

AGE-DEPENDENT STUDY OF PATHOLOGICAL PROGRESSION OF ALZHEIMER'S
DISEASE IN HIPPOCAMPAL AND CORTICAL TISSUE OF HUMAN AND AN AD
MOUSE MODEL

by

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Abstract

Degeneration of synaptic plasticity plays a critical role in Alzheimer's disease (AD) pathogenesis. Recent studies have suggested that neuroinflammation may contribute to this degeneration by disrupting the amyloid metabolism and by microglial overgrowth. This study aims to investigate the pathological progression of AD hippocampal and cortical tissues of human and an AD mouse model. To examine the amyloid and microglial activity in the human brain, we acquired brain tissues from clinically diagnosed AD and non-dementia (ND) patients. To examine this activity in the mouse model, we acquired brain tissues from age-matched wild type (WT) and AD affected (3xTg-AD) mice in 4, 6, 8 and 12-month age groups. Immunohistochemical (IHC) analysis was utilized to determine the characteristics of pathological AD hallmarks. Analysis of the human brain tissue showed an alteration in amyloid precursor protein (APP) in the hippocampus of AD patients as compared to the ND patients. The activation of microglial cells in the AD patients was increased, which indicates neuroinflammation. Results of mouse brain tissue analysis indicated a heightened proliferation of microglial cells in the CA3 region ($*p<0.05$) of the hippocampus in the 6-month-old 3xTg-AD male mice. Additionally, results indicated hyper-proliferation of microglial cells in multiple regions of the hippocampus in 8-month-old 3xTg-AD female mice, ($*p<0.05$). Moreover, increased presence of amyloid burdened neurons was observed in both the cortical and amygdala regions of 4, 6, 8, and 12-month age groups of 3xTg-AD mice as compared to their age-matched WT. Phosphorylated tau protein (pTau) was additionally found to be increased in the 3xTg-AD male mice as compared to their 3xTg-AD female counterparts in multiple age groups. Neuroinflammation and aberrant activity of microglial proliferation contribute to the progression of AD. The ability to understand, and therefore modulate, neuroinflammation may be a promising approach for prevention of progression in AD.

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Introduction and Literature Review

Alzheimer's Disease Overview:

With a growing and aging population, the impact of AD is increasing exponentially. Currently, approximately one in ten people over the age of 65 suffer from AD, and it is the sixth leading cause of death in the United States (Alzheimer's Association, 2019) (National Institutes of Health, 2016). In 2019, the financial burden from AD in the US is expected to reach \$290 billion (Alzheimer's Association, 2019) (National Institutes of Health, 2016). Since it was first identified in 1906 by Dr. Alois Alzheimer upon examination of the brain tissue of a patient who had died from an unknown mental illness, understanding of AD has improved significantly (Alzheimer's Association, 2019) (National Institutes of Health, 2016). It is now known to cause progressive atrophy of the hippocampus causing detriment to integral human functions such as memory and learning (Alzheimer's Association, 2019) (National Institutes of Health, 2016).

AD has been determined to have three major forms of physiological symptoms that present in the brain including amyloid-beta ($A\beta$) plaques, neurofibrillary tangles, and neuroinflammation. Representations of these hallmarks from Heinrich and colleagues are shown in Figures 1B, 1C, and 1D (2008). In the ND state, APP is an intracellular protein generally thought to play a role in protection from infections, promotion of injury recovery, maintenance of the blood brain barrier, and regulation of synaptic function (Brothers, Gosztyla, & Robinson, 2018). However, in the AD state, APP is incorrectly cleaved by β -secretase and γ -secretase enzymes, respectively, allowing it to present extracellularly and play a major role in the misfolding of the protein, thus allowing it to form clumps of oligomers which aggregate to form insoluble $A\beta$ plaques (Brothers, Gosztyla, & Robinson, 2018). Neurofibrillary tangles occur when the protein tau, an essential component in stabilization of microtubules and therefore the cytoskeleton of the neuron, becomes hyperphosphorylated (Nature Neuroscience, Animation: Inside Alzheimer's Disease). This compromises tau's ability to interact with the microtubules of the neuron, and therefore compromises the structural integrity of the cell, allowing for neurofibrillary tangles to form (Nature Neuroscience, Animation: Inside Alzheimer's Disease). Finally, neuroinflammation occurs as a result of heightened activity of microglial cells, the primary form of immune system defense in the central nervous system (CNS), which are responding to the presence of plaques and to neuronal death (O'Shea, Gadina, & Siegel, 2013).

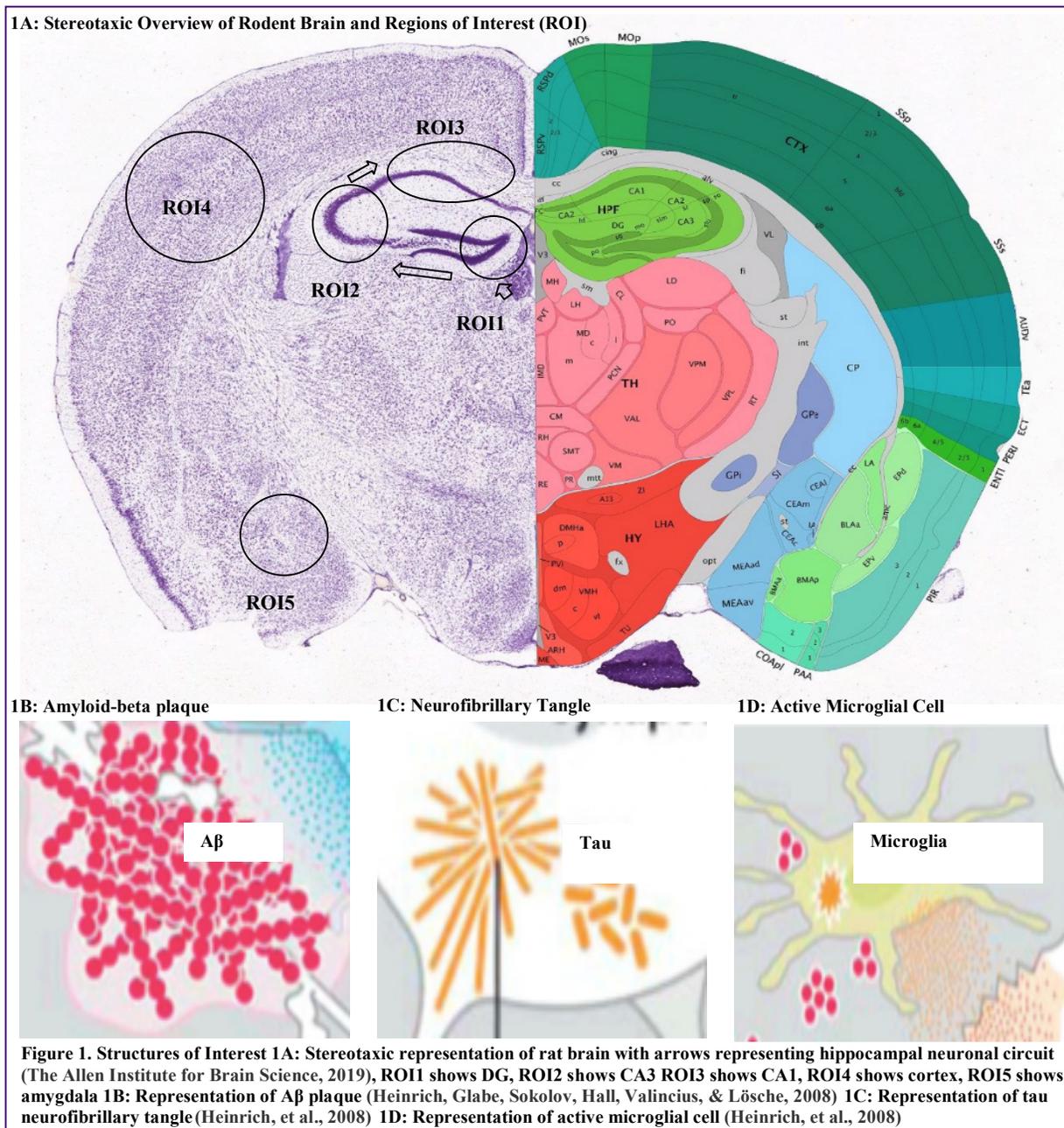
Microglial cell activation results in the release of cytokines, which then leads to an inflammatory response. These three physiological symptoms contribute both individually and cumulatively to a weakening of synaptic plasticity and overall impairment of neuronal communication ability.

Hippocampal Complex and Amygdala:

The limbic system, involved in emotional and behavioral responses especially as it relates to survival, presents with some of the most severe pathology in the AD-affected brain. This makes this system, and its significant structures of the hippocampus and the amygdala, of particular interest in defining a better understanding of AD progression and potential therapeutic interventions. Because of its responsibility for higher-order processing, the cortex is also a general area of interest in AD study. Degradation of overall functioning of the hippocampus and the amygdala and their respective roles in memory and emotional processing correlate with some of the most prominent clinical symptoms in AD patients.

The hippocampus is a vital structure for development and maintenance of long-term memory, memory consolidation, encoding, retrieving, and processing information as it relates to time and space. Extensive interconnectivity throughout the structure allows for connection of information associated with various senses, with various modalities, and throughout various forms of memories. AD patients present with significant atrophy of the hippocampus, leading to detrimental and early impairment of the aforementioned processes. The hippocampus is composed of subregions. A notable region of heightened interest as it relates to AD is the hippocampus proper, consisting of central neural circuitry in the dentate gyrus (DG), the CA3 region, and the CA1 region. Regions of interest are shown in Figure 1A, adapted from The Allen Institute of Brain Science (2019). The DG is significant in that it is one of the few sites in the adult brain capable of undergoing neurogenesis (Hamilton & Rhodes, 2015). Towards the beginning of the neuronal circuit in the hippocampus proper, the interconnectivity of neuronal communications allows the DG to play a major role in consolidation of multiple sensory inputs and association between stimuli (Hamilton & Rhodes, 2015). It is thought to tag new information as novel through distinguishing incoming stimuli from previous experiences (Langston, Stevenson, Wilson, Saunders, & Wood, 2010) (Hamilton & Rhodes, 2015). The neuronal circuit then continues to the CA3 region of the hippocampus proper, where interconnectivity of neuronal communication again allows for association between stimuli (Langston, Stevenson,

Wilson, Saunders, & Wood, 2010). The intrinsic network of recurrent collaterals suggests CA3 is potentially involved in pattern completion, which allows for whole memory retrieval through matching of incoming stimuli to familiar experiences even if only a portion of the originally encoded stimuli is presented (Langston, Stevenson, Wilson, Saunders, & Wood, 2010). The neuronal circuit of the hippocampus proper then approaches the CA1 region for end-point processing, and through the simultaneous receipt of information about current events and previous experiences, CA1 is largely thought to be a mismatch detector for information and play a role in temporal context (Langston, Stevenson, Wilson, Saunders, & Wood, 2010).



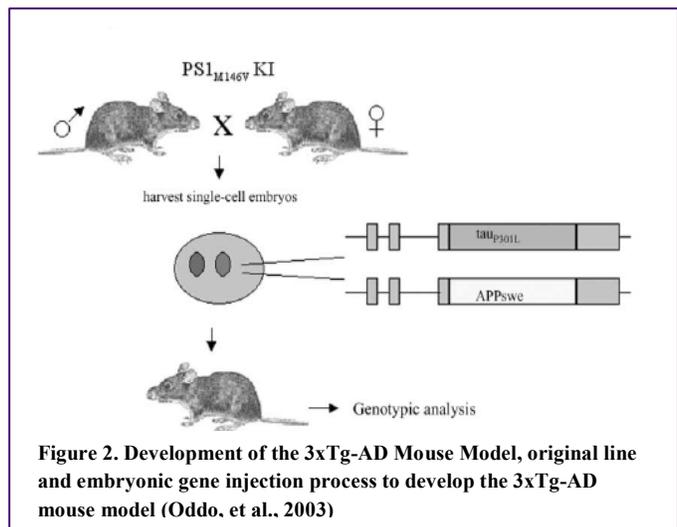
Project Overview:

The goal of this study is to investigate the pathological progression of AD in hippocampal and cortical tissues in a human and 3xTg-AD mouse model. The ethical limitations associated with human tissue include the inability to manipulate certain experimental variables. For this reason, the study assessed AD and ND age-matched human tissue at a single point in time. The 3xTg-AD mouse model allowed for study of progression of AD hallmarks in varying ages of mice, and sub-sequentially for the investigation of the potential timeline of pathological progression in this model. Illumination of the details of this progression allows this investigation to serve as a benchmark to further evaluate ongoing studies related AD. Additionally, it has the potential to shed light on an optimal timeframe for therapeutic targets of various pathologies of AD in the 3xTg-AD model. Adding to the understanding of the relative time-dependence of AD progression, this study allows for more targeted investigations as the underlying mechanisms behind AD and potential future interventions.

Materials and Methods

3xTg-AD Mouse Model:

The 3xTg-AD mouse model contains three genetic mutations that contribute to the development of AD: PS1_{M146V}, APP_{Swe}, and tau_{P301L} (Oddo, Caccamo, Shepherd, Murphy, Golde, Kaye, & LaFerla, 2003). As compared to other AD mouse models, 3xTg-AD offers the benefit of combining mutations for both A β , PS1_{M146V} and APP_{Swe}, as well as mutations for tau, tau_{P301L} (Oddo, et al., 2003). This allows for investigation as it relates to potential interactions between portions of the pathology (Oddo, et al., 2003). This model was developed by obtaining a PS1_{M146V} line and microinjecting the remaining two genes of interest into embryos, as shown in Figure 2 (Oddo, et al., 2003). The group confirmed the effectiveness of the model by documenting sequential pathology



progression as follows: loss of synaptic density and synapse number, A β deposits, and neurofibrillary tangles, respectively (Oddo, et al., 2003).

Study Groups:

Utilizing the 3xTg-AD model as the experimental group, we obtained hippocampal tissue from both one male and one female mouse sacrificed at each of the following age groups: 4-months, 6-months, 8-months, and 12-months. Each mouse of the experimental group had an age and gender matched WT counterpart. These age groups were chosen because of their comparability to the early to late stage progression of AD presentation in humans, relative to the average lifespan of a mouse model. For human tissue analysis we utilized donated tissue from age-matched individuals consisting of one AD patient and one ND patient.

IHC Staining and Quantification:

Hippocampal brain tissue of the aforementioned study groups was sectioned. These tissues were then stained via IHC with use of a sequential chamber or pep pen for hallmarks of interest. Mouse on mouse (M.O.M.) kit was used for staining of the 4G8 mouse antibody. This was utilized to visualize A β . A rabbit kit was used for the Iba-1 and pTau antibodies. These were utilized to visualize neurofibrillary tangles and neuroinflammation, respectively. All antibodies were at 1:500. Images were taken of the areas of interest, DG, CA3, CA1, cortex, and amygdala, and then quantified using an average value of presentation of each hallmark from three randomly placed boxes in each respective region of interest.

Results

Human AD and ND Patients:

As shown in Figure 3A and 3B, a comparison of intracellular APP and extracellular amyloid in AD and ND patients, showed significantly higher (***) presence of intracellular APP in the hippocampus of the ND

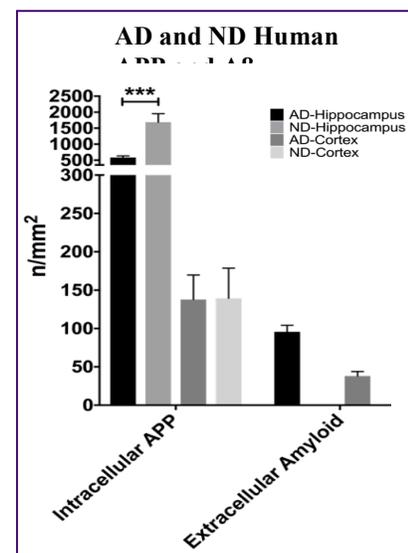
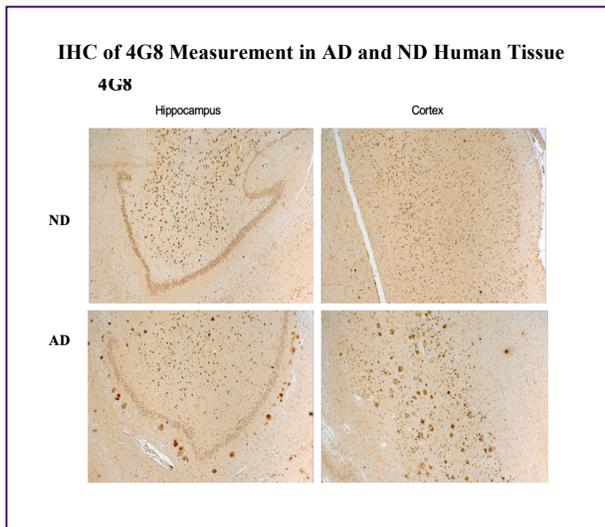


Figure 3A. (Above) Comparison of human AD and ND intracellular APP and A β , values represent mean \pm SEM, *p<0.0001 3B. (Below) Images from IHC stain of 4G8 measurement of intracellular APP and extracellular A β of human AD and ND patients**

patient than in the hippocampus of the AD patient. In humans, intracellular APP is indicative of a healthily functioning protein. Extracellular A β only presented in the AD patient, as was

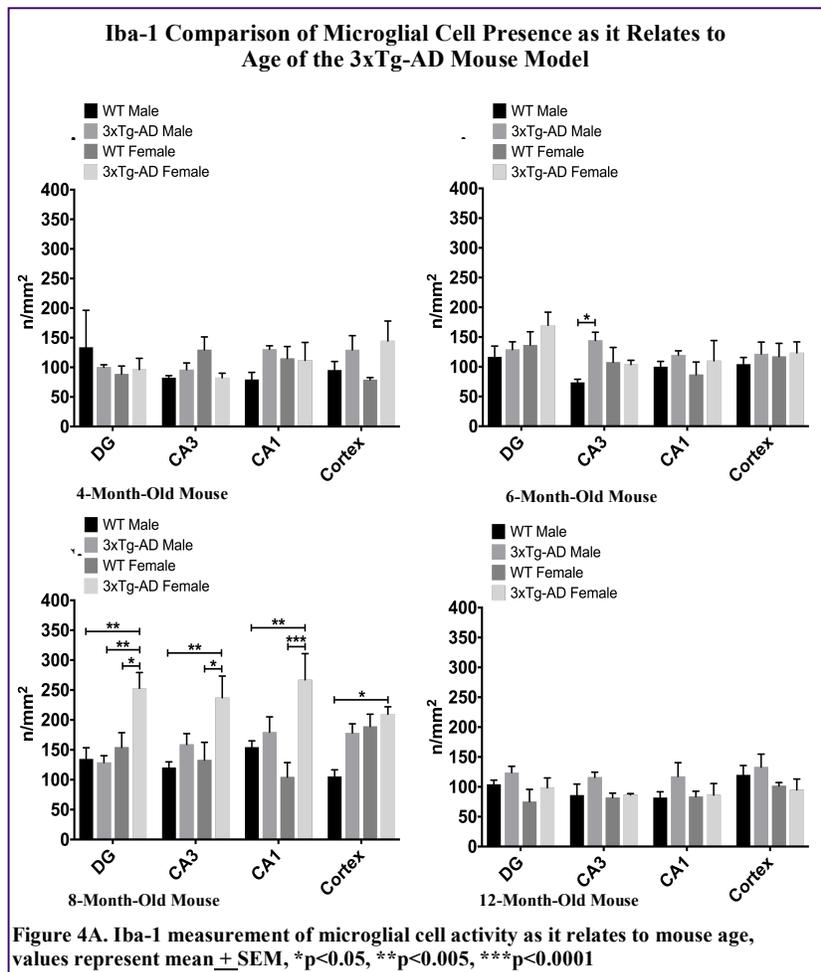


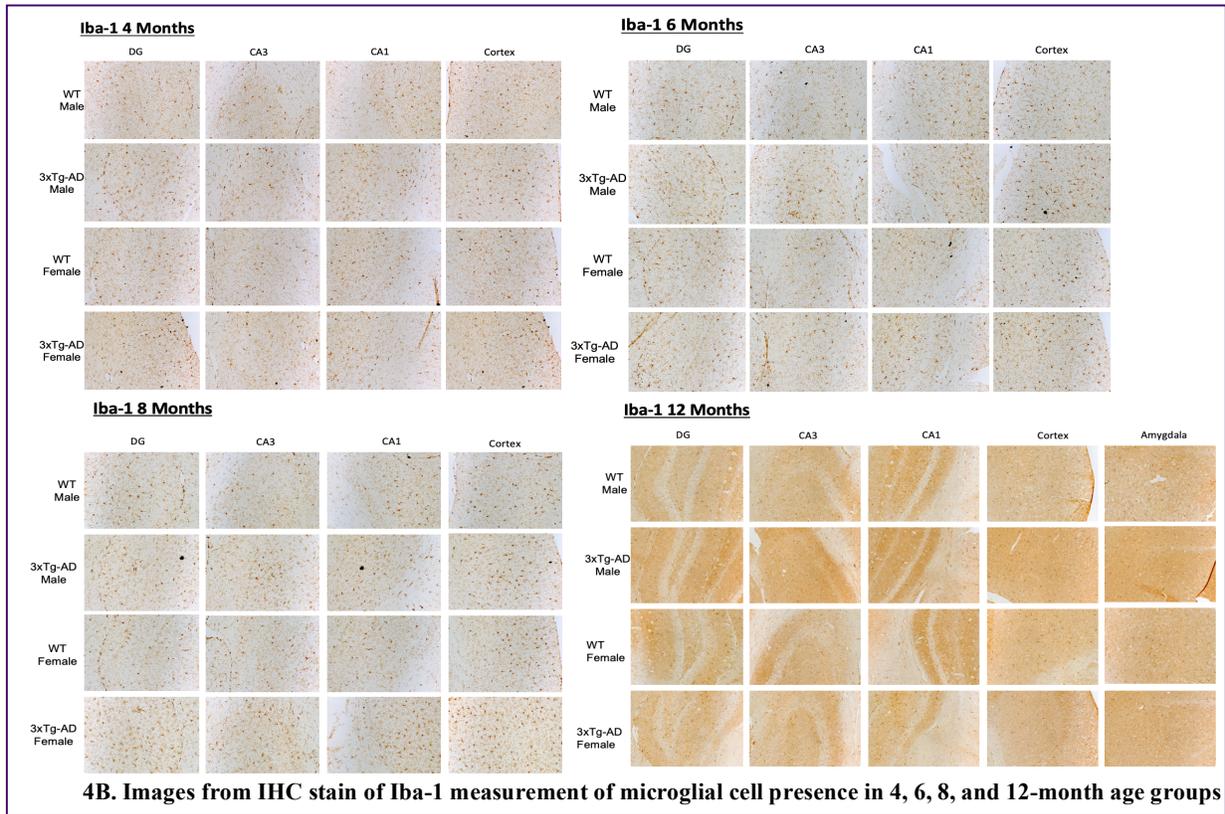
anticipated. Likewise, results were significant in the hippocampus, a major area impacted by AD.

Iba-1 Comparison of Microglial Cells:

As shown in Figures 4A and 4B, a comparison of Iba-1 measurement of microglial cell activity as it relates to mouse age showed no significant difference in quantity at 4-months-of-age. At 6-months, the 3xTg-AD

male mouse model showed significantly higher microglial cell presence (* $p < 0.05$) than its WT control at the CA3 region. At 8-months, significant differences presented at the DG between the 3xTg-AD female and the WT female (* $p < 0.05$) and between the 3xTg-AD female and the 3xTg-AD male (** $p < 0.005$). At the CA3 region in the 8-month group, there was a significant difference in quantity of microglial cells between the 3xTg-AD female and the WT female (* $p < 0.05$). At the CA1 region in the 8-month group, significant differences presented between 3xTg-AD

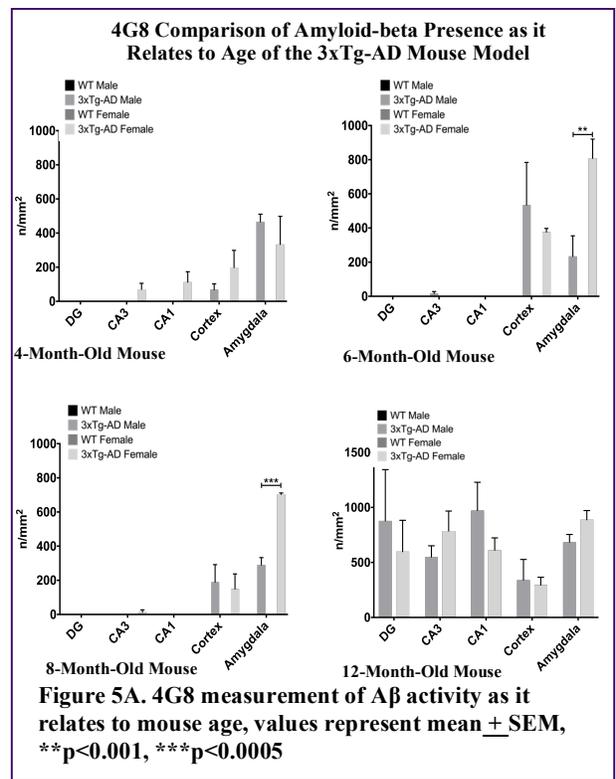




female and its control (** $p < 0.0001$). At the cortex of the 8-month group, there was no significant differences between the experimental groups and their respective controls, or between the sexes of the 3xTg-AD mice. Interestingly, at 12 months, mice showed no significant difference of microglial cell presence.

4G8 Comparison of Amyloid-Beta Presence:

As shown in Figures 5A and 5B, a comparison of 4G8 measurement of A β presence showed no significant difference at 4-months between the 3xTgAD female and the 3xTg-AD male. At 6-months, the model showed a significantly higher amount of A β in the 3xTg-AD female amygdala than its male counterpart (** $p < 0.01$) At 8-months, the model also showed a significantly higher amount



in the 3xTg-AD female amygdala than its male counterpart (** $p < 0.0005$) At 12 months, the female and male 3xTg-AD mice showed no significant difference of A β presence. A β , as

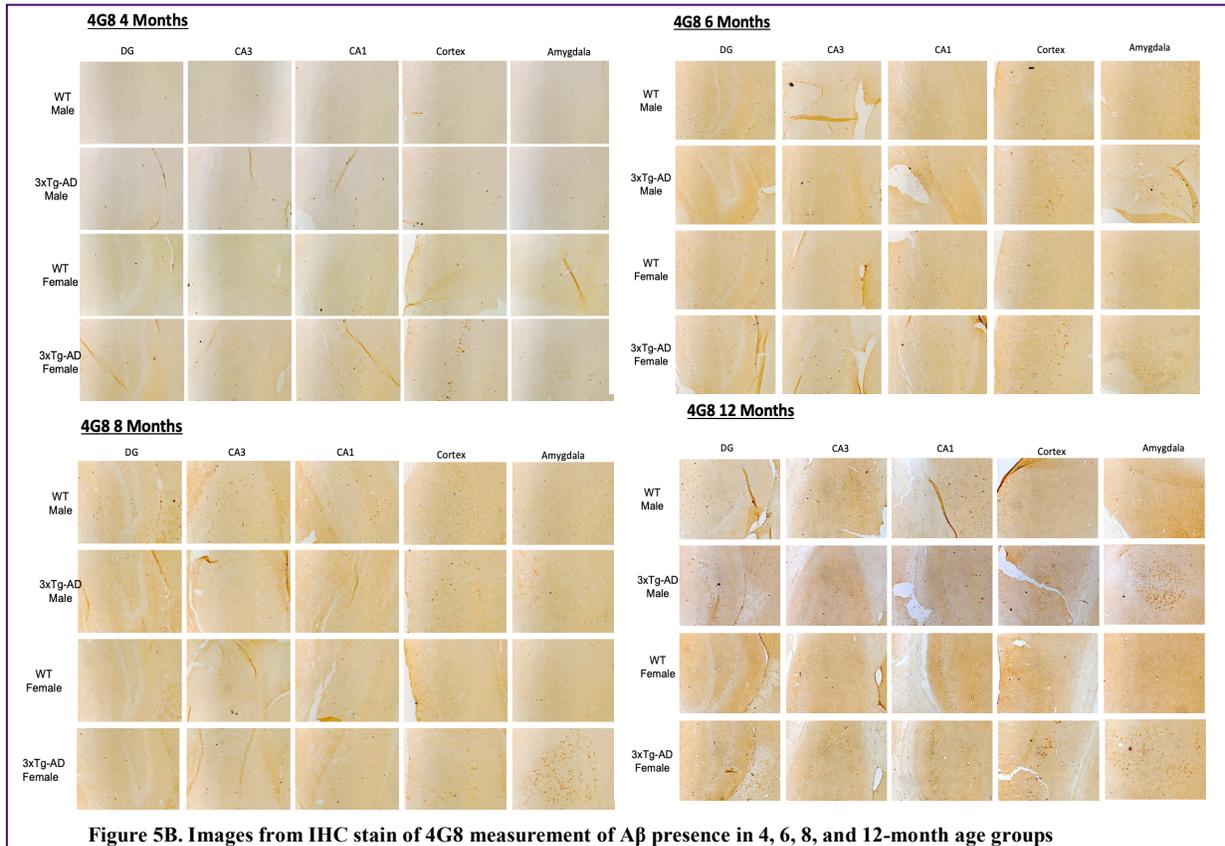
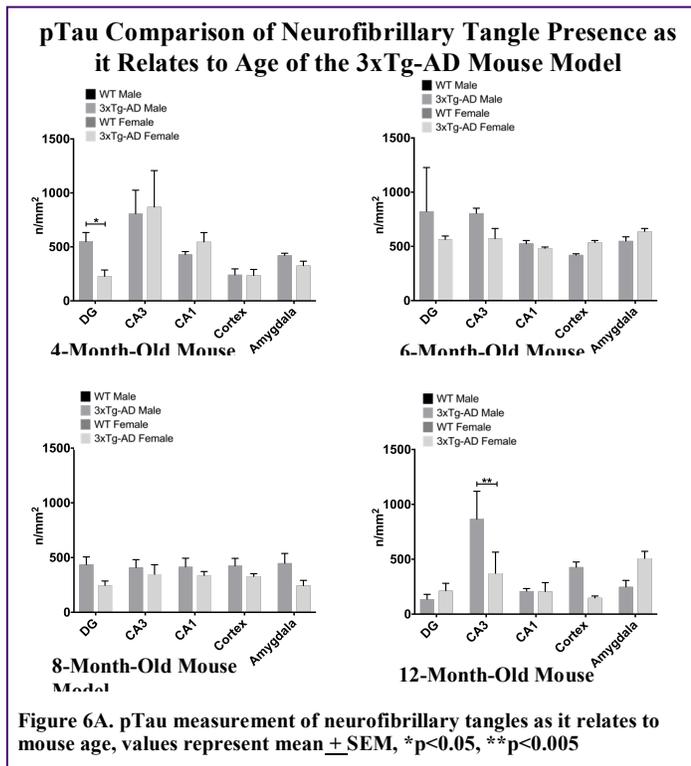


Figure 5B. Images from IHC stain of 4G8 measurement of A β presence in 4, 6, 8, and 12-month age groups

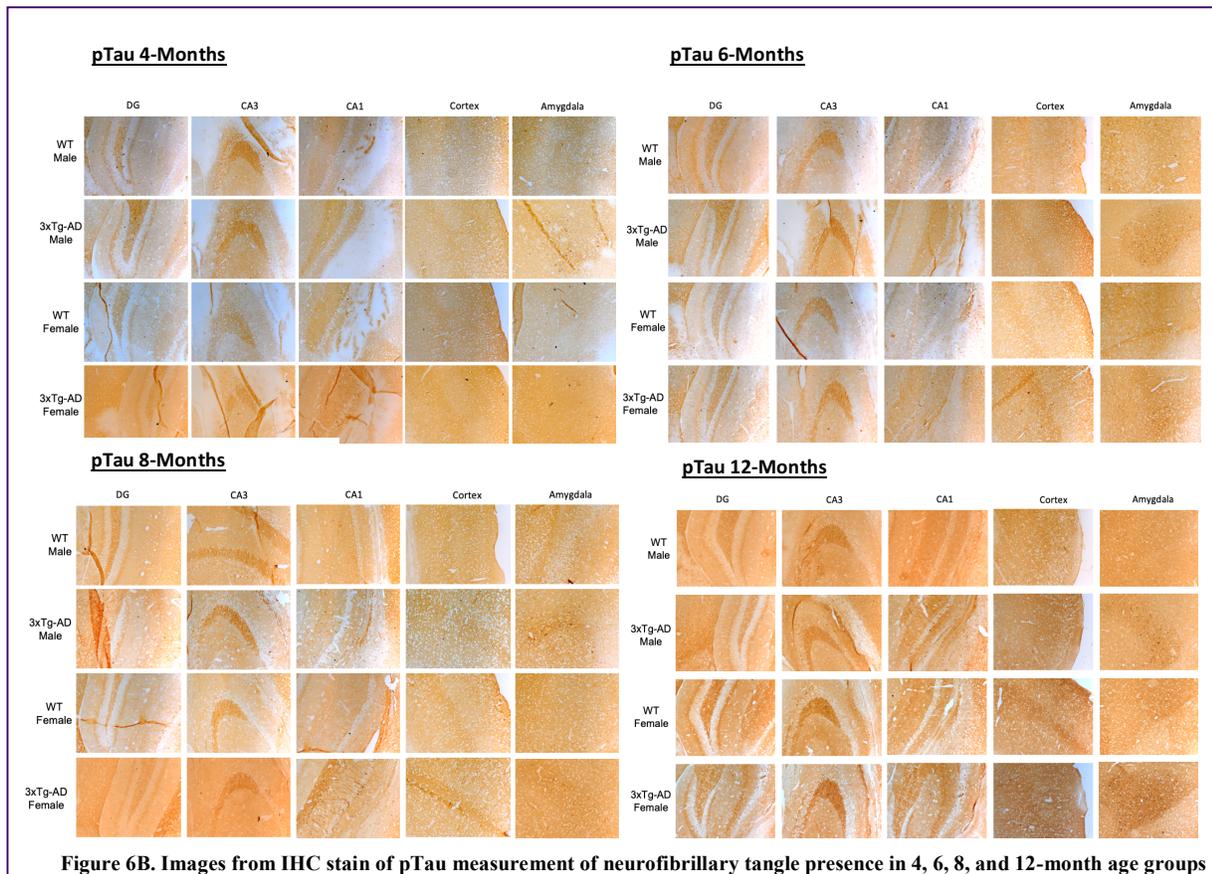
expected, was only present in the 3xTg-AD model.

pTau Comparison of Neurofibrillary Tangle Presence:

As shown in Figures 6A and 6B, a comparison of pTau measurement of neurofibrillary tangles at 4 months showed significantly higher ($*p < 0.05$) presence of pTau in 3xTg-AD male than its female counterpart in the DG. At 6-months, no significant difference was found in the level of pTau presence



between 3xTg-AD male and female. At 8-months, no significant difference was found in the level of pTau presences between 3xTg-AD male and female. At 12 months, the 3xTg-AD male



showed significantly higher (** $p < 0.005$) presence of pTau in the CA3 region than its female counterpart. pTau, not found in healthy mice, was only present in the 3xTg-AD model.

Discussion

Analysis of tissue from human patients found significantly higher intracellular APP in the ND patient hippocampus as compared to the AD patient hippocampus. Intracellular APP has not been incorrectly cleaved and is indicative of a healthily functioning protein. Thus, these findings were in line with expectations. Significant difference in the hippocampus of the two patients, and not the cortex, is in line with memory-related symptoms that present in AD patients. Extracellular APP, protein that has been cleaved and is susceptible to forming A β plaques, is symptomatic of AD pathology and, as expected, was found only in the AD patient.

Quantification of microglial cells showed significantly higher presentation in various groups of the 3xTg-AD model after 6-months of development. Additionally, there was a significantly higher presence of microglial cells in some of the 3xTg-AD female models than their male counterparts. A β is not present healthy WT mouse model. It did present at a significantly higher rate in the amygdala of the 6-month 3xTg-AD females and 8-month 3xTg-AD females as compared to their 3xTg-AD male counterparts of similar age. Likely neurofibrillary tangles, as indicated by the presence of pTau, were also not expected to present in the WT mice. These were found at a significantly higher rate in the 3xTg-AD male as compared to the 3xTg-AD female of similar age as early as 4 and as late as 12-months of age.

Interesting observations include the increased pathology present in the amygdala of the 3xTg-AD affected mice. Upon this observation, amygdala pictures and quantifications were added to the investigation. Future studies could benefit by adding to the data from the amygdala in the areas assessed before prior to this observation. 3xTg-AD mice show increased aggression and trends in agitated behavior, which could be a likely result of this damage in the amygdala. In addition to quantification, there were interesting observations in the difference of morphology, size, and distribution in presentation of the hallmarks of interest in respective groups.

Conclusion

Neuroinflammation, as shown through microglial cell presence, was indicated at 6-months and further indicated at 8-months. Interestingly, there was no significant difference in microglial cell levels in the 12-month age group. This could potentially indicate an eventual breakdown of the body's attempted immune response and desensitization as AD pathology progresses.

A higher prevalence of microglial activity and A β in the results of many of the 3xTg-AD female mice, as compared to the 3xTg-AD male mice of the same age group could be indicative of a parallel between the gender differences in the mouse model as compared to gender differences seen in clinical patients, as females show higher rate of presentation of AD as compared to their male counterparts (Alzheimer's Association, 2019). It could be of interest to investigate the apparent reverse response in the pTau present in male 3xTg-AD mice. Further investigation of pathology as it relates to gender differences would be of additional interest.

Overall, this investigation contributes to the field by enhancing to knowledge and understanding of trends in presentation and activity of AD pathological hallmarks as the disease progression, potentially increasing the effectiveness of studies of mechanisms of disease progression and potential intervention.

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