MONASH University

The Role of Glutamate in the Pathogenesis of Acquired Epilepsy in Alzheimer's Disease

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Abstract

Background: Alzheimer's disease (AD) may increase the risk of developing epilepsy up to 10-fold, compared to healthy age-matched controls. However, the pathological mechanism which underlies this increased risk is poorly understood. Since the disruption to glutamate homeostasis in the brain has been implicated in both AD and epilepsy, certain glutamatergic pathways might play mechanistic roles in the pathogenesis of epilepsy in AD. I hypothesised that AD pathologies caused disruption to the glutamate-glutamine cycle which leads to higher risk of developing epilepsy.

Aim & Hypothesis: This thesis aimed to elucidate the mechanism that links AD pathologies to an increase in seizure susceptibility using Tg2576 mouse model of AD, which was previously reported to be more susceptible to seizure, compared to WT mice. I hypothesised that mutant human APP overexpression disrupts the glutamate-glutamine cycle in the brain of Tg2576 mice by altering the expression levels of key proteins such as glutamate transporter-1 (GLT-1) and glutamine synthetase (GS), which then contribute to the pathogenesis of epilepsy.

Experiments: Metabolomic analysis was performed in the first study to explore early changes in the metabolomic profile of the samples acquired from 6-month-old (mature) Tg2576 mice, as this age represents the early stage of human mutant APP overexpression pathology. Hippocampal, cortical and serum samples from mixed-sex Tg2576 and WT mice were analysed via liquid chromatography-mass spectrometry. Then, metabolomic analyses and weighted correlation network analysis (WGCNA) were performed. Evidence to demonstrate that pathways such as glutamate metabolism, lipid metabolism and oxidative stress were affected at an early age in Tg2576 mice was provided.

In the second experiment, changes in the levels of key proteins in glutamate-glutamine cycle were investigated in Tg2576 and kindled mice via western blot. Mature Tg2576 mice displayed significantly lower levels of GLT-1 and GS proteins in their brains, compared to WT mice. Moreover, kindling significantly increased the levels of GLT-1 and glutaminase (GLS) proteins in the brain of WT mice, compared to sham. Thus, the disruption to glutamate-glutamine cycle was observed in both AD and epilepsy models.

The final experiment investigated the effects of ceftriaxone (GLT-1 expression enhancer) on the seizure susceptibility phenotype of Tg2576 mice. Ceftriaxone increased the expression level of GLT-1 in the hippocampus of naïve Tg2576 mice, compared to saline. However, the same effect was not observed in kindled Tg2576 mice. This suggests that the effect of kindling on the expression level of GLT-1 may overpower the effect of ceftriaxone treatment in Tg2576 mice. Interestingly, ceftriaxone demonstrated an anti-seizure but not anti-epileptic effect on kindled Tg2576 mice, compared to saline treated Tg2576 and WT mice. This suggests that targeting the glutamate-glutamine cycle may be beneficial against the high seizure susceptibility phenotype associated with Tg2576 mice.

Conclusion: The disruptions to glutamate-glutamine cycle and glutamate metabolism pathway in Tg2576 mice were observed at an early age, which coincided with their enhanced seizure susceptibility phenotype. Pharmacologically targeting key proteins in the glutamate-glutamine cycle has the potential to reduce the risk of seizures in AD, as demonstrated by ceftriaxone.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications during enrolment

Peer-reviewed papers published during candidature

1. **Dejakaisaya H.**, Kwan P. & Jones N.C. (2021). Astrocyte and glutamate involvement in the pathogenesis of epilepsy in Alzheimer's disease. *Epilepsia*. 00;1-9. https://doi.org/10.1111/epi.16918. PMID: 33971019.

2. **Dejakaisaya, H.**, Harutyunyan, A., Kwan, P., & Jones, N. C. (2021). Altered metabolic pathways in a transgenic mouse model suggest mechanistic role of amyloid precursor protein overexpression in Alzheimer's disease. *Metabolomics: Official journal of the Metabolomic Society*, 17(5), 42. https://doi.org/10.1007/s11306-021-01793-4. PMID: 33876332.

3. Cole B.A., Johnson R.M., **Dejakaisaya H.**, Pilati N., Fishwick C.W.G., Muench S.P., Lippiat J.D. (2020). Structure-Based Identification and Characterization of Inhibitors of the Epilepsy-Associated $K_{Na}1.1$ (KCNT1) Potassium Channel. *Iscience*, 23(5):101100. https://doi.org/10.1016/j.isci.2020.101100. PMID: 32408169.

Conference presentation

1. **Dejakaisaya H.**, Liu S., Silva J, Kwan P. & Jones N.C. Loss of GLT-1 and glutamine synthetase are associated with early stage of Alzheimer's disease in mice. Poster Presentation, *Epilepsy Society of Australia Annual Scientific Meeting* (2019). Sydney, Australia.

2. **Dejakaisaya H.**, Kwan P. & Jones N.C. Role of Glutamate in the Pathogenesis of Acquired Epilepsy in Alzheimer's disease. Poster Presentation, *CNS Disorders: Advanced Diagnostics and Treatment Conference* (2019). Melbourne, Australia.

3. **Dejakaisaya H.**, Liu S., Silva J, Kwan P. & Jones N.C. Loss of GLT-1 and glutamine synthetase are associated with early stage of Alzheimer's disease in mice. Oral Presentation, *Student of Brain Research Symposium* (2019). Melbourne, Australia.

4. **Dejakaisaya H.**, Harutyunyan A., Kwan P. & Jones N.C. Metabolomics profiling of the brain revealed potential mechanisms associating Alzheimer's disease to higher seizure susceptibility in mice. Poster Presentation, *Epilepsy Society of Australia Annual Scientific Meeting* (2020), Virtual.

5. **Dejakaisaya H.**, Harutyunyan A., Kwan P. & Jones N.C. Metabolomics profiling of the brain revealed potential mechanisms associating Alzheimer's disease to higher seizure susceptibility in mice. Poster Presentation & Data Blitz, *Epilepsy Melbourne Annual Symposium* (2021). Melbourne, Australia.

6. **Dejakaisaya H.**, Harutyunyan A., Kwan P. & Jones N.C. Metabolomics profiling of the brain revealed potential mechanisms associating Alzheimer's disease to higher seizure susceptibility in mice. Poster Presentation, *13th Asian and Oceanian Epilepsy Congress* (2021). Virtual.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer reviewed journals. The core theme of the thesis is the role of glutamate and astrocyte in the pathogenesis of epilepsy in Alzheimer's disease. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Neuroscience at the Alfred Precinct under the supervision of Prof. Patrick Kwan and A/Prof. Nigel Jones.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of Chapter 2 and 3, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author(s), Monash student Y/N
2	Astrocyte and glutamate involvement in the pathogenesis of epilepsy in Alzheimer's disease	Published	80%. Concept and writing the manuscript	1) Nigel Jones, review and editing, input into manuscript 10% 2) Patrick Kwan, review and editing, input into manuscript 10%	No No
3	Altered metabolic pathways in a transgenic mouse model suggest mechanistic role of amyloid precursor protein overexpression in Alzheimer's disease	Published	80% Concept, experiments, data collection, data analyses and writing the manuscript	1) Anna Harutyunyan, Data analyses, review and editing, input into manuscript 10% 2) Nigel Jones, review and editing, input into manuscript 5% 3) Patrick Kwan, review and editing, input into manuscript 5%	No No No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: *Hattapark Dejakaisaya* **Date:**

The undersigned hereby certifies that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:**

Acknowledgements

The immense amount of support that I have received from my family and friends during my candidature will forever be appreciated, especially since COVID-19 has impacted everyone's lives so much. Thus, I would like to dedicate this thesis to the wonderful people who believe in me and my potential from the start.

Above all, my family has always been my rock and my source of inspiration. Their unwavering love and faith have instilled me with the confidence and the bravery much needed for this PhD journey. This thesis would never have happened without the backing of my mom, dad and brother. I am extremely fortunate to be a part of this family. I love you very much.

To friends and colleagues that cheered me on as I jumped over many hurdles throughout my journey, thank you. To Nice, I greatly appreciate your support. You filled my days with so much love and joy. I also will never forget how we attempted to contact each other when I was on a trip to Uluru and there was literally zero phone reception. Looking forward to exploring many more parts of the world with you. To my "Friday Night Madness" mates. To RX, we have been an unstoppable team since the first day and my candidature was definitely more pleasant having you as a friend and a laboratory mate. I will always remember our weekly pilgrimage to Ebgames. AH and ZL, you guys made my life in Melbourne a lot brighter with your personalities. I am genuinely grateful to have friends such as yourselves in my life. May the force be with you and hopefully we can have a "Just Dance" night again in the future and know that you will have a friend whenever you visit Thailand. Special mention to Naomi Sedai, you were one of the best desk mates I could have asked for and I really do enjoy sharing my desk and tea with you. Tai'shar Manetheren!

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Chapter 1 – Introduction

Alzheimer's disease (AD) and epilepsy are two incurable neurological disorders that have been recognised for over a century[1] or in epilepsy's case, millennia.[2] There is evidence to suggest that the pathologies of AD can lead to an increase in the risk of developing epilepsy phenotype in patients.[3-5] It was found that spontaneous seizures were developed in 20% of the patients population a few years after the onset of AD symptoms.[3] In addition, 23% of the patients with severe AD developed at least 1 spontaneous seizure, while the healthy controls developed none.[4] Another study confirmed that AD increased the risk of seizures by 6-fold, compared to gender- and age-matched healthy controls.[5] Subsequently, more evidence to support the hypothesis that AD increases the risk of developing epilepsy has emerged[6, 7], which encouraged researchers to investigate the mechanism that links these two classic neurological disorders together. Despite that, this mechanism is still not elucidated. I hypothesised that glutamate plays an important role in increasing the risk of developing epilepsy in AD. Before the potential mechanisms and pathways that contribute to the pathogenesis of epilepsy in AD are discussed, it is important to review the backgrounds of these disorders individually. In this chapter, an overview of the current knowledge on these two serious neurological disorders is provided. Furthermore, the knowledge areas that are presently lacking in this field of research are reviewed.

1.1) Alzheimer's disease

The prevalence of AD is rising globally as the numbers of older population (>65 years old) in many countries, including Australia[8] and USA [9], continue to grow, despite the coronavirus disease 2020 pandemic.[10] It is predicted that 50 million people in the world have dementia, of which approximately 450,000 are Australian, and it is evident that AD is the most prevalent type of dementia, being responsible for more than 50% of all the cases.[11-14] In addition, AD is placing a global financial burden of approximately 1 trillion US dollars annually.[13] It is forecasted that the total number of worldwide population suffering from dementia will be increased to 152 million by the year 2050 and the financial burden will be doubled by 2030.[13] AD can be categorised into 2 types: 1) sporadic; and 2) familial. Approximately 90% of all AD cases are sporadic late-onset AD (LOAD), while approximately 2-3% of the cases are familial (FAD), which is associated with the early-onset AD (EOAD).[15] There is still no effective treatment that can modify or cure AD. This highlights the importance of discovering novel treatment targets and therapeutic windows that will modify or reverse the progression of AD pathologies.

1.1.1) Phenotypes & symptoms

AD is defined by progressive deterioration in 2 or more cognitive domains, such as episodic memory and executive function, which results in the impairment of daily task performance.[16] During the early stages of AD, patients will typically suffer from short-term memory loss, impairment in executive functions, spatial disorientation[17] and depressive symptoms[18], while potentially being unaware of the illness.[19, 20] This might be because the pathologies of AD affect the frontal and the temporal lobes before spreading to other brain regions, such as the neocortex.[21] Furthermore, AD may enhance the mortality rate associated with cardiovascular, respiratory and infectious complications in the patient up to 40%.[22] In severe cases, the patient will not be able to perform daily tasks independently and will be fully reliant on others. Affected individuals will also have reduced ability to socialise, gain new knowledge, recognise family members and/or have a fully compromised working memory[23], in combination with many behavioural symptoms, such as hallucinations.[24] Eventually, the patient with severe AD will die from complications related to aspiration, infection or inanition.[25]

1.1.2) Hypothesised mechanisms & risk factors

There are 2 main neuropathological hallmarks for AD, namely the deposition of the amyloid-β (Aβ) plaques and the neurofibrillary tangles (NFTs) caused by hyperphosphorylated tau protein aggregation.[21, 26]

Amyloid cascade hypothesis

Aβ deposition in the brain of AD patient is considered to be the clear and detectable pathology, preceding other known pathologies such as NFTs.[26] Thus, the amyloid cascade hypothesis has been the primary hypothesis when discussing the pathological mechanism of AD.[27] Aβ deposits are formed in the brain of AD patients due to the enhancements in the production and aggregation propensity of the Aβ peptides, which is correlated to an increase in neurodegeneration.[27, 28] The inherited forms of mutations associated with FAD are one of the factors that cause an increase in the Aβ deposits formation in the brain.[27] These inherited forms of mutations may consist of missense mutations in the genes that are involved in the production of Aβ peptide, such as amyloid precursor protein (APP) and presenilin 1 or 2. Another factor that may cause an increase in Aβ deposit formation is the sporadic mutation in genes that are involved in the Aβ peptide clearance mechanism, such as apolipoprotein, which will be discussed later in this chapter.

With the discovery of genes that are associated with FAD in the 1990s, many groups of researchers have begun studying families that are affected by the mutations in these

genes, hoping to gain insights on Aβ pathologies.[29-32] It was found that the majority of the family-linked mutations that increased the risk of EOAD are associated with the production of APP.[29, 33] The Swedish mutation is the most characterised form of FAD mutation and it was revealed that this K670N and M671L double substitution mutations took place in the APP coding region on exon number 16.[34] These mutations increase the production of βAPPs in the central nervous system (CNS) by enhancing the probability that APP will be cleaved by the β-secretase.[35-37] This increase in the production of βAPPs may lead to an increase in the production of the pathological Aβ species as illustrated in Figure 1.1. Briefly, after the APP was cleaved by β-secretase, the C-terminal fragment-β (CTF-β) and the βAPPs would be produced, a process which would be followed by the cleavage of CTF-β by γ-secretase complex.[38, 39] The process of γ-secretase cleavage typically produce $A\beta_{38}$, $A\beta_{40}$ or $A\beta_{42}$ as the product, with the main difference among these Aβ products being their lengths. In human, approximately 90% of all Aβ in the brain is in the form of $A\beta_{40}$.[40] However, the Aβ product that is most likely to form Aβ plaque in AD is the $A\beta_{42}$, not the $A\beta_{40}$.[41] There is evidence from the literature to suggest that $A\beta_{42}$ has the highest aggregation propensity due to its hydrophobic property; thus, it has the highest probability to initiate the Aβ plaque deposition process shown in Figure 1.2.[42, 43] In addition to mutations in the gene that encodes for APP, it was also discovered that mutations in *PSEN1* and *PSEN2* genes may also result in an increase in $A\beta_{42}$ production.[44, 45] The *PSEN1* and *PSEN2* genes encode for presenilin 1 and 2 proteins, respectively, and these proteins are important components of the γ-secretase complex. Investigating how the mutations in these genes lead to the pathogenesis of AD will be important for drug discovery.

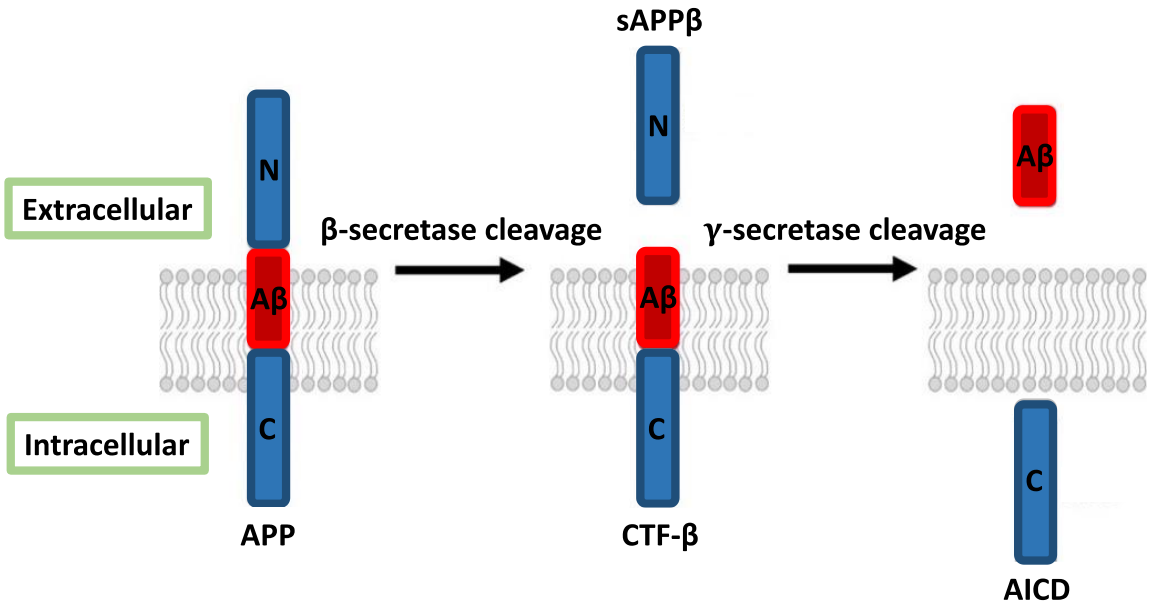

Figure 1.1. Amyloid-β (Aβ) peptide formation pathway. First, the amyloid precursor protein (APP) is cleaved by the β-secretase to produce a soluble-APPβ (sAPPβ) and a C-terminal fragment-β (CTF-β). Then, the CTF-β is cleaved by the γ-secretase to produce an amyloid intracellular domain (AICD) and an Aβ peptide. Modified from O'Brien & Wong, 2011.[39]

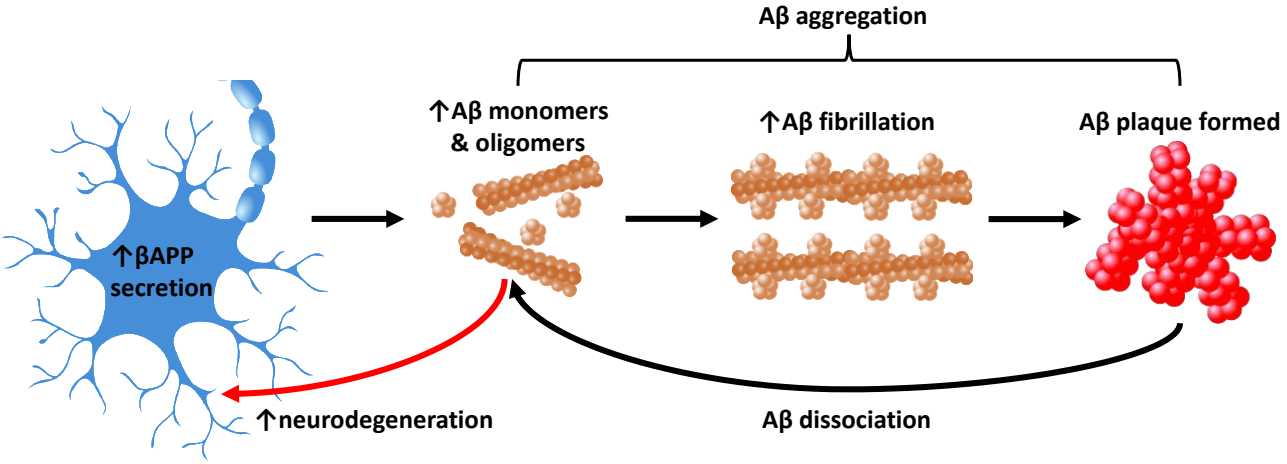

Figure 1.2. Aggregation pathway of the Aβ peptide in Alzheimer's disease (AD). The increase in the production of aggregation prone $A\beta_{42}$ peptide in AD leads to Aβ plaque deposition. Aβ plaques then act as reservoirs for the toxic Aβ oligomers, which may damage the adjacent neurons and cause neurodegeneration.

There is an ongoing debate on whether the oligomer, protofibril, mature fibril or the plaque form of $A\beta_{42}$ is the most pathological in AD. The structural differences among each form of $A\beta_{42}$ have been illustrated by Chen and colleagues.[46] Evidence from an *in vitro* study using human neuroblastoma cells[47] and animal studies[48, 49] suggests that the oligomeric form of Aβ is the most neurotoxic, compared to other forms.[50, 51] It was demonstrated that $A\beta_{42}$ oligomers were 10-fold more neurotoxic than the $A\beta_{42}$ fibrils and 40-fold more neurotoxic than the monomers.[47] Moreover, the Aβ oligomers treatment caused more disruption to the cognitive function in rats, compared to monomers.[48] The neurotoxicity of Aβ oligomers has been reported to be mediated by the mechanism which involves N-Methyl-D-aspartate receptor (NMDAR), cellular prion protein and Fyn.[49, 50] Importantly, the glutamatergic pathway has also been linked to the Aβ pathology and this will be discussed in detail in Chapter 2. Despite the fact that Aβ oligomers may be the most pathological species of Aβ, the neurotoxicity of other forms of Aβ, such as protofibril[52] and fibrillar[53] Aβ, cannot be neglected. It was shown that rare Dutch (E22Q) and Arctic (E22G) mutations in the Aβ peptide sequence encourage the productions of protofibril and fibrillar Aβ which are more toxic than the wild-type (WT) forms of these proteins.[47] Furthermore, Aβ protofibril has the ability to increase the excitability of the cell membrane, compared to low molecular weight Aβ.[52] Evidence from an *in vitro* animal study suggests that neurons located within 15μm of the fibrillar Aβ deposits also displayed abnormalities, such as smaller spine density, compared to neurons farther away.[53] Investigating the mechanisms that mediate the neurotoxicity of Aβ oligomers and other forms of Aβ is important for the discovery of novel treatment for AD as the correlation between the amount of Aβ plaques and the severity of cognitive impairment in AD patients is weak, which may suggest that the Aβ plaque is merely a bystander in Aβ pathology.[38, 40]

In contrast, Aβ has demonstrated beneficial physiological functions in the CNS, such as protecting the CNS from infections and aiding the recovery of the brain after injury.[54] Furthermore, evidence from an *in vitro* study which involved cortical neurons culture acquired from rats demonstrated that $A\beta_{42}$ monomer may provide neuroprotective effect against insulin deprivation.[55] Despite these beneficial effects, disruptions to the homeostasis of Aβ which result in an increase in the production or decrease in the clearance of Aβ may lead to the pathogenesis of AD.[56]

Since mutations in the APP gene, which result in an increase in Aβ, may play a role in FAD, it is important to discuss the physiological role of APP protein. Although the normal physiological function of APP is not well elucidated, different strains of APP-knockout

models were created to decipher these putative mechanisms.[57-60] The evidence suggested that APP-knockout models did not suffer from any detrimental phenotypes [58-60], however, knocking out amyloid-like protein 1 and 2 along with the APP gene proved to be perinatally lethal.[61, 62] This finding implies that the animal cannot survive when there is no compensatory mechanism for the lack of APP in the brain. APP-knockout models also revealed age-related memory deficits, impaired learning, lack of locomotor activity, reduced forelimb grip strength and loss of 10-20% body weight, compared to WT.[58, 59, 63] One study investigated the axonogenesis property of the βAPP-knockout mice using an *ex vivo* method and it was revealed that the neurons acquired from βAPP-knockout mice displayed lower cell viability and had reduced branching ability, compared to WT.[64] On the contrary, it was experimentally shown through *in vivo* and *ex vivo* methods that the secreted form of βAPPs promotes neural growth and protects neurons from Ca^{2+}-induced excitotoxicity.[65-67] Therefore, it is evidenced that APP and βAPPs have beneficial physiological functions, but when the balance between soluble APP and βAPPs is disrupted, toxic Aβ build-up may occur.[39]

Despite the fact that Aβ cascade hypothesis has been around for more than 25 years, the mechanism of how Aβ peptide and Aβ deposits trigger a cascade that leads to the pathogenesis of AD is still controversial.[27, 68] The most up-to-date version of the amyloid cascade hypothesis suggests that $A\beta_{42}$ oligomers, not monomers or plaques, act as the trigger that can start a cascade of intracellular signalling, while other pathways such as hyperphosphorylation of tau and neuroinflammation translate these signals into neurodegeneration and cognitive impairment.[27, 50, 68-70] Overall, more investigations in both pre-clinical and clinical scenes need to be done to generate more evidence to determine which form of Aβ is the most neurotoxic and which form of Aβ has the highest probability of triggering the downstream targets that can launch an unstoppable cascade of deleterious pathways observed in AD.

Neurofibrillary tangles

NFT, which consists of hyperphosphorylated tau protein, is another major pathological feature observed in AD patients. In AD, NFTs develop in topographical stages as described by Braak and colleagues.[71, 72] As AD progresses, NFT moves from Stage I (least severe) to Stage VI (most severe). During Stage I, the location of NFTs is confined to the transentorhinal region, but as this pathology developed to Stage VI, NFTs would become visible in most areas of the brain, including the striate and parastriate areas.

Tau protein is coded by the *Microtubule Associated Protein Tau (MAPT)* gene and, under normal physiological condition, it acts as a microtubule structure stabiliser and

facilitates axonal transportations in the CNS.[73, 74] Furthermore, tau modulates different intracellular signalling pathways via its ability to bind to various partners.[75] In AD, tau pathology is believed to be caused by an imbalance between the activities of kinases and phosphatases on tau protein.[76] Kinases that are associated to tau protein such as glycogen synthase kinase 3 (GSK-3β), cyclin-dependent-like kinase-5, protein kinase A and mitogen-activated protein kinase are reported to have increased activities in AD[77-79], while a phosphatase such as protein phosphatase 2 (PP2A) has a decreased activity.[80] PP2A is the regulator of tau phosphorylation and it was revealed that the inhibition of PP2A's activity led to an increase in the amount of phosphorylated tau protein in the brain.[81] Phosphorylation of tau encourages the aggregation of dissociated tau in the neuron soma, which may increase the risk of NFT. NFT is formed when, hyperphosphorylated tau and normal tau polymerises into paired helical filaments (PHF) mixing with straight filaments.[82, 83]

The imbalance between the activities of kinases and phosphatases on tau protein is hypothesised to be the downstream effect of Aβ pathology, based on the evidence that the Aβ plaques are typically detected before NFTs in AD patients.[26, 33] On the other hand, it is also hypothesised that Aβ pathology relies on the presence of hyperphosphorylated tau to inflict neurodegeneration, as it was found that the level of neurotoxicity correlating to the Aβ pathology was mitigated significantly in tau-knockout rodent and cell culture models.[84-87] Thus, tau pathology plays an important part in mediating neurodegeneration in AD and may contribute to the progression of the disease, despite the fact that NFTs are typically detected later than Aβ plaques. Furthermore, the effect of phosphorylated tau overexpression in the brain has also been investigated. It was found that phosphorylated tau overexpression alone may cause neurodegeneration via the direct inhibition of neuronal axogenesis and microtubule structure destabilisation.[88] However, it has been reported that tau protein hyperphosphorylation alone is not harmful enough to cause symptoms related to AD as the neurodegenerative pathways associated with phosphorylated tau may also depend on the expression levels of tau binding partners, such as axonal transport mediator or Src kinase such as Fyn, to cause axonal transport dysfunctions and cognitive impairments.[75, 89-94] Although, NFTs are reported to be caused by Aβ pathology, it was demonstrated that small tau aggregates can migrate from one neuron to an adjacent one via nanotubes and exosomes to act as aggregation seeds, independent of the Aβ pathology.[95] This piece of evidence suggests that NFTs can form and potentially cause neurotoxicity without relying on the Aβ pathology. However, when tau pathology is acting synergistically with Aβ pathology, the neurotoxicities of both pathologies are amplified.[84]

To investigate the impact of tau pathology on AD, homozygous tau-knockout mouse models have been developed. Evidence generated from these models demonstrated that the complete knockout of tau protein did not lead to developmental impairment[96-98]; however, the tau-knockout mice displayed cognitive and motor function impairments[99, 100], possibly due to the intracellular accumulation of iron species in the brain.[101] These findings suggest that there might be a compensatory mechanism for tau deficiency, although more evidence is needed to support this theory. Furthermore, transgenic mouse models that overexpress mutant human tau, such as the FTDP-17 model, were developed to study the effects of NFT on the brain.[102] It was found that these human mutant tau transgenic models display aspects of human AD pathologies, such as axonal swelling and dystrophic neurites; therefore, these models provide evidence in support of the theory that tau pathology contributes to the progression of AD.[102] It is also worth noting that these models express mutant human tau protein instead of murine tau, as the latter cannot form NFT due to its structure, which is different from human tau proteins.[103] It has been shown that the amino acid sequences of the longest isoforms of human and murine tau proteins are 89% identical and the main structural difference is located in the N-terminal end, where human tau contains 11 amino acids that are not present in murine tau.[103]

In conclusion, evidence from the literature suggests that Aβ pathology occurs prior to NFTs pathology in AD. However, both pathologies may need to act in concert for AD to progress with enough pace to cause neurological symptoms.[69, 71, 84, 104] There are 3 main theories on how Aβ peptide and phosphorylated tau may interact to exacerbate the symptoms of AD: 1) Aβ causes the hyperphosphorylation of tau directly; 2) phosphorylated tau acts as an intermediate for Aβ pathology to propagate toxicity; 3) soluble Aβ oligomers and phosphorylated tau together target the same pathway involving organelles such as mitochondria to propagate the toxicity.[84] Due to the complexity of the interplays between Aβ and tau, the exact mechanism that causes neurodegeneration in AD still need elucidation. The potential of tau as the treatment target for AD will be discussed in the “diagnosis and current treatments” section.

Other risk factors

It is well documented that Aβ plaques and NFTs are the 2 hallmark features of AD; however, there are other risk factors that play crucial roles in the development and progression of AD. One of the most well-known risk factor of AD is ageing and the chance that a person over the age of 65 years old will develop AD is as high as 30%.[21] Other reported factors that may increase the risk of AD include female gender, low educational

attainment, diabetes mellitus, mid-life hypertension, smoking, mid-life obesity, sleep deprivation[105], stroke[106, 107] and depression.[21, 108]

Importantly, apolipoprotein E ε4 (ApoE4) has also been identified as one of the genetic risk factors for LOAD. It was shown that AD patients have higher ApoE4 expression level in the CNS compared to the healthy controls.[109] The risk of developing LOAD increased from 20% to 80% when an individual carries 2 ApoE4 alleles instead of the "normal" ApoE3 alleles.[108, 110] Under normal physiological condition, ApoE3 is the most common isoform of apolipoprotein, contributing to approximately 77% of all the ApoE expressions, while the ApoE4 and ApoE2 are typically expressed at approximately 15% and 8% respectively.[111] The main function of apolipoprotein is to facilitate the transport of cholesterol and other lipids via cell-surface lipoprotein receptor binding.[112] It was also demonstrated that ApoE4 may directly interact with both Aβ and tau peptides; thus, ApoE might be one of the neurotoxicity mediators for the pathological hallmarks of AD.[90, 111, 113] However, the mechanism in which the ApoE4 interacts with AD pathologies and how it increases the risk of AD is still poorly understood. Various human ApoE4 knock-in mouse models, such as the E4FAD, have been generated to elucidate this mechanism and it was reported that ApoE4 may cause neurodegeneration via Aβ-dependent and Aβ-independent mechanisms.[114] It was also demonstrated that the E4FAD mice had higher amount of Aβ in their brains, compared to ApoE2/3 (E2/E3FAD).[115] Furthermore, evidence from E4FAD mice suggests that the expression of ApoE4 is associated with the reduction in the cerebral blood flow, which implies that ApoE4 may be linked to the vascular hypothesis of AD.[116]

The vascular hypothesis of AD is another candidate for explaining the pathophysiology of AD. In this hypothesis, disruption to the vasculature of the brain may contribute to the pathogenesis of AD by accelerating the mechanism that cause the deposition of Aβ and lead to neurodegeneration.[117, 118] It was reported that neurovascular diseases, such as stroke and atherosclerosis, may enhance the risk of developing AD in human patients up to 3-fold, compared to healthy controls.[107, 119] Evidence from preclinical studies also suggests a link between the reduction in cerebral blood flow and the increase in Aβ load in the brain.[120, 121] The vascular hypothesis of AD and other hypothesised mechanisms are gaining attention because it is becoming apparent that focusing solely on the amyloid hypothesis of AD will not yield a disease-modifying therapy for AD. The present and the future of AD treatment will be discussed in the following section.

1.1.3) Diagnosis & current treatments

AD can only be diagnosed with certainty via post-mortem staining methods, such as modified Bielschowsky silver stain, to identify the Braak & Braak stage of AD that the patient was in at the time of death.[26, 122] The lack of diagnostic tools together with the fact that there are overlapping symptoms between AD and other types of dementia, such as frontotemporal lobe dementia, mean that AD patients are commonly misdiagnosed.[123, 124] However, there are now more advanced methods for detecting changes in the CNS that are associated with AD pathologies, which may allow a more precise diagnosis of AD patients while they are still alive. These methods include cerebrospinal fluid (CSF) analysis, physical exams, mental status tests, brain imagings and blood tests.[125-127] Brain imaging with positron emission tomography (PET) scan together with a tracer, such as Pittsburgh compound B, has been successful at detecting Aβ aggregates in the patients before the onset of the AD symptoms.[128, 129] Furthermore, blood tests provide a minimally invasive method for measuring biomarkers associated with AD pathologies, such as the plasma Aβ level which correlates to the level of Aβ in the CNS of an individual.[126] More recently, Glutamate Chemical Exchange Saturation Transfer (GluCEST) protocol for Magnetic Resonance Imaging (MRI) was proposed as a new non-invasive diagnostic method for neurodegenerative diseases, such as Parkinson's disease.[130] Since AD is a complex disease with a wide spectrum of symptoms, different diagnostic tools might be combined to increase the accuracy of the diagnosis.

Currently available treatment for AD consists of 6 drugs that are approved by the Food and Drug Administration: 1) Donepezil; 2) Galantamine; and 3) Rivastigmine, which are cholinesterase inhibitors; 4) Memantine, which is a NMDAR antagonist; 5) a combination of Donepezil and Memantine; and the last one being 6) aducanumab, which is an anti-amyloid antibody.[131, 132] Since AD patients are known to suffer from memory and learning deficits, reduced choline acetyltransferase enzymatic activity and lack of acetylcholine were thought to have major roles in the disease's pathology.[133] This theory was based on the evidence which suggests that reduction in the activity of choline acetyltransferase – an enzyme responsible for the production of acetylcholine – and acetylcholine are linked to cognitive impairments.[134-136] This led to the development of inhibitors that target the cholinesterase – an enzyme responsible for the breaking down acetylcholine – as treatments for AD.[137] Despite that, it was found that cholinesterase inhibitors only alleviate the symptoms of AD and they do not slow down the progress of the disease or provide any disease modifying effect.[138] As for memantine, it is known that the overactivation of NMDAR is one of the pathological features of AD[139, 140]; therefore, memantine was designed to

ameliorates the symptoms of AD via the reduction of NMDAR activation.[141] Since NMDAR is a glutamatergic receptor, this imply how important the glutamatergic pathway is in the pathogenesis of AD. The involvement of the glutamatergic pathway will be discussed further in Chapter 2. Lastly, the first human monoclonal antibody treatment that targets Aβ oligomers and fibrils for the treatment of AD was approved in 2021, despite the uncertainty that surrounds the amyloid hypothesis of AD.[142-144] It was shown that aducanumab improved the disease outcome of AD patients through two controlled trials; thus, the approval of aducanumab was accelerated.[145] The approval of aducanumab provided an alternate treatment option for patients suffering from late stage of AD.

In the future, targeting earlier therapeutic windows with a combination of treatments to address different aspects of AD pathologies should be considered. Presently, there are many potential treatments for AD in the development pipeline, and they target different aspects of AD.[146, 147] Aβ peptide and its associated secretases, such as β and γ, are the pharmacological targets of choice and a lot of efforts have been put toward clearing the Aβ plaque from the brain of the patients suspected to be suffering from AD.[148, 149] Different approaches for removing the Aβ plaques from the brain had been explored, including humanised antibody [150, 151] and secretase blockers.[152, 153] Additionally, the potential of phosphorylated tau as a target for AD treatment cannot be ignored, and in fact, efforts have already been put into blocking the NFTs formation via immunotherapy[154, 155], but the clinical trials were unsuccessful.[156] Furthermore, compounds that target ApoE4 also yielded high efficacy in clinical trials, yet they still failed to reach the market.[157]

Despite the fact that novel treatments demonstrated encouraging results in animal studies, there is still a lack of evidence to show that newer treatments are more beneficial than the current treatments in human AD patients.[158, 159] One reason behind the repeated failure of the drugs that targets the Aβ plaques and NFTs is that the drugs are being administered too late in the disease stage, which means that irreversible damages in the brain has already been done.[160] Dead neurons simply cannot be revived by removing Aβ plaques or NFTs; therefore, removing Aβ plaques and NFTs may not cure AD. On the other hand, Aβ plaques and NFTs may represent epiphenomena rather than being the direct causes of AD.[142, 161] The mechanisms that involve Aβ plaques and NFTs may aggravate the disease but not necessarily cause it, which may explain why most clinical trials that target these pathologies continued to fail. Furthermore, major hurdles that the secretase inhibitors and the antibody-based treatments have to overcome are the low substrate specificity nature of the secretase and the blood-brain barrier (BBB).[148, 162] However, the hope for antibody-

based treatment has been boosted by the emergence of the focused ultrasound technology. This technology transiently permeates the BBB just so that the typically BBB impermeable-molecule may enter the CNS and reach its intended target.[163]

1.2) Epilepsy

Epilepsy is a debilitating neurological disorder that affects individuals across all age ranges. Epilepsy manifests as unprovoked and recurrent seizures caused by the brain neuronal network hyperexcitability. It is estimated that epilepsy affects 65 million people around the world with approximately 80% of the epilepsy patients being located in developing countries.[164] In Australia, there are more than 250,000 people living with this serious neurological disorder, and it is forecasted that 3-3.5% of the population will be affected by epilepsy at some point in their lives.[164] Furthermore, epilepsy may decrease the quality of life of the patient by at least 10-fold in countries with lower average income than developed countries.[165] The quality of life of the patient is also highly dependent on the quality of life of the family members; therefore, the support from family plays an important part in reducing the psychological symptoms associated with epilepsy.[166]

1.2.1) ***Phenotypes & symptoms***

According to the International League Against Epilepsy, epilepsy is classified into 4 categories: 1) focal; 2) generalised; 3) combined generalised & focal; and 4) unknown.[167, 168] Focal seizure is when only a hemisphere of the brain is affected by the abnormal discharge resulting in a seizure, while generalised seizure is when abnormal synchronisation of neuronal network affects the brain bilaterally. Unknown seizure is when there is not enough evidence to rule the seizure into either generalised or focal category.[169] Regarding the most common type of epilepsy in human, a study in France has demonstrated that focal epilepsy is the most common type, occurring in 62% of the epileptic subject population, while the most commonly affected region of the brain is the temporal lobe, as this region was implicated in approximately 66% of the epilepsy cases.[170] In addition to recurrent seizures, epilepsy patients also commonly suffer from comorbidities, such as cerebrovascular disease, cardiovascular disease and neuropsychiatric disorders.[171] Diagnosis of these comorbidities is very important as it will determine how the patient should be treated. Further, comorbidities such as dementia and stroke may also have bidirectional relationships with epilepsy.[171] Temporal lobe epilepsy and AD affect similar regions of the brain; thus, there may be more links between AD pathologies and the increase in the risk of epilepsy in the patients than currently elucidated.

1.2.2) ***Main causes & risk factors***

It is known that excessive and hypersynchronous neuronal discharge caused by an imbalance between excitatory and inhibitory activities in the brain may result in seizures and if these seizures became recurrent, then epileptogenesis – the birth of epilepsy – may occur.[172] There have been a vast number of studies on the mechanisms that underlie epileptogenesis in human and, concisely, there are currently 6 main aetiology categories: 1) structural; 2) genetic; 3) infectious; 4) metabolic; 5) immune; 6) unknown.[167] While idiopathic epilepsy is responsible for the majority of the cases[173], the most common non-genetic aetiological cause of epileptogenesis is traumatic brain injury (TBI).[174, 175] It was reported that TBI patients have up to 7% increased chance of developing post-traumatic epilepsy (PTE).[176] In severe cases that involve post-war injuries, the patient may have up to 53% higher chance of developing PTE.[177, 178] TBI often leads to structural lesion in the brain[179, 180], which may increase the risk of 25pilepsy.[172] As for the genetic cause of epilepsy, mutations that occur in genes which encode for ion channels, neuronal receptors, transcriptional factors and enzymes have been identified.[181] So far, mutations in more than 30 genes have already been reported and linked to an increase in the risk of developing epilepsy.[182] For the infectious cause of epilepsy, there is evidence from the literature to suggest that bacterial, parasitic and viral infections may increase the risk of epileptogenesis.[183] Metabolic causes of epilepsy consist of serious neurological disorders that are associated with deficiencies in enzymes which are responsible for regulating the level of excitatory and inhibitory neurotransmitters such as glutamate and amino butyric acid (GABA) respectively.[184] Finally, epilepsy can also be caused by autoimmune diseases, such as encephalitis, especially when the antibodies target the neuronal receptors for excitatory neurotransmitter, for example the NMDAR.[181] Interestingly, dementia has also been identified as one of the risk factors for epilepsy.[185] Although these factors ultimately result in the hyperexcitability of the brain, their underlying pathways can be different. Seizure prone brains become more susceptible to overexcitation by specific stressors, which can be different from one person to another due to the genetic and environmental factors.[186] One of the factors that may increase the risk of epileptogenesis in patients is the increase in extracellular glutamate accumulation in the CNS caused by impaired glutamate uptake mechanisms.[187] The accumulation of extracellular glutamate level in the brain has also been reported in AD[188]; therefore, this may be one of the potential link between epilepsy and AD that shall be discussed further in this thesis.

Many cases of epilepsy are still reported as unknown due to its complexity[189]; therefore, additional mechanisms related to the pathogenesis of epilepsy need to be

identified in order to reveal more aetiologies of epilepsy. Towards this end, animal models of epilepsy will be an important tool for identifying novel causes and risk factors of epilepsy. Examples of the currently available animal models of epilepsy include electric kindling of the brain[190], chemical kindling of the brain with pentylenetetrazol (PTZ)[191], kainic acid model [192] and pilocarpine model.[193] Kindling refers to a process in which seizures are repeatedly induced via a stimulus in order to incrementally increase the severity of the seizure after each induction until the severity reaches plateau.[194] The duration of the seizure is expected to lengthen and the intensity of the behavioural response during a seizure is expected to be more severe with each repeated induced seizure.[194] The animal may eventually develop spontaneous seizures as the result of kindling.[194] Therefore, kindling model of epilepsy may provide valuable information on the physiological and anatomical changes associated with epileptogenesis. Additionally, these animal models of epilepsy allow researchers to gain insights on aetiologies and effects of epilepsy by giving researchers the ability to precisely control the timing and the location of the seizure, collect tissues at specific stages of the disease development and generate important data to guide future human studies.

1.2.3) ***Diagnosis & current treatments***

A person can be diagnosed with epilepsy after they experienced: 1) 2 unprovoked seizures at least 24 hours apart; 2) 1 unprovoked seizure and enhanced risk of developing recurrent seizure during a period of a decade; or 3) confirmation of epileptic syndrome via diagnostic tools.[169] The gold standard tool for epilepsy diagnosis is electroencephalography (EEG), as this tool can detect abnormal neuronal activities, which is a commonly observed feature in epileptic patients. Further, using interictal video/EEG in conjunction with imaging techniques, such as brain MRI, may reduce the chance of misdiagnosis. Additionally, blood and CSF analyses may also be utilised to confirm any suspicion on the seizure types or to identify comorbidities.[169]

It is important to diagnose the patient with the correct type of epilepsy as each type requires a different treatment approach. Anti-epileptic drugs (AEDs) such as valproate, lamotrigine, carbamazepine and levetiracetam have different efficacies on different types of epilepsy.[195] However, drugs like levetiracetam can be efficacious against more than 1 type of epilepsy when compared to other existing AEDs. There is also less adverse effects associated with the use of levetiracetam when compared to valproate and carbamazepine.[196] Additionally, levetiracetam possesses a lower treatment withdrawal rate without sacrificing the seizure freedom rate, compared to other standard AEDs.[196] One of the most recently approved AED, Brivaracetam, has demonstrated higher specificity for its target than levetiracetam and causes even less adverse effects in the patients.[197] Despite

the availability of newer and more efficacious AEDs, drug-resistant epilepsy may still emerge when the patient is not responding to adequate doses of at least 2 appropriate AEDs. A study conducted in Singapore reported that approximately 21.5% of the patient population demonstrated drug-resistant epilepsy and the age group that had the highest risk was the 40-49 years old group.[198] After the patient is diagnosed with drug-resistant epilepsy, treatment methods such as epilepsy surgery, neurostimulation and diet treatments can be considered.[199]

Despite the availability of an array of AEDs, there is no drug that can cure or modify epilepsy; therefore, it is important to investigate the aetiologies of epileptogenesis so that earlier therapeutic windows can be revealed. In the future, gene therapy may also be an option for the treatment of epilepsy in human [200] ;however, this therapy has yet to reach clinical trial stage.

1.3) Interplay between Alzheimer's disease and epilepsy

After a substantial amount of evidence on the relationship between AD and the increase in the risk of developing epilepsy has emerged, researchers has begun to use rodent models of AD to investigate this connection.[201]

1.3.1) ***Epilepsy phenotype in AD rodent models***

A landmark study by Palop and colleagues revealed that neural network dysfunction was observed in the hAPP-J20 mouse model of AD.[202] This suggests that AD pathologies, such as Aβ overexpression, can be associated with an increase in the risk of developing epilepsy. This study demonstrated that hAPP-J20 mice exhibited higher susceptibility to pentylenetetrazol-induced seizures and higher death rate associated with induced-seizure, compared to non-transgenic controls.[202] It was also revealed that freely behaving hAPP-J20 mice frequently exhibited synchronised discharges which affected the whole brain, while the controls exhibited none.[202] To further support the hypothesis that AD pathology can cause abnormally high neuronal activities in mouse models of AD, gamma oscillation was investigated and the results revealed higher level of network activity synchronisations in AD models.[203] The evidence from the literature also suggests that other mouse models of AD are susceptible to induced seizures as they displayed more severe seizure phenotypes in response to electrical and pharmacological induced seizures, compared to WT mice.[204-206] Therefore, mouse models of AD have the potential to reveal the mechanistic links between AD pathology and the increase in the risk of epilepsy.

One of the most well-characterised and widely used mouse model of AD is the Tg2576 transgenic mouse model, first generated by Hsiao and colleagues in 1996.[207-210] Tg2576 mice harbour the Swedish mutation on the *APP* gene (K670N/M671L), resulting in the overexpression of mutant human APP. The reason why Tg2576 model was designed to overexpress mutant human APP instead of the mouse APP is because the β-secretase preferably binds to the phenylalanine at position 10 on the mouse APPβ, while in human, the β-secretase binds to the aspartate at position 1 as depicted in Figure 1.3.[208] This causes the β-secretase to produce a shorter Aβ product with lower aggregation propensity from the mouse APPβ and a longer Aβ product with higher aggregation propensity from the human APPβ. Tg2576 mice overexpress mutant human APP since they were born and the Aβ pathology occurs over time as shown in Figure 1.4. Briefly, an increase in Aβ level in the brain is typically detected after 5 months, while the Aβ plaques in the entorhinal cortex, hippocampus and other areas can be detected from approximately 9 months onwards.[208] After 6 months, Tg2576 mice would have progressive memory and learning deficits relatable to cognitive deficits in human AD patients. Additionally, Tg2576 mice demonstrate sensorimotor deficits in the balance beam and string tasks, especially in aged Tg2576 mice.[211] Furthermore, interictal spikes can be observed in Tg2576 mice via EEG from approximately 5 weeks of age onwards, which may indicate a higher susceptibility to develop epilepsy.[212] The main advantages of using Tg2576 mice are that they are well-characterised and that their colonies are easy to manage. However, there are some disadvantages associated with this model such as it takes a long time to show any AD phenotypes, such as cognitive deficits, and this model only demonstrates the effect of mutant human APP overexpression, not the other AD pathologies.

This thesis will focus on the effects of mutant human APP overexpression on the glutamatergic pathway in the brain. Chapter 2 of this thesis will discuss the potential role of glutamate in the pathogenesis of epilepsy in AD in detail. This will give us insights on how the disruption of glutamatergic pathways might link AD pathology to an increase in the risk of developing seizures. Since evidence from the literature suggests that Tg2576 mice are more prone to induced-seizures[213] than the controls, this model will be suitable to be used to answer this question.

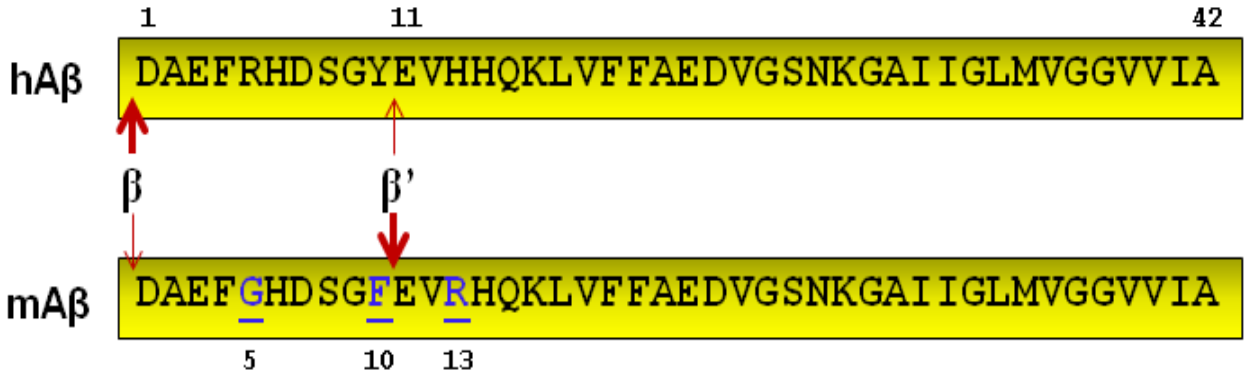

Figure 1.3. The differences between the location of cleavage by the β-secretase on the Aβ amino acid sequences from human (hAβ) and mouse (mAβ). Bold arrows = preferred site of binding (modified from Hall & Roberson, 2011).[208]

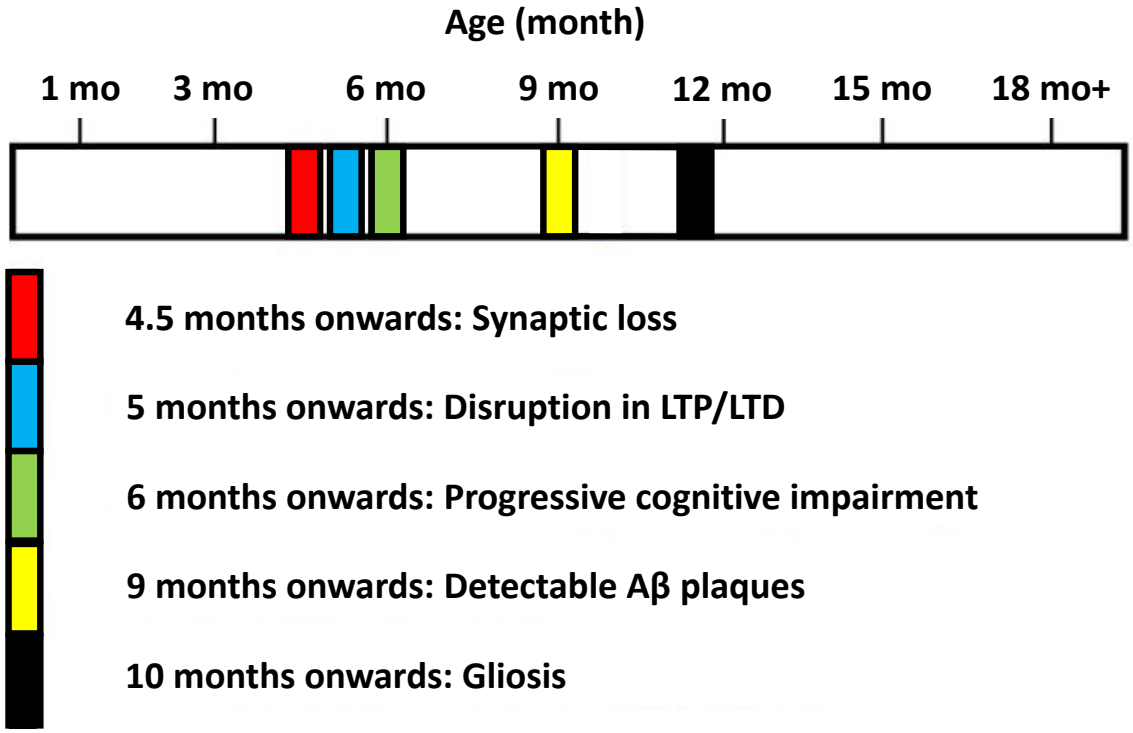

Figure 1.4. Main pathological events timeline of the Tg2576 mouse model of Alzheimer's disease. (LTP = Long-term Potentiation; and LTD = Long-term Depression). (Modified from ALZFORUM, 2019).[208, 214]

1.4) Current challenges in elucidating the mechanism that links AD pathologies to an increase in the risk of epilepsy

The mechanistic link between AD pathology and the increase in the risk of epilepsy is still poorly understood and elucidating this link may allow us to prevent the pathogenesis of seizures in AD. Additionally, the relationship between AD and epilepsy may be bidirectional; therefore, identifying the common mechanisms between these neurological disorders will be beneficial for both epilepsy and AD patients. It will also be important to investigate this mechanism in both animal and human studies. The first challenge is associated with the use of animal models, as animal models of AD only provide insights from certain pathology of AD, not the whole spectrum of pathologies like in human AD. The second challenge is associated with human study and human sample acquisition as AD can only be diagnosed with certainty post-mortem and human brain sample acquisition is not practical, compared to animal study. In animal studies, brain samples can be collected at a specific timepoint via euthanisation and brain extraction. On the other hand, brain sample acquisition procedure in human studies remains invasive[215]; thus, brain sample collection may not be preferable for the subject.

Secondly, despite many potential AD and epilepsy treatments being in the development pipeline over the last few decades, a treatment with disease-modifying effect is still unavailable. As discussed earlier in this chapter, the available treatments only alleviate the symptoms and just prolong the inevitable disease progression. For AD, this can partly be attributed to the clinical trial failures associated with the potential treatments. The potential treatments are being administered to patients who are already experiencing the symptoms of AD, which suggests that irreversible damages to the brain had been done. This implies that the ability to diagnose AD before the patient starts experiencing the symptoms is the key to a successful clinical trial for AD treatment. In the future, using a combination of drugs with different targets, such as Aβ and glutamatergic pathway, at the right dose[148] during an early therapeutic window may be considered. Therefore, it will be important to elucidate how AD pathologies alter the brain during the early stage of the disease.

With the treatment of epilepsy in AD, there is evidence to show that some of the currently available AEDs can cause cognitive impairment as an adverse effect in the patients and this can potentially aggravate the symptoms of AD.[216] Development of AEDs with different mechanisms of action than the existing compounds is warranted. Pharmacological compounds, such as perampanel (AMPA-receptor antagonist)[217], that can act on glutamatergic pathways may potentially be beneficial to the symptoms of both epilepsy and AD. To target the glutamatergic pathway, compounds such as glutamate transporter-1 (GLT-1) expression enhancer, glutamate secretion blocker or glutamate receptor inhibitors may

also be considered. By using a pharmacological compound with a unique target but known mechanism of action, more insights on the pathogenesis of acquired epilepsy in AD may be gained.

1.5) Aims and chapter overviews

The overall aim of this thesis is to elucidate the pathways that link AD pathologies to an increase in the risk of epileptogenesis. The roles of glutamate and astrocyte are of particular interest as both were implicated in epilepsy and AD. I hypothesised that disruption to the glutamate homeostasis by AD pathologies leads to an increase in the risk of developing epilepsy. Chapter 1 introduced this topic by discussing the evidence from the literature on the epidemiological data of epilepsy in AD patients. Then, the background on AD and epilepsy were discussed in detail. Chapter 2 will then discuss the roles of glutamate and astrocyte in the pathogenesis of epilepsy in AD via a published critical review article.

The first aim of this thesis is to gain insights on whether the glutamatergic pathways in the brain have been altered by the early stages of mutant human APP overexpression pathology. As will be discussed in Chapter 2, the disruption to the glutamatergic pathways may be one of the mechanisms that contribute to the increase in seizure susceptibility in AD. I hypothesised that metabolomic pathways and metabolite levels associated with glutamate are altered during the early stage of AD. It is known that Tg2576 mice are more susceptible to seizure, compared to WT[204]; therefore, metabolomics analyses were performed on brain and serum samples acquired from mature Tg2576 mouse model of AD to address this aim. Published results from the metabolomics analysis of Tg2576 mice will be presented in Chapter 3.

The second aim is to investigate how the glutamate-glutamine cycle is involved in the pathogenesis of epilepsy in AD. In Chapter 4, the effects of mutant human APP overexpression on the glutamate-glutamine cycle will be investigated. The amygdala kindling model of epilepsy was also utilised to investigate the effect of epileptogenesis on the expression levels of key proteins in glutamate-glutamine cycle. The results will provide insights on potential pharmacological targets from the glutamate-glutamine cycle for the treatment of epilepsy in AD.

Lastly, this thesis aimed to investigate how epileptogenesis affects the glutamate-glutamine cycle in Tg2576 mice. This would elucidate whether glutamate-glutamine cycle is one of the links between AD pathologies and the increase in the risk of developing epilepsy. Additionally, the potential of key proteins in the glutamate-glutamine cycle, such as GLT-1 transporter, to be the treatment target for epilepsy in AD will also be investigated. Chapter 5 of this thesis will investigate this aim.

Chapter 6 will discuss challenges encountered during each experiment and will discuss how each experiment may be improved in future studies. It will also discuss future directions based on insights gained from the experiments performed in this thesis. Finally, Chapter 6 will discuss the implications of the discoveries made in this thesis.

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Chapter 2 – Astrocyte and glutamate involvement in the pathogenesis of epilepsy in Alzheimer's disease

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2.1) Preamble to review paper

Building on what was discussed in Chapter 1, this published review paper critically considered the evidence from human and animal studies on how astrocytes and glutamatergic pathways are strong candidate mechanisms that link Alzheimer's disease (AD) pathologies to an increase in the risk of developing epilepsy.

Briefly, it discussed how AD pathologies and epileptic seizures disrupt the normal functions of astrocytes and the glutamate-glutamine cycle. The mechanism that links the disruption of astrocytic functions and the glutamate-glutamine cycle to an increase in the risk of developing epilepsy was also discussed. Additionally, this review discussed the role of proinflammatory cytokines in disrupting the astrocytic functions under AD conditions. Finally, this review discussed the potential of key proteins in the glutamate-glutamine cycle as treatment targets for epilepsy in AD.

2.2) **Review paper**

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Epilepsia

CRITICAL REVIEW – INVITED COMMENTARY

Astrocyte and glutamate involvement in the pathogenesis of epilepsy in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) can increase the risk of epilepsy by up to 10-fold compared to healthy age-matched controls. However, the pathological mechanisms that underlie this increased risk are poorly understood. Because disruption in brain glutamate homeostasis has been implicated in both AD and epilepsy, this might play a mechanistic role in the pathogenesis of epilepsy in AD. Prior to the formation of amyloid beta (Aβ) plaques, the brain can undergo pathological changes as a result of increased production of amyloid precursor protein (APP) and Aβ oligomers. Impairments in the glutamate uptake ability of astrocytes due to astrogliosis are hypothesized to be an early event occurring before Aβ plaque formation. Astrogliosis may increase the susceptibility to epileptogenesis of the brain via accumulation of extracellular glutamate and resulting excitotoxicity. Here we hypothesize that Aβ oligomers and proinflammatory cytokines can cause astrogliosis and accumulation of extracellular glutamate, which then contribute to the pathogenesis of epilepsy in AD. In this review article, we consider the evidence supporting a potential role of dysfunction of the glutamate-glutamine cycle and the astrocyte in the pathogenesis of epilepsy in AD.

KEYWORDS

Alzheimer's disease, amyloid beta, astrogliosis, epileptogenesis, glutamate, neuroinflammation, seizures

1 | INTRODUCTION

1.1 | The bidirectional relationship between Alzheimer's disease and epilepsy

The prevalence of Alzheimer's disease (AD) continues to rise globally as the proportion of the older population in many countries continues to grow. Currently, 50 million people in the world are living with dementia, which is resulting in a global financial burden of ~1 trillion US dollars annually.[1] It is predicted that by the year 2050, there will be 150 million people living with dementia and the financial burden will double by 2030.[1] AD is the most prevalent type of dementia, contributing ~60%–70% of cases.[1]

In addition to deteriorating cognitive function, many AD patients also experience spontaneous seizures and epilepsy. It has been reported that AD patients have up to 10-fold higher risk of developing epilepsy compared to healthy age-matched controls,[2–5] although it should be noted that, in some of these reports, a diagnosis of AD was made from clinical observations, and so the enrolled patients would presumably also include cases of non-AD dementia. In addition, the

relationship between AD/dementia and epilepsy is bidirectional: many patients with epilepsy also experience cognitive disturbances similar to dementia patients,[6,7] and epilepsy patients (also including a heterogeneous collection of epilepsy subtypes) have a 2-fold greater risk of dementia[8] compared with controls. Despite such evidence supporting elevated risk of epilepsy in AD patients, the mechanisms involved in the pathogenesis of epilepsy in this population have not been elucidated. Moreover, there is still no cure for AD or epilepsy and the current treatments are symptomatic with no disease-modifying effect. Understanding the mechanisms of epileptogenesis in AD would potentially aid the development of targeted preventive or disease-modifying therapies.

Toward this end, animal models of AD can be powerful tools to investigate the links between AD and epilepsy. Although rodent models do not display the full AD pathology spectrum, they allow us to isolate and investigate the impact of individual pathologies associated with AD on brain physiology, which is largely not practical in humans. It has been reported in multiple studies that mouse models of AD that overexpress mutant human amyloid precursor protein (APP) have a higher mortality rate than the nontransgenic littermate[9,10] and this may be caused by unprovoked seizures.[11,12] Indeed, spontaneous seizures[13–15] and seizure-like events, such as interictal spikes,[16] have been observed in several mouse models of AD. It was also reported that mouse models of AD with mutant APP overexpression exhibit neuronal hyperactivity, especially in the area near the amyloid beta (Aβ) plaque.[17] Recently, different studies have confirmed that rodent models of AD have higher susceptibility to kainic acid,[18] pentylenetetrazole,[19] and kindling-induced seizures[20] compared to their wild-type (WT) littermates. These findings support the use of rodent models of AD to investigate the pathogenesis of epilepsy in AD.

1.2 | The glutamate-glutamine cycle

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system (CNS) and is responsible for nearly all excitatory synaptic activities in the brain. The movement and metabolism of glutamate is tightly controlled by the glutamate-glutamine cycle. This cycle involves uptake of extracellular glutamate into nearby astrocytes (driven by glutamate transporter 1 [GLT-1]), astrocytic conversion of glutamate to glutamine (via the glutamine synthetase [GS] enzyme), transfer of glutamine to the extracellular milieu via the SNAT3 transporter, and then into the pre-synaptic neuron via the SNAT1 transporter,[21] and finally conversion of glutamine into glutamate by glutaminase (GLS), an enzyme highly enriched in the mitochondria of the pre-synaptic neurons.[22] The rapid uptake and conversion of glutamate by astrocytes prevents prolonged post-synaptic glutamatergic receptor activation, which if left unchecked, could trigger excitotoxicity and seizures; thus extracellular glutamate must be removed promptly after release.[22–24] Changes in the function or expression of any of these key glutamate-glutamine proteins would be anticipated to cause a shift in the equilibrium of extracellular glutamate[25] (Figure 1A). In this review, we propose that the glutamate-glutamine cycle plays a role in the pathogenesis of epilepsy in AD. Although other mechanisms may be very relevant,[26,27] the following section will discuss the evidence from the literature on the role of alterations to the glutamate-glutamine cycle in the pathogenesis of AD and epilepsy.

Key Points

- Astrogliosis in Alzheimer's disease can lead to the changes in the expression levels of proteins that are important for the glutamate-glutamine cycle.
- Amyloid beta (Aβ) oligomers can decrease the expression levels of GLT-1 and GS proteins in the brain leading to an increase in seizure susceptibility.
- Neuroinflammation in Alzheimer's disease can cause a loss of function in astrocytes, resulting in an increase in seizure susceptibility.

2 | THE GLUTAMATE-GLUTAMINE CYCLE: A LINK BETWEEN ALZHEIMER'S DISEASE AND EPILEPSY?

It is known that dysregulation of glutamate in the brain can contribute to the pathogenesis of both AD[28,29] and of epilepsy.[24] However, the mechanism that links AD pathologies—such as Aβ, APP, and tau—to the dysregulation of glutamate and epileptic seizure susceptibility in patients is not elucidated. Because AD is not associated with just one pathology but rather a combination of many, such as Aβ, tau phosphorylation, and neuroinflammation,[30] it is important to investigate the physiological target that these pathologies may have in common. We propose here that AD pathologies can increase the risk of seizure by disrupting the functional role of astrocytes in the glutamate-glutamine cycle, which leads to abnormal glutamate homeostasis.

The main physiological function of the astrocyte is to support and protect neurons by providing nutrients and regulating neurotransmitter levels. Astrocytes are heavily involved in maintaining glutamate homeostasis in the CNS via the tripartite synapse structure, which consists of a pre-synapse, a post-synapse, and an astrocytic process[31] (Figure 1A).

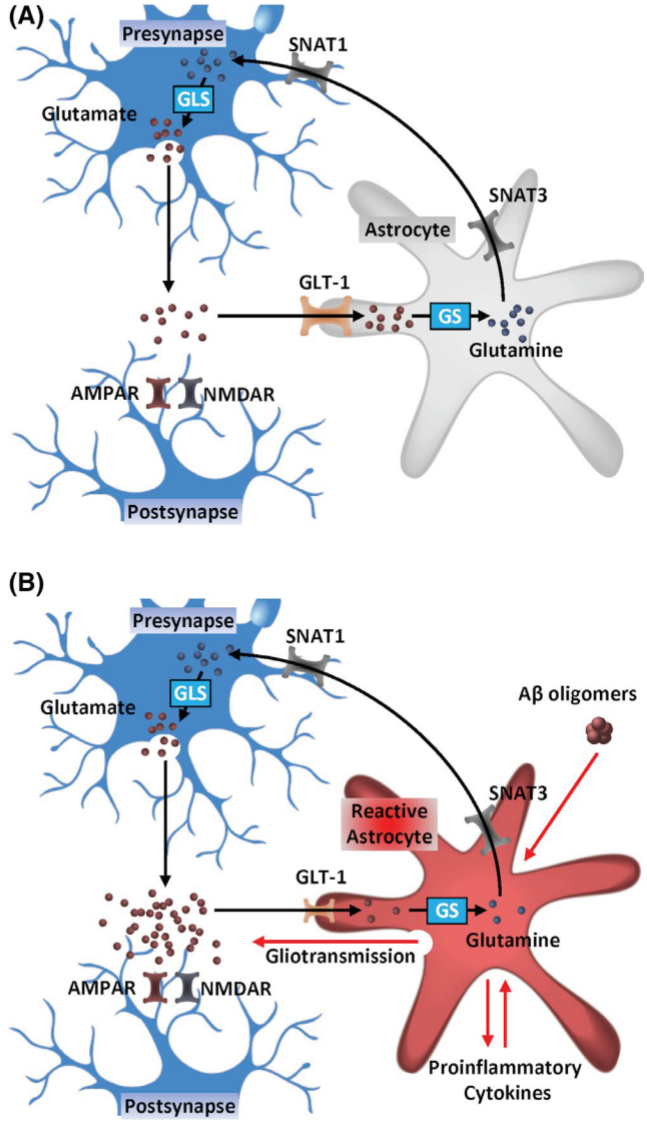

FIGURE 1 A simplified schematic of the tripartite synapse illustrating the glutamate-glutamine cycle (A) under normal circumstances and (B) in the presence of Alzheimer's disease (AD) pathology–associated molecules such as amyloid beta (Aβ) oligomers and proinflammatory cytokines. (A) In homeostatic conditions, glutamate is taken up into astrocytes via GLT-1, converted to glutamine by glutamine synthetase (GS), and transported back to the presynaptic terminal via SNAT proteins. Glutamine is then converted back to glutamate, ready to be released upon neuronal stimulation. (B) In AD, astrocytes undergo structural and functional transformation through a process called astrogliosis. These transformations lead to reduced expression of glutamate-glutamine cycle–associated proteins such as GLT-1 and GS, thus decreasing the rate of extracellular glutamate uptake and glutamine synthesis. In addition, astrogliosis results in the release of proinflammatory cytokines such as IL-β, and gliotransmitters such as glutamate, into the synaptic cleft. Together, this alters the fidelity of the glutamate-glutamine cycle, resulting in accumulation of extracellular glutamate in the synaptic cleft, and potentially leading to synaptic hyperexcitability and increases in seizure susceptibility

Changes in the expression levels of astrocyte-specific protein such as GLT-1 and GS may therefore lead to dysregulated glutamate homeostasis. Astrogliosis is a process triggered by CNS damage and/or disease involving activation and proliferation of astrocytes. In such conditions, the cells undergo striking morphological, metabolic, and functional transformations as part of the host's endogenous defense mechanism to minimize damage and restore homeostasis. Astrogliosis and glutamate homeostasis dysfunction have been associated with the pathogenesis of AD[29,32] and of epilepsy.[33,34] Astrogliosis may occur in response to AD pathologies such as Aβ,[29] which can then disrupt normal astrocytic glutamate uptake.[35] Because astrocytes are the most important regulator of extracellular glutamate,[36] disruption in glutamate uptake via astrogliosis may result in neurotransmitter accumulation in the synaptic cleft. Below, we discuss mechanisms that AD pathologies—in particular Aβ species—interact with astrocytes and the glutamate-glutamine cycle, and how this may lead to increased seizure susceptibility (Figure 1B).

2.1 | Interaction between amyloid beta and the glutamate-glutamine cycle increases risk of seizure

Aβ is one of the major hallmarks of AD, and it has been reported that this pathology is associated with astrogliosis.[37–39] Rodent models of AD that overexpress Aβ demonstrate an astrogliosis phenotype, and this typically precedes formation of Aβ plaques.[40] This might mean that Aβ can affect the glutamate-glutamine cycle before plaque formation, with evidence from animal studies also suggesting that cognitive and neuronal network impairments are observed prior to formation of Aβ plaques.[41–43] In addition, astrocytes (and microglia) are involved in the clearance and degradation of Aβ.[44] In AD, this clearance is decreased due to the overproduction of Aβ and/or impairments in the phagocytic capability of glial cells.[45,46] This accelerates the accumulation of Aβ protein in the brain, which can further enhance reactive astrogliosis. This section will discuss the interaction between Aβ and astrocytes and how associated mechanisms can affect the glutamate-glutamine cycle. Below, we further elaborate on alternate mechanisms relating to reactive astrocyte release of proinflammatory cytokines, which also modulate excitability.

2.2 | Changes in GLT-1 and GS expression levels are associated with higher seizure susceptibility

There is evidence from human studies[47–50] and rodent models[51,52] suggesting that GLT-1 gene and protein expression is

reduced by AD pathologies. In human temporal lobe epilepsy (TLE), one study reported a significant increase in GLT-1 protein and mRNA in hippocampus samples collected from patients with TLE.[55] This increase in expression might be caused by a compensatory mechanism that results in the up-regulation of GLT-1 expression to counteract elevated glutamate levels associated with seizures.[55] However, others studies failed to identify differences in GLT-1 protein expression in neocortex and temporal cortex of patients, compared to the healthy controls.[53,54] Despite these findings, data from human study are currently lacking and the effect of epilepsy on GLT-1 expression in humans remains to be clarified.

On the other hand, there is extensive evidence from rodent models to support the hypothesis that loss of GLT-1 could mediate an increase in seizure susceptibility in AD. This pathway involves the presence of oligomeric Aβ, which can reduce expression of GLT-1 in rat astrocyte cultures via the transcriptional regulator nuclear factor of activated T-cells (NFAT).[56] In addition, evidence from ex vivo studies provided insights into the functional interactions between AD pathologies and GLT-1 function. One study demonstrated that Aβ oligomers decrease astrocytic glutamate uptake via the promotion of GLT-1 internalization from the surface of the astrocyte.[57] In addition, Aβ oligomers could significantly inhibit glutamate uptake in rat astrocytic cultures via an increase in oxidative stress and ubiquitination of GLT-1.[57,58] The consequences of reduced GLT-1 appear to be detrimental: cross-breeding APP/PS1 mice (a prominent AD mouse model) with heterozygous GLT-1 mice resulted in significantly accelerated cognitive decline, compared to APP/PS1 with WT GLT-1 levels.[59] Furthermore, enhancing brain GLT-1 expression with β-lactam antibiotic such as ceftriaxone improved cognitive function in mouse models of AD.[52,60] Together, these reports suggest that AD pathologies, such as Aβ and mutant human APP overexpression, may decrease expression of GLT-1 in the brain and that this loss of GLT-1 expression correlates with cognitive decline. Furthermore, astrocytic dysfunction and consequential changes in glutamate homeostasis may take place in the presymptomatic stages of AD, without relying on the presence of Aβ plaques.[37]

Because astrocytic GLT-1 is important for synaptic glutamate uptake, sustained and progressive loss of GLT-1 expression due to AD pathology could increase the risk of seizure. There is well-established evidence from rodent models to support the association between the loss of GLT-1 expression and epileptic seizures. A landmark study by Tanaka et al[61] demonstrated that GLT-1-deficient mice display a lethal spontaneous seizure phenotype, and a subsequent study also confirmed that GLT-1 knock-out mice develop spontaneous seizures after 2 weeks, with only 50% of the knock-out mice surviving for 8 weeks.[62] In addition, inhibiting GLT-1 function with dihydrokainic acid increases extracellular glutamate and epileptiform activity in rats.[63] Furthermore, selectively knocking out astrocytic GLT-1 in mice significantly reduced glutamate uptake from the extracellular space, and this was accompanied by lower survival rate of knock-out mice due to seizures.[64] On the contrary, selectively knocking out neuronal GLT-1 did not produce any abnormal neuronal discharge or seizure.[64] This evidence supports the theory that astrocytic, rather than neuronal, GLT-1 plays an essential role in the prevention of synaptic overexcitation and seizures. On the other hand, transgenic mice with enhanced astrocytic GLT-1 expression display lower acute mortality and less-frequent seizures caused by pilocarpine-induced status epilepticus (SE) compared to WT mice.[65] One time-course study investigated the effect of kainic acid–induced SE on the expression of GLT-1 in mice. This study found that there was a transient increase in the GLT-1 expression 1 day after SE, followed by a significant decrease in the expression 4–7 days after SE.[66] Despite that, GLT-1 expression returned to the baseline level in the majority of hippocampal regions 30 days after SE.[66] These results suggest that expression of GLT-1 is modified as a consequence of SE, perhaps as compensation for the immediate increase in glutamate release caused by SE. From the current evidence, it appears that loss of GLT-1 expression can be associated with AD pathologies, such as Aβ and mutant APP overexpression, and that this may increase the seizure susceptibility due to dysregulation of glutamate homeostasis.

In addition to changes in GLT-1 levels, evidence also suggests that GS, the enzyme responsible for metabolizing glutamate to glutamine, is involved in the pathology of both AD and epilepsy. In human AD patients, the density of Aβ plaques is anti-correlated with GS protein levels in the brain,[67] and there is also significant loss of enzymatic activity of GS compared to age-matched controls.[68] Changes in GS protein have also been associated with epilepsy (reviewed in[69]). In human mesial TLE patients, decreases in GS protein expression in hippocampus have been found compared to healthy controls, and this was accompanied by significantly lower GS enzymatic activity.[70,71] Furthermore, genetic mutations that lead to GS enzyme deficiency are associated with seizures in newborn humans.[72]

Evidence from rodent models also support the involvement of GS in AD and epilepsy: in the 3xTg mouse model of AD, expression levels of GS were significantly decreased in the medial prefrontal cortex during the early and intermediate stages of pathology[73] and in the hippocampus during later stages.[74] Furthermore, astrocytes in proximity to Aβ plaques have lower GS expression levels than astrocytes distal to the plaques.[74] Because GS plays an important role in the conversion of glutamate to glutamine in the astrocyte, the reduction in GS protein or decreased enzymatic activity would be expected to result in a loss of astrocytic neuroprotective properties against glutamate-induced excitotoxicity.[75] With regard to epilepsy, one important study demonstrated that selectively knocking out GS from the cortex led to spontaneous

seizures in mice.[76] This study also found that GS-deficient mice had a 30% decrease in survival rate and a significant increase in astrogliosis in the brain. Of interest, knocking out the GS enzyme also resulted in a significant reduction in expression of cerebral GLT-1,[76] suggesting a compensatory mechanism that involves the alternation of GLT-1 expression to match the change in GS expression level in order to maintain normal glutamate homeostasis.

The current evidence suggests that loss of GS expression may increase seizure susceptibility, but that seizures per se may not reduce GS expression. For example, there is evidence showing a transient increase in GS expression following kainic acid[77] and kindling-induced seizures.[78] On the other hand, pilocarpine-induced SE in rats results in a gradual decrease in GS levels.[79] Although these results are not consistent, they do indicate that changes in the level of GS enzyme in the brain can be associated with epilepsy. Currently, there is not sufficient evidence to determine whether inhibiting or enhancing GS levels in the brain may be beneficial for the treatment of epilepsy.

In summary, the current evidence suggests that the expression and function of both GLT-1 and GS can be altered by AD pathologies, and that the loss of these proteins would be expected to alter glutamate homeostasis and consequently increase seizure susceptibility.

2.3 | Influence of proinflammatory cytokines on seizure susceptibility in AD

Because proinflammatory cytokines have been implicated in the pathogenesis of both AD[80] and epilepsy,[81] and these are associated with gliosis, it is also important to consider how cytokines themselves contribute to an increase in seizure susceptibility in AD. In addition to affecting the expression of proteins that are involved in the glutamate-glutamine cycle, soluble Aβ can also act as a damage-associated molecular pattern (DAMP).[82] As such, increasing soluble Aβ can exacerbate release of proinflammatory cytokines such as interleukin 1 beta (IL-1β) from glial cells, which in turn may contribute to the increase in seizure susceptibility.[82,83]

Proinflammatory cytokines released by reactive astrocytes as a consequence of astrogliosis can act through both autocrine and paracrine manners to enhance the expression of other proinflammatory cytokines.[39] For example, IL-1β and tumor necrosis factor α (TNF-α) can induce astrocytic release of IL-6.[84,85] In addition to perpetuating and accentuating the neuroinflammatory cycle, cytokines released by these cells can trigger mechanisms that also disrupt glutamate homeostasis and consequently increase in seizure susceptibility. We propose two primary mechanisms that might be relevant to this: alterations in gliotransmission, and facilitation of excitatory neurotransmission.

Gliotransmission describes the release of neurotransmitter such as glutamate from glial cells for the purpose of cell-to-cell communication. There is evidence to suggest that proinflammatory cytokines, including IL-6, can trigger the release of gliotransmitters such as glutamate from reactive astrocytes.[86–88] This release of glutamate is Ca^{2+}-dependent[89] and it has been shown that IL-6[90] can increase the influx of Ca^{2+} into astrocytes, thus increasing the release of glutamate into the synaptic cleft. The amount of glutamate released from the reactive astrocyte is sufficient to activate high affinity glutamate receptors such as the *N*-methyl-D-aspartate (NMDA) receptor,[91] and therefore this increase in glutamatergic activation can contribute to brain excitability. Whether this distinct mechanism contributes to epilepsy in AD warrants exploration.

Proinflammatory cytokines are also recognized to facilitate excitatory neurotransmission. Here, the role of IL-1β has been highlighted, since it is a potent proinflammatory cytokine that is associated with the pathogenesis of epilepsy via long-term and rapid mechanisms.[92] The long-term effects of IL-1β involve genomic events, which lead to structural and functional changes in glial and neuronal networks.[92] For example, the expression of genes that are reported to be associated with epilepsy, such as *HCN1*, can be significantly increased.[92] These changes cause neurons to be prone to excitatory synaptic transmission by neurotransmitters such as glutamate, theoretically leading to susceptibility to seizures.[93] Alternatively, the rapid mechanism involves kinases and changes in ion channel function.[81] In this mechanism, IL-1β released by the reactive astrocyte activates neuronal kinase systems such as Src tyrosine kinase via the interleukin-1 receptor type 1, which then cause ion channels such as NMDA receptor to be phosphorylated.[94] This phosphorylation can directly increase Ca^{2+} influx and decrease seizure threshold via the glutamatergic pathway,[95] leading to neuronal hyperexcitability. Therefore, the increase in the level of proinflammatory cytokines in AD may contribute to increased seizure susceptibility.

3 | FUTURE RESEARCH DIRECTIONS

In conclusion, there is converging evidence supporting the role of astrocytes and the glutamatergic pathway in the pathogenesis of epilepsy in AD. In AD, increased production of Aβ can result in astrogliosis, alterations in glutamate homeostasis, and elevated proinflammatory cytokines, which may increase the susceptibility to epilepsy. Furthermore, astrogliosis can be one of the earliest events in AD pathology triggered by Aβ oligomers, and does not rely on the presence of mature plaques. Because astrocytes play many crucial roles in the maintenance of healthy brain function, it would be

beneficial if astrocytic dysfunction in AD can be corrected, for instance by enhancing GLT-1 expression level pharmacologically, to restore glutamate uptake efficiency. Interactions and relationship between soluble Aβ and proinflammatory cytokines should also be the focus of future research, especially because the human data on this topic are lacking. Such experimental investigations are warranted, based on existing evidence.

Currently, there is no reliable early biomarker for epilepsy in AD, thus it is important to identify specific, sensitive, and stable candidates.[96] The current literature suggests that astrogliosis in AD can take place prior to Aβ plaque formation[40] and therefore investigating how this pathway is affected by the early AD pathology might enhance our chance of identifying a biomarker for the presymptomatic or the mild cognitive impairment stage of AD. This may allow AD to be diagnosed earlier, which will be critical for the prevention of seizures in AD patients. Early diagnosis of AD would also allow important data on the mechanisms that link AD to epilepsy to be generated.

Future research should also aim to detect regional in vivo level of extracellular glutamate, rather than the total level of glutamate. In the CNS, the level of intracellular glutamate is higher than the extracellular glutamate[97] and therefore changes in the level of extracellular glutamate can be overshadowed by the intracellular glutamate level when using such techniques as magnetic resonance spectroscopy or tissue homogenization. Therefore, measuring astrocytic glutamate uptake from the synaptic cleft would provide more insights into a potential compensatory mechanism that can counter the disruption in the brain glutamate homeostasis caused by the AD pathology. Furthermore, there is little evidence on the effect of AD pathologies on the expression of GLS, SNAT1, and SNAT3—other key proteins involved in the glutamate-glutamine cycle, particularly in human subjects. Thus more investigation is needed to understand the effect of AD pathologies on the glutamate-glutamine cycle as a whole. These insights on the involvement of astrocyte and glutamate-glutamine cycle may reveal novel drug targets for reducing seizure susceptibility in AD or slowing the progression of AD pathologies.

We also must consider the strengths and limitations of our current "animal models of AD." To date, these are generated almost exclusively by manipulating the genome of mice using transgenic technologies. Gene mutations identified in patients can be simply inserted into mouse DNA, oftentimes with that gene overexpressed, allowing us to examine the impact of, say, mutant APP on epilepsy susceptibility. These methods have proven invaluable in creating a greater understanding of the pathological development of plaques, and of facilitating the development of treatments targeting plaques. But they are first and foremost models of Aβ, not of AD, since the pathology of AD involves other aspects, notably neurofibrillary tangles composed of hyperphosphorylated tau, as well as substantial neuronal loss—features missing from our current models. The other primary criticism is that these are based on models of familial AD, which account for only a small proportion of all AD (sporadic forms are estimated to be >95% of all AD patients).[30] However, the field is moving in the right direction. Perhaps driven by failures of multiple clinical trials that were initiated following success in animal models,[98] there is a growing appreciation of the need to develop new models of disease, incorporating risk factors, such as apolipoprotein E gene (*APOE* ε4 and environmental cues,[99] as well as moving from a reliance on rodent models,[100] and moving away from mutations in APP and PSEN genes. In the current context, much of the evidence supporting a role for the glutamate-glutamine cycle as a mechanism driving seizure susceptibility in AD is generated from these animal models. Although this is currently compelling, future studies must also take advantage of the new wave of animal modeling to explore this hypothesis further.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Chapter 3 – Early Metabolomic Changes in Alzheimer's Disease

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3.1) Preamble to empirical paper

In Chapter 2, the potential involvement of the glutamate-glutamine cycle in the pathogenesis of epilepsy in AD was presented. Thus, this chapter utilised mass spectrometry in conjunction with the mature Tg2576 mouse model of AD to investigate whether the metabolic pathways and metabolite levels associated with the glutamate-glutamine cycle are affected by the early stage of AD pathology. I hypothesised that metabolic pathways and metabolites associated with the glutamate-glutamine cycle in the brain of Tg2576 are altered in the preclinical stage of AD. In addition to the glutamate-glutamine cycle, this experiment gave insights on other pathways that may be affected by the human mutant APP overexpression pathology. This will contribute to furthering our understanding of the interaction between the early stage of AD pathology and the metabolomic profile of the brain.

It is important to elucidate changes in the brain that occur during the preclinical stage of AD as clinical trial failures that are associated with AD drugs have been attributed to the late administration of the treatment to AD patients, as discussed in Chapter 1. Presently, the only method to diagnose Alzheimer's disease (AD) with certainty is through post-mortem autopsy of the brain[1] to detect Aβ plaques and neurofibrillary tangles (NFT). Despite the fact that novel developed diagnostic tools for AD, such as blood test for amyloid-β (Aβ)[2] and various brain imaging techniques[3, 4], are being developed, it is still important to discover a biomarker for AD that do not directly associated with Aβ plaques or NFTs. This is because neurodegeneration may occur even before the formation of Aβ plaques and NFTs.[5] Therefore, the insights gained from this chapter may also assist in the discovery of biomarkers associated with the glutamatergic pathway for the diagnosis of preclinical stage of AD.

3.2) Empirical paper

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ORIGINAL ARTICLE

Altered metabolic pathways in a transgenic mouse model suggest mechanistic role of amyloid precursor protein overexpression in Alzheimer's disease

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Abstract

Introduction The mechanistic role of amyloid precursor protein (APP) in Alzheimer's disease (AD) remains unclear.

Objectives Here, we aimed to identify alterations in cerebral metabolites and metabolic pathways in cortex, hippocampus and serum samples from Tg2576 mice, a widely used mouse model of AD.

Methods Metabolomic profilings using liquid chromatography-mass spectrometry were performed and analysed with MetaboAnalyst and weighted correlation network analysis (WGCNA).

Results Expressions of 11 metabolites in cortex, including hydroxyphenyllactate—linked to oxidative stress—and phosphatidylserine—lipid metabolism—were significantly different between Tg2576 and WT mice (false discovery rate < 0.05). Four metabolic pathways from cortex, including glycerophospholipid metabolism and pyrimidine metabolism, and one pathway (sulphur metabolism) from hippocampus, were significantly enriched in Tg2576 mice. Network analysis identified five pathways, including alanine, aspartate and glutamate metabolism, and mitochondria electron transport chain, that were significantly correlated with AD genotype.

Conclusions Changes in metabolite concentrations and metabolic pathways are present in the early stage of APP pathology, and may be important for AD development and progression.

Keywords Network analysis · Dementia · Amyloid-beta · Oxidative stress · Lipids metabolism · Glutamate

1 Introduction

At least 50 million people globally have dementia, and Alzheimer's disease (AD) is the most prevalent type of dementia (World Alzheimer Report 2018, 2018). Dementia is also the fifth leading cause of death worldwide (Masters et al., 2015). The current lack of disease modifying treatments may be because AD patients are diagnosed well into disease development, and pathology is already advanced, and this cannot be reversed (Mehta et al., 2017; Petersen, 2009). Therefore, the ability to identify and treat AD patients in the preclinical stage is critical to successfully interfere with AD development and progression. By investigating the mechanisms associated with the presymptomatic stage of AD, potential therapeutic targets and/or biomarkers may emerge.

Recently, metabolomic analysis has become a prominent tool for hypothesis generation and the study of mechanisms for a number of diseases and disorders (Dettmer et al., 2007; Nagana et al., 2013). This is mainly due to its unique ability to profile all the identifiable metabolites within the samples of interest and compare it to the reference samples (Birkemeyer et al., 2005). Liquid chromatography-mass spectrometry (LC–MS) instrumentation in particular is rapidly improving and in the past decade, it has been shown that the data generated from LC–MS has higher quality, consistency and reproducibility than it has ever been (Aebersold & Mann, 2003; Kuhn et al., 2012). Metabolomic analysis of blood samples may provide insights on the changes in metabolic processes that

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are associated with AD. However, whole blood contains cellular metabolites which may overshadow changes in the non-cellular metabolite levels and therefore it is common for metabolomic study to investigate serum or plasma metabolite levels instead of whole blood (Chaleckis et al., 2016). Additionally, recent technological advances combined with novel methodologies in metabolomics have managed to overcome biological problems associated with blood-based samples such as high variability in the metabolite concentration, thus making LC–MS a technique that provides both high specificity and sensitivity for metabolites (Anderson et al., 2004; Gonzalez-Dominguez et al., 2017; Pernemalm & Lehtio, 2014).

Access to tissue samples at specific timepoints during AD development is also an important aspect for the investigation of metabolomic pathways that are associated with the pathogenesis of AD. Currently, it is not possible to obtain samples from human patients because presymptomatic AD cannot yet be diagnosed in humans (Weller & Budson, 2018). Metabolomic profiling of transgenic mouse models of AD can provide insights into changes in the metabolic pathways that are associated with specific AD pathology, such as APP overexpression (Wilkins & Trushina, 2017). Therefore, in this metabolomic analysis study, the Tg2576 mouse model of AD was utilised. Tg2576 is one of the most characterised mouse models of AD that display APP pathology (Hsiao et al., 1996) by overexpressing human APP with the Swedish mutation (K760N/M671L). In this model, amyloid-β (Aβ) plaques are observed from approximately 9 months of age, while cognitive deficits appear from approximately 10 months old (Hall & Roberson, 2012). Aged Tg2576 displays an array of symptoms that are relevant to clinical profile of human AD patients (Van Dam & De Deyn, 2011). Here we chose to use 6-month-old Tg2576 mice, which are at a presymptomatic stage, to investigate metabolomic changes that are associated with early stages of AD development. Cortex, hippocampus and serum samples were of particular interest. The cortex is one of the first brain regions to be affected by Aβ pathology (Calderon-Garciduenas & Duyckaerts, 2017; Thal et al., 2002), and so changes in the metabolomic profile that are associated with early stage of AD pathology should be observable in this region. Additionally, since hippocampus is highly involved with cognitive functions and memory (Bird & Burgess, 2008)—classic symptoms of dementia—it would be interesting to study how human mutant APP overexpression pathology can affect the metabolomic profile in this area of the brain. We also chose to study serum in an attempt to identify potential metabolomic biomarkers associated with early stages of AD.

In addition to metabolite analysis, we also explored the utilisation of Weighted Gene Coexpression Network Analysis (WGCNA) in the context of metabolomic data. WGCNA is a powerful method that has been extensively used for transcriptomic analysis, however it can also be implemented for metabolomic characterisation (DiLeo et al., 2011; Fukushima et al., 2011; Novais et al., 2019). This open source pipeline performs coexpression analysis using the tools and concepts of graph theory. Under the premise that strongly correlated metabolites are likely to be functionally associated, WGCNA integrates the concentration variation of metabolites across samples into a network, which is then used to elucidate relationships between metabolite groups and sample traits. These metabolomic and network analyses provide a global profiling of all identifiable metabolites in samples without observational biases that are typically associated with focused studies of metabolism.

2 Methods

2.1 Animals

Six-month-old mixed-sex Tg2576 mice and c57 × SJL (WT) littermates (n = 7/group) were obtained from our colonies at the Department of Medicine (Royal Melbourne Hospital), University of Melbourne, Parkville, Australia. All experiments were conducted following approval from the Florey Neuroscience Institute Animal Ethics Committee (#15-003).

2.2 Tissue collection

Mice were euthanised via lethal injection of pentobarbitone (80 mg/kg) (Provet, VIC, Australia) and cardiac puncture was performed to collect blood from each animal. The blood was allowed to clot in an anticoagulant-free tube for 15 min at room temperature and then centrifuged at 1500×*g* in 20 °C for 10 min. The supernatant was then collected as serum and frozen with liquid nitrogen before being stored at − 80 °C until processing. The brains were rapidly dissected into cortex and hippocampus and the samples were snap-frozen in liquid nitrogen before being stored at − 80 °C until processing.

2.3 Materials

LC–MS-grade acetonitrile was from Burdick and Jackson (Muskegon, MI, USA). Reverse osmosis purified Milli-Q water used in LC–MS analysis was from Millipore water purification system (Merck, Darmstadt, Germany). Ammonium carbonate and internal standards consisting of CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate); CAPS (3-(cyclohexylamino)-1-propanesulfonic acid); PIPES (1,4-piperazinediethanesulfonic acid) and TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) were purchased from Sigma Aldrich.

2.4 Sample preparation

Frozen brain samples were crushed into powder using a steel multisample biopulveriser (Daintree Scientific, Australia) and quickly weighed, while not allowing the samples to thaw. Then 20 µl of cold extraction solvent was added per 1 mg of powdered tissue. The extraction solvent consisted of 80% methanol and internal standards at 1 µM concentration. The samples were then sonicated for 30 min in an ice bath, followed by vortexing at 4 °C for 15 min. The samples were then centrifuged at 14800×*g* in 4 °C for 10 min. Finally, 100 µl of the supernatant was transferred to LC–MS vials and stored at − 80 °C until further processing.

For serum samples, 25 µl was extracted with 100 µl of cold methanol containing 1 µM of internal standards. The samples were vortexed at 4 °C for 30 min, then centrifuged at 14800×*g* speed at 4 °C for 10 min. 100 µl of supernatant was transferred to LC–MS vials and the samples were stored at − 80 °C until the analysis. Blank samples were prepared so that background noise could be analysed. Pooled quality control sample was prepared by combining 5–10 µl of each sample for tissue and serum analysis separately.

2.5 LC–MS metabolomic analysis

Details of the LC–MS metabolomic analysis and data processing have been described previously (Creek et al., 2016). Briefly, LC–MS data was acquired on Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Waltham, Massachusetts, United States) coupled with high-performance liquid chromatography (HPLC) system Dionex Ultimate® 3000 RS (Thermo Scientific). Chromatographic separation was performed on a ZIC-pHILIC column (5 µm, polymeric, 150 × 4.6 mm, SeQuant®, Merck). The mobile phase (A) was 20 mM ammonium carbonate and (B) acetonitrile.

2.6 Data processing

The acquired LC–MS data was processed in an untargeted fashion using open source software IDEOM (Creek et al., 2012), which initially used *ProteoWizard* to convert raw LC–MS files to *mzXML* format and *XCMS* to pick peaks to convert to.*peakML* files. *Mzmatch.R* was subsequently used for the alignment of samples and the filtering (Scheltema et al., 2011). IDEOM interface in MS Excel (Creek et al., 2012) was, again, utilised for feature identification, organisation and data quality evaluation.

2.7 Raw data analysis and metabolomic profiling

Univariate and multivariate analyses were performed using Metaboanalyst 4.0 (Chong et al., 2018). The datasets were quantile normalised, log transformed (Durbin et al., 2002) and auto-scaled (van den Berg et al., 2006). These parameters ensured the best normal distribution for the data. For the univariate analysis, unpaired *t*-test was performed and fold-changes were calculated in order to identify the differences in abundance of each metabolite between Tg2576 and WT. The *p*-values acquired from the *t*-test were corrected for multiple comparisons with the Benjamini–Hochberg method (Benjamini & Hochberg, 1995), which yielded false discovery rate (FDR). The significance threshold between groups was set to FDR < 0.05.

For the multivariate analysis, principle component analysis (PCA) was performed to detect outliers and partial least-square discriminant analysis (PLS-DA) was performed to identify metabolites that were driving the separation between the groups. A metabolite with variable importance in projection (VIP) scores of > 1 was considered to be an important feature. Leave-one-out cross validation (LOOCV) was performed to examine the fit quality of the model (Westerhuis et al., 2008). In LOOCV, the difference between R^2 and Q^2 values should be approximately less than 0.3 (Eriksson et al., 2013). A metabolite was deemed as significantly affected by genotype when its FDR < 0.05 and its variable importance in projection (VIP) score > 1.

2.8 Pathway enrichment analysis

Pathway enrichment analysis was performed separately from the raw data analysis using the Metaboanalyst 4.0 online tool (Chong et al., 2018) with *Mus musculus* species (KEGG database) as reference (Kanehisa & Goto, 2000). The normalised peak intensity data from the Tg2576 samples were compared to WT littermates. To be qualified as an important pathway, the metabolic pathway must have FDR < 0.05 and the pathway impact score > 0.2.

2.9 Network analysis (WGCNA)

Weighted Correlation Network Analysis (WGCNA) is a systems biology methodology which can be used with metabolomics data to investigate the correlation between metabolites based on their abundance across the samples. This approach will identify previously unknown groups of metabolites that act in concert in the context of AD pathology. For detailed description of the WGCNA package please refer to (Langfelder & Horvath, 2008). Using the general framework of WGCNA, correlation-based networks characterising the metabolome of the Tg2576 and WT littermates were constructed. Briefly, the raw LC–MS peak intensity

values were quantile normalised and log transformed, followed by auto scaling, and the zero values were omitted by replacing them with one-half of the detection threshold value. A dendrogram was created via hierarchical clustering of the samples in order to identify and remove outliers. A series of soft thresholding values were used to determine the optimal power at which the normalised data fit the scale-free topology model. Using a soft thresholding power of 7, an adjacency matrix was constructed reflecting the pair-wise correlation coefficients between all the detected metabolites. The correlation network was then built based on the adjacency matrix, where each node corresponds to a single metabolite, and the edges between the nodes represent the correlation between the peak intensity values of the given metabolite across all samples. The metabolite modules were then identified through clustering analysis and pathway enrichment analysis was performed. We also examined the sample trait correlation (Tg2576 vs WT) to the modules identified in the network.

3 Results

3.1 Raw data analysis

The normalised metabolomic data acquired from the cortex, hippocampus and the serum can be seen in Supplementary Figs. 1, 2 and 3. Multivariate analysis, in the form of unsupervised principle component analysis (PCA) was performed on the normalised peak intensity data from each sample set. This allowed the visualisation and comparison of the individual Tg2576 and WT data from cortex, hippocampus and serum in order to identify outliers. Score plots from PCA identified one outlier from the cortex samples as can be seen in Fig. 1a—this sample was removed from subsequent analyses. Data from the hippocampus (Fig. 1b) and serum (Fig. 1c) showed appropriate data grouping, which indicated consistency in the metabolomic profiles of the samples within each group.

Supervised partial least-square discriminant analysis (PLS-DA) was performed to identify metabolites that are significantly different between groups. PLS-DA showed no overlap between Tg2567 and WT groups in all three sample types (Fig. 2). The results from PLS-DA were validated using leave-one-out cross validation (LOOCV) (Supplementary Fig. 4). The LOOCV successfully validated the PLS-DA results from the cortex samples (Supplementary Fig. 4A and Supplementary Table 1). LOOCV indicated that the multivariate analysis results from hippocampus and serum samples had high risks of being overfitted (Supplementary Fig. 4B and C) and therefore these two sets of data could not be visualised via PLS-DA.

3.2 Metabolomic profiling

In the cortex samples, PLS-DA identified 11 metabolites with VIP scores > 1 and FDR-adjusted p-value < 0.05 (Table 1). Average fold-changes were also calculated for each metabolite. Average fold-change > 1 indicates an increase in the level of the metabolite, while fold-change < 1 indicates decrease. The most significantly altered metabolite was the 3–4-Hydroxyphenyllactate (FDR = 0.045 & VIP scores = 1.97), which was found to be decreased (average fold-change = 0.60) compared to the WT control. Levels of metabolites that are linked to the glycerophospholipids pathway such as Phosphatidylethanolamine (44:3) (FDR = 0.045, VIP scores = 1.92 & average fold-change = 1.56) and Lysophosphatidylethanolamines (18:0) (FDR = 0.045 & VIP scores = 1.92, average fold-change = 1.30) were also significantly increased compared to the controls.

3.3 Pathway enrichment analysis

Four significantly enriched metabolic pathways, including Glycerophospholipid metabolism (FDR = 0.0271 & Pathway impact = 0.560) and Glycerolipid metabolism (FDR = 0.0271 & Pathway impact = 0.374), were identified in the cortical samples of Tg2576 mice (Table 1 and Supplementary Fig. 6). In addition, one significantly enriched pathway from the hippocampal samples was also identified, the sulphur metabolism pathway (FDR = 0.0237 & Pathway impact = 0.213). There were no significantly enriched pathways detected in the serum samples.

3.4 Network analysis

In order to investigate the metabolite-group correlations, WGCNA was conducted on the metabolite peak intensity data from each of the three tissues types. By hierarchically clustering all samples from each tissue type into a sample tree, we identified and eliminated one WT sample from cortical dataset (Fig. 3a), two samples (TG and WT) from hippocampal dataset (Fig. 3b) and one WT sample from serum dataset (Fig. 3c) as outliers (Supplementary Fig. 7). The coexpression networks were then constructed and 12 modules were identified in the cortical dataset, 10 modules in hippocampal dataset and eight modules in serum dataset. In the cortical dataset, three modules were positively correlated with TG samples ($p < 0.05$), and one module was negatively correlated with TG samples (Fig. 4a). In the hippocampal dataset, one module was significatly positively correlated with the TG samples (Fig. 4b). Lastly, analysis of serum dataset revealed one module that was significantly negatively correlated with TG genotype (Fig. 4c). The modules were then functionally annotated by performing pathway enrichment analysis of the member metabolites for each identified

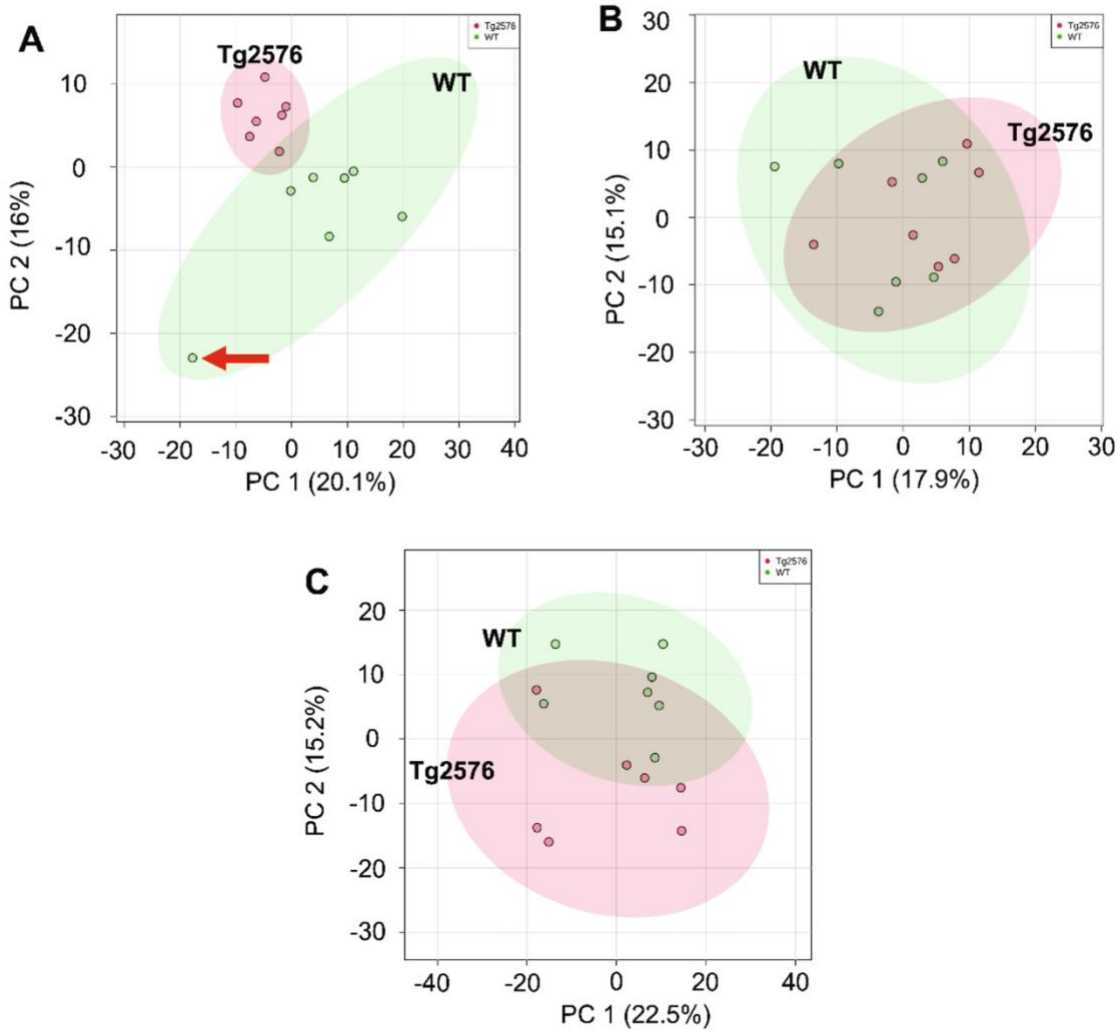

Fig. 1 Identification of the outlier using the principle component analysis (PCA) on the metabolomic data acquired from the 6-month-old Tg2576 and WT littermates. Principle component (PC) 1 and 2 were plotted against each other as these two components represent the majority of the variances **a** PCA identified one WT sample to be an outlier from the cortex samples (red arrow). The metabolomic data collected from this mouse was removed from subsequent analysis. There were no outliers in the **b** hippocampus and the **c** serum samples

module. The functional enrichment analysis revealed significant enrichment in three pathways from the cortical dataset (Table 2), two pathways from the hippocampal dataset (Table 2) and one pathway "aminoacyl t-RNA biosynthesis" (FDR = 0.0449) in the serum dataset.

4 Discussion

Currently, AD can only be diagnosed with certainty post-mortem by staining the brain for Aβ plaques and neurofibrillary tangles (McKhann et al., 1984). Because of this, identifying early mechanisms and metabolic pathways that are associated with the pathogenesis of AD might enable patients to be diagnosed before substantial brain pathology emerges, potentially facilitating therapeutic intervention. Here, 6-month-old Tg2576 mice were used, as this age represents the presymptomatic stage of the Aβ pathology in AD (Hsiao et al., 1996). Metabolomic analysis was the chosen method for this investigation due to its ability to provide global profiling of the metabolites, pathway enrichment and network correlation between disease and control samples (Fiehn, 2002).

We identified metabolites and metabolic pathways that were altered in Tg2576 mice compared with WT mice, each of which may be related to mechanisms associated with the APP pathology in AD. Firstly, we observed that metabolites which are linked to oxidative stress were significantly altered by mutant APP overexpression phenotype. The metabolite level with the most pronounced

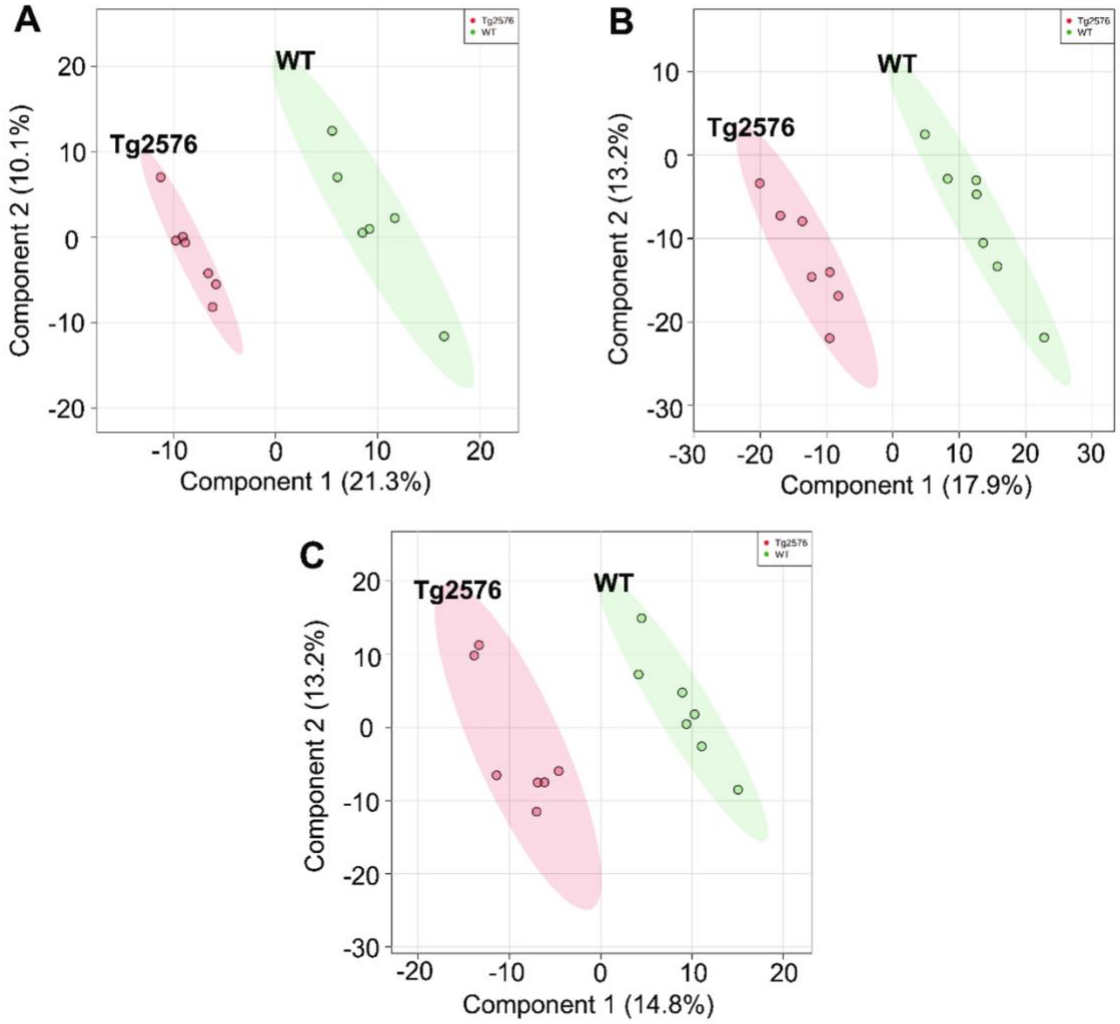

Fig. 2 Multivariate analysis using the partial least square-discriminant analysis (PLS-DA) on the peak intensity data. PLS-DA is a classification-based statistical model, which sharpens the separation between groups of observations. The PLS-DA scores plots show no overlap between the data collected from **a** cortex; **b** hippocampus and **c** serum samples of Tg2576 and WT groups, suggesting that the metabolomic profiles of the samples from Tg2576 are distinguishable from WT and therefore further analysis may be performed

difference was the hydroxyphenyllactate (KEGG ID: C03672). Hydroxyphenyllactate is classified as a phenolic compound and has been shown to exhibit anti-oxidant effects by reducing production of mitochondrial reactive oxygen species (Beloborodova et al., 2012; RiceEvans et al., 1997). We found a significant decrease in the level of hydroxyphenyllactate in Tg2576 mice. Npi-methyl-L-histidine (KEGG ID: C01152), a derivative of L-histidine, was also decreased in the cortex of the Tg2576. This metabolite is directly linked to the catabolism of carnosine (Kanehisa & Goto, 2000), and carnosine has been reported to exhibit antioxidant effects (Kohen et al., 1988). This could indicate a decrease in the synthesis of carnosine from L-histidine due to an early metabolic dysfunction in AD (Cararo et al., 2015; Fonteh et al., 2007), which may lead to an increase in oxidative stress. Finally, deoxyguanosine monophosphate (dGMP) (KEGG ID: C00362) was identified by the metabolomic analysis. Similar to hydroxyphenyllactate and npi-methyl-L-histidine, dGMP also has a role in oxidative stress (Henle et al., 1996). Furthermore, dGMP is also involved in purine metabolism and purine metabolism disorder has been reported in AD (Ansoleaga et al., 2015). It was found that the level of dGMP decreases as the AD stage progresses (Ansoleaga et al., 2015). Since Hydroxyphenyllactate, npi-methyl-L-histidine and dGMP are all associated with oxidative stress, this may indicate that the increase in oxidative stress in the brain is one of the earliest events that occurs in the presymptomatic stage of AD. The role of oxidative stress in AD is supported by the current literature (Lin & Beal, 2006; Nunomura et al.,

Table 1 The list of differentially expressed metabolites and the significantly enriched pathways between Tg2576 and WT in cortex samples

(A) Metabolite name	Average fold change	p value	FDR	VIP score	Associated pathway
3–4-Hydroxyphenyllactate	0.60	0.00025	0.045	1.97	Tyrosine metabolism
Phosphatidylethanolamine (44:3)	1.56	0.00048	0.045	1.92	Glycerophospholipids
Lysophosphatidylethanolamines (18:0)	1.30	0.00052	0.045	1.92	Glycerophospholipids
Phosphatidylserine (36:2)	1.25	0.00059	0.045	1.90	Glycerophospholipids
O-Propanoylcarnitine	0.71	0.00061	0.045	1.90	Oxidation of branched fatty acids
Npi-methyl-L-histidine	0.60	0.00066	0.045	1.90	Histidine metabolism
Phosphatidylserine (40:7)	1.28	0.00070	0.045	1.89	Glycerophospholipids
O-Butanoylcarnitine	0.65	0.00090	0.045	1.87	Fatty acyl carnitines
2′-Deoxyguanosine 5′-monophosphate	0.53	0.00097	0.045	1.87	Purine metabolism
Diglyceride (P-32:1)	1.91	0.0010	0.045	1.86	Diradylglycerols
Phosphatidylserine (44:12)	1.44	0.0011	0.045	1.86	Glycerophospholipids

(B) Metabolic pathway	p value	FDR	Pathway impact score
Glycerophospholipid metabolism	0.000757	0.0271	0.560
Glycerolipid metabolism	0.00125	0.0271	0.374
Glycine, serine & threonine metabolism	0.00233	0.0379	0.366
Pyrimidine metabolism	0.00431	0.0467	0.464

(A) A metabolite was identified as significantly different when its FDR-adjusted p-value < 0.05 and variable importance in projection (VIP) score > 1. Average fold change was calculated to identify both the amplitude and the direction of the change in the metabolite levels in the Tg2576 group compared to the WT group. The metabolic pathway that is associated with each metabolite is also listed. (B) Metabolic pathways with an FDR-adjusted p-value < 0.05 and pathway impact score > 0.2 were identified as significantly enriched. This analysis identified four significantly enriched metabolic pathways from the data acquired from the cortex samples

2001) and therefore increase in oxidative stress in the brain can be one of the earliest indicators of AD.

In addition to oxidative stress, six significantly altered metabolites that are related to lipid metabolism were also identified: three from the phosphatidylserine (PS) (KEGG ID: C02737) group; two from the phosphatidylethanolamine (PE) (KEGG ID: C00350) group; and one from the diglyceride (KEGG ID: C00165) group. PS and PE metabolites are classified as phospholipids and are involved in many important metabolic pathways such as glycine, serine, threonine and glycerophospholipid metabolism (Kanehisa & Goto, 2000). In late-onset AD, changes in the level of PS metabolites have been reported (Sarrafpour et al., 2019; Tokuoka et al., 2019; Wells et al., 1995), however these changes have not been previously observed in the presymptomatic stage of AD. In addition to their roles in metabolic pathways, PS also play important roles in neural transduction, being involved in signalling protein interactions, membrane-bound receptor modulation and regulation of exocytosis (Kim et al., 2014). PS can also increase the affinity of AMPA receptor in rat brain membranes (Baudry et al., 1991) and therefore the significant increase in the levels of PS detected here might indicate an increase in the membrane hyperexcitability in the presymptomatic stage of AD.

Lastly, metabolites that are associated with energy production (Schooneman et al., 2013) including o-butanoylcarnitine (KEGG ID: C02862) and o-propanoylcarnitine (KEGG ID: C03017) were also identified here. These two metabolites are classified as acylcarnitine and changes in the level of acylcarnitines can be an indicator of metabolic disorders (Koeberl et al., 2003; Millington & Stevens, 2011). It has been documented that metabolic dysfunction is a prominent feature in the AD brain (Blonz, 2017) and disorders such as diabetes can be associated with AD (Craft, 2009).

Pathway enrichment analysis identified five pathways that were significantly enriched in AD compared to controls. The glycerolipid metabolism pathway and the glycerophospholipid metabolism pathways were both enriched in the cortex of Tg2576 mice. This may be relevant to the glycerolipid & glycerophospholipid metabolic disorder which has been observed in AD patients (Wood, 2012). The enrichment in glycerolipid and glycerophospholipid metabolism pathways might occur because these two classes of lipids are the major constituent of neuronal and mitochondria membranes and in AD, both neurodegeneration (Masters et al., 2015) and mitochondrial dysfunction are present (Lin & Beal, 2006). This finding complements well with the results from individual metabolites, as we identified six metabolites that are associated with lipid metabolism.

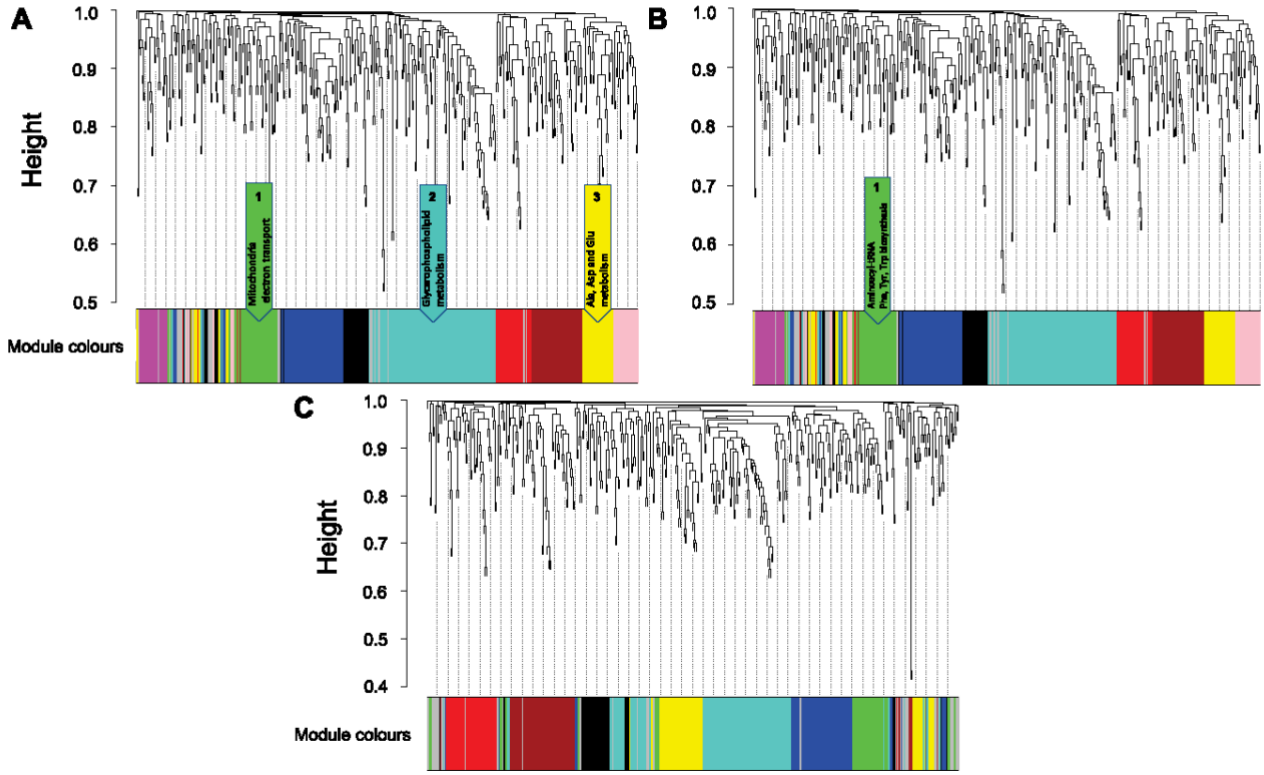

Fig. 3 Cluster dendrogram of all annotated metabolites identified in **a** cortex, **b** hippocampus and **c** serum of Tg2576. The metabolites were clustered into modules (colour coded) based on their abundance correlation across all samples in the dataset. The parameters of the adjacency matrix and the module detection were set as follows: soft thresholding power = 7; minimum module size = 10; and type of network = signed

Another pathway that was highlighted by our analysis was the glycine, serine and threonine metabolism pathway. This pathway is involved in the energy production process in the brain as it can produce metabolic intermediates that can be directly utilised by glycolysis and the Krebs cycle (Kanehisa & Goto, 2000). AD has been linked to energy production deficiency (Blonz, 2017) and the results from our metabolomics analysis support the role of energy production deficits in the presymptomatic stage of AD. Furthermore, pyrimidine metabolism pathway was also identified as significantly enriched and recently, the literature has associated pyrimidine metabolism with AD (Muguruma et al., 2020). It has been demonstrated that AD pathology could alter pyrimidine metabolism (Pesini et al., 2019) and indeed, pyrimidine metabolism plays a big role in mitochondria (Wang, 2016). Therefore, the impairment in pyrimidine metabolism might indicate mitochondrial dysfunction in AD (Murphy & Hartley, 2018).

In the hippocampus samples, sulphur metabolism pathway was identified as significantly enriched. This discovery is supported by the current literature, which demonstrated that the disruption in sulphur metabolism is linked to AD (Griffin & Bradshaw, 2017; Townsend et al., 2004). It has been reported that the levels of sulphur-containing amino acids such as cysteine were increased in the hippocampal region of AD patients (Xu et al., 2016). Additionally, the increase in plasma homocysteine have been associated with AD (Townsend et al., 2004) and the rise in plasma homocysteine level may precede AD symptoms (Seshadri, 2006). Here it can be seen that the results from the cortex and hippocampus are different. This is possibly because at 6-month-old, the cortex of Tg2576 is more affected by APP overexpression than the hippocampus.

In agreement with the results from metabolite analysis and pathway enrichment analysis, WGCNA identified mitochondrial dysfunction and glycerophospholipid metabolism to be positively correlated with AD genotype (cortex, green and turquoise modules, Table 2). Mitochondrial electron transport chain is important for the production of adenosine triphosphate (ATP) and reactive oxygen species (ROS) (Zhao et al., 2019) and therefore, dysfunction in this pathway could indicate energy deficit and overproduction of ROS. Moreover, early APP pathology is associated with altered lipid metabolism as evidenced in this study. Metabolites associated with glycerophospholipid metabolism pathway were significantly different in Tg2576 mice compared

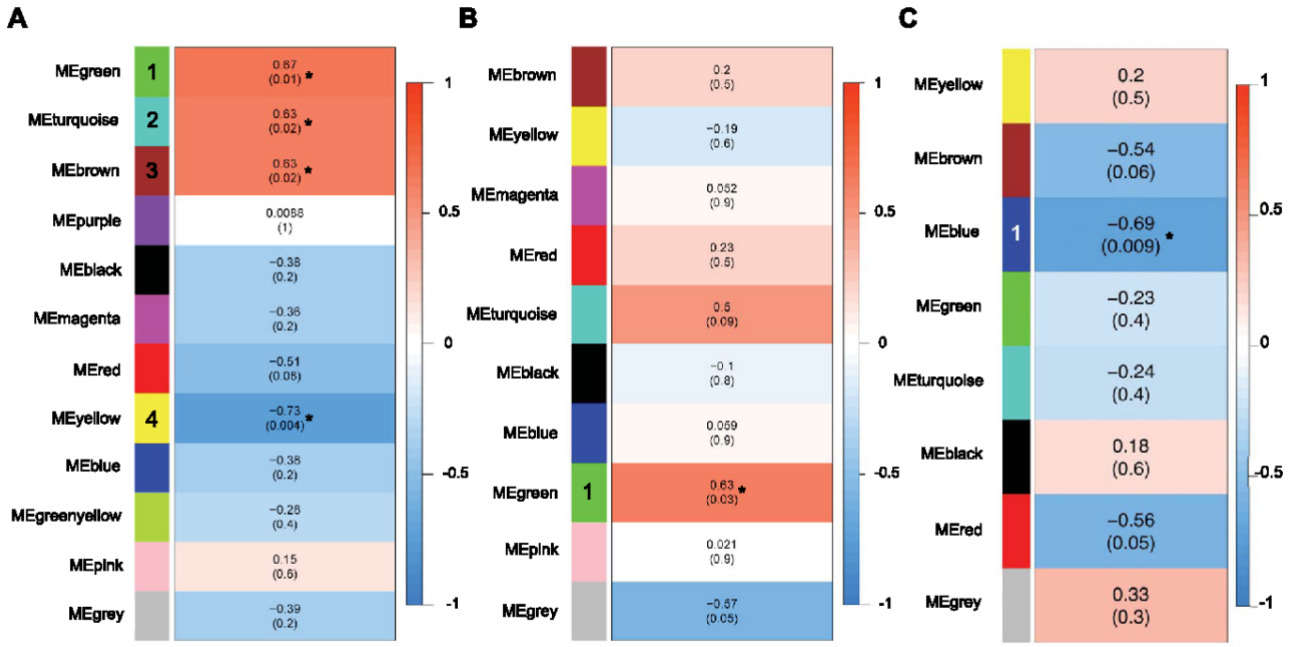

Fig. 4 The relationship between sample traits and modules in **a** cortex, **b** hippocampus and **c** serum datasets. The number in each module represent the positive or negative Pearson correlation, with the associated *p-value* in brackets, between the module eigengene (ME) and Tg2576 samples. The green, turquoise, brown and yellow modules in the cortex, the green module in hippocampus and the blue module in the serum were significantly correlated with the AD genotype

Table 2 The metabolic pathways in the (A) cortex and the (B) hippocampus that are significantly correlated to the AD genotype as calculated by WGCNA

Module colour	Metabolic pathway	p value	FDR
(A)			
Green	Mitochondria electron transport chain	0.00042	0.041
Turquoise	Glycerophospholipid metabolism	0.00044	0.037
Yellow	Alanine, aspartate and glutamate metabolism	0.000063	0.0053
(B)			
Green	Aminoacyl-tRNA biosynthesis	0.00022	0.019
Green	Phenylalanine, tyrosine and tryptophan biosynthesis	0.00089	0.037

The significance threshold was set to FDR < 0.05

to WT. Additionally, at the metabolic pathway level, glycerophospholipid and glycerolipid pathways were identified as significantly enriched.

Alanine, Aspartate and Glutamate metabolism (cortex, yellow module, Table 2) has also been identified to be negatively correlated with AD genotype. While alanine and aspartate are very important in the CNS, there is no clear evidence of their association with the pathogenesis of AD (Ellison et al., 1986; Kori et al., 2016). On the other hand, changes in glutamate levels are known to be highly associated with AD, motivating the use of memantine as a therapeutic option, which targets the glutamatergic pathway (McShane, et al., 2019). This finding is very important because it implies that the change in the level of glutamate in AD may happen during the presymptomatic stage of AD. Therefore, pathways that regulate glutamate homeostasis in the brain, such as the glutamate-glutamine cycle (Danbolt, 2001), may have the potential to be an early pharmacological target for the treatment of AD. The current literature would suggest that astrocytic glutamate transporters such as glutamate transporter 1 might be such candidate for drug target (Fontana, 2015).

Finally, aminoacyl-transfer RNA biosynthesis and phenylalanine, tyrosine and tryptophan biosynthesis were identified as signinficantly positively correlated with AD samples (hippocampus, green module, Table 2). Changes in the level of aminoacyl-transfer RNA has been associated with AD and mild cognitive impairment (MCI) in the literature (Ding et al., 2005; Xu et al., 2019). Similarly, changes in the levels of phenylalanine, tyrosine

and tryptophan biosynthesis have also been reported in AD (Griffin and Bradshaw, 2017). These results suggest that pathways involving amino acid metabolism could be affected early in the APP pathology. The functional annotation of the modules identified by WGCNA as well as pathway enrichment analysis was limited by the small size of metabolite databases and ambiguities in identifiers/aliases for particular isomers of a given metabolite as well as the small sample size of this study.

5 Conclusion

We have identified several metabolites that are linked to lipid metabolism and oxidative stress which are affected early in disease development in the Tg2576 mouse model of AD which exhibits mutant human APP overexpression pathology. Additionally, we report here that pathways such as Alanine, Aspartate and Glutamate metabolism are associated with AD and these findings can be the future basis of research in this area. However, we found no significant change in the metabolomic profile of the serum samples. This might mean that any metabolite change in the serum may not be detectable in the early stage of APP pathology. The results from this investigation have identified potential targets for future research focusing on metabolic disturbances. This might lead to new approaches to diagnose and treat patients in the presymptomatic stage of AD. It also allowed for new hypotheses on the mechanisms and pathways involved in the pathogenesis of AD to be generated and provided more insights into the current hypotheses.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11306-021-01793-4.

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Authors' contributions HD conceptualized and designed the study, prepared the material and collected the data. Analysis were performed by HD and AH. The first draft of the manuscript was written by HD and all authors commented on previous versions of the manuscript. PK and NJ provided the resources, supervision and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials The metabolomics and metadata reported in this paper are available via Metabolights (www.ebi.ac.uk/metabolights/MTBLS2280) study identifier MTBLS2280 (Haug et al., 2020).

Declarations

Conflict of interest The authors have no conflict of interest—either financial or otherwise—associated with this research.

Research involving human and/or animal participants This article does not contain any studies involving human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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3.3) Supplementary figures for empirical paper

Supplementary Figures for "Altered metabolic pathway in a transgenic mouse model suggest mechanistic role of APP overexpression in Alzheimer's disease"

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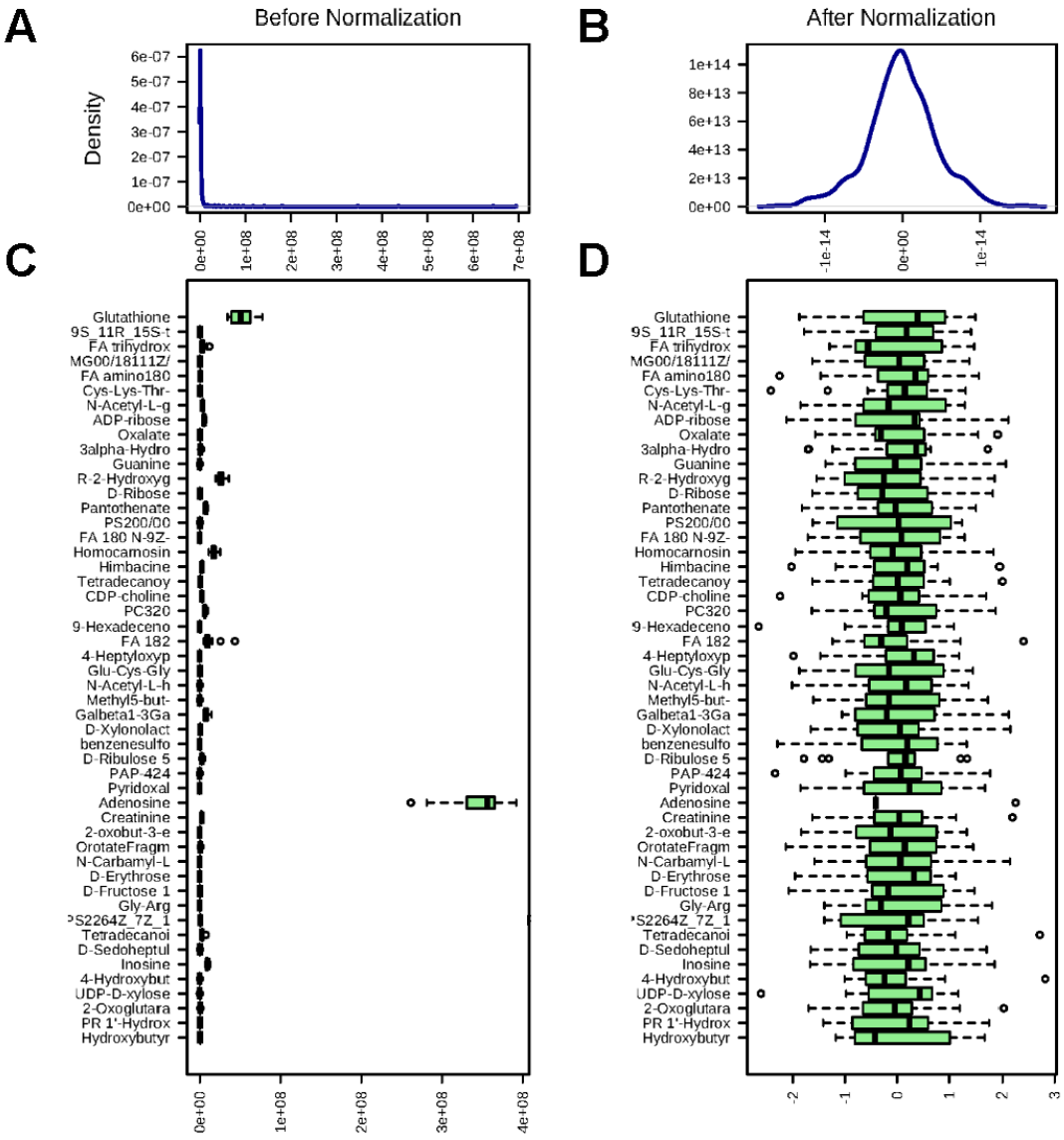
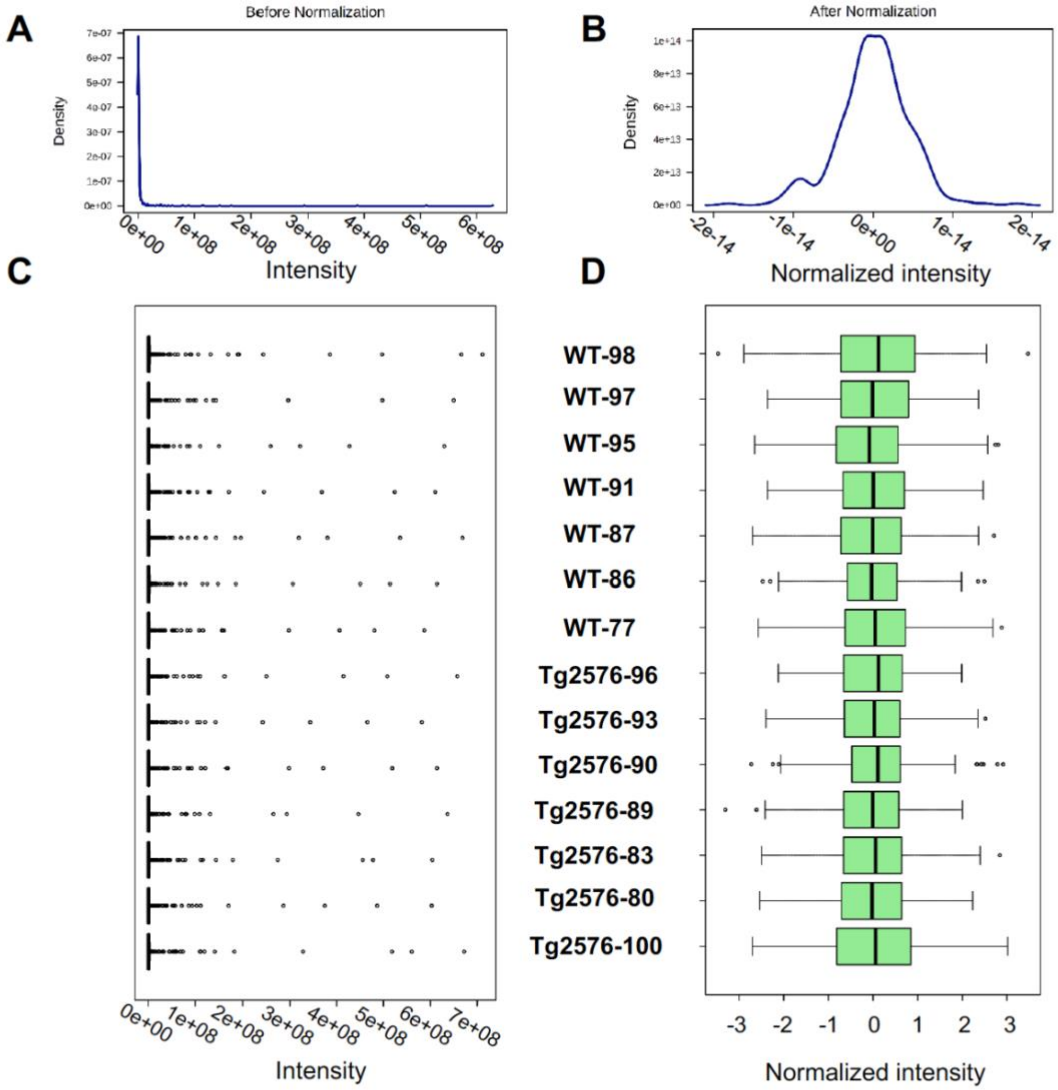
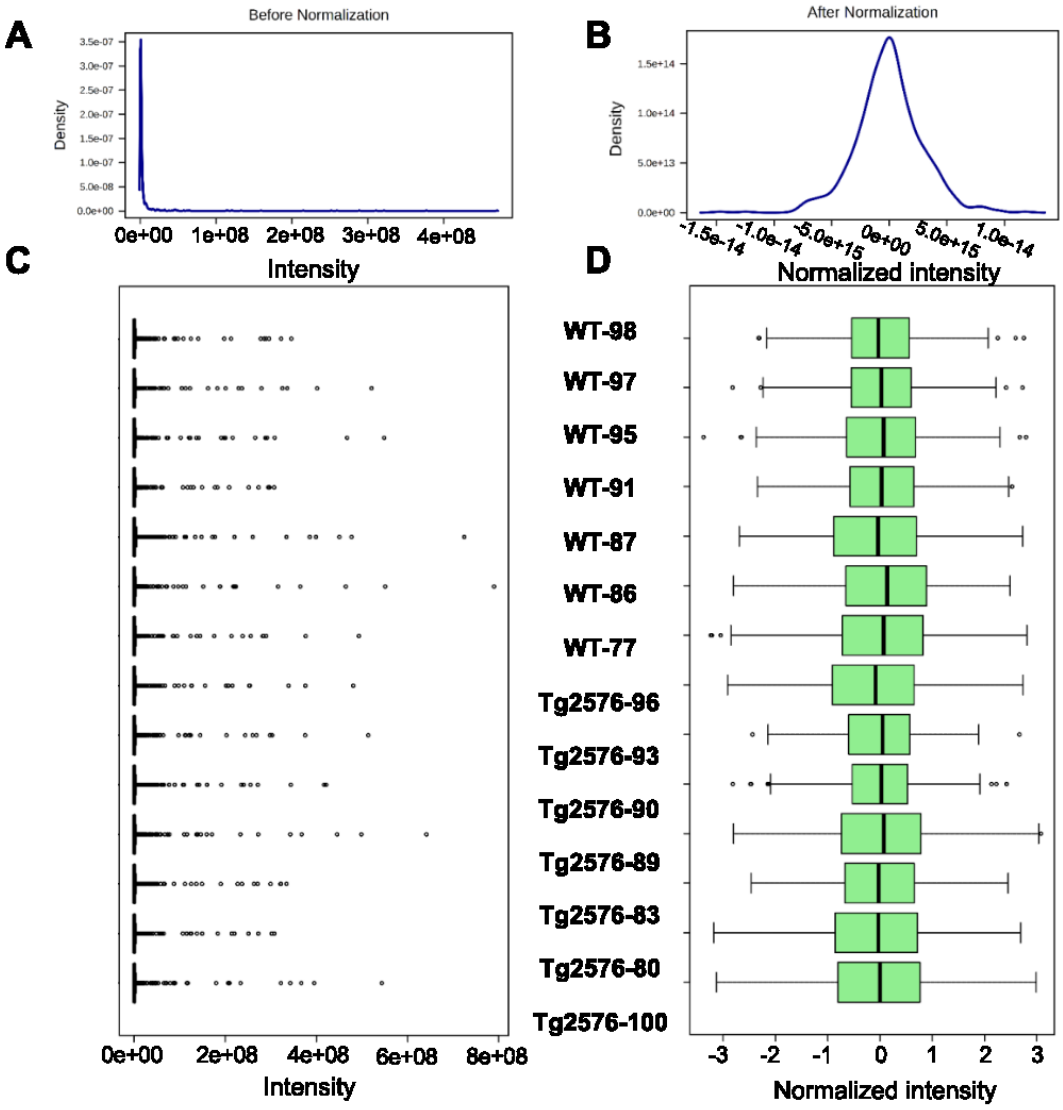


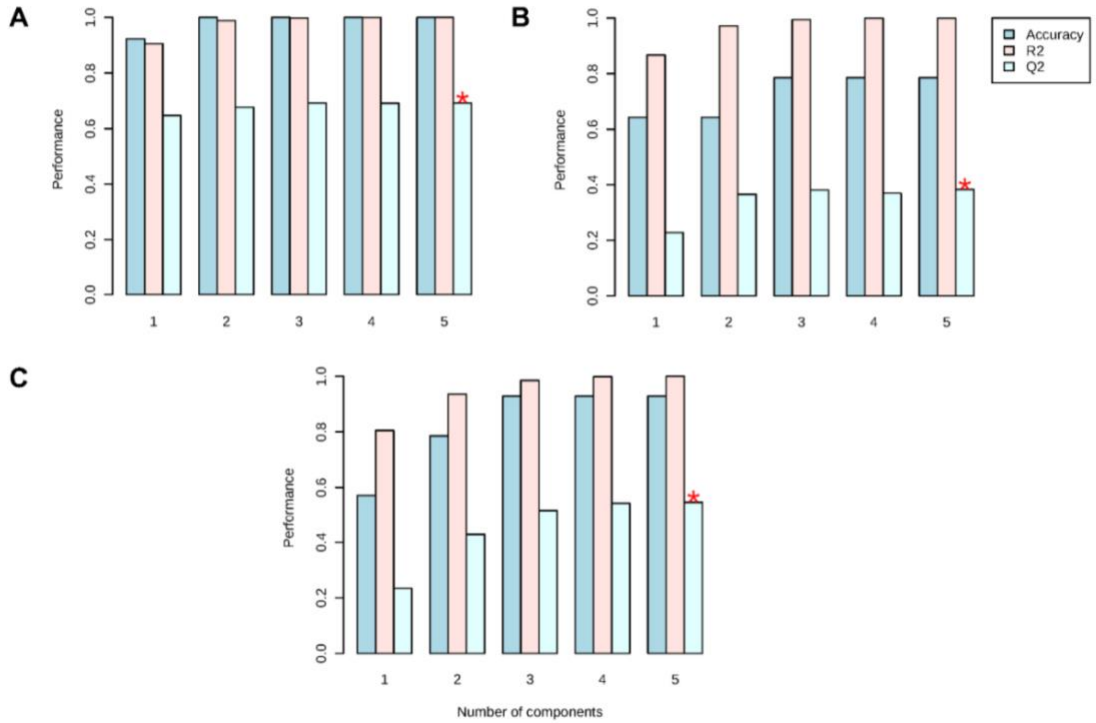
Supplementary figure 1. Kernel density plots and box plots before and after data normalisation for the cortex samples. The data was quantile normalised, log transformed and auto-scaled. The kernel density plots showed the distribution of the data A) before and B) after the normalisation steps. After the normalisation step, it can be seen that the data was normally distributed. The boxplots show the peak intensity data distribution for the metabolites C) before and D) after the normalisation. Only 50 representative metabolites from the cortex samples were shown due to the space limit.



Supplementary figure 2. Metabolomic data normalisation of the hippocampus sample. The data was quantile normalised, log transformed and auto-scaled. The kernel density plots showed the distribution of the data A) before and B) after the normalisation steps. After the normalisation step, the data was normally distributed. Boxplots show the peak intensity data distribution for the metabolites in each sample C) before and D) after normalisation.



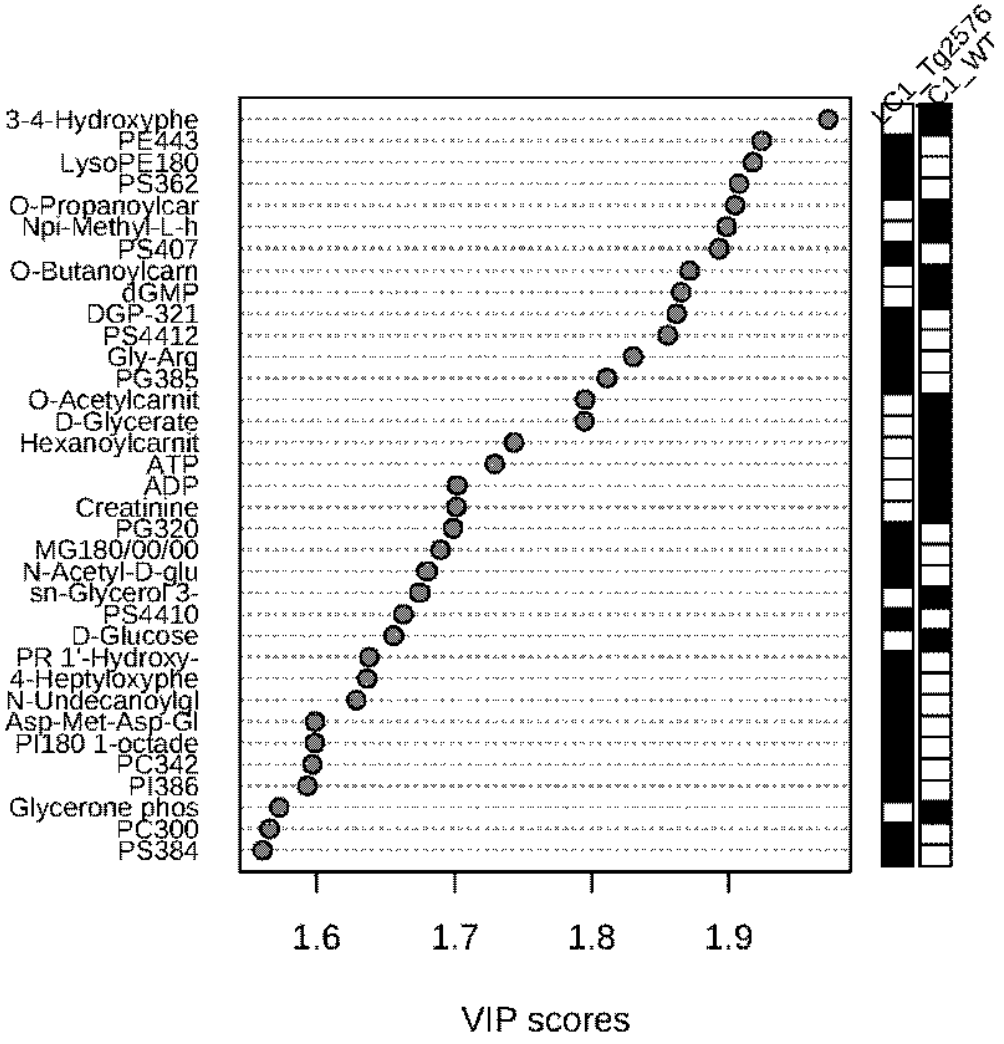
Supplementary figure 3. Metabolomic data normalisation of the serum sample. The data was quantile normalised, log transformed and auto-scaled. The kernel density plots showed the distribution of the data A) before and B) after the normalisation steps. After the normalisation step, the data was normally distributed. Boxplots show the peak intensity data distribution for the metabolites in each sample C) before and D) after normalisation.



Supplementary figure 4. Validation of the results from the partial least-square discriminant analysis (PLS-DA). The leave-one-out cross-validation (LOOVC) test was used for validating the results from the PLS-DA of A) cortex; B) hippocampus and C) serum samples. When the difference between R^2 and Q^2 is ≤ 0.3, the component is considered validated. * indicate the component with the highest fitting accuracy. It could be seen that only the results from the cortex was validated with the LOOVC.

Components	1	2	3	4	5
R^2	0.9050	0.9883	0.9981	0.9998	0.9996
Q^2	0.6704	0.6774	0.6919	0.6911	0.6920

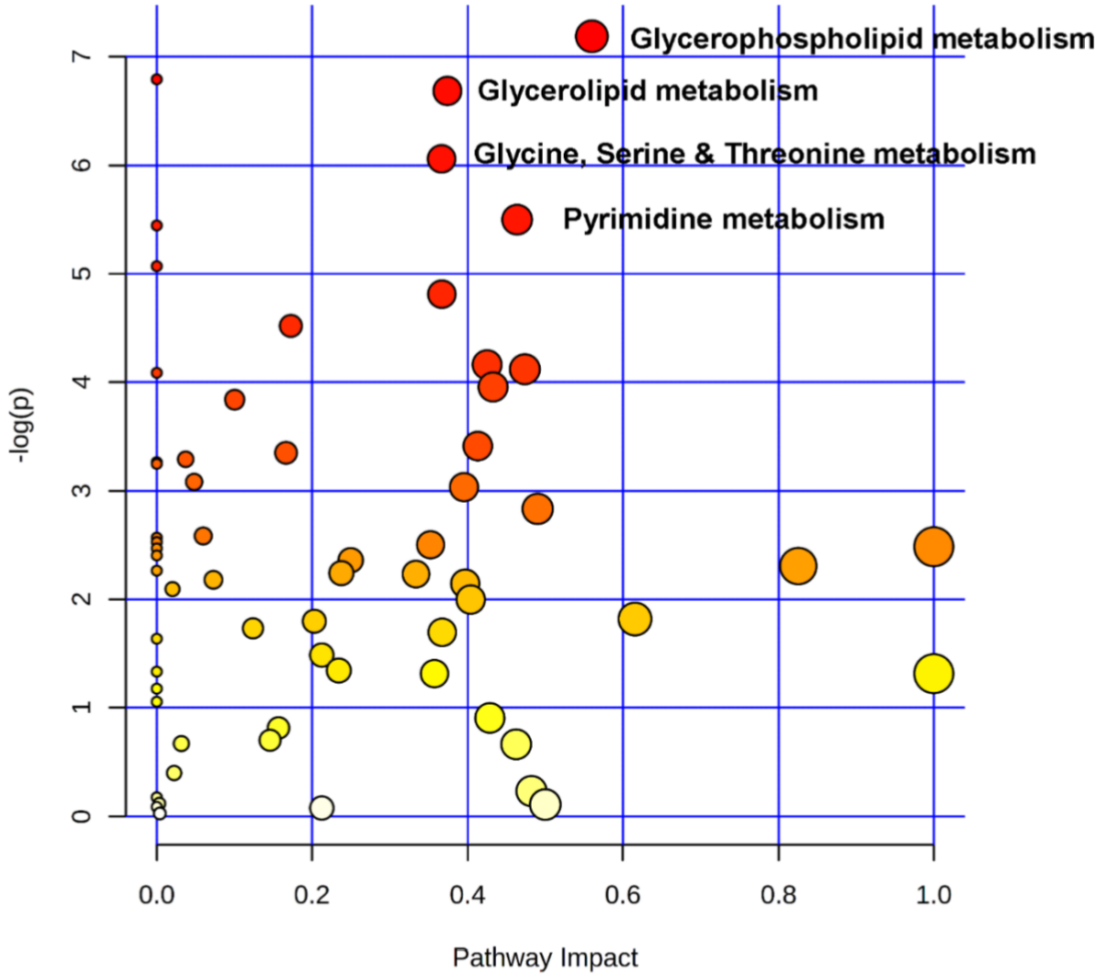
Supplementary table 1. Leave-one-out cross-validation (LOOVC) outputs on the results from the partial least-square discriminant analysis of the cortex samples. LOOVC validated the from the cortex samples.



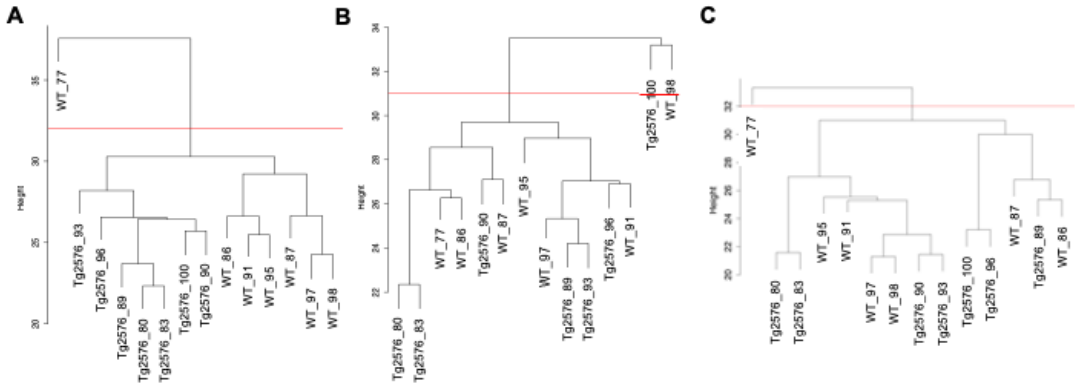
Supplementary figure 5. Metabolites that are driving the separation between Tg2576 and WT samples. Variable importance in projection (VIP) scores for each metabolite from the cortex samples was acquired from the PLS-DA analysis. A metabolite was considered to be significant in this model when the VIP score associated with the metabolite is greater than 1. This figure only shows 35 metabolites due to the space limit.

Metabolite name	*P*-value	FDR
3-4-Hydroxyphenyllactate	0.00025	0.045
Phosphatidylethanolamine (44:3)	0.00048	0.045
Lysophosphatidylethanolamines (18:0)	0.00052	0.045
Phosphatidylserine (36:2)	0.00059	0.045
O-Propanoylcarnitine	0.00061	0.045
Npi-Methyl-L-histidine	0.00066	0.045
Phosphatidylserine (40:7)	0.00070	0.045
O-Butanoylcarnitine	0.00090	0.045
2'-Deoxyguanosine 5'-monophosphate	0.00097	0.045
Diglyceride (P-32:1)	0.0010	0.045
Phosphatidylserine (44:12)	0.0011	0.045

Supplementary table 2. Significantly different metabolites from the cortex of Tg2576 compared to WT. T-test has identified 11 significantly different (FDR adjusted *p-value* < 0.05) metabolites in the cortex of 6-month-old Tg2576 compared to the WT littermate.



Supplementary figure 6. Significantly enriched pathways in the cortex of Tg2576 compared to WT. According to pathway enrichment analysis, metabolic pathway with FDR adjusted p-value < 0.05 and pathway impact > 0.2 would be identified as the significantly enriched pathway. In this study, the pathway enrichment analysis had identified 4 significantly enriched pathways from the cortex samples.



Supplementary figure 7. Outlier identification for WGCNA. The A) cortex, B) Hippocapus and C) serum samples from each animal were hierarchically clustered and categorised into dendrograms. Then the cutreeStatic function of WGCNA package (cutHeight = 31) was used to eliminate the samples that branched off the main sample tree at >31 height (red line).

3.4) Reference

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Chapter 4 – Disruption in glutamate-glutamine cycle is associated with increased seizure susceptibility in Tg2576 mouse model of Alzheimer's disease

Preamble to Chapter 4

This chapter independently explored the effects of AD pathology and epilepsy on key proteins in the glutamate-glutamine cycle. This will give valuable insights on how glutamate-glutamine cycle are affected under different conditions. The narrative of Chapter 4 and Chapter 5 are intricately linked as Chapter 5 will investigate how AD and epilepsy together affect the glutamate-glutamine cycle and explore a potential treatment for epilepsy in AD associated with this pathway.

4.1) Abstract

Background: Individuals with Alzheimer's disease (AD) have up to 10-fold higher chance of developing spontaneous seizures, compared to healthy age-matched controls. The mechanism that links AD to epilepsy is not well established. Mechanism associated with the glutamatergic pathways, such as disruption to glutamate-glutamine cycle, has been implicated in the pathogenesis of both diseases; therefore, this may be one of the links.

Objective: This study aimed to investigate changes in the expression levels of key proteins in glutamate-glutamine cycle, such as glutamate transporter-1 (GLT-1), glutamine synthetase (GS) and glutaminase (GLS), in the context of AD and epilepsy, using the seizure prone Tg2576 mouse model of AD. I hypothesised that the expression levels of these key proteins are altered in Tg2576 mice, compared to wild-type (WT). I also hypothesised that kindling-induced seizures will alter the expressions of these key proteins in WT mice, compared to sham.

Methods: Seven aged (13 to 14-month-old) mixed-gender Tg2576 and 5 WT littermates were kindled to compare their seizure susceptibilities. The levels of key proteins in glutamate-glutamine cycle were compared between 7 matured (6-month-old) naïve Tg2576 mice and 7 WT littermates via western blot. Then, quantitative polymerase chain reaction (qPCR) was performed to measure changes in the levels of genes associated with glutamate-glutamine cycle. Finally, 6 aged WT mice were kindled and their levels of key proteins in glutamate-glutamine cycle were compared with 6 sham WT mice.

Results: Compared to WT, Tg2576 mice displayed significantly higher seizure class (median = Class V vs. Class III, $p = 0.02$) and seizure duration (mean = 54.86s vs 31.06s, $p = 0.0399$) in response to kindling. This suggests that Tg2576 mice are more prone to kindling than WT mice. Compared to WT, there were significant reductions in the levels of GLT-1 ($p = 0.0093$) and GS ($p = 0.0016$) proteins in naïve Tg2576 mice. This implies that the disruption to glutamate-glutamine cycle may play a role in enhancing the susceptibility to kindling in Tg2576 mice. No significant change in the gene expression levels of GLT-1 and GS in Tg2576 mice was observed. Finally, there were significant enhancements in the expression levels of GLT-1 protein in the cortex ($p<0.0001$) and the hippocampus ($p = 0.0075$) and GLS protein in the cortex ($p = 0.0016$) of kindled WT mice, compared to sham. This may suggest that the glutamate-glutamine cycle is involved in the mechanism that increases the brain's susceptibility to seizure.

Conclusion: Tg2576 mice are more susceptible to kindling-induced seizure than the WT. This might be due to lower baseline levels of GLT-1 and GS in Tg2576 mice, which would increase the chance of glutamate accumulation in their brains. Kindling caused the expression levels of GLT-1 and GLS to increase, which implicates their involvement in the pathogenesis of epilepsy. Therefore, the results suggest that disruption to the glutamate-glutamine cycle may be associated with the pathogenesis of both AD and epilepsy.

4.2) Introduction

Currently, there is evidence from the literature to demonstrate that AD patients have up to 10-fold higher risk of developing seizures, compared to healthy age-matched controls.[1-3] However, the mechanism that links AD pathologies to higher seizure susceptibility is not well understood. I propose that abnormal glutamate homeostasis might be a strong candidate mechanism that links AD pathologies to an increase in the risk of developing epilepsy. While other mechanisms[4] might also be relevant and cannot be neglected, they will not be the focus of this thesis.

Disruption to glutamate-glutamine cycle in the brain has been implicated in the pathogenesis of AD[5-7] and epilepsy.[8] There is evidence to show that changes in the expression levels of key proteins in this cycle are associated with lethal seizure[9] and cognitive impairment[10] phenotypes in rodent models.[11] This may imply that the disruption to glutamate-glutamine cycle plays a role in the pathogenesis of both epilepsy and AD; thus, it might be one of the common mechanisms between these two neurological disorders.

The glutamate-glutamine cycle is highly involved in the homeostasis of glutamate in the extracellular space of the brain. Astrocyte is one of the key players in the glutamate-glutamine cycle since some of the key proteins that are involved in this cycle, such as GLT-1 and GS enzyme, are exclusively expressed on the astrocytes.[12-14] The main physiological function of an astrocyte is to support and protect the neurons by providing nutrients and regulating neurotransmitter levels. Astrocytes are heavily involved in maintaining glutamate homeostasis in the CNS via tripartite synaptic structure, which consists of a pre-synapse, a post-synapse, and an astrocytic process.[15] Briefly, the astrocytic process uptakes glutamate from the synaptic cleft via GLT-1, then the glutamate is converted into glutamine via the intracellular GS enzyme. Since this process converts the neuronal-active glutamate into the neuronal-inactive glutamine, the excitatory signal propagation will be impeded. Therefore, alterations to the expression levels of GLT-1 and GS proteins may disrupt the glutamate homeostasis in the brain.[16] Furthermore, the role of reactive astrocyte formation (astrogliosis) has also been implicated in the pathogenesis of AD[6, 17] and epilepsy.[18-20] Astrogliosis may occur in response to AD pathologies, such as Aβ plaques, and this process may disrupt normal astrocytic functions, such as glutamate uptake.[21] Since astrocytes are important regulators of glutamate in the brain[14], disruption to the astrocyte's glutamate uptake efficiency may cause an accumulation of glutamate in the synaptic cleft, leading to an increase in the brain's excitability. For a detailed discussion on the interactions between AD pathologies, astrocytes and the glutamatergic pathway refer to Chapter 2.

Despite the evidence to demonstrate that alterations to the expression levels of key proteins in glutamate-glutamine cycle may lead to higher seizure susceptibility[9] and cognitive impairments[10, 22], the effects of AD pathologies on the expression levels of key proteins in glutamate-glutamine cycle have not been investigated in the context of epilepsy in AD. Thus, this study aims to reveal potential pharmacological targets for ameliorating the enhanced seizure susceptibility associated with AD. To this end, animal models of AD are powerful tools for generating insight onto the potential mechanisms that may lead to an increase in seizure susceptibility. The first advantage of using an animal model is the practicality and the ease of use. As discussed in Chapter 3, sample collection is not a challenge in animal studies because the samples can be collected at the desired timepoint with ease. On the contrary, sample collection can be more difficult to achieve in human studies due to the invasive nature of the procedures. Secondly, the effect of a particular AD pathology, such as mutant human APP overexpression, can be elucidated individually in animal models. On the other hand, this is not possible in human studies because AD involves a myriad of pathologies that interacts with each other, which may prevent the elucidation of individual mechanism.[23] Animal models of AD will generate important data needed to guide the design of future human studies on the topic of epilepsy in AD.

However, there are some limitations associated with the use of animal models. Although the ability to investigate each AD pathology individually can be advantageous in some respects, it can also be limiting. Since AD is not associated with just one pathology but rather a combination of many, the results obtained from rodent models of AD that display only one or two pathologies from AD might not be fully translatable to human studies because of this discrepancy. Additionally, the murine form of the proteins associated with AD pathologies, such as Aβ and tau, are not naturally pathological.[24] Therefore, the mutated human form of these proteins are transgenically inserted into the mouse model to produce the pathologies that are comparable to AD in humans. Furthermore, these mutated human pathological proteins are designed to be overexpressed from birth, making these models different from human AD patients. This suggests that the pathological phenotypes observed in these models of AD may be caused by the presence of the mutated human form of the protein from birth rather than the actual pathological signalling cascade observed in human AD. Despite that, data generated from animal models are crucial for elucidating the mechanism that links AD pathologies to an increase in seizure susceptibility. Additionally, when the animal model is appropriately validated and a proper control is used, then the translational impact of the data generated from the animal study is increased.[25] Animal studies also allow researchers to control aspects of the experiment which are impractical to

control in human studies, for examples time specific tissue collection and the living condition of the subjects.[25] Thus, the insights gained from animal studies are important and can be used to guide the design of future human studies.

I aimed to investigate how key proteins in the glutamate-glutamine cycle, such as GLT-1 and GS, are affected by the Aβ pathology in the brain. I also aimed to investigate whether alterations in the levels of these key proteins happen during the early stages of the Aβ pathology. To this end, Tg2576 mouse model of AD was utilised. Tg2576 model is one of the most characterised models of mutant human APP overexpression pathology[26], and there is evidence from the literature to show that Tg2576 mice are more prone to kindling than WT controls.[27, 28] Chapter 1 discussed how Tg2576 model is suitable for the study of mechanism that links Aβ pathology to an increase in seizure susceptibility. Chapter 2 discussed how Aβ pathology may disrupt the glutamate-glutamine cycle, which then leads to an increase in the risk of developing epilepsy in AD. Since the overexpression of mutant human APP may lead to Aβ pathology, the Tg2576 model became an important tool to investigate this mechanism. In this study, the cortical and the hippocampal regions of the brain were of particular interest. This was because, there is evidence to show that Aβ pathology affects the cortical region of the brain before other regions.[29, 30] Since Tg2576 mice display the Aβ pathology, investigating the cortical samples acquired from this model of AD may reveal the early effects of Aβ pathology on the glutamate-glutamine cycle. Additionally, it was shown that the entorhinal cortex is one of the first region that is involved in the reverberation of the seizure before spreading the seizure to other regions of the brain following an electrical stimulation.[31] As for the hippocampus, it is well-documented that this region of the brain is highly involved in cognition and memory.[32] Since memory deficit is closely associated to AD[23], hippocampus becomes the region of high interest in this field of research. It was also demonstrated that the cortical and the hippocampal regions are particularly susceptible to neurodegeneration.[33] Furthermore, hippocampus is one of the first regions in the brain to display epileptiform activity in response to a stimulation.[31] Thus, investigating these brain regions will give us the insights on how the glutamate-glutamine cycle is affected by the mutant human APP overexpression pathology in Tg2576 mice and how this may lead to an increase in seizure susceptibility. I propose that mutant human APP overexpression pathology alters the expression levels of key proteins in glutamate-glutamine cycle, leading to an increase in seizure susceptibility.[11]

4.3) Aims & Hypotheses

The first aim of this study was to investigate the kindling-induced seizure susceptibility of the Tg2576 mice, compared to WT littermates. This would validate the use of Tg2576 mice in this investigation. Then, I aimed to investigate the effects of mutant human APP overexpression pathology on the expression levels of key proteins in the glutamate-glutamine cycle, such as GLT-1 and GS proteins. As discussed earlier, alterations to the expression levels of these key proteins have been associated with lethal seizure and impaired cognition phenotypes. Thus, investigating how and when these alterations to the glutamate-glutamine cycle occur in the Tg2576 model may reveal novel therapeutic targets and an optimal window for the reducing seizure susceptibility phenotype in this model. Finally, I aimed to investigate the effects of kindling-induced seizures on the expression levels of key proteins in the glutamate-glutamine cycle. This would generate important insights on how this cycle may be involved in the pathogenesis of epilepsy. Chapter 5 will then investigate the effects of epilepsy in AD on the glutamate-glutamine cycle.

I hypothesised that Tg2576 mice have lower baseline expression levels of GLT-1 and GS proteins compared to WT littermates. I also hypothesised that kindled WT mice would have altered expression levels of key proteins in glutamate-glutamine cycle, compared to sham WT mice.

4.4) Methods

4.4.1) ***Animals***

For the kindling susceptibility experiments, 7 aged (13 to 14-month-old) mixed-gender Tg2576 mice and 5 c57 x SJL (WT) littermates were used. To investigate the effects of mutant human APP overexpression pathology on the expression levels of key proteins in glutamate-glutamine cycle, 7 mature (6-month-old) mixed-gender Tg2576 mice and 7 WT littermates were used. To investigate the effects of kindling on the expression levels of key proteins in glutamate-glutamine cycle, 12 aged mixed-gender WT mice were split equally into two groups: 1) kindled and 2) sham. Mice in the sham group underwent the same surgery and kindling procedures as the kindled group, but they were not electrically stimulated. The difference in the age of mice used in each experiment was due to the difference between the aims of each experiment. The rationale behind this difference will be discussed further in the “Discussion” section of this chapter. All mice were acquired from colonies at the Alfred Research Alliance (Prahran, Melbourne, Australia). All experiments were conducted in the Department of Neuroscience (Alfred Hospital), Monash University with the approval from the Alfred Research Alliance Animal Ethics Committee.

4.4.2) ***Electrode implantation***

In total, 7 Tg2576 mice and 11 WT mice underwent electrode implantation surgery. To begin the surgery, the animal was placed in an induction box supplied with 5% isoflurane mixed with oxygen at the rate of 2L/min to induce general anaesthesia. Once induced, the animal was placed on a heat pad and anaesthesia was maintained with 1-2% isoflurane mixed with 250-300ml/min of oxygen supplied via a nose cone. The depth of the anaesthesia was confirmed via toe pinch method. If the animal responded to the toe pinch, then the percentage of isoflurane in the air supply would be increased. After the animal no longer responded to a toe pinch, the head of the animal was secured with ear bars on a stereotaxic frame. Eye gel was applied to both eyes to prevent dryness during the surgery. After that, the fur on top of the scalp was removed to expose the skin. The skin was disinfected with chlorhexidine, then an incision was made along the midline of the scalp between lambda and bregma points. The connective tissues were removed with hydrogen peroxide. Once the skull surface was exposed and dried, the bregma point was marked with a pen. This point was used for zeroing the implantation coordinates as shown in Figure 4.1. Briefly, a bipolar depth electrode (Science Products, Hofheim, Germany) was implanted into the left amygdala (anteroposterior [AP]: -0.8; mediolateral [ML]: 3.1 relative to bregma; dorsoventral [DV]: -5.0 relative to dura) and a monopolar depth electrode was implanted into the right hippocampus (AP: -2.0; ML: -2.5; DV: -2.0). The implantation coordinates for the left

amygdala and the right hippocampus were validated in a separate experiment prior to the electrode implantation. Validation was done by microinjecting crystal violet dye into the brain of the euthanised Tg2576 and WT mice at the amygdala and hippocampus coordinates (Figure 4.1), then the injection sites were confirmed via histology. In addition, 3 extradural EEG recording electrodes were implanted to allow EEG recordings. The cortical and reference EEG electrodes were placed anterior to the coronal suture, while the ground electrode was placed posterior to the lambdoid suture. Furthermore, 3 anchor screws were implanted to reinforce the areas between each electrode. The electrodes were inserted into a pedestal, then dental cement and adhesive were used to shape the cap around the pedestal, thus allowing a cable to be plugged onto the electrodes. After the implantation, each animal was allowed to recover in a warm cage supplied with high water content food.

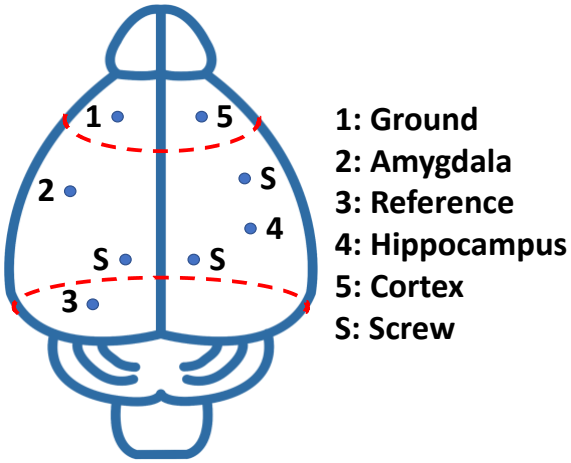

Figure 4.1. Electrode implantation coordinates for the kindling experiment. Left amygdala coordinates = Anteroposterior (AP): -0.8; Mediolateral (ML): +3.1 relative to bregma; Dorsoventral (DV): -5.0 relative to the dura. Right hippocampus coordinates = AP: -2.0, ML: -2.5; DV: -2.0. A depth electrode was implanted at location number 5 without a specific coordinate. Extradural recording electrodes were implanted at location 1 and 3 without specific coordinates to serve as ground and reference, respectively. Additionally, 3 anchor screws were implanted to increase the overall strength of the electrode cap.

4.4.3) ***Amygdala kindling procedure***

In this thesis, EEG and behavioural data were used to define a seizure. To qualify as a seizure, the EEG trace from the animal must be: 1) ≥6 seconds long; 2) ≥2x the amplitude of the baseline; 3) synchronised; 4) evolving with increasing spike frequency per minute and 5) biological, not mechanical (movements). An example of a seizure detected on the EEG can be seen in Figure 4.2. As for the behavioural evidence, this study used the Racine scale[34] to classify the severity of the seizure as follows: class I = mouth and facial automatism; class II = head nodding and jerks; class III: class II plus forelimb clonus; class IV: class III plus rearing or Straub tail, and class V = class IV plus falling over and/or jumping/running.

After 7 days of recovery from electrode implantation surgery, the after-discharge thresholds (ADT) of all mice, except the sham, were tested by stimulating the bipolar electrode once every 1 minute using the battery-operated ISO-Flex stimulus isolator (Microprobes for Life Science, Gaithersburg, MD, USA) until the stimulation triggered a seizure. The intensity of the stimulation began at 200µA and was increased with 20µA increment after each stimulation until the current reached 400µA. If the stimulation intensity reached 400µA without triggering a seizure, then the session would be restarted after 24 hours from 200µA. EEG data were recorded with the ML870 PowerLab data acquisition device (ADInstruments, Bella Vista, NSW, Australia) attached to the ML136 Animal Bio Amp signal amplifier (ADInstruments). LabChart 7 software (ADInstruments) was used for controlling the stimulation and visualising the EEG data. Seizure durations were determined from the EEG data. Once the ADTs were established, each mouse was subjected to a once-per-day stimulation at their respective ADT current. A mouse was considered fully kindled once it had 15 induced seizures. After being fully kindled, the mouse was euthanised on the day of the 15th seizure, then the brain samples were collected.

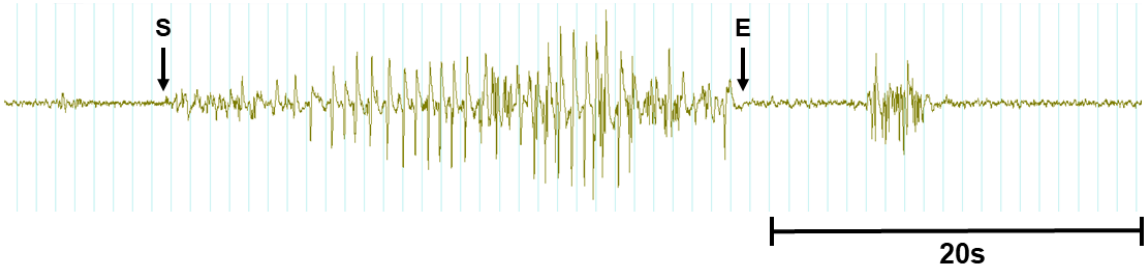

Figure 4.2. Representative trace of a spontaneous seizure recorded from a mouse. The seizure duration was approximately 30 seconds long. S = Start, E = End.

4.4.4) ***Sample preparation and western blot***

To collect the brain samples for protein analysis, the mouse was first euthanised via lethal injection of 80mg/kg of pentobarbitone (Provet, VIC, Australia). Then, the brain was extracted from the skull, transferred onto an ice-cold petri dish and washed with ice-cold saline. The olfactory bulb and the cerebellum were discarded, then the brain was separated into the left and the right hemispheres. After that, the brain was dissected into 4 slices along the coronal plane, then the cortical and the hippocampal regions were dissected. Each brain region was flash frozen with liquid nitrogen immediately after the dissection. Same procedure was applied to both hemispheres. The brain samples were then stored at -80°c until further use.

The frozen brain samples were homogenised in 5% sodium dodecyl sulphate (SDS) via the following procedures. Frozen brain sample was crushed into powder with mortar and pestle on dry ice. The powdered sample was then transferred to a 1.5ml tube, then 250µl of 5% SDS (Sigma-Aldrich, Bayswater, VIC, Australia) was added. Using a 23 gauge needle and 1cc/ml syringe, the solution was repeatedly drawn and ejected against the inside wall of the tube until the sample was broken down completely. The tube was then incubated at 100°C for 5 minutes to denature the proteins. After that, the samples were centrifuged at 13,000g for 15 minutes at 25°C. Then, the samples were stored at -20°C until further use. Protein level quantification was performed on the homogenised samples according to the protocol of the bicinchoninic acid (BCA) assay (cat#23225, Thermofisher). Briefly, the optical densities generated by the samples at 562nm were detected using the Multiskan™ FC Microplate Photometer (ThermoFisher). Then, the concentrations of the protein in the homogenised sample were determined using the straight line equation generated by the albumin standard from the assay.

The levels of the proteins of interest from each sample were determined via western blot. Two methods of western blot were utilised in this study. The first method was the traditional western blot, while the second was the robot-assisted western blot (Wes) (Protein Simple, San Jose, CA, USA). The traditional western blot was performed when the primary antibody was not compatible with the Wes system. This was the case with the GLT-1 antibody (cat#701988, ThermoFisher, Scoresby, VIC, Australia).

With the traditional western blot, GLT-1 (cat#: 701988, rabbit, conc. = 1:500, Thermofisher) and GS (cat#: ab64613, mouse, conc. = 1:10,000, Abcam, Melbourne, VIC, Australia) primary antibodies were used. GAPDH (cat#: 2118, rabbit, conc. = 1:10,000, Cell Signalling, Karrinyup, WA, Australia) and β-actin (cat#: ab8224, mouse, conc. = 1:10,000, Abcam) antibodies were used to measure the levels of housekeeping proteins. As for the

secondary antibodies, the swine anti-rabbit antibody (cat#: P0217, Agilent Technologies, Melbourne, VIC, Australia) was used for detecting the GLT-1 and GAPDH antibodies, while the goat anti-mouse immunoglobulin HRP (cat#: P0447, Agilent Technologies) was used for detecting GS and β-actin antibodies. To start the experiment, proteins from the homogenised samples were first separated according to their sizes via polyacrylamide gel electrophoresis in the Tank Blotting System (Bio-rad, Hercules, CA, USA). A 10% polyacrylamide gel required 4.2ml ultrapure water, 3.3ml 30% acrylamide/Bis solution, 2.5ml 1.5M Tris-HCl, 70µl 10% APS and 7µl of TEMED (Sigma-Aldrich). The concentration of the polyacrylamide gel could be adjusted to increase or decrease the separation between each protein band and facilitate analysis. The 100bp DNA ladder (Promega, Madison, WI, USA) was used as the reference. Gel electrophoresis was run at 80mA for approximately 50 minutes. After the separation, protein bands in the gel were transferred onto the immobilon membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), using the wet electroblotting tank system (Bio-Rad). For the transfer step, the tank was filled with an ice-cold transfer buffer (25mM TRIS, 192mM Glycine and 20% methanol) and the system ran at 300mA for 100 minutes. Once completed, the membrane was transferred to a 50ml falcon tube, then it was blocked with 25ml of 5% skimmed milk for 1 hour. The membrane was then washed twice with TBST solution (50mM Tris-Cl, 150mM NaCl, 0.1% Tween 20 (Sigma-Aldrich) with pH 7.2-7.4) and incubated overnight in 5ml of the primary antibody diluted with 5% bovine serum albumin (BSA) solution at 4°C. On the next day, the membrane was washed 3 times with TBST solution, then it was incubated in the secondary antibody diluted with 5ml of skimmed milk for 1 hour. After the incubation, the membrane was washed 4 times with TBST, then it was incubated with enhanced chemiluminescence (ECL) solution (non-commercial[35]) for 5 minutes. Gel Documentation (GelDoc) system (Bio-Rad) was used for exposing the membrane and recording photos of the protein bands. After that, the antibodies on the membrane was stripped with 50ml of stripping buffer, which contained 1g of SDS powder, 3ml of 1M Tris-HCl (pH6.8), 0.4ml of β-mercaptoethanol and ultrapure water. After the stripping process, the membrane was washed once with TBST and stored at 4°C in fresh TBST solution until further use. ImageLab (Bio-Rad) software was used for the analysis of band intensities from the photo of the membrane.

For the experiments with Wes, GLT-1 (cat#: sc-365634, conc. = 1:80, Santa Cruz, Tullamarine, VIC, Australia), GS (cat#: ab64613, mouse, conc. = 1:320, Abcam), glutaminase enzyme (GLS, cat#: ab93434, rabbit, conc. = 1:10, Abcam) and glial fibrillary acidic protein (GFAP, cat#: Z0334, rabbit, conc. = 1:20, Agilent Technologies) antibodies were used. GAPDH (cat#: 2118, rabbit, conc. = 1:200, Cell Signalling) was used as the

housekeeping protein. All consumables used in Wes experiments were supplied by ProteinSimple (San Jose, CA, USA), unless stated otherwise.

Sample preparation for Wes was performed according to the manufacturer's instructions. Briefly, protein analyses of the brain samples were performed on the Wes system using the 12-230 kDa separation modules (cat#: SM-W004). Additionally, the anti-mouse (cat#: DM-002) and/or the anti-rabbit (cat#: DM-001) detection modules were utilised to match the host species of the primary antibodies used in each experiment. Sample preparation and plate loading were performed according to the manufacturer's instructions. Briefly, the homogenised brain samples were diluted to 0.50µg/µl (final loading amount of 1.2µg) with 0.1x sample buffer (diluted from 10x sample buffer provided by the separation module). Fluorescent master mix from EZ Standard Pack 1 (cat#: PS-ST01EZ-8) was added to the diluted samples, then the samples and the biotinylated ladder from the EZ Standard Pack 1 were denatured at 95°C for 5 minutes on a heat block. Primary antibodies were diluted using the antibody diluent solution from the detection modules. The other components of the detection modules were HRP-conjugated secondary antibody, luminol and peroxide. These were pipetted into the detection plate along with the samples and the antibodies according to the manufacturer's guideline. Compass for SW (ProteinSimple) software was used for running the assay and the preliminary analysis of the protein levels.

4.4.5) ***Gene expression analysis with quantitative polymerase chain reaction***

The RNA was, first, extracted from each sample using the Rneasy Plus Mini Kit (cat#: 74034, Qiagen, Chadstone, Melbourne, Australia) according to the manufacturer's instructions. Briefly, the samples were lysed with the lysis buffer, which consisted of RLT+ reagent (Qiagen), β-mercaptoethanol and DX reagent (Qiagen). The samples were then loaded into the QIAcube (Qiagen) for automated RNA extraction and purification. After the extraction, RNA concentrations were quantified using the QIAxpert system (Qiagen). The extracted RNA was then stored at -80°C until further use.

To convert RNA to cDNA, Omniscript RT Kit (Qiagen) was used according to the manufacturer's instructions. In brief, the master mix was made by combining 10x Buffer RT, random hexamer, dNTP 5mM Mix, Omniscript Reverse Transcriptase and Rnasin (Promega). The RNA from each sample was diluted to 1µg/µl with Rnase-free water, then 7µl of the master mix was added to make the total reaction volume of 20µl. The samples were then incubated at 37°C for 1 hour in a thermocycler, then they were stored at -20°C until further use.

The samples used in quantitative polymerase chain reaction (qPCR) were prepared using the QIAgility automated PCR preparation system (Qiagen). The cDNA samples were mixed with TaqMan Fast Advanced Master Mix (cat#: 4444556, Thermofisher) and TaqMan Gene (ThermoFisher) according to the manufacturer's protocol. In this chapter, GLT-1 (assay ID: Mm00441457_m1) and GS (assay ID: Mm00725701_s1) genes were investigated in comparison to 4 reference genes consisted of TBP (assay ID: Mm01277042_m1), YWHAZ (assay ID: Mm03950126_s1), GAPDH (assay ID: Mm99999915_g1) and β-actin (assay ID: Mm02619580_g1). After the sample preparation process, the plate was transferred to the QUANTStudio 7 system (Thermofisher) to perform qPCR.

Data acquired from QUANTStudio 7 system were analysed according to the method described by Livak and Schmittgen, 2001.[36] Briefly, the cycle threshold (Ct) values from the genes of interest (GLT-1 and GS) were subtracted by the averaged Ct value of the reference genes in both Tg2576 and WT samples to obtain the ΔCt values. Then the ΔCt values from the Tg2576 group were subtracted by the ΔCt values from the WT group to obtain the ΔΔCt values and the relative expression level was calculated with $2^{(-\Delta\Delta Ct)}$.

4.4.6) ***Statistical analysis***

Statistical significance was calculated using the Prism 8 software (GraphPad, La Jolla, CA, U.S.A.). The normality of the data from each experiment was assessed by the Shapiro-Wilk test. If the data was normally distributed, then the unpaired Student's *t*-test would be used. If the data was nonparametric, then the Mann-Whitney *U* test would be used. The significance threshold for each analysis was set at $p<0.05$. All results from the western blot were normally distributed. For the kindling experiment, the seizure class results were not normally distributed but the seizure duration results were normally distributed. Normally distributed data were presented as mean ± standard error of the mean (SEM), while nonparametric data were presented as median ± interquartile range (IQR).

4.5) Results

4.5.1) ***Tg2576 mice were more susceptible to kindling-induced seizures than WT mice***

To validate that Tg2576 mice are more susceptible to kindling than WT, 7 Tg2576 mice and 5 WT mice were kindled, and their results were compared. The severity of each induced seizure was recorded as shown in Figure 4.3A. The number of animals in each group during each kindling session can be seen in Table 4.1. Visually, Tg2576 mice experienced more severe seizures than WT during the first 3 kindling sessions. In response to the first stimulation, Tg2576 mice displayed a median of Class V seizure, while the WT mice displayed a median of Class I seizure. This difference was statistically significant with $p = 0.01$ ($U = 3.5$, *Mann-Whitney*, Figure 4.3A). However, the difference in seizure class between Tg2576 and WT mice were not statistically significant from the second kindling session onwards (Figure 4.3A).

In another comparison, it was shown that the median number of stimulations needed for the WT mice to develop their first Class V seizures was 4, while all Tg2576 mice required just 1 stimulation. This suggests that Tg2576 mice are more prone to develop Class V seizures than WT mice. However, the p-value of this finding did not reach the significance threshold of $p<0.05$ ($U = 3.5$, $p = 0.0667$, *Mann-Whitney*, Figure 4.3B).

The average seizure duration from each kindling session was compared between Tg2576 and WT mice as shown in Figure 4.3C. During the first 2 kindling sessions, the average seizure duration from Tg2576 mice were visually longer than the WT. However, only the difference from the first session was significant (mean = 31.06s vs 54.86s, $p = 0.0399$, unpaired t-test), as shown in Figure 4.3C. There was no significant difference between seizure durations from the second kindling session onwards.

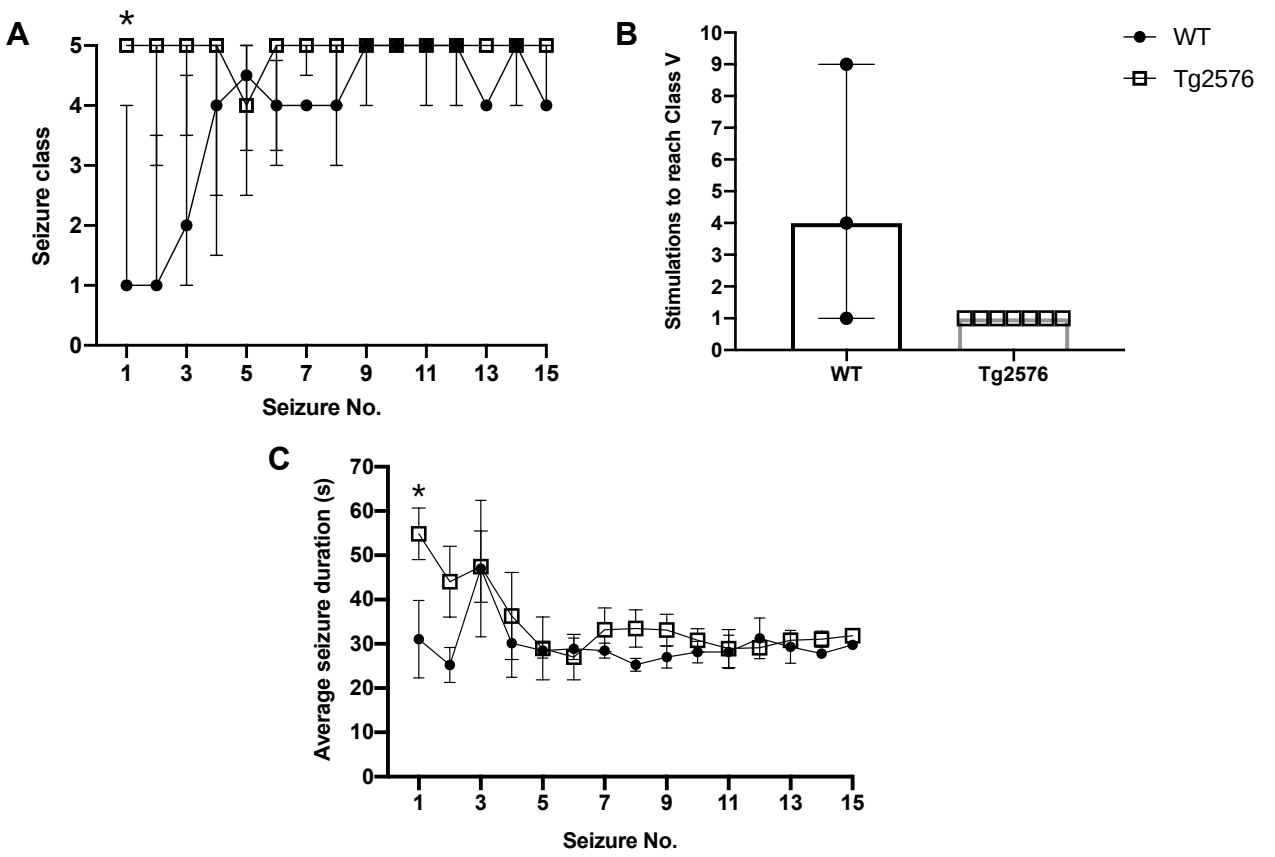

Figure 4.3. Tg2576 mice demonstrated higher kindling susceptibility than the wild-type (WT). **A)** Compared to WT, Tg2576 mice displayed higher seizure classes during the first 3 kindling sessions. There was no significant difference in seizure severity between Tg2576 and WT groups from the second session onwards. **B)** All Tg2576 mice required 1 stimulation to develop Class V seizures, while WT mice may require more than 1 stimulation. **C)** The average seizure duration from the Tg2576 group during the first kindling session was significantly longer than the WT group. There was no significant difference in the average seizure duration from the second session onwards. * $p<0.05$, medians ± IQR for **A)** and **B)**, means ± SEM for **C)**.

Seizure Number	WT	Tg2576
1	5	7
2	5	5
3	4	5
4	4	5
5	4	5
6	4	5
7	3	5
8	3	5
9	3	5
10	3	5
11	3	5
12	3	5
13	3	5
14	3	5
15	3	5

Table 4.1. Number of wild-type (WT) and Tg2576 mice during each stage of the kindling experiment. The number of animals decreased as the number of stimulations increased due to reasons such as loss of electrode cap, which resulted in euthanasia.

4.5.2) ***Reductions in GLT-1 and GS protein expressions observed in Tg2576 mice***

It was revealed that there were significant reductions in the levels of key proteins in glutamate-glutamine cycle in Tg2576 mice, compared to WT littermate, as shown in Figure 4.4. The mean GLT-1 protein level in relation to the β-actin in the cortex of Tg2576 mice was significantly decreased by 65% (p = 0.0093, unpaired t-test, Figure 4.4A & B). However, there was no significant difference between the expression level of GLT-1 in the hippocampus of Tg2576 mice, compared to WT (p = 0.767, unpaired t-test, Figure 4.4C & D).

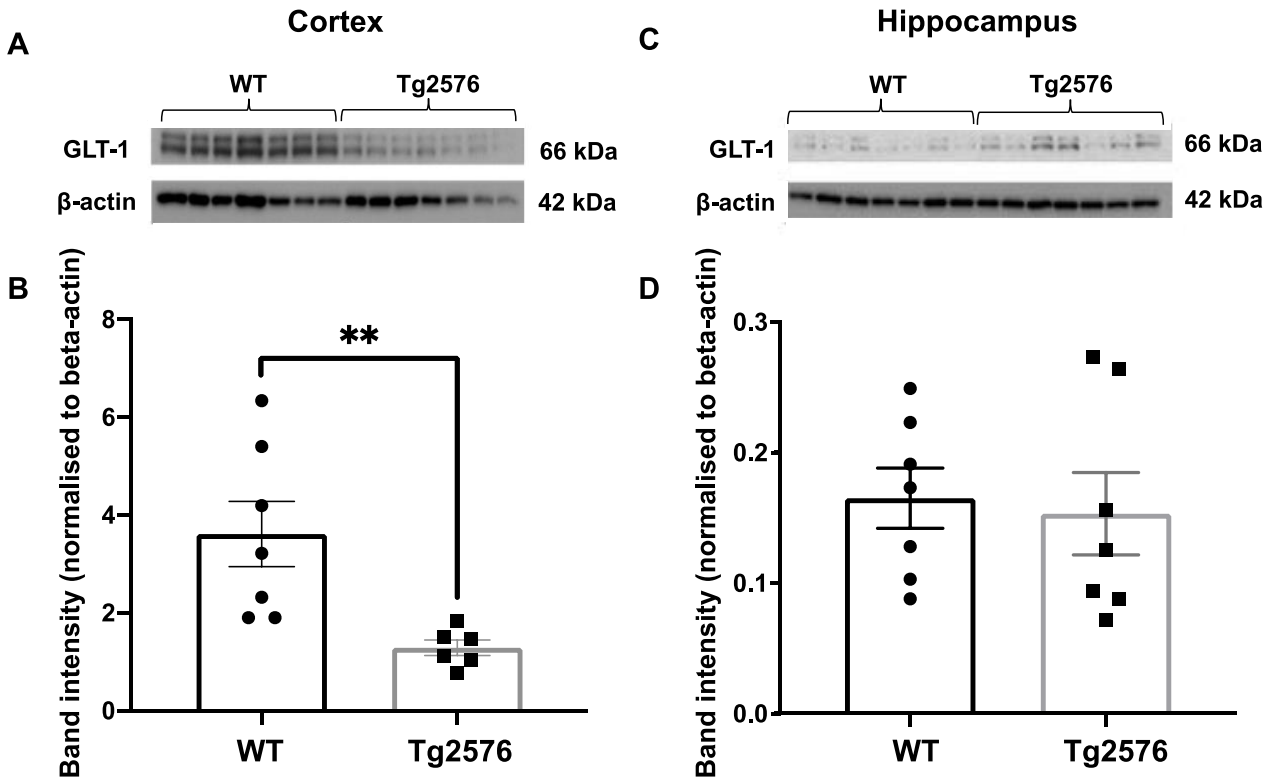

Figure 4.4. The expression level of the GLT-1 protein in the brain was significantly altered in Tg2576 mice. **A)** Clear reductions in the GLT-1 band intensities were observed in the cortex of Tg2576 mice, compared to WT. **B)** The average GLT-1 band intensity was significantly decreased in the cortex of Tg2576 mice. **C)** The clear difference that was observed in the cortex was not observed in the hippocampus of Tg2576 mice. **D)** No significant difference in the average GLT-1 band intensity was observed in the hippocampus of Tg2576 mice compared to WT. One cortical sample from the Tg2576 group was removed because the band intensities for housekeeping protein and GLT-1 from this sample were too faint to be analysed. **p<0.01, means ± SEM.

Furthermore, it was revealed that the expression level of GS protein in the cortex was significantly reduced in Tg2576 mice, compared to WT. As shown in Figure 4.5A & B the mean GS expression level was significantly reduced by 50% in the cortex of Tg2576 mice ($p = 0.0016$, unpaired t-test). On the contrary, there was a slight increase in the level of GS protein in the hippocampal samples from Tg2576 mice, compared to WT (Figure 4.5C). However, the significance of this change was only approaching the threshold with $p = 0.0589$ (unpaired t-test, Figure 4.5D).

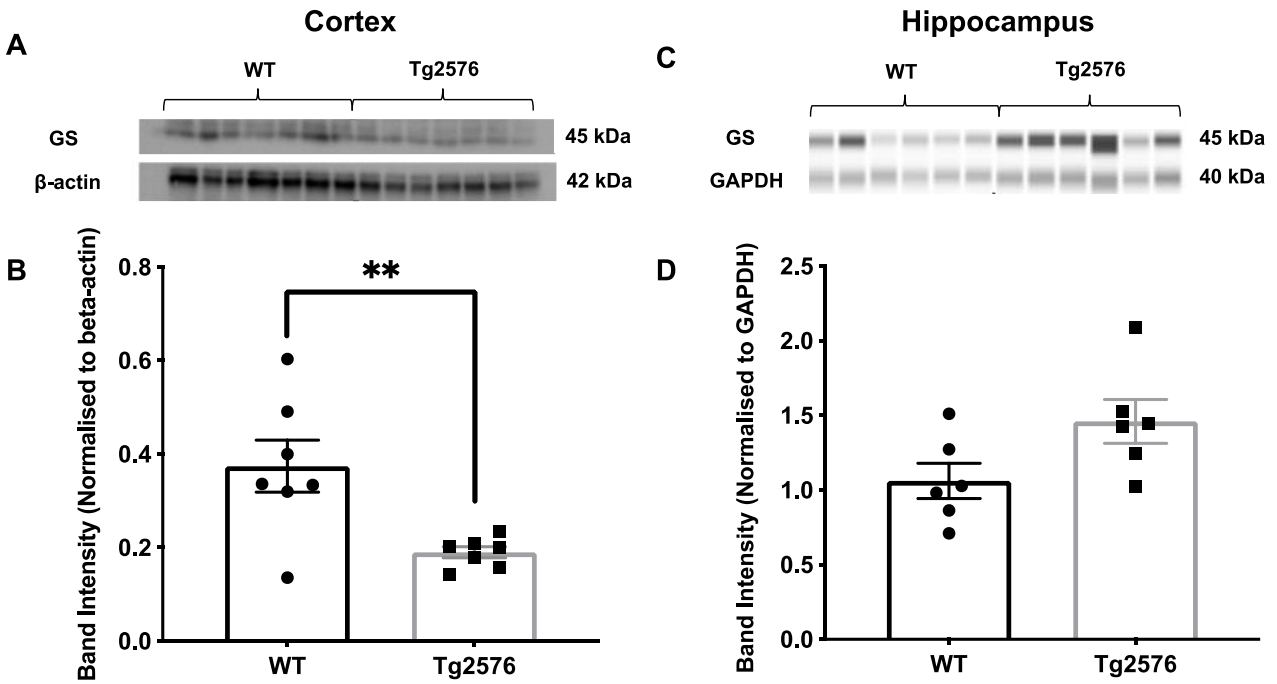

Figure 4.5. Significant reduction in the expression level of GS was observed in the cortex but not the hippocampus of Tg2576 mice. **A)** Cortical samples from WT mice displayed slightly more intense GS protein bands than Tg2576. **B)** Tg2576 mice had a significantly reduced expression level of GS in the cortex, compared to WT mice. **C)** GS protein bands from the hippocampal samples of Tg2576 mice were visually more intense than the bands from WT. **D)** The difference in the GS protein levels in the hippocampus was not statistically significant. **p<0.01, means ± SEM.

Changes in the expression levels of GLS protein were also investigated in this experiment. As shown in Figure 4.6, the average expression levels of GLS in the cortex ($p = 0.212$, unpaired t-test, Figure 4.6A & B) and hippocampus ($p = 0.310$, unpaired t-test ,Figure 4.6C & D) of Tg2576 mice were not significantly different from the WT.

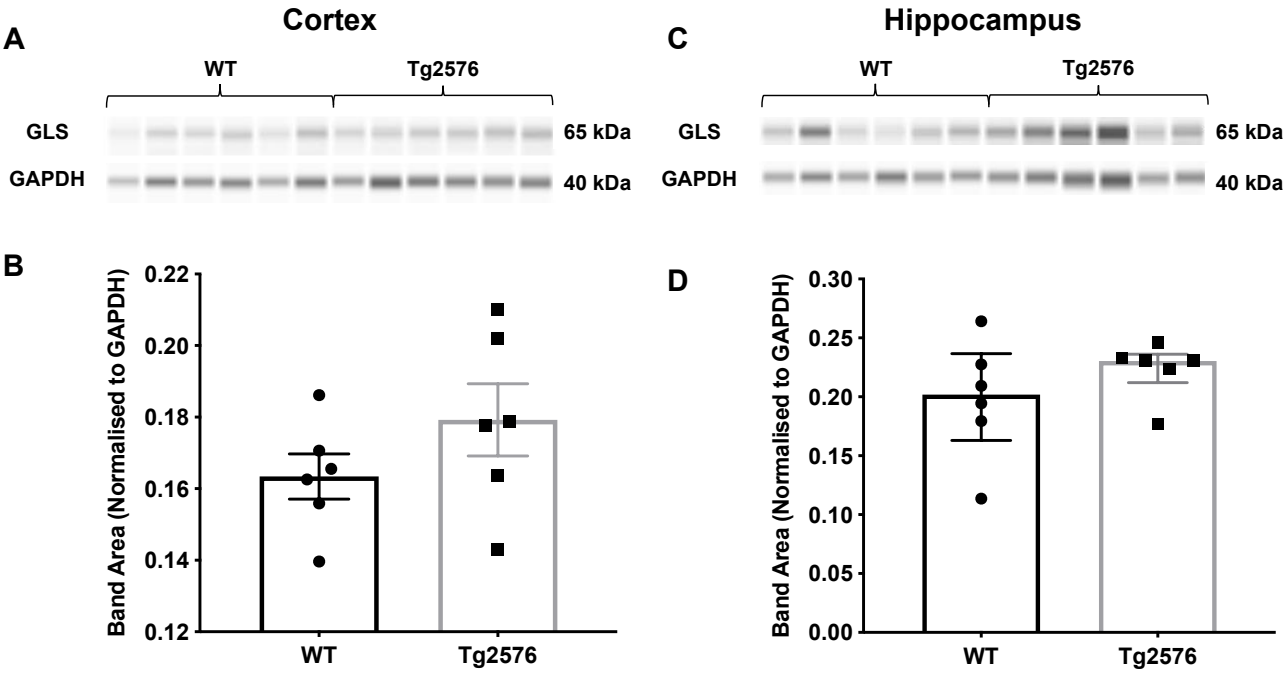

Figure 4.6. The level of GLS enzyme in the brain was not significantly altered in Tg2576 mice. **A)** Band intensities from the cortical samples did not indicate clear difference between Tg2576 mice and WT. **B)** Change in the expression level of GLS was not significant in the cortex of Tg2576 mice, compared to WT. **C)** GLS protein band intensities from the hippocampal samples of Tg2576 mice indicated a slight increase in the protein expression. **D)** The difference in the expression level of GLS in the hippocampus was not significant between Tg2576 and WT mice. Means ± SEM for **B)** and Median ± IQR for **D)**.

Finally, there was no significant difference in the expression levels of GFAP in Tg2576 mice, compared to WT. The GFAP bands seen in Figure 4.7A & B for the cortex ($p = 0.649$, unpaired t-test) and Figure 4.7C & D for the hippocampus ($p = 0.239$, unpaired t-test) did not reveal any significant difference between the groups.

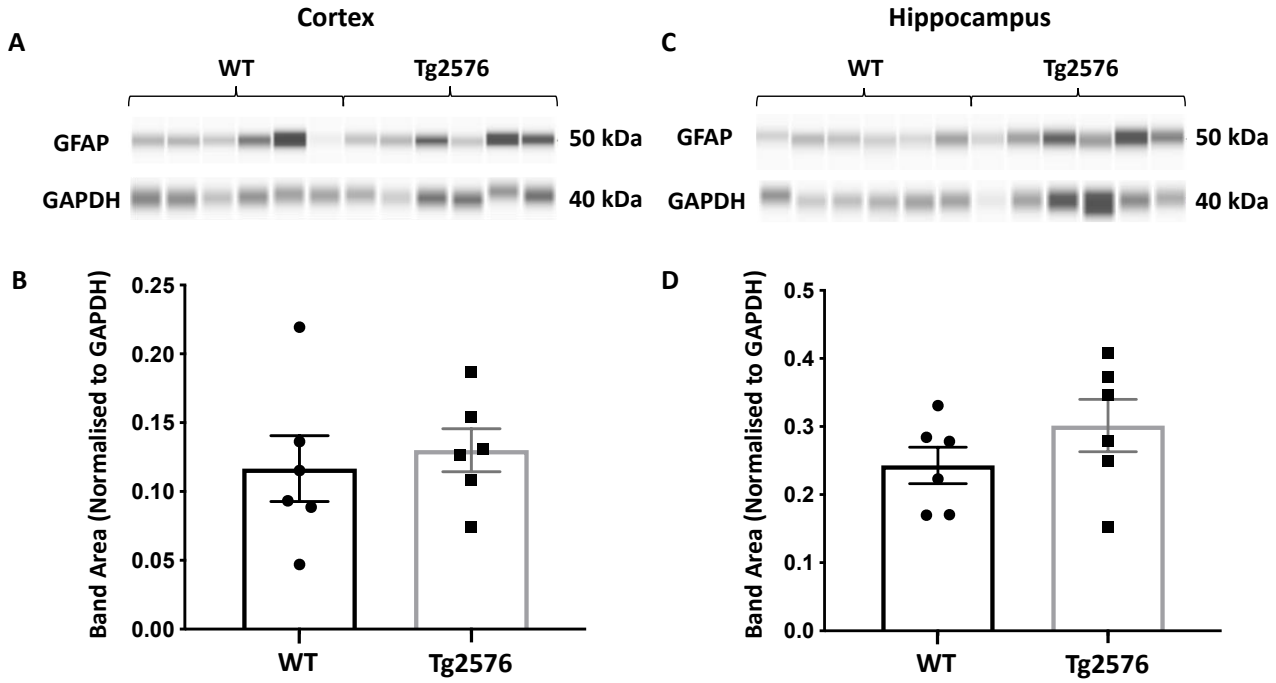

Figure 4.7. No significant difference in the expression levels of GFAP between Tg2576 mice and WT was observed. **A)** No clear difference in GFAP band intensities from the cortical samples of Tg2576 and WT mice was observed. **B)** There was no significant difference in the expression level of GFAP in the cortex of Tg2576 compared to WT mice. **C)** The band intensities of GFAP from the hippocampal samples of Tg2576 mice indicated a slight increase in GFAP expression. **D)** There was no significant difference between the expression level of GFAP level in the hippocampus of Tg2576 mice, compared to WT. Means ± SEM.

4.5.3) ***Expression levels of GLT-1 and GS genes were not altered in Tg2576 mice***

Since the expression levels of GLT-1 and GS proteins were significantly decreased in the cortex of Tg2576 mice compared to WT, I proposed that reductions in the expression levels of GLT-1 and GS genes may also be detected. It was found that there was no significant difference between the relative expression levels of GLT-1 gene ($p = 0.205$, unpaired t-test, Figure 4.8A) and GS gene ($p = 0.525$, unpaired t-test, Figure 4.8B) in the cortex of Tg2576 mice, compared to WT.

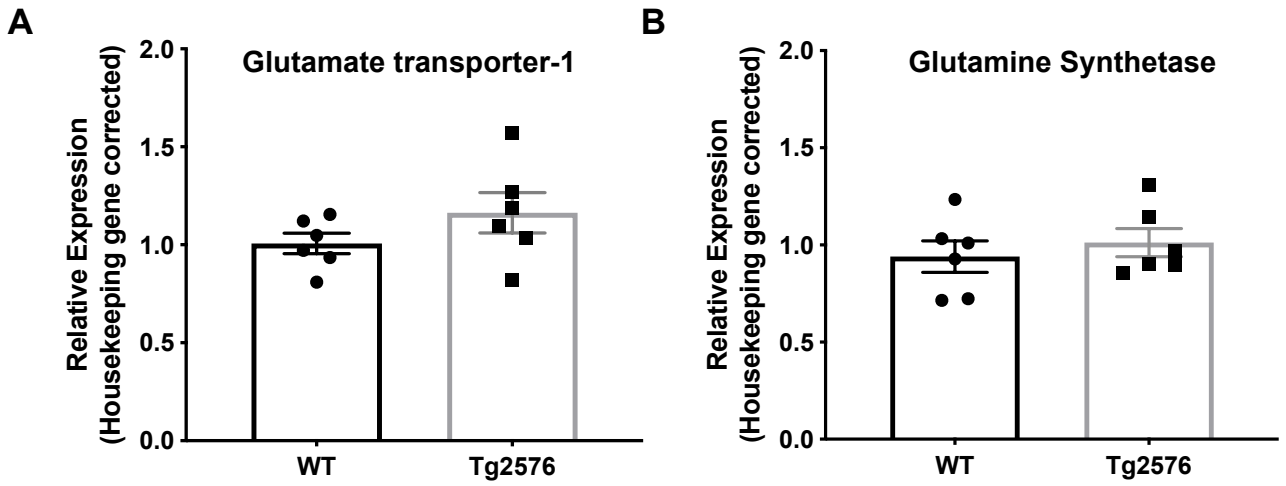

Figure 4.8. The gene expression levels of GLT-1 and GS in the cortex of Tg2576 mice were compared to WT. The relative gene expressions of **A)** GLT-1 and **B)** GS from the cortex of Tg2576 mice were not statistically different from WT mice. Means ± SEM.

4.5.4) ***Kindling altered the expression levels of key proteins in Glu-Gln cycle***

It was revealed that kindling-induced seizures significantly increased the expression level of GLT-1 protein in the cortex by 134% with $p<0.0001$, compared to sham mice (unpaired t-test, Figure 4.9A & B). In the hippocampus, kindling-induced seizures significantly increased the expression level of GLT-1 protein by 20% also, compared to sham ($p<0.01$, unpaired t-test, Figure 4.9C & D).

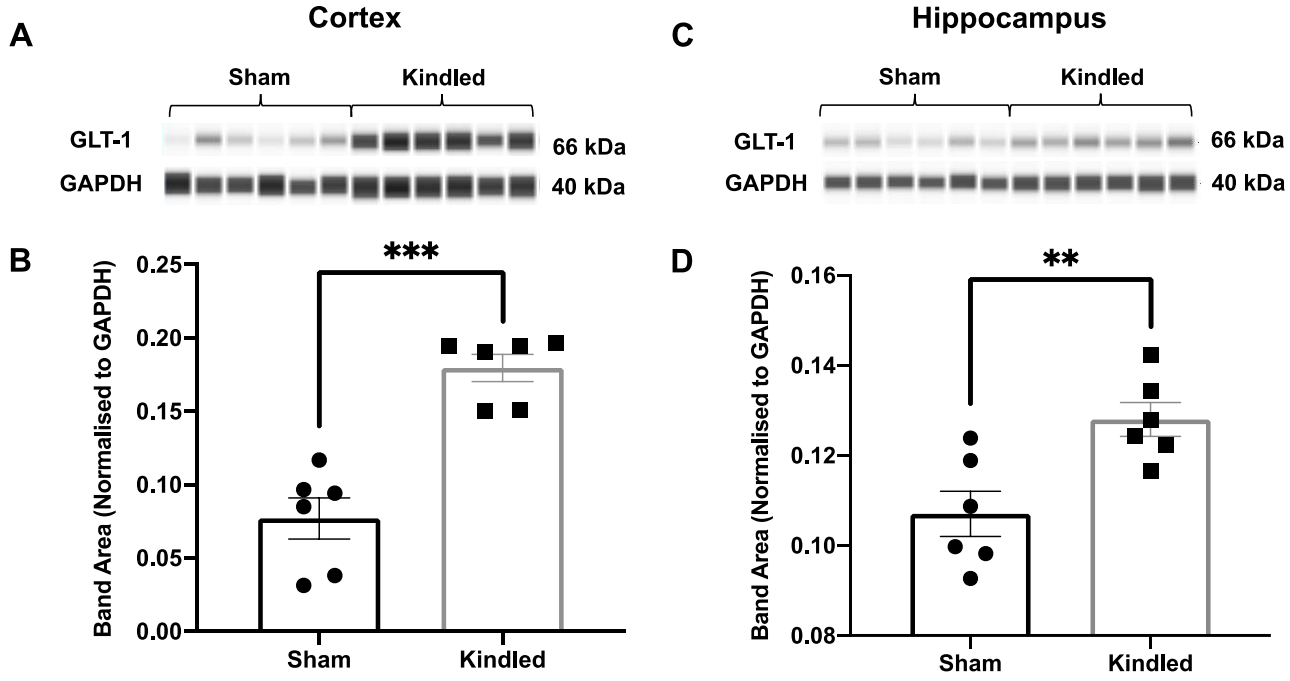

Figure 4.9. Kindling-induced seizures significantly increased the expression level of GLT-1 protein in the brain. **A)** Clear difference in the band intensities between the sham and the kindled mice was observed in the cortical samples. **B)** Significant increase in the expression level of GLT-1 protein was observed in the cortex of kindled mice. **C)** Difference in GLT-1 band intensities between kindled and sham WT mice was also observed in the hippocampal samples. **D)** The enhancement in GLT-1 protein expression level in the hippocampus of kindled mice was also significant. ***$p<0.001$, ** $p<0.01$, means ± SEM.

Kindling, however, did not significantly affect the expression levels of GS protein in both the cortex ($p = 0.832$, unpaired t-test, Figure 4.10A & B) and the hippocampus ($p = 0.626$, unpaired t-test, Figure 4.10B & D) of kindled mice compared to sham.

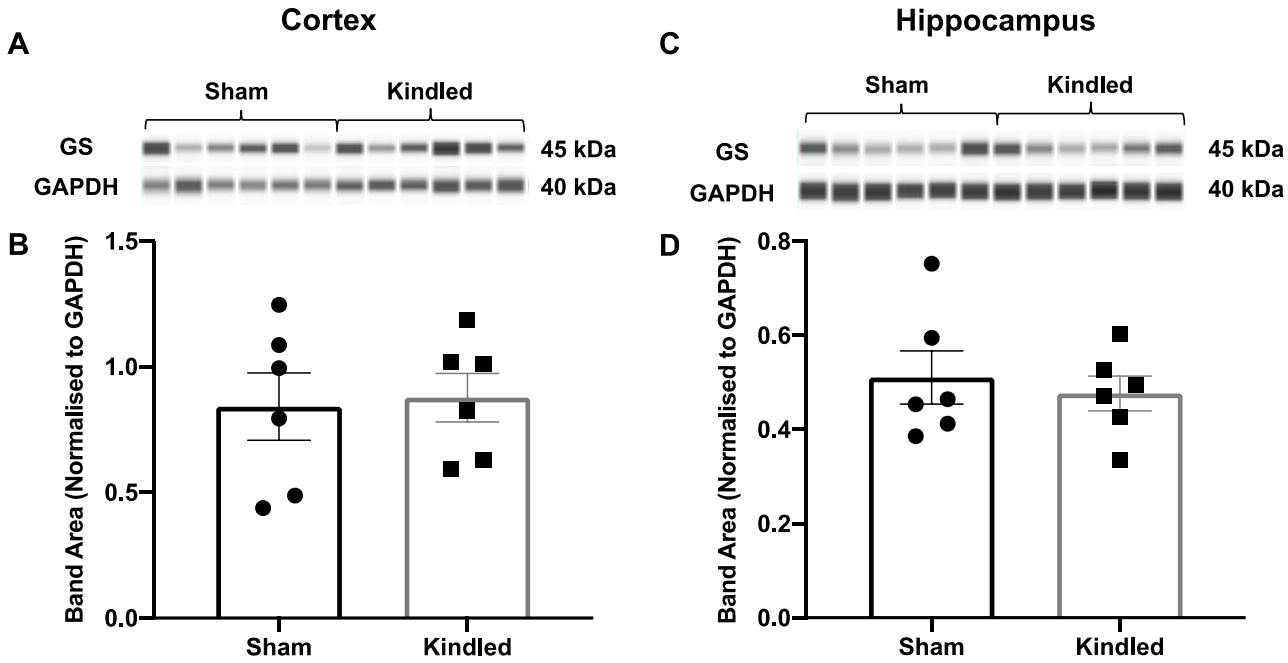

Figure 4.10. Kindling did not significantly affect the expression levels of GS protein in the brain. **A)** There was no difference in the band intensities of GS between kindled and sham WT mice. **B)** The expression level of GS protein in the cortex of kindled mice was not significantly different from sham. **C)** The band intensities of GS from the hippocampal samples also did not show a clear difference between the groups. **D)** The difference observed in the hippocampus was not statistically significant. Means ± SEM.

The expression levels of GLS protein were increased by 35% in both the cortex (Figure 4.11A) and the hippocampus (Figure 4.11C) of the kindled WT mice, compared to sham. The difference observed in the cortex was significant with $p<0.01$ ($p = 0.0044$, unpaired t-test, Figure 4.11B), but the difference in the hippocampus was only approaching the significance threshold with $p = 0.0767$ (unpaired t-test, Figure 4.11D).

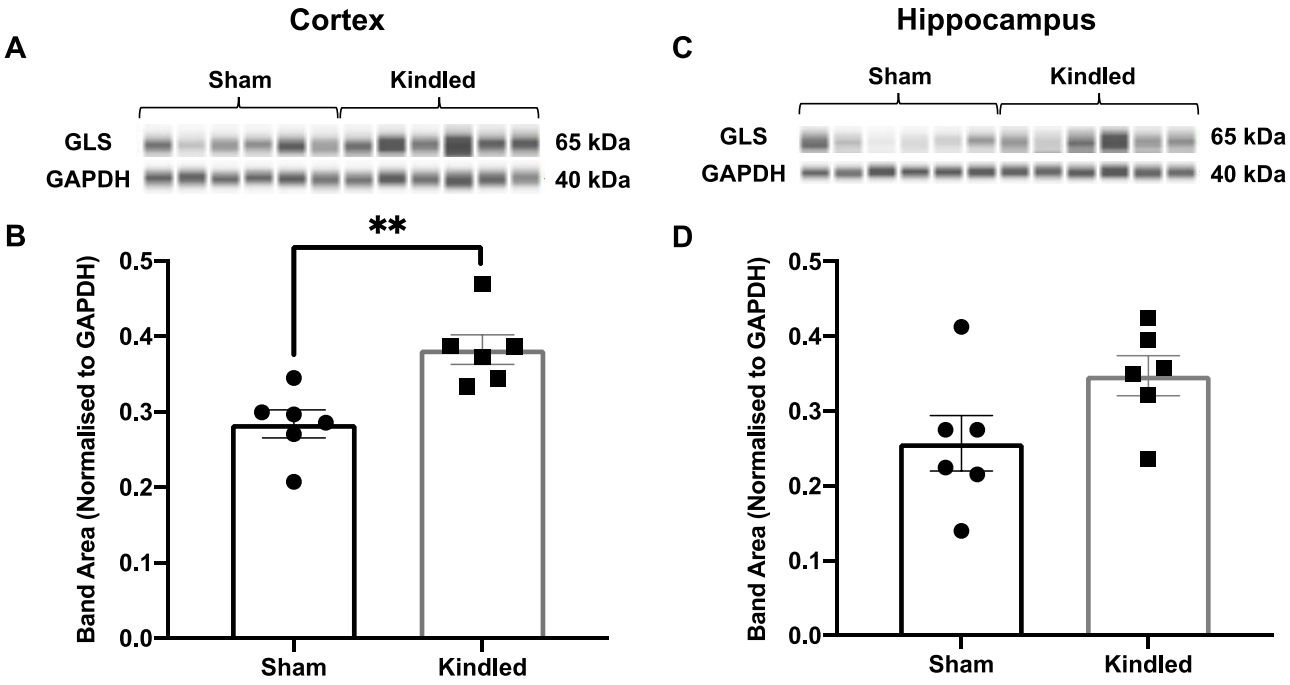

Figure 4.11. Kindling increased the expression level of GLS enzyme in the brain. **A)** The bands GLS proteins from kindled mice were more intense than from sham. **B)** The increase in the expression level of GLS in the cortex of kindled mice was significant, compared to sham. **C)** Band intensities of GLS proteins from the hippocampal samples also suggested that kindling increased the expression of GLS proteins. **D)** The difference in GLS expression levels in the hippocampus between kindled and sham mice was not significant. ** $p<0.01$, means ± SEM.

Finally, kindling numerically increased the expression level of GFAP by 11% in the cortex ($p = 0.602$, unpaired t-test) and 39% in the hippocampus ($p = 0.184$, unpaired t-test), compared to sham, as shown in Figure 4.12. However, these changes were not statistically significant.

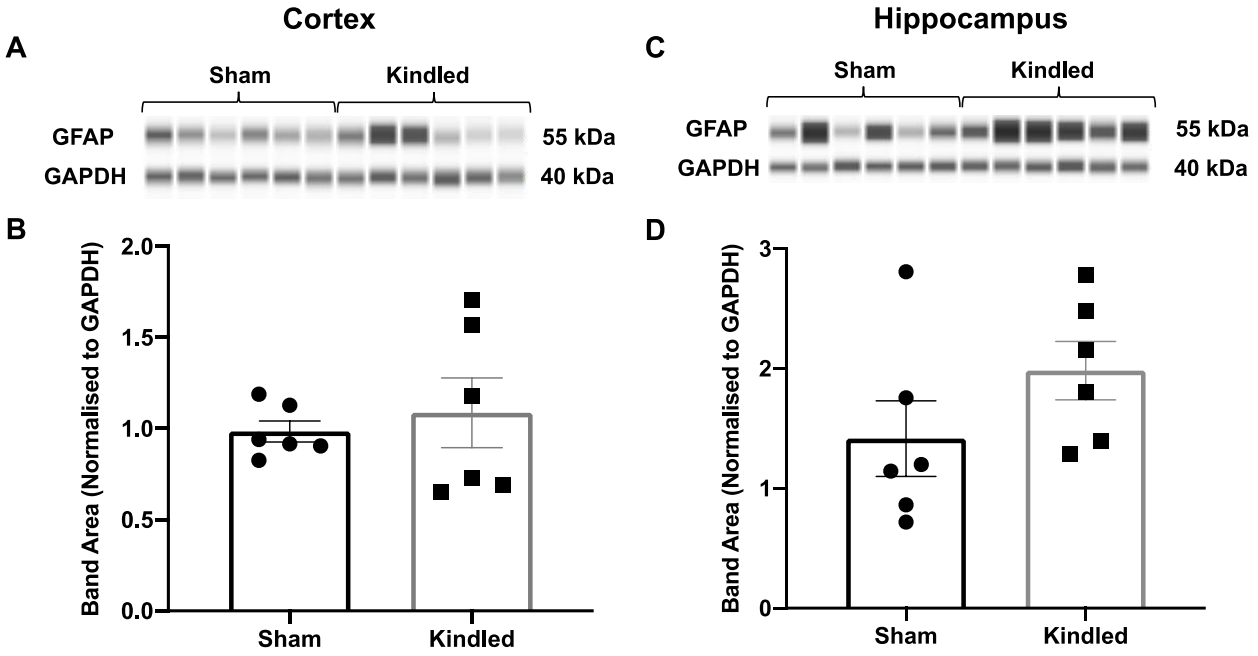

Figure 4.12. There was no difference in the level of GFAP between kindled and sham WT mice. **A)** No clear difference was observed from the band intensities of GFAP in the cortex. **B)** There was no significant change in GFAP levels between the groups in the cortex. **C)** The band intensities of GFAP from the hippocampus suggested that kindling increased the GFAP level in kindled mice. **D)** The difference in GFAP protein levels in the hippocampus between groups was not significant. Means ± SEM.

4.6) Discussion

To summarise the results from this chapter, Tg2576 mice were more susceptible to kindling-induced seizures than WT mice under the test conditions of this study, which agrees with the evidence from the literature.[37] This enhanced seizure susceptibility in Tg2576 mice might be associated with the reductions in the expression levels of key proteins in glutamate-glutamine cycle. It was revealed that 6-month-old Tg2576 mice displayed reduced baseline expression levels of GLT-1 and GS proteins in the brain, compared to WT littermates. However, these reductions only occur at protein level, not gene level. Furthermore, comparing the protein analysis results between kindled and sham WT mice revealed that kindling significantly enhanced the expression levels of GLT-1 and GLS proteins in the brain. The effects of kindling-induced seizures on the gene expressions of proteins in glutamate-glutamine cycle were not investigated in this study. The implications of each finding will be discussed in detail in this section.

4.6.1) ***Disruption in Glu-Gln cycle may lead to an increase in seizure susceptibility***

Tg2576 mice were found to be more prone to develop Class V seizures than WT littermates, which is consistent with the literature.[37] This piece of evidence may indicate that the overexpression of mutant human APP in Tg2576 mice is associated with one of the mechanisms that lead to an increase in seizure susceptibility. To elucidate this mechanism, the levels of key proteins that are associated with glutamate-glutamine cycle were compared between naïve 6-month-old Tg2576 mice and WT littermates.

The results from protein analyses of the cortical samples acquired from Tg2576 mice suggest that significant reductions in the baseline levels of GLT-1 and GS proteins are associated with mutant human APP overexpression pathology, as shown in Table 4.2. The level of GLT-1 protein in the cortex of Tg2576 mice was reduced by 65%, while the level of GS was reduced by 50%, compared to WT littermate. These results suggest that the disruption in glutamate-glutamine occurs during the early stages of mutant human APP overexpression pathology because Tg2576 mice in this experiment were 6 months old. The results also suggest that disruption to glutamate-glutamine cycle may occur before the emergence of Aβ plaque, since Aβ plaque cannot typically be detected in 6-month-old Tg2576 mice.[26] Furthermore, it was shown that the expression level of GLS protein in the brain was not significantly affected by the mutant human APP overexpression pathology under the experimental conditions of this study. This suggests that APP and its related products, such as the soluble $A\beta_{42}$ species, may affect the astrocytic components of the glutamate-glutamine cycle[38, 39] before the neuronal components. There is evidence from the

literature to demonstrate that $A\beta_{42}$ oligomers can cause the astrocytes to undergo structural, functional and morphological changes, which may explain the reductions in the expression levels of GLT-1 and GS proteins observed in Tg2576 mice.[11] As for GLS, the relationship between the expression level of GLS and AD pathologies is still poorly understood. One study demonstrated a significant increase in the expression level of GLS in the cerebral cortex of 9-month-old APP/PS1 mouse model of AD.[40] The results also indicated that this significant increase in the expression level of GLS occurred at protein level, not gene level.[40] Therefore, it is possible that the overproduction of $A\beta_{42}$ in the brain may alter the expression level of GLS. However, this alteration in GLS expression level may happen later than the reductions in the expression levels of GLT-1 and GS, as demonstrated in this chapter. In future studies, it will be important to investigate the role of GLS in the context of epilepsy in AD by using Tg2576 mice that are older than 6 months, as this enzyme has the potential to be a treatment target for epilepsy in AD due to its role in the glutamate-glutamine cycle.[11]

Protein of interest	Cortex	Hippocampus
GLT-1	↓↓ (***p=0.0093***)	n.s.
Glutamine synthetase	↓↓ (***p=0.0016***)	n.s.
Glutaminase	n.s.	n.s.
Glial fibrillary acidic protein	n.s.	n.s.

Table 4.2. Summary of the effects of mutant human APP overexpression pathology in Tg2576 mice on the levels of key proteins in glutamate-glutamine cycle. The cortical and hippocampal samples from 6-month-old Tg2576 mice were compared to the samples from WT littermates (unpaired t-test, ↓↓ = $P<0.01$, n.s. = not significant).

Reductions in the baseline levels of GLT-1 and GS proteins in the brain that were detected in this chapter suggest that disruption to glutamate-glutamine cycle might be the cause of the enhanced seizure susceptibility in Tg2576 mice. In Chapter 2, the potential mechanism that links the disruption to glutamate-glutamine cycle to an increase in seizure susceptibility was discussed in detail. Briefly, the decrease in the expression level of GLT-1 protein in the brain may translate into the decrease in glutamate uptake capability of the astrocytes.[14] This may result in an accumulation of extracellular glutamate, which then lead to an increase in the synaptic excitability.[9] Moreover, the reduction in the expression level of GS protein in the brain may also contribute to the accumulation of glutamate in the synaptic cleft.[41, 42] This is because the reduction in the expression level of GS protein may translate into the reduction in the rate of glutamate to glutamine conversion in the astrocytes. Thus, a build-up of glutamate in the astrocytes may occur, leading to an increase in the

pressure for the astrocytes to release glutamate into the synaptic cleft as gliotransmitter to maintain the intracellular glutamate balance.[43] Therefore, reductions in the baseline levels of GLT-1 and GS proteins in the brain may contribute to an increase in brain excitability in Tg2576 mice. Future studies may test this hypothesis by utilising a selective GLT-1 inhibitor, such as dihydrokainic acid[44, 45], in an experiment with a kindling model of epilepsy. For example, a group of WT mice can be treated with dihydrokainic acid prior to kindling. Then, the kindling susceptibility from this group can be compared to the results from WT mice that were treated with the vehicle. WT mice treated with dihydrokainic acid are hypothesised to be more susceptible to kindling than WT mice treated with saline.

Interestingly, significant reductions in GLT-1 and GS protein levels were only observed in the cortical samples, not the hippocampal samples of Tg2576 mice. This suggests that the effects of mutant human APP overexpression pathology might begin in the cortical area before spreading to other regions of the brain.[29] Despite that, a trend was observed that GS protein level in the hippocampus of Tg2576 mice was affected by the mutant human APP overexpression pathology in an opposite manner to the cortex. Hippocampal samples from Tg2576 mice contained higher levels of GS and GLS proteins, compared to the WT littermates. Although these changes were not statistically significant, they may suggest a compensatory mechanism in the hippocampus to counteract the reductions in GLT-1 and GS protein levels in the cortex. However, more evidence is needed to confirm this theory. On the other hand, changes in the levels of GLT-1 and GS proteins in the hippocampus may happen at a later timepoint, compared to the cortex; therefore, changes in the hippocampus were not observed in this study. Future studies should investigate the effect of age on the expression levels of key proteins in glutamate-glutamine cycle by utilising Tg2576 mice at different ages.

Finally, this study investigated changes in the GFAP levels in the brain of Tg2576 mice, compared to WT littermates. It was revealed that the expression level of GFAP in the brain of Tg2576 mice was not significantly different from WT littermates, which may suggest that there was no increase in astrogliosis in 6-month-old Tg2576 mice. This was expected due to the fact that the astrogliosis phenotype in Tg2576 model is generally detected after the animal reached 10-month-old.[46] Thus, the reductions in the baseline levels of GLT-1 and GS proteins in the cortex of Tg2576 may not be caused by an increase in astrogliosis, but rather the direct interaction between key proteins in glutamate-glutamine cycle and mutant human APP along with its downstream products, such as the soluble Aβ species.

Since the reductions in GLT-1 and GS protein levels in Tg2576 mice were significant, I also hypothesised that the expressions of the GLT-1 and GS genes would also be altered

in Tg2576 mice compared to the WT. However, no significant change in the levels of GLT-1 and GS gene expressions was detected in the cortical samples of Tg2576 mice, compared to WT. This suggests that the transcription processes for GLT-1 and GS genes were unaffected by the mutant human APP overexpression pathology in 6-month-old Tg2576 mice, which may indicate that the expressions of GLT-1 and GS proteins in the cortex of Tg2576 mice were disrupted during the translation process, not the transcription process. Therefore, this may imply that potential treatments for epilepsy in AD that target the transcription processes of GLT-1 and GS to counter the loss of these proteins may not be as effective as the treatments that target the translation processes. However, the translational processes that govern the expression of GLT-1 and GS proteins in the brain are not well-understood. On the other hand, the transcription processes that are involved in governing the expression level of GLT-1 have been elucidated. It was revealed that signalling pathways associated with the nuclear factor kappa B (NF-κB) transcriptional factor govern the expression of GLT-1 proteins.[47] Furthermore, dysregulation in NF-κB activities has been implicated in many diseases, including AD.[48, 49] Since, the pathways that are involved in the translational processes of GLT-1 have not been elucidated, targeting the transcription process is currently the only way to enhance GLT-1 expression in the brain.[50] One drug candidate for enhancing the transcriptional level of the GLT-1 protein in the brain is the β-lactam antibiotic called ceftriaxone.[51] Chapter 5 will discuss the potential of ceftriaxone in ameliorating the high seizure susceptibility phenotype in Tg2576 mice in more details. In the future, it will be important to determine which process governs the translation of GLT-1 and GS proteins and which process is more suitable to be the target for enhancing the expression levels of these proteins to decrease the seizure susceptibility in Tg2576 mice.

4.6.2) ***Kindling-induced seizures alter the expression levels of key proteins in Glu-Gln cycle***

Since it has been established that reductions in the expression levels of key proteins in glutamate-glutamine cycle, such as GLT-1[9] and GS[52], may lead to higher seizure susceptibility, it would also be important to elucidate the effects of seizures on the glutamate-glutamine cycle. This will generate insights on how levels of key proteins associated with glutamate-glutamine cycle are modulated during epileptogenesis. To this end, the expression levels of these key proteins were compared between kindled and sham WT mice. The summary for this investigation can be seen in Table 4.3.

Protein of interest	Cortex	Hippocampus
GLT-1	↑↑↑ ***(p<0.0001)***	↑↑ ***(p=0.0075)***
Glutamine synthetase	n.s.	n.s.
Glutaminase	↑↑ ***(p=0.0044)***	n.s.
Glial fibrillary acidic protein	n.s.	n.s.

Table 4.3. The summary of the effects of kindling on the expression levels of key proteins associated with glutamate-glutamine cycle. Results from kindled wild-type (WT) mice were compared to sham WT mice (unpaired t-test, ↑↑ = P<0.01, ↑↑↑ = p<0.001, n.s. = not significant).

The results revealed that kindling-induced seizures significantly increased the expressions of GLT-1 and GLS proteins in the brain. I hypothesised that the increase in GLT-1 expression observed in the brain of kindled WT mice might be one of the brain's mechanisms designed to increase the rate of glutamate uptake in the synaptic cleft. This would prevent the accumulation of extracellular glutamate and reduce the chance that overexcitation may occur. The *in vitro* and *in vivo* evidence from the literature also support this hypothesis.[51] Further, the evidence from animal studies also demonstrated that enhancing the GLT-1 protein expression level in the brain may grant neuroprotective effect against neuronal excitotoxicity.[53, 54]

However, the results from this chapter suggest that the increase in GLT-1 expression level caused by kindling-induced seizures did not provide sufficient neuroprotective effect to lower the overall seizure susceptibility of Tg2576 mice. It was shown that seizures were still triggered by kindling despite this marked increase in GLT-1 protein in the brain, and the longer the animal remained in the experiment the more severe the seizure became. This may suggest that the increase in GLT-1 expression in the brain may facilitate an increase in seizure susceptibility through an unknown mechanism. One potential mechanism might involve the disruption of the inhibitory neuron functions due to an increase in glutamate

uptake rate, which may lead to an imbalance in the excitation/inhibition of the brain and subsequently higher seizure susceptibility.[55, 56] Despite this, it has been experimentally shown that enhancing the GLT-1 expression level with ceftriaxone improved the outcome of pentylenetetrazole-induced seizures in rodent models.[57, 58] Since the evidence from the literature suggests that enhancing GLT-1 expression in the brain may provide neuroprotective effects and reduce seizure susceptibility, it is possible that the enhanced GLT-1 protein level observed in this chapter was a part of the compensatory mechanism designed to counter an increase in glutamate accumulation caused by kindling.[59] Therefore, kindling-induced seizures may enhance the seizure susceptibility of the animal via other mechanism that might not involve the alteration of GLT-1 expression.

On the other hand, the increase in GLS protein level observed in the cortex of kindled WT mice was hypothesised to be caused by the direct and/or the indirect effects of kindling as outlined in Figure 4.13. In the direct effect hypothesis, the presynaptic glutamate pools in the brain of kindled mice were exhausted as the result of induced seizures; therefore, these pools would require replenishment. This may suggest that the brain increased the level of GLS expression in order to increase the presynaptic glutamate production rate, as higher GLS expression level can be translated into higher rate of glutamine to glutamate conversion.[13] As for the indirect effect hypothesis, the enhanced level of GLS protein was consequential to the enhancement in the rate of glutamate uptake as the result of enhanced GLT-1 expression.[60] Thus, it is possible that more glutamate would need to be released from the presynapse to compensate for the increase in glutamate uptake rate in the synaptic cleft. With this said, it is important to consider other compensatory mechanisms that may counteract the increase in glutamate uptake rate in the synaptic cleft. One example is the mechanism which involves enhancements in the expression levels or the affinities of glutamatergic receptors, such as the NMDAR, to increase the sensitivity of the post-synaptic neuron to excitatory signal.[61] However, the dysregulation of compensatory mechanism might lead to brain hyperexcitability, which then contribute to the pathogenesis of AD[62, 63] and epilepsy.[8, 64] In future studies, it will be important to investigate the effects of the enhancement of GLS expression on seizure susceptibility, as this may reveal important insights on the potential of GLS as a treatment target for epilepsy in AD.

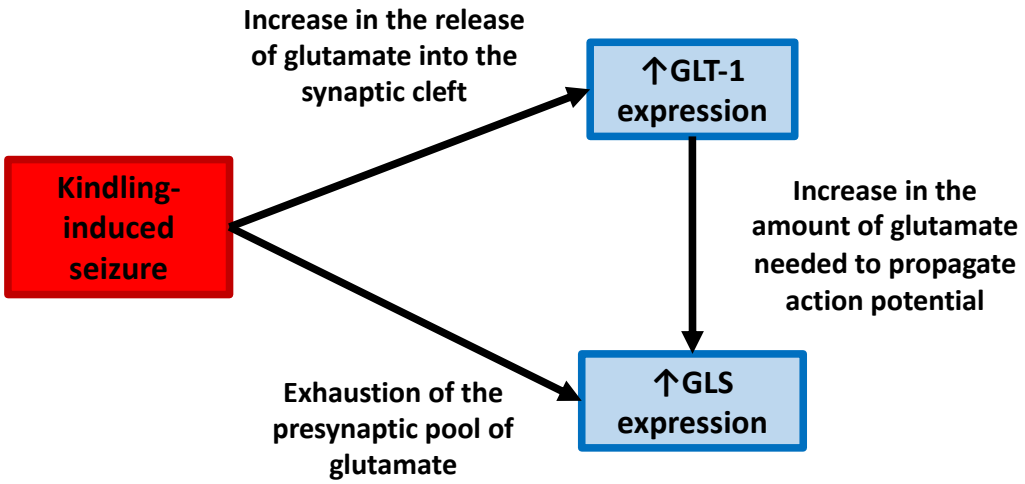

Figure 4.13. Hypothesised pathways on how kindling-induced seizures result in GLT-1 and GLS proteins expression levels enhancements in the brain of WT mice.

Despite causing a significant increase in the expression level of GLS protein in the cortex, kindling did not produce the same effect in the hippocampus. Thus, the increase in GLS expression level that was observed in the cortical samples may be caused by a specific mechanism in the brain that affects the cortical region of the brain before the hippocampal region. However, the results suggest a strong trend that kindling may also increase the GLS expression level in the hippocampal region of the brain. Therefore, the sample size should be increased in future experiments to investigate whether the expression level of GLS protein in the hippocampus can be significantly increased by kindling-induced seizures.

On the other hand, kindling-induced seizure did not affect the expression level of GS enzyme in the brain. This might be because the astrocytes prevented the accumulation of intracellular glutamate through other pathways that did not involve the modulation of GS protein level, such as releasing glutamate as gliotransmitter.[65] Therefore, the pressure to convert glutamate into glutamine might not be increased since the astrocytes could still balance the intracellular glutamate level efficiently.

Finally, kindling-induced seizures did not significantly change the level of GFAP in the cortex and the hippocampus of WT mice, which may suggest that there was no increase in the level of astrogliosis in these regions of the brain under the test conditions of this study. There is evidence from the literature to demonstrate that kindling-induced seizures may cause an increase in the level of GFAP in the brain at the stimulation site.[66-68] This suggests that changes in the level of GFAP in the brain might be the most pronounced in the amygdala region, not the hippocampal or the cortical regions. Thus, it will be important to investigate changes in the level of GFAP and other key proteins in the amygdala region in future studies to generate more insights on the relationship between epilepsy and the glutamate-glutamine

cycle. Furthermore, other astrogliosis markers, such as S100β[69], should be utilised to generate more data on the relationship between kindling-induced seizure and astrogliosis.

The results from this study suggest that protein expressions in the hippocampus were less affected by amygdala kindling than in the cortex. Changes in the GLT-1 and GLS protein levels between kindled WT mice and sham were more remarkable in the cortical samples than in the hippocampal samples. Therefore, amygdala kindling-induced seizures might affect different regions of the brain with a varying degree of severity, which is consistent with the literature.[70, 71] It has also been shown that the piriform cortex is one of the first region to be affected by amygdala kindling-induced seizures[72], while the hippocampus may not initially be affected.[73] Additionally, the difference in the number of astrocytes between the cortical and the hippocampal regions[74] might also play a role in determining the degree that the astrocytic proteins associated with glutamate-glutamine cycle, such as GLT-1 and GS, are affected by amygdala kindling-induced seizures.

4.6.3) ***Discrepancy in the ages of mice in each experiment***

In this chapter, there were discrepancies in the ages of mice used in each experiment. This was because each experiment had a different hypothesis regarding the ages of mice. For the seizure susceptibility investigation, aged (13-14-month-old) mice were used. This experiment was performed to confirm that my cohort of Tg2576 mice were more susceptible to kindling-induced seizure than the WT.[37] Aged mice were chosen based on a previous study from the literature which demonstrated that aged Tg2576 mice are more susceptible to kindling-induced seizure than WT.[37]

As discussed in Chapter 2, reductions in the expression levels of GLT-1 and GS proteins can be associated with Aβ pathology [11, 75], and they may lead to an increase in the brain's excitability.[9, 76] However, there is no evidence from the literature to elucidate how early in the Aβ pathology do these reductions first become detectable. Therefore, I chose 6-month-old Tg2576 mice and WT littermates for my experiment, as this age represents an early stage of Aβ pathology.[26] From the results of this experiment, I hypothesised that 6-month-old Tg2576 mice would exhibit increased in kindling susceptibility, compared to WT littermate and this hypothesis will be tested in future study.

After revealing that 6-month-old Tg2576 mice display reductions in the baseline levels of GLT-1 and GS proteins in their brains, I performed another experiment to investigate how kindling-induced seizure affects the levels of these key proteins. For this experiment, I chose aged WT mice, as this age is representative of the population that is the most prone to

developing AD.[23] The differences in ages of the mice in each experiment did not affect the main outcomes interpretation.

4.6.4) ***Disparity in the data within each group***

Some of the western blot results displayed disparity between each sample from the same group. For example, some of the cortical samples from WT mice displayed high GLT-1 protein signals, while some displayed low signals (Figure 4.4). The disparity might have arisen during sample preparation as the density of protein bands may be affected by variable sample degradation due to improper handling of the brain tissues.[77] For instance, the amount of time used during the brain extraction might vary from one animal to another; thus, the protein degradation in each sample may also vary. Additionally, the time it took to homogenise the samples might also contribute to this difference in protein signal. The difference between the amount of detergent (5% SDS) in each sample might also contribute to this disparity as the protein of interest might not be fully denatured; therefore, the signal from the protein of interest may be different in each sample. Finally, pipetting error may also play a part during the experiment, causing the protein concentration of each sample to be different, which might affect the signal strength of the proteins of interest from each sample.

It is also possible that the disparity in the results observed between samples in the same group might be due to gender effect, as mixed-gender mice were used in this chapter. The expression levels of key proteins in the glutamate-glutamine cycle in the brain may be associated with the concentration of glutamate. Evidence from human studies suggests that females display higher concentration of glutamate in the hippocampal region of the brain than age-matched males[78], but not in regions such as frontal white matter, frontal grey matter, parietal grey matter and basal ganglia in healthy subjects.[79] To add, GLT-1 and GS proteins are almost exclusively expressed in astrocytes; therefore, the gender effect on the number of astrocytes in the brain may directly affect the signal strength of these proteins on western blot. There is evidence from an animal study to suggest that there are more astrocytes in the dentate gyrus and the CA1 areas of the hippocampus from female mice, compared to age-matched male mice.[80] Another study which investigated the gender effect on the number of astrocytes in the medial amygdala acquired from rats reported no significant difference in the number of astrocytes between male and age-matched female.[81] These results imply that the gender effect on the number of astrocytes in the brain might be region-specific. However, these studies only used ≤10 animals per group in their studies and should benefit from increasing their samples sizes in the future. Thus, the current evidence in the literature is not convincing enough to confirm that there is a significant gender effect on the number of astrocytes which may affect the western blot results for

proteins such as GLT-1 and GS. Although significant results were still observed in this chapter, the gender effect will be taken into consideration in future studies to further reduce the disparity in the data.

To summarise the discussion, the overexpression of mutant human APP pathology in 6-month-old Tg2576 mice is associated with reductions in the expression levels of key proteins in glutamate-glutamine cycle, such as GLT-1 and GS. The reductions in the expression levels of these key proteins may aggravate AD pathology and increase the brain's susceptibility to seizure. The levels of key proteins such as GLT-1 and GLS were also significantly altered in kindled WT mice, compared to sham. This implies that the glutamate-glutamine cycle may play an important role in the pathogenesis of AD and epilepsy in Tg2576 mice.

4.7) Future Directions

It would be logical to investigate treatments for epilepsy in AD that target the glutamate-glutamine cycle, as the results from this study highlight the importance of this cycle in the pathogenesis of both AD and epilepsy. Investigating the effect of a drug that can increase the expression level of GLT-1 protein in the brain on the symptoms of epilepsy and AD will generate the data required to develop treatments for epilepsy in AD.[51] This will also provide insights on the importance of GLT-1 expression level in the mechanism that causes Tg2576 mice to be more prone to seizure than WT. Chapter 5 of this thesis will investigate this topic further.

To add, measuring the level of extracellular glutamate in the brain in real time using methods such as microdialysis would reveal how much extracellular glutamate accumulation there is in Tg2576 mice. This would also identify if there is a compensatory mechanism that can counteract the reduction in the expression level of GLT-1 protein in the brain, which will provide important data to be utilised towards drug discovery.

Changes in the expression level of GLS in the brain caused by kindling was also implicated in this chapter. Despite this, there is not much literature to elucidate the role of GLS in the pathogenesis of epilepsy and AD. Therefore, investigating the role of GLS in the pathogenesis of epilepsy in AD will be important, as GLS has the potential to be a pharmacological target that can reduce the amount of glutamate in the presynapse; thus, reducing the chance of glutamate accumulation in the synaptic cleft.

Lastly, more data from animal studies are needed to fully elucidate the mechanism that links AD pathologies to an increase in the risk of developing epilepsy. The data acquired from animal studies will help guide the design of future human studies in this field. Since

animal studies do not produce the same challenges as human studies in terms of sample acquisition, they can provide vital data. It will also be important to investigate the role of glutamate-glutamine cycle in human AD patients in the future. Additionally, animal models of AD cannot fully replicate the whole spectrum of human AD pathologies; therefore, treatments or mechanisms that were discovered from animal studies might not be fully translatable to human patients. However, without the data generated from animal studies, the discovery of novel treatments and mechanisms elucidation may be impeded. Future directions will be discussed further in Chapter 6.

4.8) Conclusion

Alterations in the levels of key proteins in the glutamate-glutamine cycle are linked to the mutant human APP overexpression pathology in Tg2576 mice and the kindling model of epilepsy. The evidence from this chapter suggests that the reduction in the expression level of GLT-1 protein occurred during the early stages of Aβ pathology and the evidence from the literature suggests that enhancing the expression level of GLT-1 protein in the brain may improve cognition in mouse model of AD.[82, 83] Thus, the reduction in GLT-1 expression level in AD may play a role in the mechanism that mediates the acceleration of AD symptoms development.

Since Tg2576 mice are more susceptible to kindling-induced seizure than WT, I theorised that lower baseline level of GLT-1 protein in Tg2576 mice is one of the factors that enhanced their seizure susceptibility. This was based on the evidence from the literature that the loss of GLT-1 protein in the brain may lead to fatal seizure.[9] However, the evidence from this chapter indicates that kindling-induced seizures are associated with a remarkable increase in the expression level of GLT-1 protein in the brain. This suggests that the increase in GLT-1 expression due to repeated brain stimulation may contribute to the increase in seizure susceptibility through an unknown mechanism, since the mice became more susceptible to seizure after each stimulation.[84] On the other hand, the increase in GLT-1 expression level detected might be a part of a compensatory mechanism that was designed to counter the increase in the release of glutamate in the brain. Future studies should investigate the effect of GLT-1 knockdown and GLT-1 upregulation on seizure susceptibility of the Tg2576 mice to determine whether GLT-1 enhancement contributes to or reduces the seizure susceptibility. Lastly, the effects of kindling-induced seizure on the expression levels of key proteins in glutamate-glutamine cycle in Tg2576 mice and the effects of GLT-1 expression level enhancement on the seizure susceptibility phenotype of Tg2576 mice will be investigated in Chapter 5.

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Chapter 5 – Glutamate transporter-1 as a pharmacological target for reducing seizure susceptibility in mouse model of Alzheimer's disease

Preamble to Chapter 5

As a continuation from Chapter 4, this chapter investigated the potential of GLT-1 gene expression enhancer called ceftriaxone in reducing the seizure susceptibility phenotype of Tg2576 mouse model of Alzheimer's disease (AD). This will provide valuable insights on how crucial the GLT-1 protein is in the pathogenesis of epilepsy in AD. Importantly, I would like to acknowledge the assistance that I received in the preparation of this chapter. During late 2020, I had to visit my home country because of compelling family reasons and was unable to return to Australia due to the COVID-19 travel restrictions. Thus, fellow PhD students Runxuan Lin and Anna Harutyunyan performed the experiments to gather raw data from 5 animals in my stead (contribution to Chapter 5: Hattapark Dejakaisaya: 95%, Runxuan Lin: 2.5%, Anna Harutyunyan 2.5%).

5.1) Abstract

Background: Individuals with Alzheimer's disease (AD) have up to 10-fold higher chance of developing seizures compared to healthy aged-matched control. The mechanism that links AD pathologies to an increase in seizure susceptibility is poorly understood. Alterations to the expression levels of key proteins in glutamate-glutamine cycle, such as glutamate transporter-1 (GLT-1), has been implicated in both AD and epilepsy. I hypothesised that pharmacologically increasing the GLT-1 protein expression level in the brain with ceftriaxone will ameliorate the high seizure susceptibility phenotype in mouse model of AD to a similar level as the WT controls.

Methods: In the pilot study, 16 naïve Tg2576 mice were put into 2 groups of 8, where one group was treated with GLT-1 enhancer (200mg/kg ceftriaxone) while another was treated with vehicle (saline) for 7 consecutive days. In the main experiment, 5 Tg2576 mice were treated with a daily dose of 200mg/kg ceftriaxone (Tg-Ceft), 8 Tg2576 mice were treated with saline (Tg-Sal) and 6 WT mice were treated with saline (WT-Sal). All mice used in this chapter were aged (12 to 14 months old) and mixed-gender. Kindling was performed on all mice in the main experiment. Western blot was used to measure changes in the levels of key proteins in glutamate-glutamine cycle in the cortical and hippocampal samples collected from each animal.

Results: Ceftriaxone treatment significantly increased the expression level of GLT-1 protein in the hippocampus of Tg2576 mice *($p = 0.0177$)*, which validated the use of ceftriaxone in this model. Compared to saline, ceftriaxone did not significantly ameliorate the high seizure susceptibility phenotype of Tg2576 mice, as it was revealed that seizure classes from Tg-Ceft group were not significantly different from Tg-Sal group throughout the experiment ($p>0.99$). However, there was also no significant difference in seizure classes between Tg-Ceft and WT-Sal groups at any time during the experiment ($p>0.99$). On the other hand, the Tg-Sal group displayed significantly higher seizure classes than the WT-Sal group during the first 3 kindling sessions ($p = 0.02$, 0.02 and 0.01 respectively). This suggests that ceftriaxone treatment may reduce the seizure susceptibility of Tg2576 to the level between Tg2576 and WT mice. Protein analysis results suggest that when Tg2576 mice were kindled, ceftriaxone did not manage to alter the expression of key proteins in the glutamate-glutamine cycle.

Conclusion: Ceftriaxone is effective at enhancing the expression of GLT-1 in the brain of the Tg2576 mouse model of AD. However, ceftriaxone did not ameliorate the high seizure susceptibility phenotype in Tg2576 mice under the test conditions of this study. Despite that, the results suggest a trend that ceftriaxone treatment may have an anti-seizure effect but not anti-epileptogenic effect on Tg2576 mice.

5.2) Background

AD may increase the risk of seizures in patients up to 10-fold, compared to healthy age-matched controls.[1-4] Despite that, the mechanism that links AD pathologies to an increase in the risk of developing seizures, and consequently epilepsy, has not been elucidated.

Reductions in the expression levels of key proteins in glutamate-glutamine cycle in the brain, such as GLT-1, have been implicated in Alzheimer's disease.[5-9] GLT-1 is an important protein that is mainly expressed on the astrocytes[10, 11] and its main function is to uptake glutamate from the synaptic cleft after it was released from the pre-synaptic stores.[12-14] The decrease in the expression level of GLT-1 protein in the brain may translate into an increased risk of extracellular glutamate accumulation and aberrant glutamate homeostasis, as discussed in Chapter 2. It is also evident that disruptions to the expression levels of key proteins in glutamate-glutamine cycle play a role in cognitive impairment in AD[15, 16] and these disruptions are associated with the Aβ pathology.[17-19] Moreover, one of the existing AD treatments, memantine, acts specifically on the glutamatergic pathway to alleviate the symptoms of the disease.[20] This highlights the importance of the glutamatergic pathway in the pathogenesis of AD and the potential of GLT-1 as a pharmacological target for the treatment of AD.

The involvement of glutamate-glutamine cycle, especially the GLT-1 protein, has also been implicated in the pathogenesis of epilepsy.[12] Since the main function of GLT-1 is to uptake glutamate from the synaptic cleft and terminate the excitatory signal propagation[21], reduced GLT-1 expression in the brain may lead to higher risk of extracellular glutamate accumulation and synaptic hyperexcitability.[22] It has been shown that loss of GLT-1 in the brain may lead to fatal seizure phenotype in mice.[12] Therefore, disruption to the glutamate-glutamine cycle is one of few pathways that have been implicated in the pathogenesis of both AD and epilepsy. Hence, it might be the one of the links between AD pathologies and higher risk of developing seizures and possibly epilepsy.

Since the results from Chapter 4 suggested that alteration to the expression level of GLT-1 protein is associated with the pathogenesis of both AD and epilepsy, targeting GLT-1 protein pharmacologically may provide beneficial effects toward reducing seizure susceptibility under AD condition. Enhancing the expression level of GLT-1 protein in the brain is hypothesised to ameliorate the high seizure susceptibility phenotype associated with lower baseline level of GLT-1 expression caused by AD pathologies (See Chapter 2). One of the potential candidates for enhancing the expression level of GLT-1 protein in the brain is the third-generation cephalosporin antibiotic called ceftriaxone.[23] It has been shown in the

literature that treating mouse models of AD with a daily dose of 200mg/kg of ceftriaxone for 7 consecutive days significantly increased the level of GLT-1 expression in the hippocampus[24, 25] via transcription enhancement associated with the nuclear factor κB (NF-κB) pathway.[26] Experimental evidence also confirmed that ceftriaxone is highly soluble in saline[27, 28] and that it increased the GLT-1 expression level in the brain when administered via intraperitoneal (IP) injection.[25] This implies that ceftriaxone possesses the ability to cross the blood-brain barrier (BBB) effectively.[29, 30] Compared to other cephalosporin antibiotics, ceftriaxone has a particularly long biological half-life at 8 hours, making this drug more clinically relevant, as the doses can be given less often.[31] Evidence from animal studies also suggests that 200mg/kg dose of ceftriaxone improved cognitive functions in rodent model of AD.[24, 25, 32] However, the effect of ceftriaxone on kindling-induced seizure susceptibility in rodent AD model is not yet investigated, hence it will be investigated in this chapter.

Here, the amygdala kindling model of epilepsy was utilised to allow the investigator to precisely control the timing and the site of seizure onset, which can be advantageous for the study of the effect of a drug on the seizure susceptibility.[33] As for the rodent model of AD, there is evidence from the literature to demonstrate that Tg2576 mouse model of AD is more susceptible to kindling-induced seizure, compared to their WT littermates.[34] This was investigated and confirmed in Chapter 4; thus, Tg2576 mice were utilised in this chapter. The Tg2576 model displays mutant human APP overexpression pathology of AD, and it was confirmed in Chapter 4 that there was a decrease in the baseline level of GLT-1 expression in the brain of Tg2576 mice. Therefore, investigating the effect of ceftriaxone on kindled Tg2576 mice will provide important insights on how GLT-1 expression enhancement may potentially ameliorate the high seizure susceptibility phenotype associated with the mutant human APP overexpression pathology.

5.3) Aim & Hypotheses

I aimed to investigate the effect of ceftriaxone treatment on the high seizure susceptibility phenotype of the Tg2576 model. I hypothesised that Tg2576 mice treated with ceftriaxone will be significantly more resistant to kindling-induced seizures, compared to Tg2576 mice treated with saline. Additionally, Tg2576 mice treated with ceftriaxone will not be significantly more susceptible to kindling-induced seizure, compared to WT littermates treated with saline.

5.4) Methods

5.4.1) ***Animals***

Aged (12 to 14 months old) mixed-gender Tg2576 mice and c57 x SJL (WT) littermates were acquired from the colonies at Alfred Research Alliance, Prahran, Melbourne, Australia. Studies were performed in the Department of Neuroscience (Alfred Hospital), Monash University. All experiments were conducted with the approval from the Alfred Research Alliance Animal Ethics Committee.

5.4.2) ***Pilot study***

Initially, a pilot study was performed on a cohort of naïve Tg2576 mice to investigate if ceftriaxone (Cayman Chemical, Ann Arbor, MI, USA) can increase the expression level of GLT-1 protein in the brain. A total of 16 aged mixed-gender Tg2576 mice were used for this experiment. All mice were housed based on their gender in a standard cage with food and water available *ad-libitum*. The mice were put into 2 groups of 8, with each group consisting of 4 males and 4 females. The treatment group was administered intraperitoneally (IP) daily with a dose of 200mg/kg of ceftriaxone dissolved in saline, while the control group was administered with saline. Each group was treated for 7 consecutive days. For the treatment group, 20mg/ml of ceftriaxone in saline was freshly prepared each day. To achieve the daily dose of 200mg/kg, each animal was weighed and 0.1ml/10g of 20mg/ml ceftriaxone was administered according to the weight of the animal. For the control group, 0.1ml/10g of saline was administered instead of ceftriaxone. At the end of the last day of treatment, each mouse was euthanised and the brain samples were collected for protein analysis.

5.4.3) ***Experimental design***

For the main experiment, 13 aged mixed-gender Tg2576 mice and 6 WT littermates were used. There were 10 female and 9 male mice that met their endpoints in this experiment. The experiment was performed according to the timeline shown in Figure 5.1. Briefly, the animals were randomly assigned into 3 groups, which were: 1) WT mice treated with saline (WT-Sal, 2 males and 4 females); 2) Tg2576 mice treated with saline (Tg-Sal, 3 males and 5 females) and 3) Tg2576 mice treated with ceftriaxone (Tg-Ceft, 4 males and 1 female). Saline and ceftriaxone treatment doses in the main experiment were identical to the pilot study. Briefly, 200mg/kg of ceftriaxone and 0.1ml/10g of saline doses were used. The investigators were blinded to the genotypes, treatments and groups of the animal. The mice were treated according to their group for 7 consecutive days after recovering from electrode implantation surgery, then they would undergo kindling while still being treated daily according to their groups to maintain the effects of the treatment.

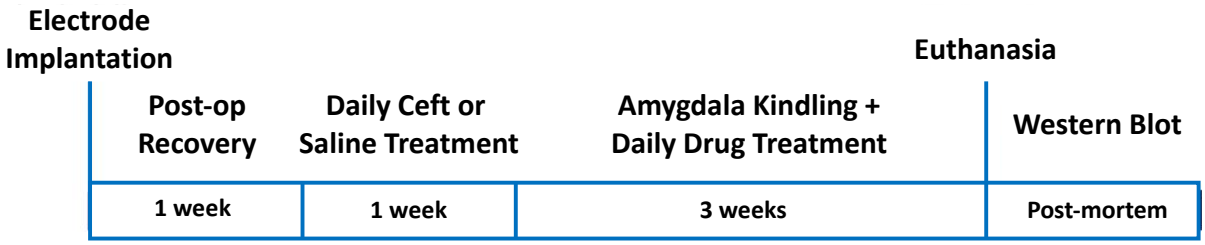

Figure 5.1. Timeline for the experiment designed to investigate the effect of ceftriaxone treatment on the high seizure susceptibility phenotype of Tg2576 mice.

5.4.4) ***Kindling procedure***

Electrode implantation surgery was performed on all mice in the main experiment. Briefly, a bipolar depth electrode (Science Products, Hofheim, Germany) was implanted into the left amygdala (anteroposterior [AP]: -0.8; mediolateral [ML]: 3.1 relative to bregma; dorsoventral [DV]: -5.0 relative to dura), 3 extradural electrodes were implanted along with 2 anchor screws at the locations shown in Figure 5.2. Detailed methods for electrode implantation surgery was described in Chapter 4.

For this experiment, each mouse was considered to be fully kindled once it had 5 Class V seizures or 15 total induced seizures, whichever occurred first. The primary outcome measured the class of seizure displayed by the animal in response to each stimulus, while the secondary outcome measured the number of stimulations needed for each animal to develop its first Class V seizure according to the Racine scale.[35] Once fully kindled, each mouse was euthanised at the end of the day and the brain samples were collected for protein analysis.

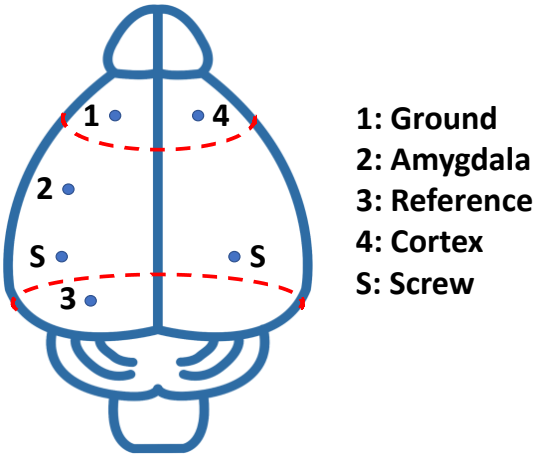

Figure 5.2. Electrodes were implanted at the locations shown to enable amygdala kindling and electroencephalogram recording of the brain.

5.4.5) ***Protein analysis***

All mice were euthanised via lethal injection with pentobarbitone (80mg/kg) (Provet, VIC, Australia) and their brains were extracted according to the protocol described in Chapter 4. Briefly, the cortical and the hippocampal samples were extracted from the animal, then they were frozen at -80°C until protein analysis. Western blot was performed to measure changes in the levels of key proteins in glutamate-glutamine cycle as described in Chapter 4. In short, this chapter used Wes (Protein Simple, San Jose, CA, USA), which is a robot-assisted western blot, to measure the levels of GLT-1 (cat#: sc-365634, conc. = 1:80, Santa Cruz, Tullamarine, VIC, Australia), GS (cat#: ab64613, mouse, conc. = 1:320, Abcam, Melbourne, VIC, Australia), GLS (cat#: ab93434, rabbit, conc. = 1:10, Abcam) and GFAP (cat#: Z0334, rabbit, conc. = 1:20, Agilent Technologies, Melbourne, VIC, Australia) proteins, with GAPDH (cat#: 2118, rabbit, conc. = 1:200, Cell Signalling, Karrinyup, WA, Australia) as the housekeeping protein.

5.4.6) ***Data analysis***

Statistical significance was calculated using Prism 8 software (GraphPad, La Jolla, CA, U.S.A.). Initially, the normality of each set of data was determined by the Shapiro-Wilk test of normality**.** Normally distributed data were presented as mean ± standard error of the mean (SEM), while nonparametric data were presented as median ± interquartile range (IQR). For the kindling experiment, ANOVA test was used to determine the statistical significance of the normally distributed data sets, then Tukey's multiple comparison test was performed as the post-hoc test to identify the significance of the difference between each group. If the data were not normally distributed, then ANOVA with Kruskal-Wallis nonparametric test would be used, followed by Dunn's test for multiple comparison and the adjusted p-values would be reported. In this chapter, all the western blot results were normally distributed, while all the results from the kindling experiment were nonparametric. The significance threshold for all analyses was set to $p<0.05$. The western blot results from the kindling experiment in this chapter were presented as percentage compared to control.

5.5) Results

5.5.1) ***Ceftriaxone enhanced GLT-1 expression in the brain of Tg2576 mice***

First, the effect of 200mg/kg of ceftriaxone treatment on the expression level of GLT-1 protein in the brain of Tg2576 mice was investigated. Ceftriaxone treatment did not significantly increase the expression level of GLT-1 protein in the cortex of Tg2576 mice ($p = 0.3357$, unpaired t-test, Figure 5.3A & B) compared to saline treatment. However, ceftriaxone significantly increased the expression level of GLT-1 protein in the hippocampus of Tg2576 mice by 30% ($p = 0.0177$, unpaired t-test, Figure 5.3C & D).

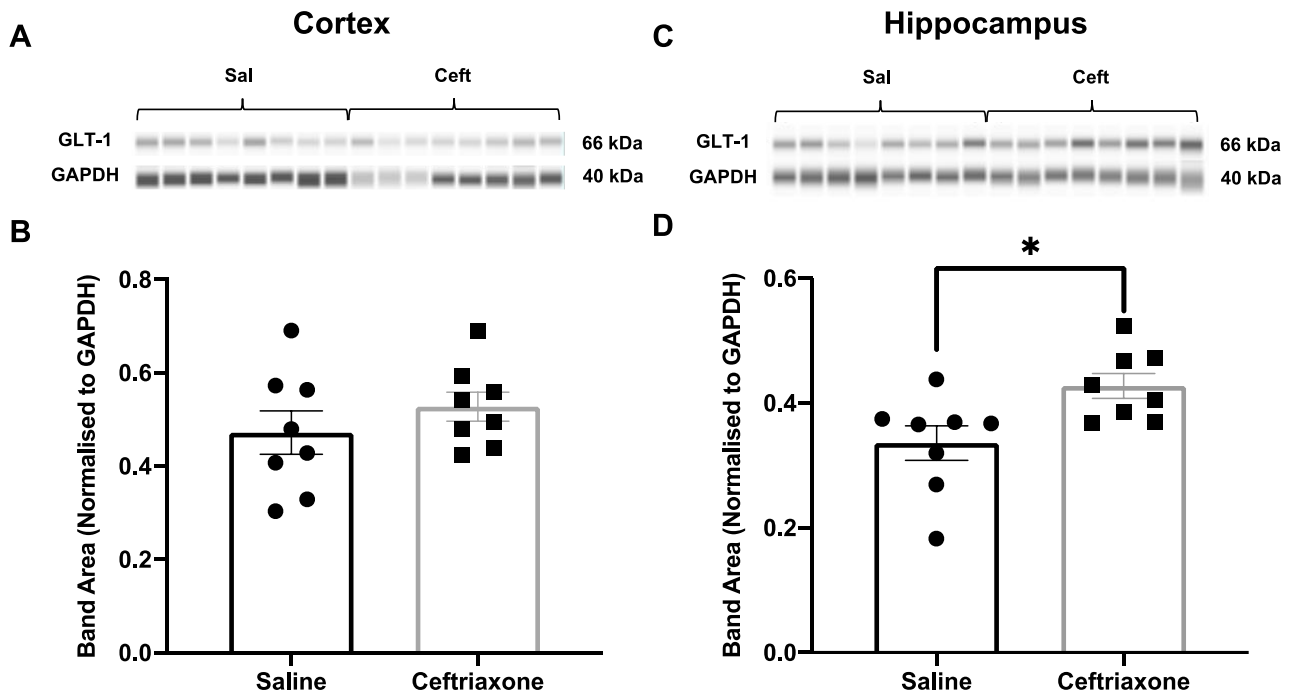

Figure 5.3. Ceftriaxone significantly increased the expression level of GLT-1 protein in the hippocampus, not the cortex of Tg2576 mice, compared to the saline treatment. **A)** The band areas of GLT-1 protein (66 kDa) from the cortex did not indicate a clear change linked to the ceftriaxone treatment. **B)** Slight increase in the GLT-1 protein expression level in the ceftriaxone group was not significant. **C)** In the hippocampus, the normalised intensities of GLT-1 protein bands did not display a clear difference between the groups. **D)** However, ceftriaxone treatment did significantly enhance the GLT-1 protein expression level in the hippocampus. *$p<0.05$, data = means ± SEM.

5.5.2) ***Ceftriaxone enhanced GLS but not GS and GFAP in Tg2576***

Other than its impact on the expression level of GLT-1 protein, the impact of ceftriaxone on the expression levels of GS, GLS and GFAP were also investigated. It was evident in Figure 5.4A & B that ceftriaxone significantly increased the expression level of GLS protein in the cortex by 12% ($p = 0.0105$, unpaired t-test). This was the first time that a significant change in GLS expression level in the brain has been linked to the effect of ceftriaxone treatment. However, the same effect of ceftriaxone was not observed in the hippocampal samples ($p = 0.762$, unpaired t-test, Figure 5.4C & D).

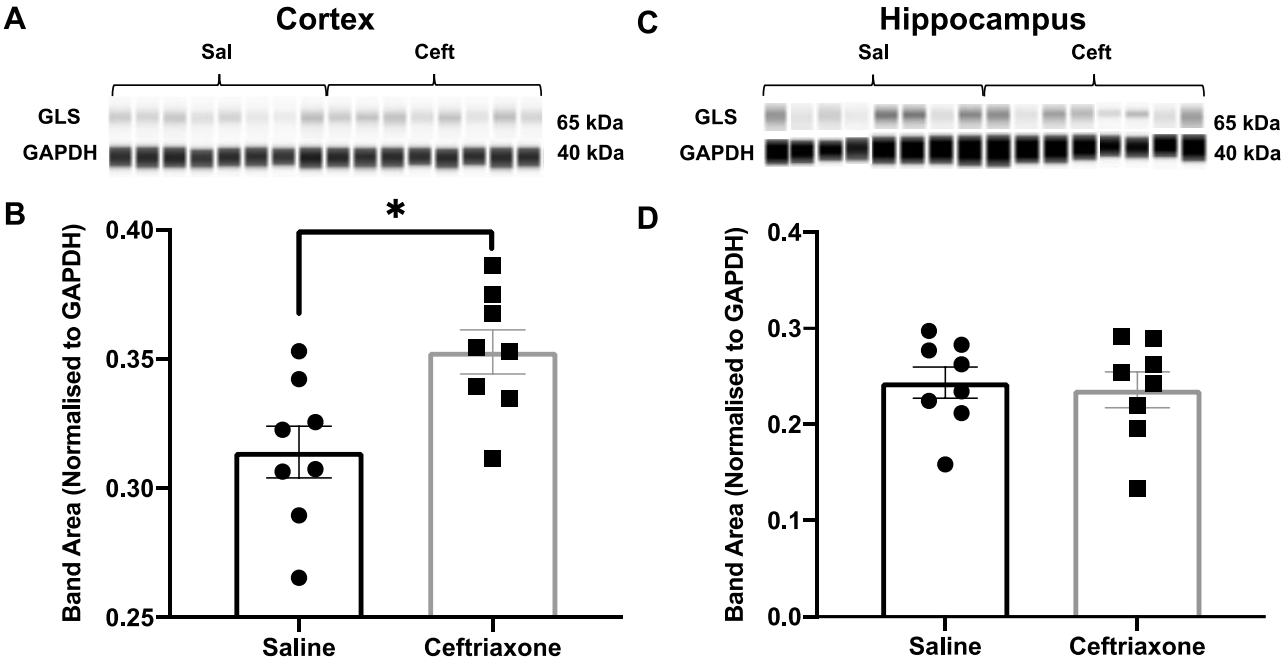

Figure 5.4. Ceftriaxone significantly increased the expression level of GLS protein in the cortex, not in the hippocampus of Tg2576 mice. **A)** The band areas of GLS protein (65 kDa) from the cortex were normalised to their corresponding band areas of GAPDH protein (40 kDa). **B)** Ceftriaxone treatment significantly increased the GLS protein expression in the cortex of Tg2576 mice. **C)** No clear difference in the band areas of GLS protein was observed in the hippocampal samples between the groups. **D)** Ceftriaxone treatment did not significantly alter the level of GLS protein in the hippocampus. *$p<0.05$, data = means ± SEM.

Ceftriaxone also did not alter the level of GS protein expression in the brain of Tg2576 mice, as shown in Figure 5.5. No significant change in the level of GS proteins was observed in the cortex ($p = 0.736$, unpaired t-test, Figure 5.5A & B) and in the hippocampus ($p = 0.419$, unpaired t-test, Figure 5.5C & D) of Tg2576 mice treated with ceftriaxone, compared to saline treatment.

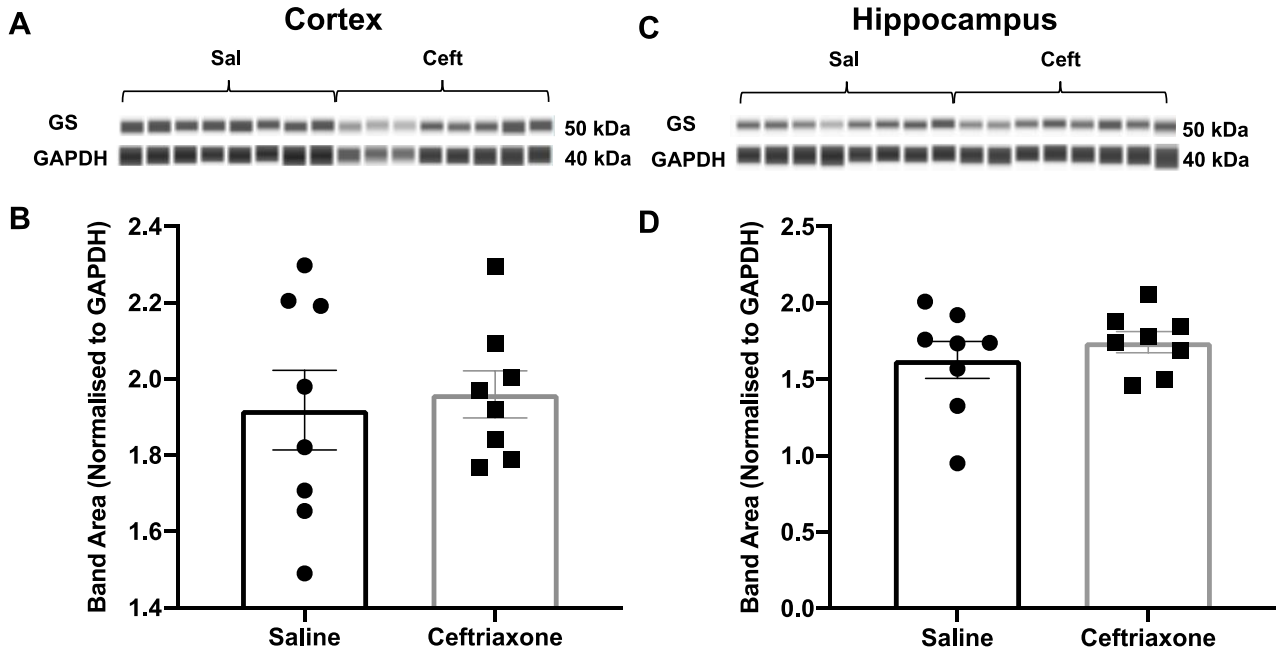

Figure 5.5. Ceftriaxone treatment did not increase the level of GS protein in the brain of Tg2576 mice, compared to saline treatment. **A)** The band intensities of GS protein (50 kDa) from cortex did not show a clear difference between the groups and **B)** this change did not reach the significance threshold. **C)** No change in the GS level was observed in the hippocampal samples. **D)** There was no significant difference in the level of GS protein in the hippocampal samples between the groups. Data = means ± SEM.

As for the GFAP expression level, it was revealed that ceftriaxone treatment decreased the level of GFAP in the cortex by 11% (Figure 5.6A & B). On the contrary, ceftriaxone slightly increased the level of GFAP in the hippocampus by 17% (Figure 5.6C & D). Despite that, changes in the level of GFAP in the cortex ($p = 0.188$, unpaired t-test) and the hippocampus ($p = 0.405$, unpaired t-test) caused by ceftriaxone were not statistically significant under the test conditions.

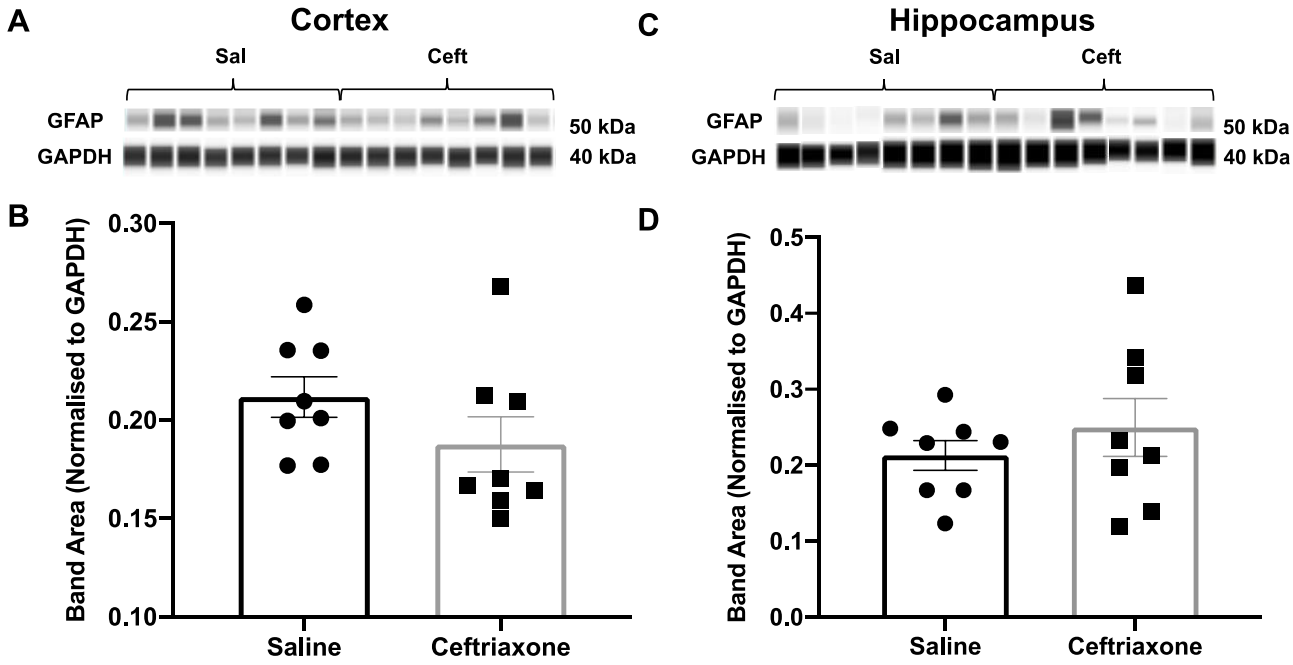

Figure 5.6. Ceftriaxone did not significantly alter the level of GFAP in brains of Tg2576 mice, compared to saline treatment. **A)** The band intensities did not show a clear difference between groups in the cortex. **B)** Ceftriaxone treatment did not significantly decrease the level of GFAP in the cortex. **C)** There was no clear difference in band intensities between the groups in the hippocampus. **D)** No significant difference in GFAP level was identified in the hippocampus. Data = means ± SEM.

5.5.3) ***Ceftriaxone did not significantly improve kindling outcomes in Tg2576 mice***

It was evidenced in Figure 5.7A that there was no significant difference in seizure severities when the results from Tg-Ceft group was compared to Tg-Sal and WT-Sal groups (adjusted-p>0.05, Kruskal-Wallis with Dunn's post-hoc). It was also revealed in Figure 5.7A that seizure classes from Tg-Sal group were significantly higher than the WT-Sal group during the first 3 kindling sessions (adjusted-p = 0.0212, 0.0152 and 0.0143 respectively, Kruskal-Wallis with Dunn's post-hoc). There was no significant difference between Tg-Sal and WT-Sal groups from the third session onwards (adjusted-p>0.05, Kruskal-Wallis with Dunn's post-hoc, Figure 5.7A).

Interestingly, it was observed that during the first stimulation session both Tg-Ceft and WT-Sal groups had an identical median seizure class of Class IV (adjusted-p>0.99, Kruskal-Wallis with Dunn's post-hoc, Figure 5.7B), which was lower than the Tg-Sal group (Class V). The difference between Tg-Sal and WT-Sal groups was significant (adjusted-p = 0.0212, Kruskal-Wallis with Dunn's post-hoc, Figure 5.7B), but the difference between Tg-Ceft and Tg-Sal groups was not statistically significant (adjusted-p = 0.206, Kruskal-Wallis with Dunn's post-hoc, Figure 5.7B).

The number of stimulations needed for each animal from each group to develop its first Class V seizure was also investigated, as shown in Figure 5.7C. Tg-Sal mice were significantly more susceptible to kindling than WT-Sal mice (median = 1 stimulation vs 4 stimulations, p=0.0393, Kruskal-Wallis with Dunn's post-hoc). Tg-Ceft mice also required a median of 1 stimulation to develop their first Class V seizures, which was not statistically different from both Tg-Sal (adjusted-p>0.99, Kruskal-Wallis with Dunn's post-hoc) and WT-Sal (adjusted-p = 0.179, Kruskal-Wallis with Dunn's post-hoc) groups as shown in Figure 5.7C.

The results from this experiment suggest that a daily dose of 200mg/kg of ceftriaxone did not significantly ameliorate the high kindling susceptibility phenotype of Tg2576 mice under the test conditions of this study. The numbers of animals from each group during each stage of kindling were summarised as shown in Table 5.1. One limitation of note was after the fifth session, the number of animals in the Tg-Ceft group was too small to be analysed statistically. Therefore, the data after the fifth session was not included in the statistical analyses of this experiment.

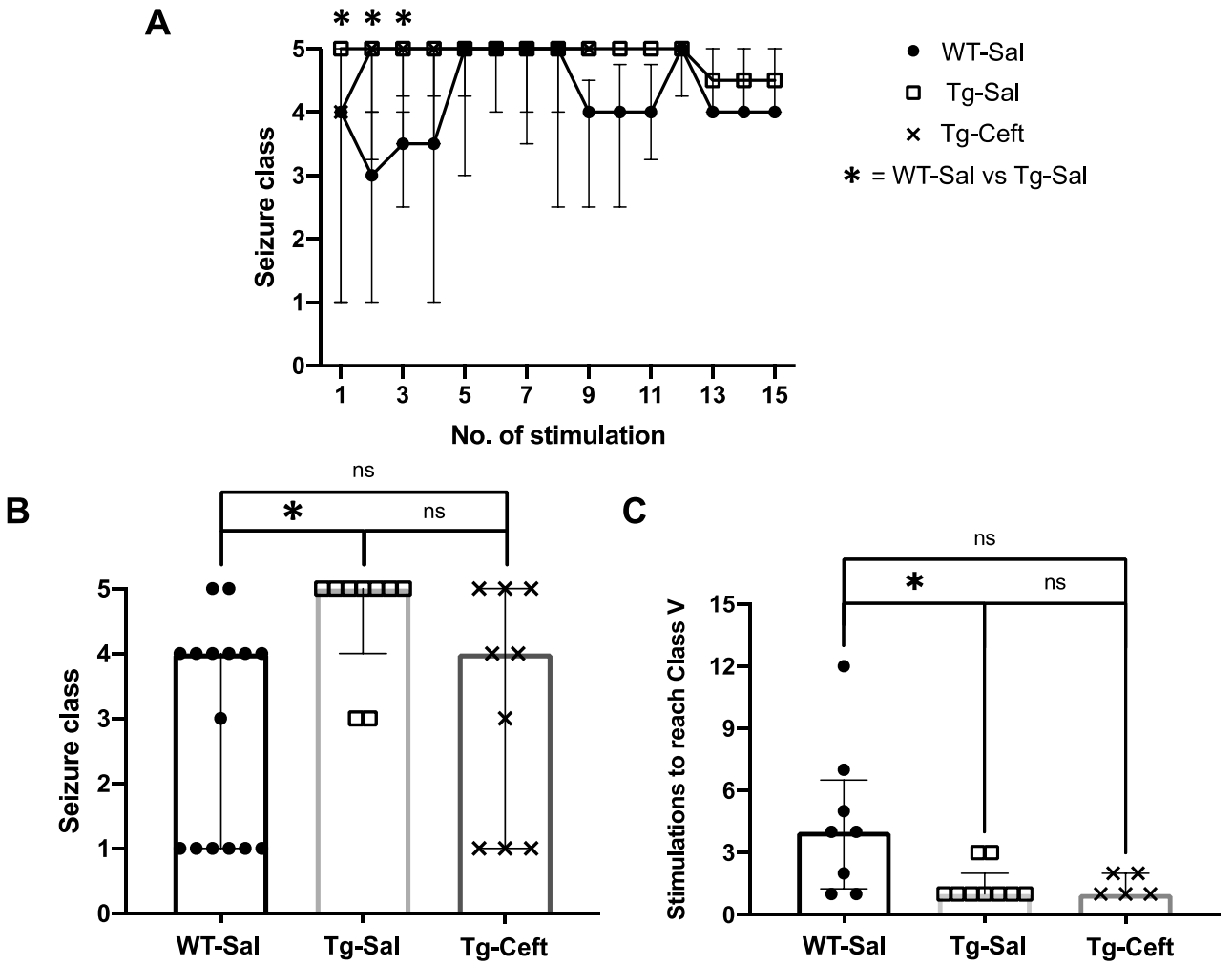

Figure 5.7. The effect of ceftriaxone treatment on the high seizure susceptibility phenotype of Tg2576 mice (Tg-Ceft), compared to saline treatment (Tg-Sal) and WT littermates (WT-Sal). **A)** Seizure evolution chart to compare the median seizure classes from each group during each stage of the amygdala-kindling experiment. **B)** Median seizure classes from each group during the first kindling session were compared. **C)** The differences between the kindling susceptibilities of each group were compared. *p<0.05 (WT-Sal vs Tg-Sal), data = medians ± IQR, ANOVA with Kruskal-Wallis test and Dunn's test for multiple comparison. Data gathered after the fifth kindling session onwards was not included in the descriptive analyses due to the small sample size in Tg-Ceft group (<4 animals).

Stim No.	WT-Sal	Tg-Sal	Tg-Ceft
1	15	9	9
2	11	9	6
3	10	8	5
4	10	8	4
5	7	8	4
6	6	7	2
7	6	4	1
8	5	3	1
9	5	3	1
10	4	2	0
11	4	2	0
12	4	2	0
13	1	2	0
14	1	2	0
15	1	2	0

Table 5.1. Number of mice from WT-Sal, Tg-Sal and Tg-Ceft groups after each session of amygdala kindling-induced seizure.

5.5.4) ***Ceftriaxone did not alter the levels GLT-1, GS, GLS and GFAP in kindled mice***

The expression levels of GLT-1 and GLS proteins were expected to be increased in the Tg-Ceft group due to the effect of ceftriaxone treatment. The results shown in Figure 5.8 demonstrated that there were no significant changes in the levels of any key proteins in the cortical samples between all groups. The samples were collected from 6, 8 and 5 mice from WT-Sal, Tg-Sal and Tg-Ceft groups respectively.

Compared to the WT-Sal group, the mean level of GLT-1 protein expression from the Tg-Sal group was reduced by 7%, as shown in Figure 5.8A ($p = 0.787$, ANOVA with Tukey's post-hoc). Also, the mean level of GLT-1 protein expression in the Tg-Sal group was 9% lower than the Tg-Ceft group ($p = 0.703$, ANOVA with Tukey's post-hoc, Figure 5.8A). Finally, there was no difference in GLT-1 expression level between Tg-Ceft and WT-Sal groups ($p = 1$, ANOVA with Tukey's post-hoc).

With the expression level of GS protein in the cortex, there was no notable difference between WT-Sal and Tg-Sal groups ($p = 1$, ANOVA with Tukey's post-hoc, Figure 5.8B). However, the mean level of GS expression in Tg-Ceft group was 20% ($p = 0.191$, ANOVA with Tukey's post-hoc) and 19% ($p = 0.177$, ANOVA with Tukey's post-hoc) higher than WT-Sal and Tg-Sal respectively, as shown in Figure 5.8B.

The expression levels of GLS from Tg-Sal and Tg-Ceft groups were 13% ($p = 0.262$, ANOVA with Tukey's post-hoc) and 16% ($p = 0.211$, ANOVA with Tukey's post-hoc) higher than WT-Sal, respectively, as shown in Figure 5.8C. There was no significant difference in the GLS expression levels between Tg-Sal and Tg-Ceft groups ($p = 1$, ANOVA with Tukey's post-hoc, Figure 5.8C).

Lastly, the mean GFAP expression levels in Tg-Sal and Tg-Ceft groups were 15% ($p = 0.850$, ANOVA with Tukey's post-hoc) and 25% ($p = 0.691$, ANOVA with Tukey's post-hoc) higher than the WT-Sal group, respectively, as shown in Figure 5.8D. There was also no significant difference in the GFAP expression levels between Tg-Sal and Tg-Ceft groups ($p = 1$, ANOVA with Tukey's post-hoc, Figure 5.8D).

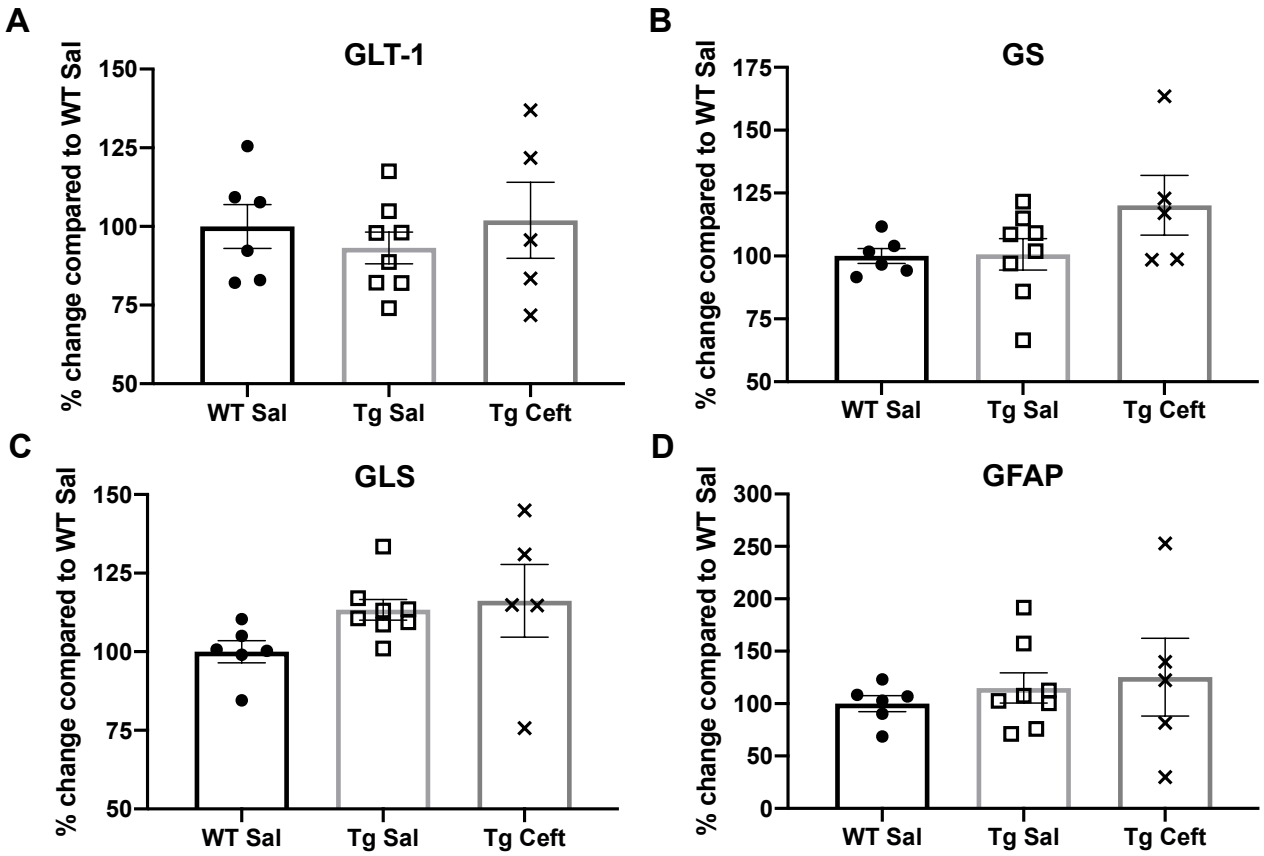

Figure 5.8. Ceftriaxone treatment did not significantly alter the level of any key proteins in the glutamate-glutamine cycle in the cortex. **A)** Glutamate transporter-1 (GLT-1) **B)** Glutamine synthetase (GS) **C)** Glutaminase (GLS) **D)** Glial fibrillary acidic protein (GFAP). ANOVA with Tukey's post-hoc test, data = means ± SEM.

Similarly to the results from the cortical samples, there was also no significant difference in the expression levels of key proteins in the hippocampal samples between any groups. The mean GLT-1 expression levels in the Tg-Sal and Tg-Ceft groups were decreased by 12% ($p = 0.572$, ANOVA with Tukey's post-hoc) and 8% ($p = 0.826$, ANOVA with Tukey's post-hoc), respectively, compared to WT-Sal group (Figure 5.9A). There was no significant difference between GLT-1 protein expression levels between Tg-Ceft and Tg-Sal groups in the hippocampus ($p = 1$, ANOVA with Tukey's post-hoc, Figure 5.9A).

With the GS protein expressions, the results from the hippocampus were very similar to the cortex. In Tg-Ceft group, the mean level of GS expression was increased by 21% ($p = 0.194$, ANOVA with Tukey's post-hoc), while in Tg-Sal group it was decreased by 5% ($p = 0.868$, ANOVA with Tukey's post-hoc), compared to WT-Sal group (Figure 5.9B). The difference between Tg-Ceft and Tg-Sal groups was 27% and this change was approaching the statistical significance threshold with $p = 0.0681$ (ANOVA with Tukey's post-hoc, Figure 5.9B).

For GLS, the expression level was decreased by 10% ($p = 0.729$, ANOVA with Tukey's post-hoc, Figure 5.9C) in Tg-Ceft group and increased by 7% ($p = 0.816$, ANOVA with Tukey's post-hoc, Figure 5.9C) in the Tg-Sal group, compared to WT-Sal group. There was a 17% less GLS protein expression in the Tg-Ceft group compared to the Tg-Sal group; however, this difference was not significant ($p = 0.368$, ANOVA with Tukey's post-hoc, Figure 5.9C).

Finally, it was found that there was a 57% increase in the mean level of GFAP in the Tg-Ceft group compared to the WT-Sal group ($p = 0.132$, ANOVA with Tukey's post-hoc, Figure 5.9C). The GFAP level in the Tg-Ceft group was also 52% higher than the Tg-Sal group ($p = 0.150$, ANOVA with Tukey's post-hoc, Figure 5.9C). On the other hand, there was no difference in the mean GFAP expression level between the Tg-Sal group and WT-Sal group ($p = 1$, ANOVA with Tukey's post-hoc, Figure 5.9C).

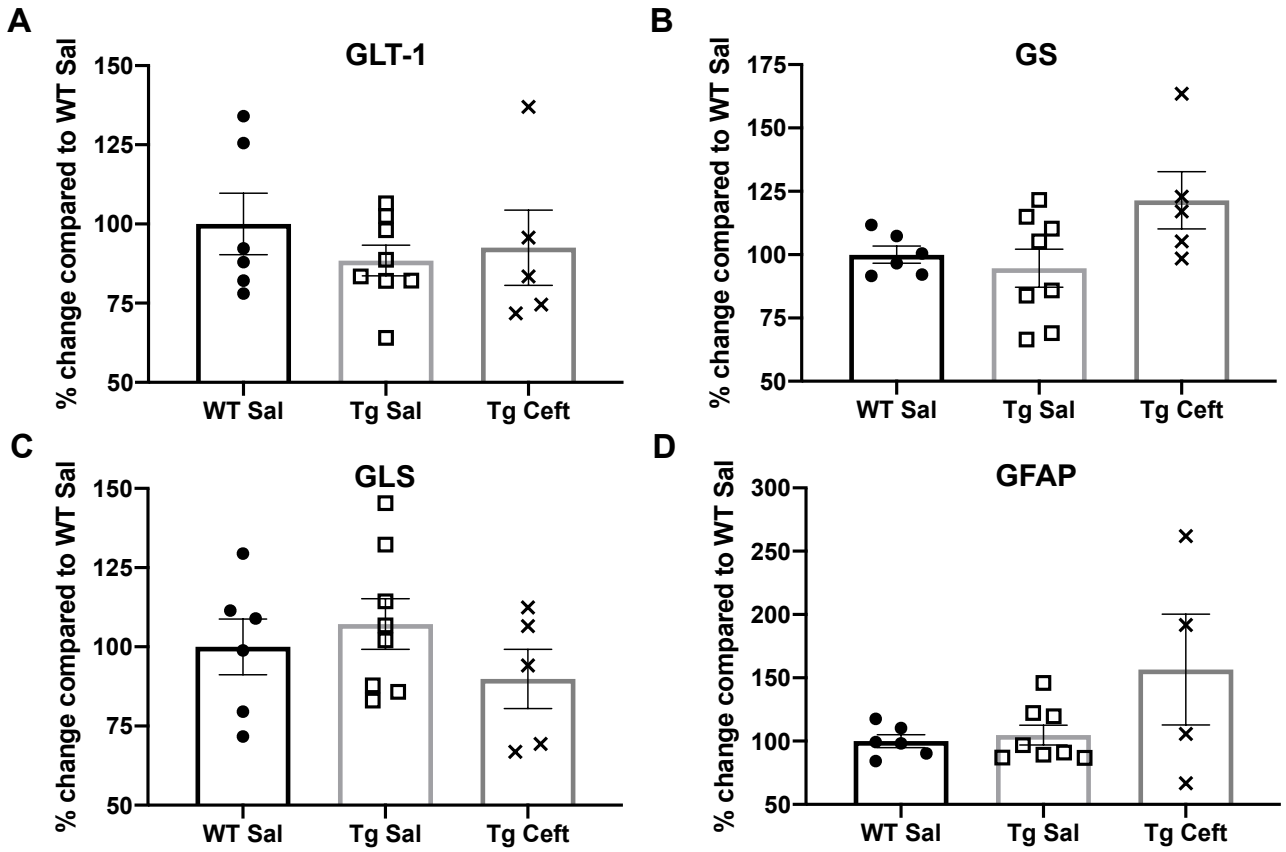

Figure 5.9. Ceftriaxone treatment did not significantly alter the expression levels of key proteins in the glutamate-glutamine cycle in the hippocampus. **A)** Glutamate transporter-1 (GLT-1) **B)** Glutamine synthetase (GS) **C)** Glutaminase (GLS) **D)** Glial fibrillary acidic protein (GFAP). One hippocampus sample data point from the Tg-Ceft group was removed from the GFAP level investigation because the bands were too faint to analyse. P>0.05, ANOVA with Tukey's post-hoc test, data = means ± SEM.

5.5.5) ***Kindling diminished the effects of ceftriaxone on GLT-1 and GLS levels***

It was revealed that ceftriaxone treatment significantly increased the levels of GLT-1 and GLS proteins in the brain of naïve Tg2576 mice, not the kindled Tg2576 mice. Ceftriaxone treatment significantly increased the expression level of GLT-1 in the hippocampal samples of naïve Tg2576 mice by 25% ($p = 0.0177$, unpaired t-test, Figure 5.10A), not in the kindled Tg2576 mice ($p = 0.938$, ANOVA with Tukey's post-hoc, Figure 5.10B). Ceftriaxone treatment also significantly increased the expression level of GLS protein in the cortical samples of naïve Tg2576 mice by 12% ($p = 0.0105$, unpaired t-test, Figure 5.11A), not in the kindled Tg2576 mice ($p = 1$, ANOVA with Tukey's post-hoc, Figure 5.11B).

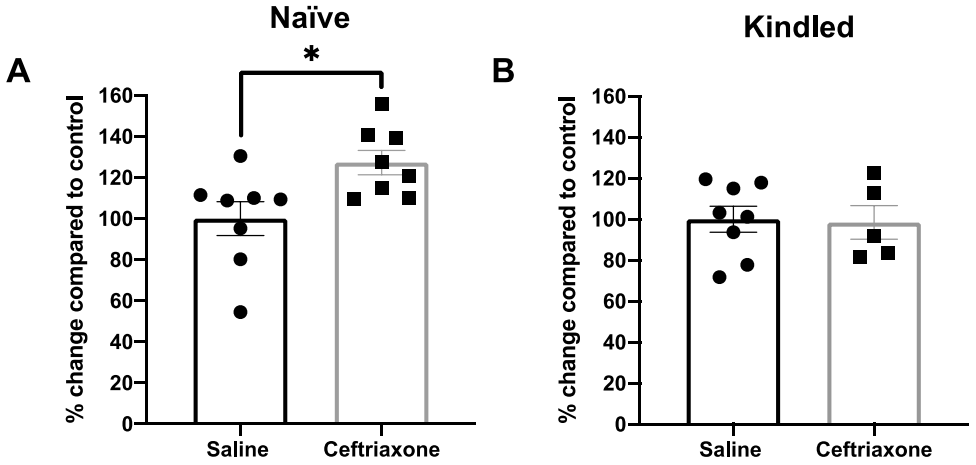

Figure 5.10. The effect of ceftriaxone treatment on the expression level of GLT-1 protein in the hippocampal samples of **A)** naïve Tg2576 and **B)** kindled Tg2576 mice, compared to saline treatment. *p<0.05, data = means ± SEM, data presented as the percentage of control (saline).

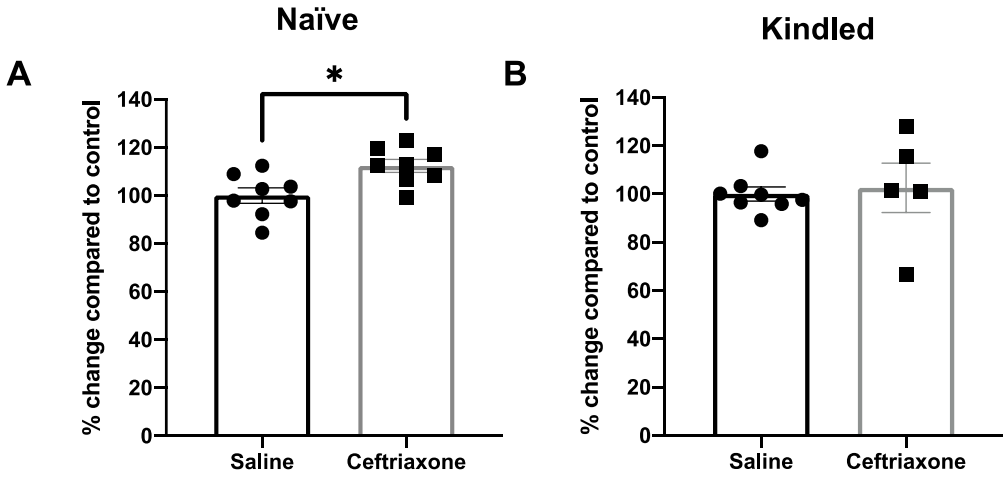

Figure 5.11. The effect of ceftriaxone treatment on the expression level of GLS in the cortical samples of **A)** naïve Tg2576 and **B)** kindled Tg2576 mice, compared to saline treatment. *p<0.05, data = means ± SEM, data presented as the percentage of control (saline).

5.6) Discussion

To summarise, a daily single dose of 200mg/kg of ceftriaxone caused an increase in the expression levels of GLT-1 and GLS proteins in the brains of Tg2576 mice. The results from this chapter suggest that ceftriaxone treatment may reduce the severity of the seizure associated with the initial stimulation, compared to saline treatment. However, ceftriaxone did not reduce the overall kindling susceptibility of the Tg2576 mice. Ceftriaxone also did not significantly alter the levels of key proteins in glutamate-glutamine cycle in kindled Tg2576 mice. This section will discuss these key findings.

5.6.1) ***Ceftriaxone increased the expression levels of GLT-1 and GLS in Tg2576 mice***

The summary of the effects of ceftriaxone treatment on the expression levels of key proteins in glutamate-glutamine cycle can be seen in Table 5.2. As hypothesised, daily treatment with 200mg/kg of ceftriaxone for 7 consecutive days significantly increased the level of GLT-1 protein expression in the brain of Tg2576 mice, compared to saline. However, this increase was only significant in the hippocampal region, not the cortical region. This finding is consistent with the findings from previous studies which showed that ceftriaxone treatment could significantly increase the expression level of GLT-1 in the hippocampus in mouse models.[24, 25] On the contrary, there is no substantial evidence from the literature to show that ceftriaxone can increase the level of GLT-1 protein in the cortical region of the brain. Future studies should investigate the potential of ceftriaxone in enhancing the expression level of GLT-1 protein in the cortical region of the brain in Tg2576 mice further. This will be important as a significant reduction in the expression level of GLT-1 in the cortex of Tg2576 mice was observed in Chapter 4 and the reduction in GLT-1 expression level may lead to higher seizure susceptibility in the brain.[14]

It is also possible that the difference in gender may have caused the effectiveness of ceftriaxone on the expression level of GLT-1 protein in the brain to vary. However, the results from the pilot study in this chapter demonstrated that ceftriaxone significantly increased the brain's expression level of GLT-1 protein in the mixed-gender Tg2576 mice group, compared to saline. This is consistent with the evidence from the literature as it was showed that ceftriaxone is effective at enhancing the expression level of GLT-1 in mice, regardless of the gender.[24]

Protein of interest	Cortex	Hippocampus
GLT-1	n.s.	↑ ***(p = 0.0177)***
Glutamine synthetase	n.s.	n.s.
Glutaminase	↑ ***(p = 0.0105)***.	n.s.
Glial fibrillary acidic protein	n.s.	n.s.

Table 5.2. The effects of ceftriaxone treatment on the expression levels of key proteins in glutamate-glutamine cycle. The cortical and hippocampal samples were acquired from aged Tg2576 mice treated with 200mg/kg, compared to aged Tg2576 mice treated with saline. ↑ = $p<0.05$, unpaired t-test.

In addition to the enhanced expression level of GLT-1 in the brain, it was also revealed that ceftriaxone treatment significantly enhanced the expression level of GLS protein in the cortex of Tg2576 mice. This interesting change might be a part of a compensatory mechanism that was triggered by the increase in GLT-1 protein level in the brain due to ceftriaxone treatment, as depicted in Figure 5.12. Briefly, increased GLT-1 protein expression would result in an increase in glutamate uptake rate from the synaptic cleft into the astrocyte[36], which would lead to higher demand for glutamate in the pre-synaptic neuron. Therefore, the expression level of GLS protein would be enhanced to replenish the pre-synaptic glutamate pool. This mechanism might be necessary for the maintenance of glutamate homeostasis within the tripartite synapse environment.[37, 38] To add, the enhancements in the expression levels of GLS and GLT-1 proteins were observed in kindled WT mice in Chapter 4, suggesting similarities in the mechanism between the effects of ceftriaxone treatment and kindling-induced seizures on the glutamate-glutamine cycle. Thus, the results in this chapter may suggest that ceftriaxone can trigger the brain's defense mechanisms against overexcitation by enhancing the expression level of GLT-1 in the brain before the seizure occurs.[23, 39] Therefore, ceftriaxone may have the potential to reduce the occurrence of seizure associated with the reduction in GLT-1 expression level in the brain. Interestingly, the increase in GLS expression level observed in this study only occurred in the cortical region, not the hippocampal region. This may suggest that the mechanism that triggered the increase in GLS expression is localised in the cortical region. Therefore, it would be important to investigate the role of GLS in the pathogenesis of epilepsy further to elucidate this mechanism. For future studies, investigating how GLS inhibition affects the seizure susceptibility will yield important insights on the role of GLS in the pathogenesis of epilepsy. Small molecule inhibitors for GLS, such as C968 [40], may be utilised for this purpose.

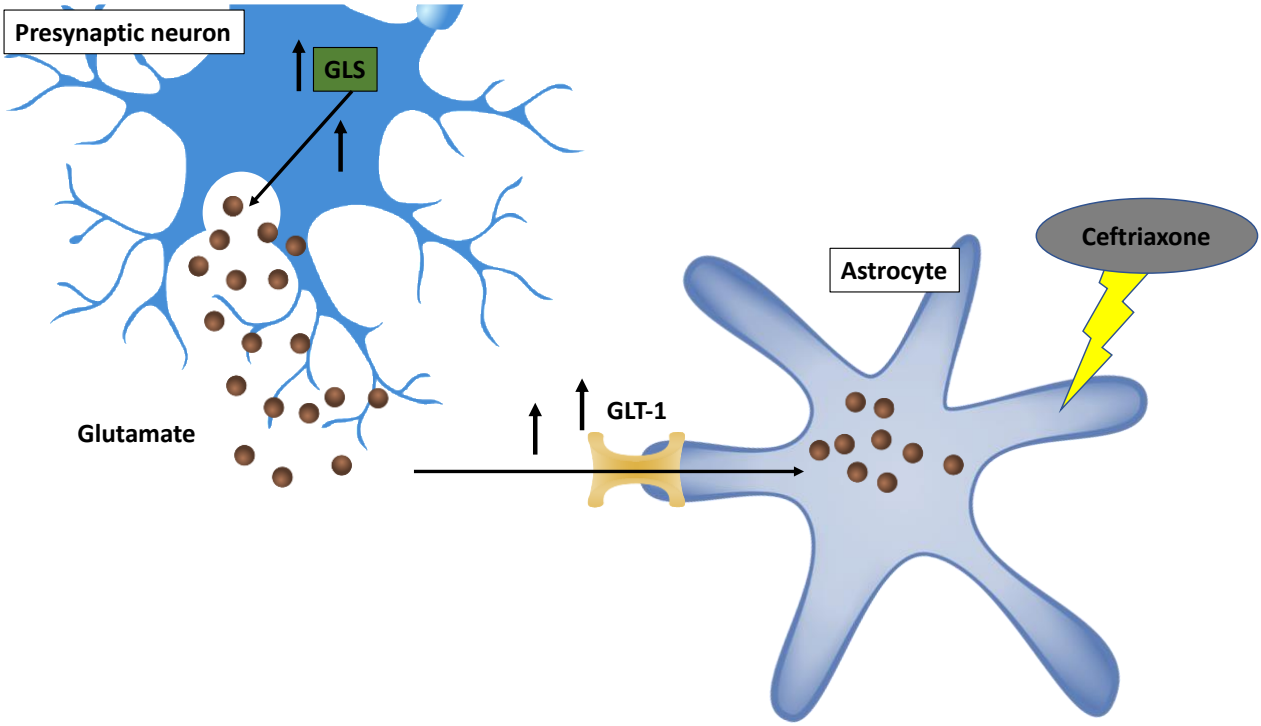

Figure 5.12. The effects of ceftriaxone on the expression levels of GLT-1 and GLS proteins in the brain. Ceftriaxone significantly increased the expression levels of GLT-1 in the brain, which resulted in an increase in glutamate uptake from the synaptic cleft into the astrocyte. This hypothetically led to an increase in the demand of glutamate in the pre-synapse, as glutamate is being removed from the synaptic cleft at a quicker rate than usual. Thus, this caused an increase in the level of GLS in the brain of Tg2576 mice to match this increase in the demand for glutamate in the pre-synapse.

As for GS, ceftriaxone also did not significantly alter the level of this enzyme in the brain of Tg2576 mice under the test conditions of this study. This may suggest that the significant increase in GLS expression level that was observed in this study was caused by a higher demand for glutamate in the pre-synapse rather than the increase in glutamine level.[41] Since ceftriaxone may not increase the production glutamine, there may be no increase in the pressure for GLS to rebalance the glutamine level and convert glutamine into glutamate. To test this hypothesis, future study should utilise methods such as microdialysis[42] to investigate how ceftriaxone treatment may affect the level of glutamine in the brain.

Finally, the results from this chapter suggest that ceftriaxone treatment did not significantly affect the level of GFAP protein in the brain of Tg2576 mice, as expected. This may indicate that ceftriaxone does not affect the astrogliosis phenotype in Tg2576 mice under the test conditions. In future studies, other markers for astrogliosis, such as the S100-β[43], should be used to further support this statement. The effect of ceftriaxone treatment on the high kindling seizure susceptibility phenotype of Tg2576 mice observed in this study will be discussed in the following section.

5.6.2) ***Ceftriaxone as an anti-seizure drug but not an anti-epileptogenic drug***

In this chapter, mice from the Tg-Ceft group were hypothesised to be less susceptible to kindling-induced seizure than the Tg-Sal group. Further, the Tg-Ceft group was also expected to display a similar level of seizure susceptibility to the WT-Sal group. However, the results revealed that ceftriaxone treatment did not ameliorate the high seizure susceptibility phenotype of the Tg2576 mice. Despite that, interesting observations were made regarding the effects of ceftriaxone treatment on Tg2576 mice. It was demonstrated that Tg-Ceft mice displayed less severe seizures (Class IV) in response to the first stimulus during kindling, compared to Tg-Sal mice (Class V). Additionally, the Tg-Ceft group displayed an identical seizure class as the WT-Sal group (Class IV) in response to the first stimulus. This may suggest that the increase in GLT-1 expression level in the brain as the result of ceftriaxone treatment may provide neuroprotective effects against induced seizure[23] that occurred in response to the first stimulus. However, it was also observed that seizure classes of the Tg-Ceft group from the second kindling session onwards were identical to the Tg-Sal group. Moreover, ceftriaxone treatment did not significantly reduce the number of stimulations needed to trigger the first Class V seizure in Tg2576 mice. Thus, the results from this experiment revealed a trend which suggests that ceftriaxone may provide an anti-seizure effect, not anti-epileptogenic effect on Tg2576 mice under the test conditions. There are 3 hypothesised reasons to support this statement.

Firstly, amygdala kindling can cause the brain to undergo significant metabolic and physical changes[33], which may diminish the beneficial effects of ceftriaxone treatment. Despite that, there is evidence from the literature to demonstrate the beneficial effects of ceftriaxone in pentylenetetrazole (PTZ) kindling model of epilepsy, such as delaying the onset of the seizure, decreasing seizure severity and reducing the incidence of death associated with seizure.[44, 45] As discussed, ceftriaxone also displayed seizure severity reducing effect in electric kindling model in this chapter; however, the effect size of ceftriaxone, which was observed in this chapter, was smaller than hypothesised. This discrepancy between the effect sizes of ceftriaxone against electric kindling and PTZ kindling models might be due to the difference in the nature of the stimulus.[46, 47] Compared to electric-kindling model, the mechanism that leads to convulsions in PTZ kindling model might be more sensitive to changes in the expression level of GLT-1. Thus, ceftriaxone treatment was more effective at reducing seizure susceptibility in the PTZ model than the electric-kindling model.

Secondly, a daily single dose of 200mg/kg ceftriaxone used in this study might not be sufficient to provide a lasting neuroprotective effect against amygdala kindling-induced seizures. Thus, higher doses of ceftriaxone or repeated doses might be needed to increase the duration and the effect size of ceftriaxone treatment. Furthermore, the diminishing neuroprotective effect of ceftriaxone that was observed in this chapter might be caused by a compensatory mechanism designed to counteract the increase in the expression level of GLT-1 in the brain. The results from the pilot study suggest that the effect of 200mg/kg of ceftriaxone on the expression of GLT-1 was detectable after 7 days of treatment. However, it is not known whether this effect is detectable after 30 days, which was the duration of the main experiment in this chapter.

Finally, the number of animals in the Tg-Ceft group was relatively small and may not provide enough power to detect the full effect of ceftriaxone treatment in this study. The small sample size also affected the normality of the data distribution, which reduced the power of the statistical analysis. In future studies, larger sample size should be considered to increase the power of the analysis and increase the chance of detecting the full effect of ceftriaxone treatment on the seizure susceptibility of Tg2576 mice.

5.6.3) ***Mechanism behind the neuroprotective effect of ceftriaxone***

To investigate the mechanism on how ceftriaxone provide neuroprotective effect and potentially reduce the severity of the first seizure in Tg2576 mice, this study compared the effects of ceftriaxone on the expression levels of GLT-1 and GLS proteins between naïve and kindled Tg2576 mice. It was revealed that the effects of ceftriaxone on the expression

levels of GLT-1 and GLS proteins in the brain were only observed in naïve Tg2576 mice, not kindled Tg2576 mice. There is evidence from animal studies to demonstrate that NF-κB pathway plays an important role in the mechanism that mediates kainate-induced seizures.[48, 49] Since ceftriaxone also act through the NF-κB pathway[26], induced seizures may have diminished the effects of ceftriaxone in Tg2576 mice by competing for the NF-κB pathway and activating this pathway to the maximum level before ceftriaxone could activate it. The results from chapter 4 support this statement as it was shown that kindling-induced seizures significantly increased the levels of GLT-1 and GLS proteins in the brain. Based on this line of evidence, I hypothesised that kindling-induced seizures elevated the expression levels of GLT-1 and GLS in the brain to the maximum level; thus, their expression levels cannot be enhanced any further by ceftriaxone.

On the other hand, the NF-κB pathway may be involved in a compensatory mechanism designed to reduce the overexcitation of the brain.[50] In Chapter 4, I hypothesised that kindling-induced seizures triggered a compensatory mechanism which involved the enhancement in the expression level of GLT-1 protein in the brain. Since ceftriaxone induced a similar effect on the expression levels of GLT-1 to kindling, I hypothesised that ceftriaxone treatment may prime the brain to defend itself against overexcitation via the enhancement of the expression level of GLT-1 protein.

Regarding GS and GFAP, ceftriaxone treatment did not significantly alter the expression levels of these proteins. Compared to WT-Sal, the increase in the expression level of GS observed in the Tg-Ceft group was approaching but did not reach the significance threshold. In the future, investigating how the expression level of GS is affected by the increase in the expression level of GLT-1, may yield important insights on the potential of GS as an additional drug target to GLT-1 in preventing seizures in AD.[51] As for GFAP, there is evidence from the literature to demonstrate that ceftriaxone treatment caused a decrease in the expression level of GFAP in the brain following traumatic brain injury.[51] In the future, other markers for astrogliosis, such as S100-beta[52], and older Tg2576 mice with clear astrogliosis phenotype[53] should be utilised to further investigate the effect of ceftriaxone on GFAP expression level in the brain. This investigation may reveal the potential of ceftriaxone treatment for ameliorating the astrogliosis phenotype in Tg2576 mice.

Another important point to discussion is that there were mice which were euthanised before they reached the experimental endpoint during the experiment. In this chapter, the criterion for a mouse to reach its endpoint and be considered fully kindled was clear, as seen in the methods. For example, the mouse that died overnight after the first kindling session would not be considered fully kindled; thus, this mouse would not be included in the final protein analysis. To add, a similar endpoint criterion was used in Chapter 4, and it was

shown that this criterion was robust enough to allow the measurements of the main experimental outcomes, such as detecting changes in the expression levels of key proteins in glutamate-glutamine cycle.

Finally, it must be noted that mice from the WT-Sal group were expected to display a Class I to Class II seizure in response to the initial stimulus.[34] However, the WT-Sal group in this study displayed a Class IV seizure. One reason that might explain this phenomenon was the enhanced level of stress in each animal, which may be caused by the daily experimental procedures. There is evidence from the literature to demonstrate that restraining and injecting methods may increase the plasma corticosterone level, heart rate and body temperature in the animal.[54] Each mouse from the main experiment of this chapter underwent IP injection before kindling on a daily basis until the experiment was over. This increased stress level may interfere with the results since stress has been associated with increased seizure susceptibility.[55, 56] Therefore, it will be important to allow the animal to rest for at least 1 hour after the injection before proceeding with kindling in future experiments.

5.7) Future directions

It will be important to generate more data on the ability of ceftriaxone to ameliorate the symptoms of AD and epilepsy from animal studies in order to guide the design of future human studies on this topic. Ceftriaxone and other β-lactam antibiotics, such as penicillin and amoxicillin, possess the ability to promote the gene expression of GLT-1 in the brain, which signify a class effect.[23] Currently, evidence from the literature suggests that ceftriaxone causes an increase in GLT-1 expression through the mechanism which involves the activation of NF-κB pathways, as it was shown that removing the NF-κB binding sites from the GLT-1 promotor resulted in a dampened GLT-1 expression enhancing effect of ceftriaxone.[26] It was also reported that ceftriaxone may activate the NF-κB pathways via the degradation of IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and p65 translocation.[26, 57] Furthermore, the presence of Akt (protein kinase B) was crucial for the mechanism that led to an increase in GLT-1 expression through the NF-κB pathway as the interaction between mTor (mechanistic target of rapamycin) and Akt contributed to the promotion of GLT-1 expression.[58] As discussed, seizures may activate the NF-κB pathway and interfere with the effects of ceftriaxone in the brain.[48, 49] Therefore, investigating the effect of ceftriaxone on different models of epilepsy will be important for determining if ceftriaxone is the most suitable GLT-1 expression enhancer for treating epilepsy in AD. Other GLT-1 expression enhancers that are not β-lactam antibiotics include riluzole[59] and dexamethasone.[60]

After a substantial amount of data on the effects of GLT-1 enhancement has been generated via animal studies, it will be important to translate these results to human studies. It is known that rodent AD models can only partially imitate AD pathologies; thus, the effects of ceftriaxone seen in AD models might not be fully translatable to what will happen in humans. However, the evidence gathered from this chapter suggests that ceftriaxone may provide beneficial effects against the symptoms of epilepsy in AD rodent models. This will be discussed further in Chapter 6. Additionally, developing a more specific GLT-1 expression enhancer will also be valuable. The new drug should decrease the number of adverse effects related to ceftriaxone[61] and increase the efficacy of GLT-1 expression enhancement. This may increase the clinical relevance of the drug further.

It will also be important to investigate the effects of higher dosage and longer treatment duration of ceftriaxone in AD rodent models. The effect of ceftriaxone on the expression level of GLT-1 protein in the brain should be investigated at different timepoints, such as after 30 days of daily treatment, to establish whether ceftriaxone becomes less effective overtime. This will generate more pharmacological data on the effect of ceftriaxone on the high seizure susceptibility phenotype in AD models. Furthermore, studying the effect of ceftriaxone on different models of AD, such as human tau overexpression model[62, 63], will reveal if ceftriaxone can provide neuroprotective effect against overexcitation under different AD pathologies. Chemoconvulsants[45] and genetic models of epilepsy can also be utilised to gather more information on the anti-seizure effect of ceftriaxone on different epilepsy models.[64]

Lastly, measuring the amount of extracellular glutamate in the brain in real-time via methods such as microdialysis and microsensors[65] will allow the investigation of the degree that ceftriaxone treatment is altering the rate of glutamate uptake in the brain. This would give us the insight on whether enhancing the level of GLT-1 in the brain alone will be sufficient to rebalance the glutamate level or are there other compensatory mechanisms that also need to be addressed.

5.8) Conclusion

The evidence uncovered in this chapter provided insights on how pharmacologically enhancing the GLT-1 expression level in the brain with ceftriaxone may reduce the seizure susceptibility of Tg2576 mouse model of AD. The results demonstrated a trend that ceftriaxone treatment may provide an anti-seizure effect, not anti-epileptogenic effect to the brain against amygdala kindling-induced seizure in Tg2576 mice. However, the effects of ceftriaxone on the kindling susceptibility of Tg2576 mice that were observed in this chapter were not significant. Since the evidence from the literature suggests that ceftriaxone

treatment has the potential to ameliorate the symptoms of AD[24, 25] and epilepsy[23], it will be worthwhile to investigate the effects of ceftriaxone on the high seizure susceptibility phenotype of different mouse models of AD further. To add, there is currently no disease modifying treatment for epilepsy in AD so it will be important to elucidate the mechanism that links these two serious neurological disorders and identify targets that can impede the progress of both diseases in one shot.

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Chapter 6 – General Discussion

6.1) Summary of main findings

This project contributed to the field of epilepsy in AD by generating important data from the Tg2576 model of AD. Chapter 1 reviewed the literature in the field of AD and epilepsy and placed the groundwork for further discussion on the topic of the mechanism that has the potential to link AD pathologies to an increased risk of developing epilepsy. Chapter 2 then elucidated the potential roles of glutamate and astrocytes in the mechanism that links AD pathologies to an increased risk of developing epilepsy through a published critical review paper.[1] The evidence from human and animal studies on the role of glutamate-glutamine cycle in the mechanism that links AD pathologies to an increased risk of developing epilepsy was also critiqued.[2-4] AD pathologies, such as mutant human APP overexpression, disrupt the glutamate-glutamine cycle by reducing the expression levels of astrocytic proteins such as GLT-1 and GS. This disruption may lead to higher seizure susceptibility as it may increase the chance of extracellular glutamate accumulation in the synaptic cleft.[5, 6] Therefore, disruption to the glutamate-glutamine cycle may be one of the mechanisms that link AD pathologies to an increase in the risk of developing epilepsy.

Since it is evident that the alterations to the expression levels of key proteins in glutamate-glutamine cycle may play a part in the pathogenesis of AD, I aimed to investigate how early these alterations may occur under AD condition. My published metabolomics study (see Chapter 3) revealed how metabolites in the brain were affected by the mutant human APP overexpression pathology in mature Tg2576 mice.[7] It was demonstrated that glutamate metabolism was significantly enriched in mature Tg2576 mice, compared to WT. It was also identified that lipid metabolism and pathways associated with oxidative stress were significantly enriched in Tg2576 mice. Since Aβ-plaque is not typically present in mature Tg2576 mice, changes in the metabolomic profiles that were observed were not hypothesised to be caused by the final stage of Aβ pathology.[8] I hypothesised that the mutant human APP and/or the soluble Aβ species derived from APP caused the metabolomic changes that were observed in mature Tg2576 mice. This hypothesis was also supported by the evidence from the literature as it was shown that the soluble Aβ species are typically more toxic than the Aβ plaque.[9, 10] Finally, this study generated valuable insights on potential early therapeutic targets and the optimum treatment window for epilepsy in AD.

Chapter 4 then attempted to elucidate the potential of glutamate-glutamine cycle as the link between AD pathologies and the increased risk of epilepsy. Key proteins in the glutamate-glutamine cycle were found to be significantly altered by the pathogeneses of

both AD and epilepsy. It was revealed that the expression levels of GLT-1 and GS proteins were significantly reduced in mature Tg2576 mice, compared to WT. The results suggested that the disruption to glutamate-glutamine cycle was one of the earliest events in the brain that is associated with mutant human APP overexpression pathology. Although there was conflicting evidence from the literature regarding the effect of seizures on the expression level of GLT-1[11-13], the results from Chapter 4 demonstrated that the expression level of GLT-1 protein was significantly enhanced in kindled mice, compared to sham. The contradictory nature of the results from different studies can be attributed to the variance among different epilepsy models and the timing of the tissue collection. I hypothesised that the significant increase in GLT-1 expression level might be one of the compensatory mechanisms which was triggered in response to seizures. The results from this Chapter 4 also confirmed that Tg2576 mice were more susceptible to kindling-induced seizure than the WT mice.[14] Thus, key proteins in glutamate-glutamine cycle, such as GLT-1, have the potential to be early pharmacological targets for reducing seizure susceptibility in Tg2576 mice.

Based on the evidence that was discussed in Chapter 2 to 4, I hypothesised that enhancing the expression level of GLT-1 protein in the brain will mitigate the high seizure susceptibility phenotype in Tg2576 mice. This hypothesis was tested in Chapter 5, and it was revealed that Tg2576 mice treated with a daily dose of 200mg/kg of ceftriaxone were not significantly more susceptible to kindling than WT mice treated with saline. On the other hand, the results from Tg2576 mice treated with saline were consistent with the results from Chapter 4, as these mice were significantly more susceptible to kindling induced seizure than WT. The results suggested that Tg2576 mice treated with ceftriaxone were not as susceptible to kindling as Tg2576 mice treated with saline. However, after the first seizure was triggered, the severity of the induced seizure in Tg2576 mice treated with ceftriaxone returned to a similar level as the saline group. This meant that the neuroprotective effect of ceftriaxone might not be long lasting when given under the condition tested. Additionally, the sample sizes in each group were small; thus, future studies need to increase the sample size to further investigate the effects of ceftriaxone on the seizure susceptibility of Tg2576 mice. In conclusion, enhancing the expression level of GLT-1 in the brain may provide a level of protection against seizures in mouse models of AD. This gave us insights on the potential role of GLT-1 as a pharmacological target for reducing seizure susceptibility in AD.

6.2) Implications

Results from this project have important implications in the field of epilepsy and AD. The evidence discussed in my published critical review in Chapter 2 implied that the glutamate-glutamine cycle may be involved in the mechanism led to the pathogenesis of epilepsy in AD.[1] Evidence to demonstrate that alterations to the expression levels of key proteins in glutamate-glutamine cycle, such as GLT-1, led to lethal seizure and cognitive impairment phenotypes in animal models was discussed. Furthermore, the evidence to support the role of astrogliosis in the disruption of glutamate-glutamine cycle and how this might lead to the pathogenesis of AD[15, 16] and epilepsy[17, 18] was discussed. The evidence implied that pharmacologically targeting the glutamate-glutamine cycle may be beneficial against the pathogenesis of both AD and epilepsy.

There is a lack of literature on the potential treatments for epilepsy under AD condition. Therefore, it will be important to generate more data in this field using the insights gained from this thesis. Existing anti-epileptic drugs (AEDs) and AD drugs that target the glutamatergic pathway should be investigated to reveal potential treatments that will ameliorate the symptoms of epilepsy and AD. One of the approved AEDs that has the potential to treat epilepsy in AD is perampanel. Perampanel is the first selective and non-competitive AMPAR (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) antagonist to be used for the treatment of epilepsy.[19, 20] The AMPA receptor is a glutamate receptor which is coupled to an ion channel that mediates the excitatory signal propagation by allowing the influx of Ca^{2+} into the neuron.[21] Thus, inhibiting AMPARs may reduce the excitability of the brain. The side effects of perampanel are also generally well-tolerated. The pharmacokinetic profile of perampanel is characterised by fast absorption with an oral bioavailability of approximately 100% and an average elimination half-life of 10.5 hours.[19] Thus, it will be important to investigate whether perampanel can ameliorate the symptoms of AD in future studies.

Memantine is another potential candidate for the treatment of epilepsy in AD and it works by preventing the influx of Ca^{2+} into the neurons through NMDAR inhibition.[22] Memantine has been approved for the treatment of moderate to severe AD patients, as there is evidence to show that memantine improves the cognitive and behavioural impairment symptoms in AD patients, as well as provides neuroprotective effects to the brain.[22, 23] As for the pharmacokinetic profile of memantine, it has the oral bioavailability of approximately 100% with the biological half-life of approximately 70 hours.[23] The side effects of memantine are well-tolerated.[22] Since memantine acts through the glutamatergic pathway, it will be interesting to investigate the potential of memantine as the treatment for epilepsy under AD

conditions. Despite these beneficial effects, the risk-benefit relationship of memantine in AD must be considered as memantine is a symptomatic treatment, not disease modifying treatment.[24]

It was also implied in Chapter 2 that decrease in the expression levels of key proteins in glutamate-glutamine cycle that was observed under the condition of AD may lead to higher seizure susceptibility. Therefore, drugs that can enhance the expression levels of these key proteins may have the potential to decrease the chance that AD patients will develop epilepsy. Ceftriaxone, which is a cephalosporin antibiotic, has demonstrated the ability to enhance GLT-1 protein expression level in the brain in animal studies.[25] There is evidence from the literature to suggest that enhancing GLT-1 expression level may reduce cognitive impairments in mouse models of AD. [26, 27] Regarding epilepsy, it has been shown that the lack of GLT-1 protein in the brain may lead to lethal epileptic seizure.[3] Additionally, ceftriaxone treatment significantly reduced the seizure severity and the number of deaths associated with seizure in mice.[28, 29] The pharmacokinetic profile of ceftriaxone is characterised by negligible metabolism and the elimination half-life of approximately 7 hours.[30] However, ceftriaxone is only bioavailable when administered through intravenous or intramuscular injections as it has less than 1% bioavailability when taken orally.[31] Based on the evidence that ceftriaxone may provide beneficial effects against the symptoms of AD and epilepsy, this drug has the potential to be used as a treatment for epilepsy in AD; thus, further investigation is warranted.

Published results from Chapter 3 implied that mutant human APP overexpression pathology triggered metabolomic changes in the brain of Tg2576 mice before the formation of Aβ-plaque.[7] Pathways such as glutamate metabolism[32, 33], lipid metabolism[34, 35] and oxidative stress[36, 37] were identified as significantly enriched in mature Tg2576 mice. The results implied that these significantly enriched pathways have the potential to contain biomarkers or treatment targets for the early stage of Aβ pathology. Importantly, changes in glutamate metabolism was implicated, which meant that the glutamatergic pathway has the potential to be an early treatment target in AD. Thus, drugs that act on the glutamatergic pathway are hypothesised to provide beneficial effects against the symptoms of epilepsy and AD during the early stages of AD. The results also implicated that the pathways associated with lipid metabolism and oxidative stress are affected early in AD. Hence, antioxidant treatments may provide beneficial effects against the pathogenesis of both AD and epilepsy. However, antioxidant as a preventive treatment for AD[38] and epilepsy[39] is still underutilised, mainly due to the clinical trial failures.[40, 41] AD is a complex disorder with more than one pathologies, which implies that reducing oxidative stress alone may not be

sufficient to prevent AD. Therefore, utilising antioxidative therapy along with other therapies that target different aspects of AD may be more effective at preventing AD.

As for the lipid metabolism, it was reported in the literature that some of the approved AEDs, such as carbamazepine, may alter the plasma lipid levels in epilepsy patients.[42] This finding implies that it will be important to consider how a drug interacts with the lipid profiles in the brain before approving the drug to be used in the treatment of epilepsy in AD.

In Chapter 4, it was found that the expression levels of key proteins associated with the glutamate-glutamine cycle were significantly altered by kindling-induced seizures and mutant human APP overexpression pathology. Additionally, it was confirmed that Tg2576 mice were more susceptible to kindling than the WT.[14] The results from Chapter 4 along with the evidence from the literature[4, 43] support the hypothesis in Chapter 2 that the glutamate-glutamine cycle is disrupted in AD, which may lead to higher chance of developing epilepsy. This finding implies that pharmacologically targeting the glutamate-glutamine cycle may reduce the seizure susceptibility in Tg2576 mice. The reductions in the expression levels of GLT-1 and GS proteins in Tg2576 mice suggest that the treatments that can correct these changes may be beneficial against the symptoms of epilepsy [5, 44] and AD.[22, 32]

Finally, evidence from Chapter 5 demonstrated that pharmacologically enhancing the GLT-1 expression level in the brain with ceftriaxone may be beneficial for ameliorating the high seizure susceptibility phenotype in Tg2576 mice. As mentioned earlier in this section, there is evidence from the literature to support the use of ceftriaxone as the treatment for epilepsy and AD.[26-29] This thesis also revealed that ceftriaxone reduced the seizure severity of the first induced seizure in Tg2576 mice, compared to saline. Furthermore, Tg2576 mice treated with ceftriaxone were no longer more susceptible to kindling than the WT; thus, ceftriaxone treatment did demonstrate an anti-seizure effect. Despite that, ceftriaxone did not demonstrate an anti-epileptogenic effect, since it did not reduce the number of stimulations needed to trigger the first Class V seizure in Tg2576 mice, compared to saline. However, this study did generate important evidence to support the role of GLT-1 protein expression enhancer, such as ceftriaxone, in treating epilepsy in AD and lay the groundwork for future experiments regarding the treatment of epilepsy in AD through the glutamatergic pathway.[44]

To summarise, the results generated by this thesis together with the evidence from the literature imply that disruption to the glutamate-glutamine cycle is associated with the pathogeneses of AD and epilepsy.[1, 2, 5] This finding also implies that glutamate-glutamine cycle has the potential to be a part of the mechanism that links AD pathologies to an increase in the risk of developing epilepsy. Therefore, pharmacologically targeting key proteins in the

glutamate-glutamine cycle, such as GLT-1 and GS, may provide beneficial effects against the symptoms of epilepsy in AD. This thesis initiated the investigation on the potential role of ceftriaxone as the treatment for epilepsy in AD that will pave the way for future studies on this topic.

6.3) Limitations

The first limitation of this study was associated with the use of the animal model of AD. Since human AD is not caused by just one pathology but a combination of many, animal models cannot fully recapitulate the full spectrum of AD. Therefore, discoveries made using the Tg2576 model in this thesis might not be fully translatable to future human AD studies. However, with proper validation and controls, the reproducibility of the results gathered from animal models can be increased, leading to a higher translational impact.[45] Animal models of AD also allow specific aspects of AD to be studied, as well as allow researchers to perform timepoint studies with invasive methods, such as brain tissue collection, which are not practical in human studies.[46] Because of these advantages, animal models can assist in generating experimental data that may elucidate the underlying mechanisms that are involved with a certain AD pathology. Therefore, it will be important to generate more data from different animal models of AD, such as the tau pathology model[47], and investigate the effects of different AD pathologies on the glutamate-glutamine cycle. These data will guide the design of future human studies and determine how involved is the glutamate-glutamine cycle in the pathogenesis of epilepsy in AD. After that, it will be important to investigate changes in the expression levels of key proteins in glutamate-glutamine cycle in human brain samples acquired from AD patients with epilepsy, as this will bridge the results between animal and human studies. It is worth noting that without animal models of AD, the discovery of novel pathways and treatments in this field will be slow-moving. Therefore, animal and human studies must be used synergistically to optimise the rate that new discoveries in the field of epilepsy in AD are made.

Secondly, there were technical limitations regarding the electrode caps that were used for the kindling experiments. These caps were surgically implanted onto the scalp of each mouse for the experiments in Chapter 4 and 5. Tg2576 mice used in this thesis had thinner skulls than the WT as they were bred based on the c57 x sjl mice and it has been shown in the literature that loss of bone density is associated with the c57 mice.[48] This meant that there was a higher chance of the breaking of the crown in Tg2576 mice, resulting in euthanisation. For future experiments, different configurations for the electrode cap will be considered to reduce the chance of losing the cap. Commercialised stimulation cable made from plastic may be used to reduce the overall weight of the cable; thus, this change will

lower the chance that the cable will pull the cap away from the skull. Additional modifications to the cable such as an attachment on top of the cage that will allow the cable to manoeuvre freely will also be considered. This will reduce the amount of stress on the cap, decrease the chance that the cable will be entangled and allow the animal to roam with less restriction. A change from the screw-on cap system to a magnetic or a clip-based system will also be considered, as this will reduce the amount of force required to plug and unplug the cable from the cap. Change of the background strain for Tg2576 mice may also be beneficial since the thinness of the skull and tumour development susceptibility associated with our colony contributed to the loss of animals in this study.

As a carry-over effect from the limitation discussed above, the final number of mice included in the analyses became smaller than the initial number. Therefore, the analyses might not provide enough power to detect small changes. For example, the number of WT mice used in the seizure susceptibility study was 3; therefore, the results associated with this experiment became insignificant. Small sample sizes were also caused by other limitations associated with the use of animal models. For instance, the ageing of the mice cannot not be rushed; therefore, it took a long time before each mouse arrived at the desired age. Secondly, loss of animals could occur at any time due to the nature of the experiment and it was impractical to replace the animals that required premature euthanisation during an experiment. In the future, the risk of premature euthanisation should be taken into consideration and this risk should be compensated with the appropriate starting number of animals. Furthermore, a better experimental design will also help reduce the chance of losing an animal during the experiment.

Third, it is possible that the gender-effect may have caused some disparities in the expression levels of key proteins in the glutamate-glutamine cycle within each group, which were reflected on the western blot results. However, as discussed in Chapter 4 and touched on in Chapter 5, there was a lack of evidence to support the significance of gender-effect on the expression levels of key proteins in glutamate-glutamine cycle. Furthermore, the effect of ceftriaxone on the expression level of GLT-1 was significant in the mixed-gender Tg2576 mice group. This finding suggested that the gender-effect on the efficacy of ceftriaxone may not be significant. Despite this finding, future studies should still address the potential gender-effect on the efficacy of ceftriaxone and the expression levels of key proteins in glutamate-glutamine cycle by performing a pilot study. The pilot study should mimic the conditions of the main experiment and compare the outcomes between age-matched male and female subjects to rule out the potential gender-effects.

Finally, the use of Racine behavioural scale to score the severity of induced seizure was also a limitation. Racine scale[49] has the maximum score of Class V, which limited how the data was statistically analysed. Descriptive statistics became more challenging to perform as the seizure severity in most animals reached the maximum seizure class of Class V after a number of stimulations. In future experiments, a modified Racine scale similarly to what was used by Palop and colleagues in 2007 will be considered.[50] This modified Racine scale categorises 8 stages of seizures based on the behaviour of the animals: Class 0 = normal exploratory behaviour; Class I = immobility; Class II = generalised spasm, tremble, or twitch; Class III = tail extension; Class IV = forelimb clonus; Class V = generalised clonic activity; Class VI = bouncing or running seizures; Class VII = full tonic extension and Class VIII = death.[50]

6.4) Future research directions

It will be important to generate more data from animal studies on the topic of glutamate-glutamine cycle in the pathogenesis of epilepsy in AD to guide the design of future human studies. Despite the fact that the use of animal models may lead to limitations associated with the translational aspect of the results, there is a lack of literature on the mechanism that links AD to epilepsy. By generating more insights on this mechanism from animal studies, future human studies can be better designed. Improving the design of future human studies will be important because the main challenge that is associated with human studies is the difficulty in obtaining the samples. Therefore, data generated from animal studies will assist in determining the best timepoint to collect valuable samples from human subjects. By investigating the human brain samples acquired from human AD patients, insight on how the full spectrum of AD pathologies may affect the glutamate-glutamine cycle and cause an increase in seizure susceptibility will be generated. The evidence gathered from human studies on this topic may reveal novel drug targets and allow novel treatments for epilepsy in AD to be discovered.

Regarding the metabolomic studies, utilising aged (>12-month-old) Tg2576 mice will reveal valuable insights into how the metabolomic profile of the brain is affected by the later stage of mutant human APP overexpression pathology. Mature Tg2576 mice were used in this thesis, and this gave us insight on how mutant human APP overexpression pathology affected the brain before the formation of Aβ plaque. Therefore, using the older Tg2576 mice will give us insights on how metabolomic pathways identified in mature Tg2576 might be exacerbated in aged Tg2576 mice. Additionally, it will be important to investigate whether the concentrations of the metabolites, such as glutamate and glutamine, in the brain of aged Tg2576 mice are altered as this may reveal the optimal therapeutic window for the potential

treatment targets identified by the metabolomic study in the hope of reducing seizure susceptibility in AD.

Furthermore, developing a specific GLT-1 enhancer will be beneficial for future research on this topic, as there is evidence to support the role of GLT-1 in the pathogenesis of epilepsy and AD in animal models. The specific enhancer may cause the effects of GLT-1 enhancement to be more pronounced, since the results may not be contaminated by the adverse effects associated with the current GLT-1 enhancer, such as ceftriaxone.[51] Moreover, existing AEDs that target the glutamatergic pathway, such as perampanel, should also be tested under AD conditions. Since the mechanisms of action of these existing drugs are well characterised, they will generate more insights on the involvement of the glutamatergic pathway in the pathogenesis of epilepsy in AD. Other than perampanel, the anti-seizure and anti-epileptogenic effects of memantine, which is an approved treatment for AD, should also be investigated. As discussed earlier, memantine acts through the glutamatergic pathway to improve the cognitive outcomes of AD patients; thus, using memantine to treat epilepsy in AD patients may provide beneficial effects on the symptoms of both diseases.

Another potentially beneficial course of action will be to add the “WT mice treated with ceftriaxone” group to future studies based on Chapter 5 of this thesis. This group will provide insights on whether ceftriaxone can reduce the kindling susceptibility in WT animals. It will also allow the investigation of the mechanism of action of ceftriaxone in the kindling model of epilepsy without the interference from the effects of mutant human APP overexpression pathology associated with the Tg2576 model, giving us clearer understanding on how GLT-1 enhancement provides neuroprotective effect to the brain and reduce the overall seizure susceptibility.

It will also be intriguing to investigate the effect of ceftriaxone in more severe mouse models of AD, especially the models that display both the mutated human APP and mutated human tau pathologies, such as the 3xTg[52] and the J20 x rTg4510 models.[53] Such models of AD may better represent human AD than the Tg2576 model. Using the tau/APP models will also reveal important insights on whether ceftriaxone can reduce the seizure susceptibility phenotype when both tau and APP pathologies are present. Furthermore, it will also be important to investigate how ceftriaxone affects the cognitive function of tau/APP mice as this may reveal whether enhancing the GLT-1 protein expression in the brain can improve the cognitive outcomes when both tau and APP pathologies are present. Thus, this investigation will increase the translatability of the results for future human studies.

Finally, investigating changes in the level of extracellular glutamate in the brain of Tg2576 mice in real-time will reveal the significance of the disruption to glutamate-glutamine cycle on the glutamate homeostasis. Since it has been shown in Chapter 4 that the disruption to the glutamate-glutamine cycle is involved in the pathogenesis of both epilepsy and AD, it will be important to determine if this disruption may translate into changes in the level of glutamate in the extracellular space. Detection techniques such as microdialysis will allow such investigation.[54] If no significant change in the level of extracellular glutamate is detected during the later stage of AD pathology, then this may suggest that there is a compensatory mechanism counteracting the effects of glutamate-glutamine cycle disruption. Thus, this investigation may indicate if other glutamatergic pathways are involved in rebalancing the glutamate level in the extracellular space under AD condition, allowing a more thorough elucidation of the glutamatergic pathways that are involved in the pathogenesis of epilepsy in AD.

6.5) Conclusion

The evidence to support the role of the glutamate-glutamine cycle disruption in the pathogenesis of epilepsy in AD has been demonstrated in this thesis. The evidence from human and animal studies on the role of glutamate-glutamine cycle in the pathogenesis of epilepsy in AD was thoroughly discussed in Chapter 2. Through the use of metabolomic analyses, Chapter 3 demonstrated that glutamate metabolism is one of the earliest pathways to be altered by the mutant human APP overexpression pathology in Tg2576 mice. Then, Chapter 4 revealed that the alterations in the expression levels of key proteins in glutamate-glutamine cycle, such as GLT-1 and GS, by the mutant human APP overexpression pathology might be associated with an increase in seizure susceptibility in Tg2576 mice. Finally, it was demonstrated in Chapter 5 that pharmacologically enhancing the GLT-1 expression level in the brain of Tg2576 mice with ceftriaxone may have provided an anti-seizure effect in these mice. Therefore, pharmacologically targeting GLT-1, which is a key protein in glutamate-glutamine cycle, may provide neuroprotective effect against excitotoxicity under AD conditions. Data gathered from this thesis will pave the way for future studies in the field of epilepsy in AD.

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Appendices

Appendix A

Abstract: Epilepsy Society of Australia Annual Scientific Meeting (2019). Sydney, Australia

Title: Loss of GLT-1 and glutamine synthetase are associated with early stage of Alzheimer's disease in mice

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Alzheimer's disease (AD) can increase the risk of epileptogenesis up to 10-fold in patients, compared to healthy age-matched controls. However, the underlying mechanisms leading to this increased risk have not been discovered.

OBJECTIVES: Here we proposed that changes in the brain occurring early in the AD disease process contribute to a susceptibility to epileptogenesis. Early disruption in the brain's glutamate homeostasis has been reported in both epilepsy and AD and therefore this study aimed to explore the potential role of glutamate in the pathogenesis of acquired epilepsy in AD. It also aimed to identify potential early biomarkers for acquired epilepsy in AD.

METHODS: Brain tissue was excised from 6 month-old Tg2576 AD mice along with their wild-type (WT) littermate. Western blotting and mass spectrometry were performed on the extracted brain samples.

RESULTS: Tg2576 mice had significantly lower amounts of GLT-1 and Glutamine synthetase in the cortex, compared to the WT ($p<0.01$). Results from Mass spectrometry have shown that metabolites such as glutamate and glutamine have the potential to be the early biomarkers for acquired epilepsy in AD.

CONCLUSION: The results show disruptions to the glutamate-glutamine cycle in Tg2576 mice, suggestive of impairment in astrocytic function. These findings support the hypothesis that the brain's glutamate homeostasis is affected early in AD and that this might lead to a higher susceptibility of the brain to epileptogenesis via the extracellular glutamate spill-over in the synaptic cleft. The findings from the metabolomics analysis also suggest that there are changes in different brain's metabolites early in AD.

Appendix B

Abstract: CNS Disorders: Advanced Diagnostics and Treatment Conference (2019). Melbourne, Australia.

Title: Role of Glutamate in the pathogenesis of acquired epilepsy in Alzheimer's disease

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Alzheimer's disease (AD) can increase the risk of epileptogenesis up to 10-fold in the patient, compared to healthy age-matched controls. However, the relationship between acquired epilepsy and AD is yet to be elucidated. Here we proposed that changes in the brain that occur early in the AD pathology may lead to this higher susceptibility to epileptogenesis. Disruption in the brain's glutamate homeostasis has been reported in both diseases; therefore, it has the potential to link the two diseases together. This study aimed to explore the potential role of glutamate in the pathogenesis of acquired epilepsy in AD. It also aimed to identify potential early biomarkers or a diagnostic tool for acquired epilepsy in AD. Six-month-old Tg2576 AD mice along with their wild-type (WT) littermate were utilized in this study. The cortex and the hippocampus were extracted from the animal, then western blotting and mass spectrometry were performed. Tg2576 had significantly lower amounts of GLT-1 and Glutamine synthetase in the cortex, compared to the WT. Additionally, mass spectrometry has shown that metabolites such as glutamate and glutamine have the potential to be the early biomarker for acquired epilepsy in AD. The results suggest that the astrocytic function could be impaired early in AD, and this includes the glutamate-glutamine cycle. This impairment might lead to a higher susceptibility of the brain to epileptogenesis via the excessive extracellular glutamate. The findings from the metabolomics analysis also suggest that there are changes in different brain's metabolites early in AD.

Appendix C

Abstract: Student of Brain Research Symposium (2019). Melbourne, Australia.

Title: Loss of GLT-1 and glutamine synthetase are associated with early stage of Alzheimer's disease in mice

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Alzheimer's disease (AD) can increase the risk of epileptogenesis up to 10-fold in patients, compared to healthy age-matched controls. However, the underlying mechanisms leading to this increased risk have not been discovered.

We proposed that changes in the brain occurring in the early AD stage contribute to a susceptibility to epileptogenesis. Early disruption in the brain's glutamate homeostasis has been reported in both epilepsy and AD and therefore this study aimed to explore the potential role of glutamate in the pathogenesis of acquired epilepsy in AD. It also aimed to identify potential early biomarkers for acquired epilepsy in AD.

Cortex tissue was excised from 6 month-old Tg2576 AD mice along with their wild-type (WT) littermate. Western blotting and mass spectrometry were performed on the extracted cortex samples.

Tg2576 mice had significantly lower amounts of GLT-1 and Glutamine synthetase in the cortex, compared to the WT ($p<0.01$). Results from Mass spectrometry have demonstrated that metabolites that are involved in important pathways such as lipid metabolism and glycolysis have the potential to be the early biomarkers for acquired epilepsy in AD.

The results show disruptions to the glutamate-glutamine cycle in Tg2576 mice, suggestive of impairment in astrocytic function. These findings support the hypothesis that the brain's glutamate homeostasis is affected in AD prior to Aβ plaque formation, and this might lead to epileptogenesis via the extracellular glutamate spill-over in the synaptic cleft. The findings from the metabolomics analysis also suggest that there are changes in the brain's metabolomic pathways early in AD.

Appendix D

Abstract: Epilepsy Society of Australia Annual Scientific Meeting (2020). Virtual.

Title: Metabolomics profiling of the brain revealed potential mechanisms associating Alzheimer's disease to higher seizure susceptibility in mice.

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Patients with Alzheimer's disease (AD) have up to 10-fold increased risk of epilepsy, compared to healthy age-matched controls. The underlying mechanisms leading to this increased risk are unclear.

OBJECTIVE: The aim of this study was to identify metabolic pathways that are altered in the early stage of amyloid precursor protein (APP) pathology to generate hypotheses regarding mechanisms associated with increased epilepsy risk in AD. The highly seizure-prone Tg2576 mouse model of AD is an appropriate tool for investigating changes in the brain associated with mutant APP overexpression.

METHODS: Metabolomics data was collected from the cortex and hippocampus of 6-month old Tg2576 mice (n=7) along with their wild-type (WT) littermate (n=7) via liquid chromatography-mass spectrometry. Univariate, multivariate and pathway enrichment analyses were performed on MetaboAnalyst and the weighted correlation network analysis was performed using R.

RESULTS: We identified 11 metabolites (adjusted-p < 0.05, variable importance in projection score > 1) significantly affected by APP overexpression in the cortex. Pathway enrichment analysis yielded 4 significantly enriched metabolic pathways from the cortex and 1 from the hippocampus (adjusted-p < 0.05, pathway impact > 0.2). Network analyses identified 5 pathways that were significantly correlated (adjusted-p < 0.05) with AD genotype. Our analyses suggest that oxidative stress, lipid and amino acid metabolism pathways could be affected early by the APP pathology.

CONCLUSION: This study identified changes in metabolite levels and metabolic pathways that are linked to the early stage of the APP pathology in AD. These findings provide new insights into the early disruption to the metabolic processes in the CNS caused by the APP pathology, which might increase the seizure susceptibility of the brain.

Appendix E

Abstract: Epilepsy Melbourne Annual Symposium (2021). Melbourne, Australia.

Title: Metabolomics profiling of the brain revealed potential mechanisms associating Alzheimer's disease to higher seizure susceptibility in mice.

Dejakaisaya H[1], Harutyunyan A[2], Kwan P[1,2], Jones NC[1,2]

[1]Department of Neuroscience, Monash University;

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Patients with Alzheimer's disease (AD) have up to 10-fold increased risk of epilepsy, compared to healthy age-matched controls. The underlying mechanisms leading to this increased risk are unclear.

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CONCLUSION: This study identified changes in metabolite levels and metabolic pathways that are linked to the early stage of the APP pathology in AD. These findings provide new insights into the early disruption to the metabolic processes in the CNS caused by the APP pathology, which might increase the seizure susceptibility of the brain.

Appendix F

Abstract: 13th Asian and Oceanian Epilepsy Congress (2021). Virtual.

Title: Metabolomics profiling of the brain revealed potential mechanisms associating Alzheimer's disease to higher seizure susceptibility in mice.

Dejakaisaya H[1], Harutyunyan A[2], Kwan P[1,2], Jones NC[1,2]

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Patients with Alzheimer's disease (AD) have up to 10-fold increased risk of epilepsy, compared to healthy age-matched controls. The underlying mechanisms leading to this increased risk are unclear.

PURPOSE: The aim of this study was to identify metabolic pathways that are altered in the early stage of amyloid precursor protein (APP) pathology to generate hypotheses regarding mechanisms associated with increased epilepsy risk in AD. The highly seizure-prone Tg2576 mouse model of AD is an appropriate tool for investigating changes in the brain associated to mutant APP overexpression.

METHODS: Metabolomics data was collected from the cortex and hippocampus of 6-month old Tg2576 mice (n=7) along with their wild-type (WT) littermate (n=7) via liquid chromatography-mass spectrometry. Univariate, multivariate and pathway enrichment analyses were performed on MetaboAnalyst, and the weighted correlation network analysis was performed using R.

RESULTS: We identified 11 metabolites (adjusted-$p < 0.05$, variable importance in projection score > 1) significantly affected by APP overexpression in the cortex. Pathway enrichment analysis yielded 4 significantly enriched metabolic pathways from the cortex and 1 from the hippocampus (adjusted-$p < 0.05$, pathway impact > 0.2). Network analyses identified 5 pathways that were significantly correlated (adjusted-$p < 0.05$) with AD genotype. Our analyses suggest that oxidative stress, lipid and amino acid metabolism pathways, such as glutamate, could be affected early by the APP pathology.

CONCLUSION: This study identified changes in metabolite levels and metabolic pathways that are linked to the early stage of the APP pathology in AD. These findings provide new insights into the early disruption to the metabolic processes in the CNS caused by the APP pathology, which might increase the seizure susceptibility of the brain.