.5mM EGTA, 1% Triton-X-100, 0.1% Sodium

deoxycholate, 0.1% SDS, 140mM NaCl dissolved in ddH_2O, along with protease inhibitor, Na_3VO_4, NaF and PMSF). The samples were then centrifuged for 10 minutes at 10000g, 4°C. The supernatant was collected and was used for performing ELISA for IL-1β. Samples were analysed for IL-1β using mouse IL-1β ELISA kit as per manufacturer's protocol (Quantikine ELISA, catalog# ML B00C).

Western Blot

5mg of tissue samples were collected from the frozen brain tissues and homogenized in 100µl urea buffer with PI. 1:4 dilutions were performed in the urea buffer with protease inhibitor for the western blot. Proteins were separated in 12% Bis-Tris Gel (Thermo Fisher Scientific) and then transferred to PVDF membrane (Bio-Rad). The membranes were dried, activated and blocked using 5% non-fat milk (Labscientific Inc.) in TBS buffer (20mM Tris-HCl, 150mM NaCl) for 1hr at room temperature. The membranes were probed with appropriate primary antibodies overnight at 4°C. The primary antibodies used were provided in Table 3.1. Goat anti-mouse IgG HRP conjugated and goat anti-rabbit IgG HRP conjugated secondary antibodies (Thermo Fisher Scientific, #31430 and 31460, 1: 1,000) were used and the images were collected on a Bio-Rad Chemidoc Imaging Software. Image Lab software (Bio-Rad) was used for analysing the bands obtained.

Table 3.1: Antibodies used in Western Blot

Primary Antibody	Dilution	Secondary Antibody	Company/Catalog#
APP	1:5000	Mouse	Millipore/MAB348
APP CTF	1:1000	Mouse	Biolegend/802803
β-Secretase (BACE1)	1:1000	Rabbit	Proteintech/1287-1-AP
CTSD	1:2000	Rabbit	Proteintech/21327-1-AP
CTSB	1:1000	Rabbit	Proteintech/12216-1-AP

Neprilysin (MME, CD10)	1:750	Rabbit	Proteintech/18008-1-AP
IDE	1:1000	Mouse	Santa cruz/sc393887
P-tau (s396)	1:1000	Mouse	Cell Signalling Technology/ 9632S
P-tau (s404)	1:1000	Rabbit	Cell Signalling Technology/ 20194T
Total Tau (tau46)	1:1000	Mouse	Cell Signalling Technology/ 4019S

Quantification of Microglial Activity

1. Calculation of Convex hull area

The area encompassed by the entire microglial cells was found by determining the area of the polygon that is created by straight lines that join the most distal points of the microglia branches.

2. Calculation of Iba1 density

To determine the density, the number of microglial cells in a given area was counted. This number was divided by the total area sampled to get the density. The value was multiplied by 10^6 to get density per mm^2.

CHAPTER 4

RESULTS

4.1 Verifying the expression of mutant APP in the transgenic mice models

To study about the Aβ plaques in APP mutant mice models, we used 5x FAD mice that co-express three APP mutations [Swedish (K670N/M671L), Florida (I716V) and London (V717I)] along with two PS-1 mutations (M146V, L286V). Oakely et al., have demonstrated that mice model with these mutations have increased Aβ42 production leading to increased plaque formation and eventually neurodegeneration. The genotypes were confirmed with standard PCR, (Figure 4.1) represents a gel image showing (from left to right), wild type: 100 bp ladder (5µl); nonTg (6 lanes, no band at 377 bp) and APP mutant: 100 bp ladder (5µl); 5x FAD (6 lanes) with bands at 377 bp. Therefore, the genotype results show an evidence for the expression of mutant APP in the mice models.

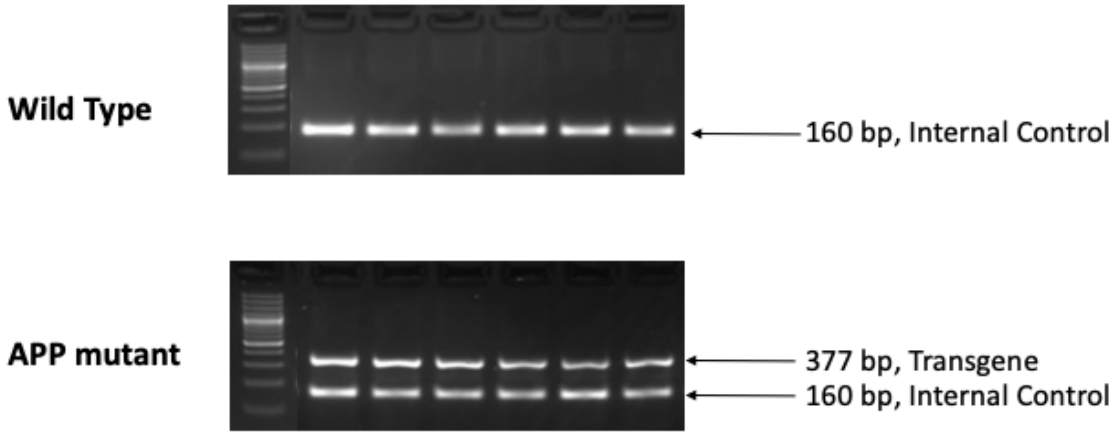
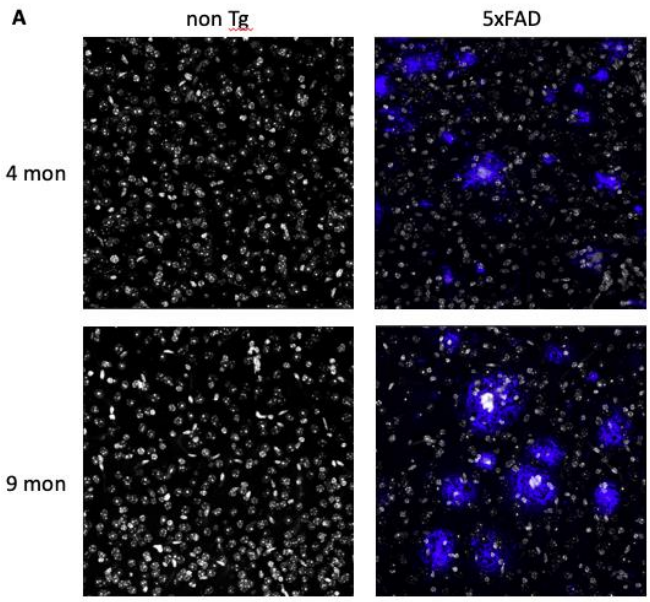


Figure 4.1: Verifying the expression of mutant APP in the transgenic mice model. PCR for confirming 5X FAD genotype: Lane 1: 100 bp ladder, Lane 2-7: wild type, Lane 8: Ladder, Lane 9-14: 5x FAD.

4.2 Aβ plaques in 5xFAD mice models

To check for the presence of Aβ plaques in the AD mice model, we performed immunostaining on brain tissue slices collected from 4 and 9 month old wild type and 5xFAD mice. As seen (Figure 4.2 (A)), there are no plaques in the wild type mice models and the 5xFAD mice have intensive plaques with microglial cells surrounding them. Also, there are increasing plaques in the 9 month mice model when compared with their 4 months old counterparts. We also performed ELISA to examine the levels of Aβ40 and Aβ42 using 4 month old brain tissue samples. The results show higher levels of Aβ42 over Aβ40 demonstrated by the ratio of Aβ42/Aβ40.



B

ELISA for Aβ

Mice Model	Aβ40		Aβ42	
	In PBS (pg/µg protein)	In Guanidine (pg/µg protein)	In PBS (pg/µg protein)	In Guanidine (pg/µg protein)
Wild Type	nil	nil	nil	nil
5xFAD	0.5534451	505.075202	1.28105644	2317.885317

Aβ42/Aβ40 ratio (5xFAD)	
In PBS	In Guanidine
2.314695	4.589189

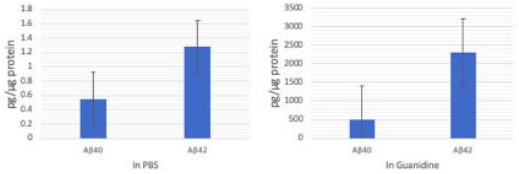


Figure 4.2: Aβ plaques in 5x FAD mice models. (A) Immunostaining of Aβ in 4 and 9 months old wild type and 5x FAD mice models. (B) ELISA to check the level of Aβ40 and Aβ42 in 4 months old wild type and 5x FAD mice model along with the quantitative analysis of Aβ40 and Aβ42 in PBS and Guanidine.

4.3 APP Processing in AD mice models

To study APP processing in the 5xFAD mice, we performed western blot using the tissue samples collected from 2, 4 and 9 months old 5xFAD mice. Amyloid precursor protein from which Aβ is derived is shown and the expression of APP is similar in all the 3 age groups. We looked for the presence of BACE1 (Beta site APP Clearing Enzyme) as its action on APP is the first step in Aβ production. Also, we compared the level of two C-terminal fragments (CTF99 and CTF83) generated by α- and β-secretase respectively. In all the 3 age groups, mice exhibit more CTF99 production than that of CTF83 indicating that the action of β-secretase is favored. Also, expressions of BACE-1 and APP CTF99 remain unaltered across all the 3 age groups.

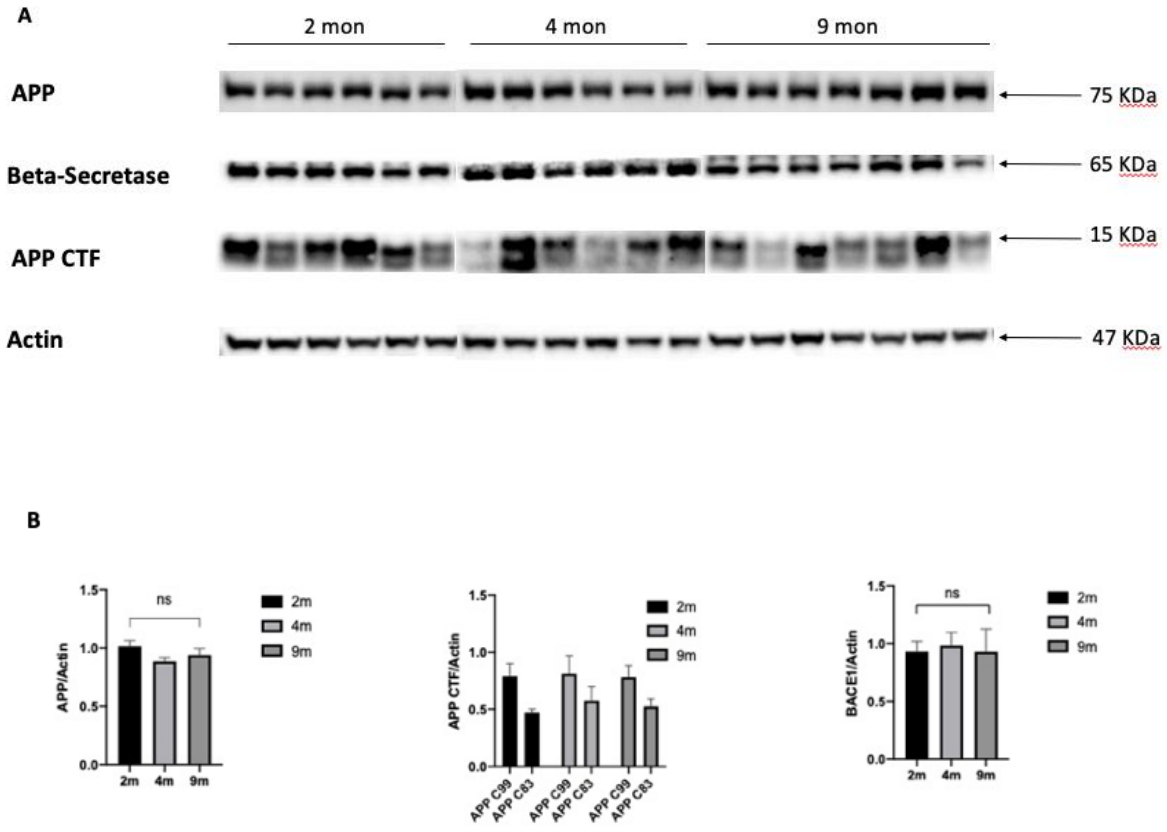


Figure 4.3: APP processing in AD mice models. (A) Western Blot showing the expression of (a) APP, (b) APP CTF, (c) BACE1 and (d) β-actin in 2, 4 and 9 month old mice models. (B) Quantitative analysis of (a) APP, (b) APP CTF, (c) BACE1 in 2, 4 and 9 month old mice models. All quantification done using β-actin as the loading control.

4.4 Aβ Clearance in APP mice models

Aβ clearance has an important role in reducing the accumulation of Aβ plaques in the normal brain and glial cells take main part in this process. We mainly focused on the microglial cells and the enzymes produced by them in Aβ clearance for our study. We performed western blot using the tissue samples collected from 2, 4 and 9 months old 5x FAD mice models, to study the expression of the proteases involved in Aβ clearance. Cathepsin D and B are lysosomal peptidases present in the microglia and are found to reduce Aβ to less toxic forms, intracellularly. NEP and IDE produced in activated microglia and play a role in degrading extracellular located Aβ. It has been found that the expression and activity of many of these enzymes decline with age and also depend on the stage of the disease. We measured the expression of these enzymes in the brain tissues and found that their expression remains almost same in the 3 different age groups.

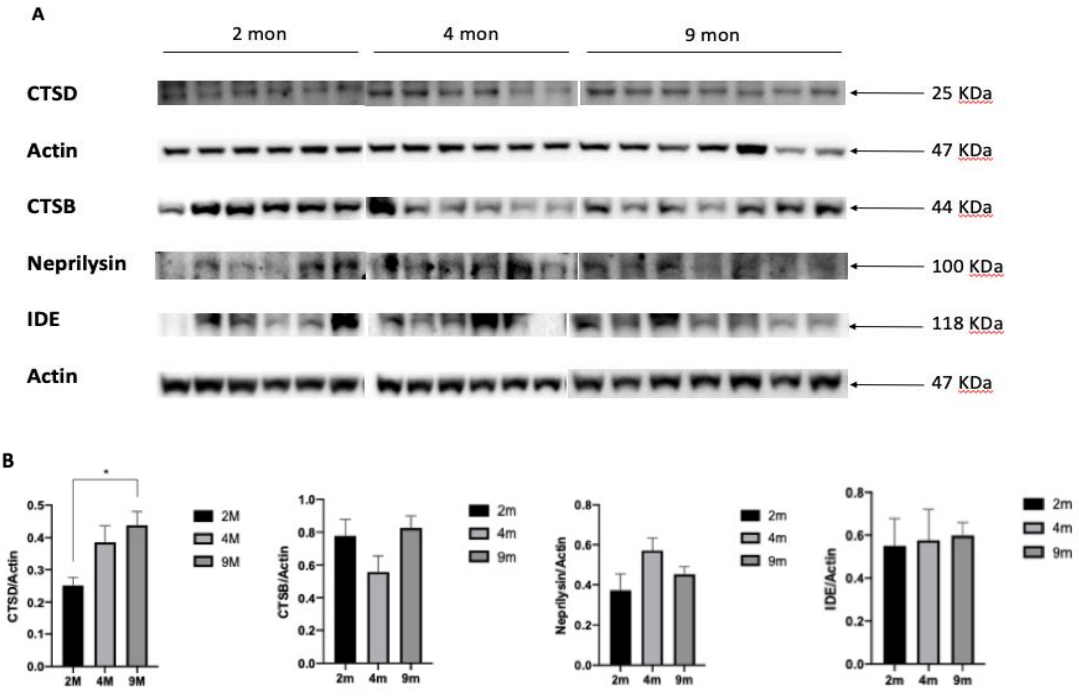


Figure 4.4: Aβ clearance in APP mice models. (A) Western Blot showing the expression of (a) CTSD, (b) β-actin and (a) CTSB, (b) Neprilysin, (c) IDE and (d) β-actin in 2, 4, and 9 months old mice models. (B) Quantitative analysis of (a) CTSD, (b) CTSB, (c) Neprilysin and (d) IDE in 2, 4, and 9 months old mice models. All quantification done using β-actin as the loading control. (n=6 per genotype, **P<0.029 using one-way ANOVA followed by Bonferroni posttest)

4.5 Effect of APP mutation on Tau Phosphorylation

We performed western blot using the tissue samples from 5xFAD mice to check for the protein levels of total tau and phosphorylated tau. Depending on the extracellular Aβ peptide, an association between tau pathology and App processing has been found in AD mice models with transgenes encoding mutant forms of APP and PS1 (Moore et al., 2015). We examined the level of tau and phosphorylated tau in 3 different age groups of 5xFAD mice and found that there was no significant difference in the levels of the tested protein (both total and phosphorylated) between the three groups. We examined tau phosphorylation at AA396 and 404, because phosphorylation of those sites often occurs early in the Alzheimer's disease and are found to be associated with Aβ toxicity. The western blot results indicate that the level of phosphorylated tau proteins remains almost same in all the three age groups, suggesting that amyloidosis is the major age-dependent brain pathology in 5xFAD mice.

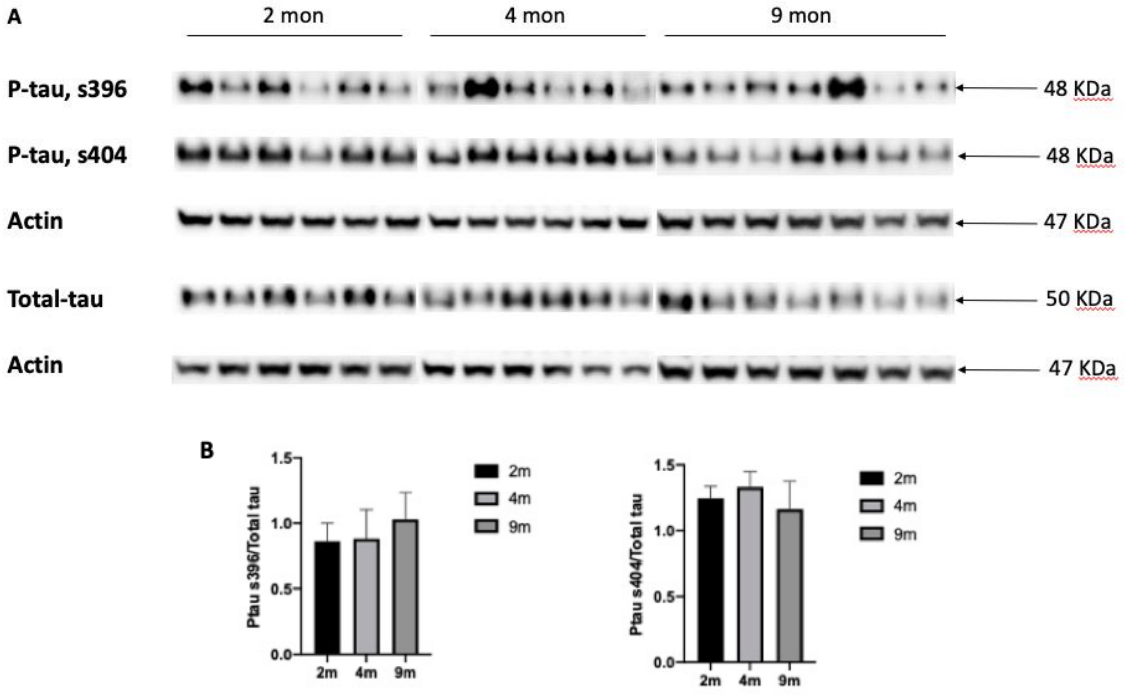
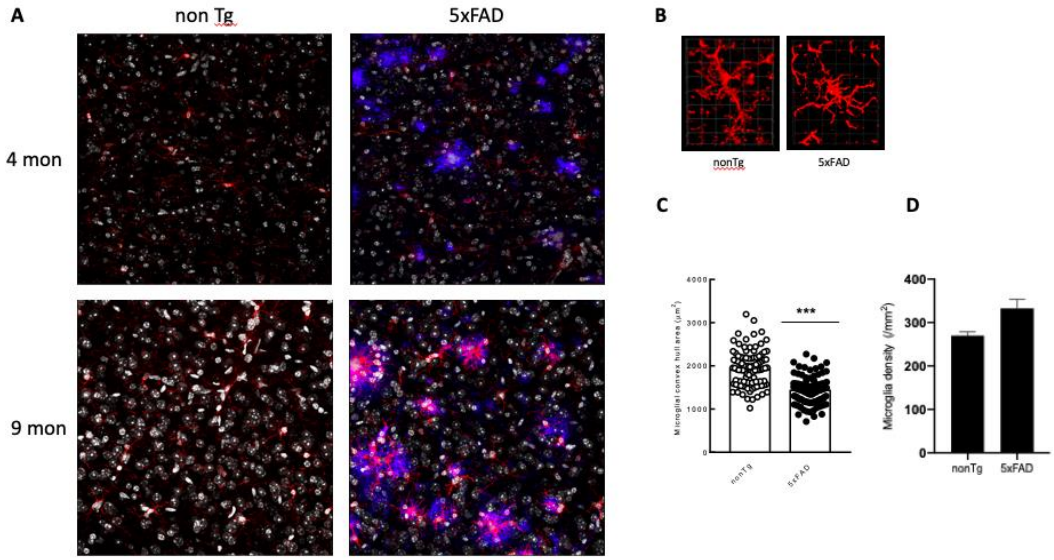


Figure 4.5: Effect of APP mutation on Tau phosphorylation. (A) Western Blot showing the expression of phosphorylated tau (a)s396, (b) s404, (c) β-actin and (a) total tau and (b) β-actin in 2, 4 and, 9 months old mice models. (B) Quantitative analysis of phosphorylated tau (a)s396 and (b) s404 in 2, 4 and, 9 months old mice models. Both phosphorylated tau are quantified using total tau.

4.6 Inflammation in APP mice models

Neuroinflammation mediated by various pro-inflammatory cytokines and chemokines, including IL-1 family of cytokines that are activated by inflammasomes, play an important role in progressive neurodegeneration and behavioral impairment that are characteristic symptoms of AD (Singhal et al., 2014). Increased density and reduced size with morphological changes in microglial cells indicate that they are in activated state in AD and there is an upregulation in the release of inflammatory signal in activated microglia. So, we performed immunofluorescence staining of microglial cells with Iba1 to examine their association with amyloid plaques and also to analyse their morphological changes in the brain slices of nonTg and 5xFAD mice (Figure 4.6(A, B)). To determine the change in size and shape of the microglia, we calculated convex hull area of the microglial cells in 9 months old nonTg and 5xFAD mice (Figure 4.6(C)). Quantification results showed that there is an increase in the density of microglial cells in the 9 months old 5xFAD mice when compared to their nonTg counterparts (Figure 4.6(D)). The results indicated that the microglia of 5xFAD are reactive in the brain. ELISA was performed to examine the levels of IL-1β using the brain tissue samples collected from 4 and 9 months old nonTg and 5xFAD mice. The results obtained showed that there is a significant increase in the level of the pro-inflammatory cytokine, IL-1β, indicating the role of neuroinflammation in AD pathogenesis in 5xFAD mice.



E

ELISA FOR IL-1β

Mice Model	4 months old (pg/µg protein)	9 months old (pg/µg protein)
nonTg	0.02934	0.300074
5xFAD	0.041873	1.326966

F

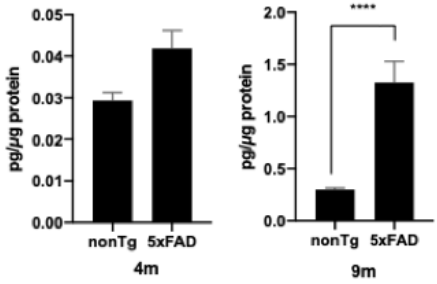


Figure 4.6: Inflammation in APP mice models. (A) Immunostaining of Iba1 and Aβ in 4 and, 9 months old wild type and 5xFAD mice models. (B) Immunostaining of Iba1 in 9 months old wild type and 5xFAD mice models. (C) Quantitative analysis of area encompassed by the microglia cells in 9 months old wild type and 5xFAD mice models (n=100 microglia per group). (D) Quantitative analysis of number of microglia cells per mm^2 in 9 months old wild type and 5xFAD mice. (E) ELISA to check the level of IL-1β in 4 and 9 months old wild type and 5xFAD mice. (F) Quantitative analysis of IL-1β in 4 and 9 months old wild type and 5xFAD mice (n=5 per genotype, **P<0.029 using one-way ANOVA followed by Bonferroni posttest).

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CHAPTER 5

DISCUSSION

Alzheimer's disease is characterized by the presence of amyloid plaques and neurofibrillary tangles in the brain. Mutations in amyloid precursor protein present near the secretase cleavage sites are identified to contribute to the generation of high levels of Aβ. Also, mutations in PS1 that performs catalytic activity in γ-secretase diverts the APP processing towards the generation of Aβ. 5xFAD mice with mutations in both APP and PS1 have very high amounts of Aβ in the brain and are developed mainly to contribute to AD research, by helping in understanding more about the role of plaques in AD pathogenesis. For our study, we have used 5xFAD mice of different ages to study the processing and clearance of Aβ in the brain and also about neuroinflammation in AD mice models.

Our genotyping data shows the presence of mutant APP gene in the mice models used for this study. We identified the presence of amyloid plaques in the brain of 5xFAD mice using immunofluorescence staining and ELISA experiments. As suggested in previous studies, our data indicates that the aggregation and size of the plaques increase with age. Furthermore, our data suggests that the 5xFAD mice primarily produce Aβ42, a more toxic Aβ isoform. Since Aβ42 is capable of forming fibrils faster than Aβ40, this explains the fast build-up of amyloid plaques in mouse brains. Of note, such a high ratio of Aβ42 to Aβ40 is prominent in familial AD patients. Therefore, our findings support the use of 5xFAD mice to recapitulate brain amyloidosis in familial Alzheimer's disease.

APP processing by β-secretase is the first step in the production of Aβ. So we looked at the level of BACE-1 enzymes in the mice models and found that the enzyme expression in same at all the ages. In AD, activity of β-secretase on APP is high when compared to that of α-secretase. This is confirmed by our data by showing the presence of a higher level of CTFβ, generated by β-secretase activity, than that of CTFα, generated by α-secretase. From our data, we found that the

expression of BACE-1 and APP CTF is similar in all the three different age groups of 5xFAD mice, indicating active APP processing starting from the early age of the mice.

There is an interplay between APP processing and amyloid plaque clearance in the brain, which helps in avoiding the accumulation of Aβ. Since a tight interaction between the amyloid plaques and the microglia cells is usually found in the brain, we checked for the expression of microglial enzymes to study their role in Aβ clearance. Cathepsin B and D are intracellular enzymes, present in the lysosomes of microglia. They mainly participate in degrading the Aβ that are phagocytosed by microglial cells. Neprilysin and IDE are peptidases belonging to the same family and are produced by activated microglia. Neprilysin is a membrane protein and usually degrades extracellular Aβ. IDE, on the other hand, is a secreted protein and is also involved in degrading extracellular Aβ. Many studies have suggested that there is a decrease in the expression level for these enzymes with age, leading to the accumulation of plaques. But our data suggests that the level of these enzymes are almost similar in all the three age groups of AD mice model.

A relationship between Aβ and tau phosphorylation, leading to the high levels of neurofibrillary tangles have been identified in Alzheimer' disease. Increase in Aβ levels is found to increase tau phosphorylation via GSK-3β, which usually phosphorylates tau at s396 and s404 sites. So, we looked at the expression of total tau and tau phosphorylated at these sites. When compared to the total tau, we did not find significant difference in the level of tau phosphorylated at s404 and at s396 sites. Also, we did not find any change in their levels between the three age groups.

Neuroinflammation plays an important role in the progressive neurodegeneration and other physiological symptoms, characteristic of AD. Microglia, being the major source for inflammatory signalling molecules, when activated, secrete these molecules in high level. The cytokine, IL-1β, is involved in various immune responses and is mainly produced by microglia in an inflammasome-mediated manner. We performed immunofluorescence staining to check for the association of activated microglia near the amyloid plaques in 4 and 9 month old 5xFAD mice.

Our findings suggest intensive microglia activation, associated with the plaques, in 9 month old 5xFAD mice than their counterparts at 4 months old. The absence of plaques and activated microglia in the brain of nonTg mice supports the deleterious effect of Aβ deposition on microglia activation. We looked for changes in the morphology of microglia in 9 month old nonTg and 5xFAD mice. We also determined the area encompassed by the entire microglial cells and their density in the brain. The data obtained showed that the microglia had less, short branches, the area of the cells were also small and there were more microglial cells per area in the brain of 5xFAD mice, indicating that they are in activated form. we also found that there is an increase in the level of expression of pro-inflammatory IL-1β in 4 and 9 months old 5xFAD mice when compared to their nonTg counterparts. Thus, microglia activation and the resulting increase in the level of the pro-inflammatory cytokine, IL-1β causes neuroinflammation in 5xFAD mice.

Finally, our studies suggest that activation of microglia and its role in the release of pro-inflammatory cytokines play a major role in AD pathogenesis, with age-related brain Aβ accumulation. The level of APP processing and Aβ clearance is same in all the three different age groups of AD mice model. But, there is an age-dependent increase in amyloid plaques. This is accompanied by an increase in the association of activated microglia with the plaques, indicating the association of neuroinflammation with AD progression.

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BIOGRAPHICAL SKETCH

Bhavatharini Udhayakumar was born in Tamil Nadu, India in November 1996. After completing her high school education, Bhavatharini joined Government College of Technology, Coimbatore, India for the undergraduate program in Industrial Biotechnology. She received a Bachelor of Technology in Industrial Biotechnology in 2018. She immediately went on to pursue her master's in Cell and Molecular Biology at The University of Texas at Dallas in 2018. She is currently a final year master's student at The University of Texas at Dallas.

CURRICULUM VITAE

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EDUCATION

The University of Texas at Dallas (Expected) May 2020
MS Cell and Molecular Biology (Thesis) GPA:3.698/4

Government College of Technology (GCT), Coimbatore, India July 2014 to April 2018
B.Tech Industrial Biotechnology (Fasttrack) GPA: 8.7/10

TECHNICAL SKILLS

Instruments	: Spectrophotometer, Haemocytometer, Light Microscope
Techniques	: Genotyping, PCR, Western Blot, ELISA, Cell Culturing, Gene-Cloning including infusion and transformation, Immunofluorescence staining, Nucleic acid Extraction, Gel Extraction, Tissue Sectioning, Tissue sample collection, Mice euthanasia
Tools & Applications	: MS Word, MS PowerPoint, MS Excel, Image J, GraphPad Prism, LabVIEW

RELEVANT COURSES	ONLINE (MOOC) COURSES
Immune Biology	Introduction to the Biology of Cancer
Molecular Biology	The Science of Stem Cells
Cell Biology	
Biochemistry	

EXPERIENCES

- **GRADUATE STUDENT RESEARCHER, USA** (September 2018-Present)

  Gained hands-on experience with PCR, genotyping, Western Blot, Immunofluorescence staining, cloning work including infusion and transformation and handling AD mice models. Currently studying the role of amyloidopathy in AD mice models.

- **GRADUATE TEACHING ASSISTANT, USA** (January 2019-Present)

  Effectively assisted and taught "Introduction to Biology Laboratory" to undergraduate students. Responsibilities include but are not limited to reagent preparation, cell culturing and grading reports and exams. Demonstrated basic biology techniques to the students. Conducted and taught molecular and cell biology techniques to the students.

- **INTERNSHIP AT NOVOZYMES, India** (December 2017-May 2018)

  Assisted in purifying mannanase and α-amylase using HPLC and performed stability assays using urea. Validated different strategies for kinetic assays for both the enzymes and determined the efficient one for enzyme assay development. Performed fluorescent assays to determine the efficiency of the enzymes in detergents.

- **INTERNSHIP AT SUNGLOW BIOTECH, India** (April 2017)

  Responsibilities included media preparation, preparation of plant tissues, inoculation, pre-rooting and transplantation of regenerated plant, on different commercial plant varieties

ACADEMIC PROJECTS

- **KINETIC STUDIES ON α-AMYLASE VARIANTS:** Performed kinetic studies using 96-well plates for about 100 variants using both natural substrates and commercially available insoluble substrates and determined kinetic parameters using Microsoft Excel Solver function to determine the efficient variant
- **REVIEW ON INDUSTRIAL APPLICATIONS OF EXTREMOZYMES:** Performed an extensive study on the types and applications of extremophiles
- **MODELING OF DNA AND RIBOSOMES:** Designed a study model of three types of DNA to understand their difference and a model of ribosome to demonstrate translation

AWARDS AND ACHIEVEMENTS

- Received "Certificate of Appreciation" for 3 consecutive years for consistent academic performance in undergraduate program

EXTRA-CURRICULAR ACTIVITIES

- Literary and Debate Society, Member September 2014-May 2018