Translational investigations of the intersection between Alzheimer’s Disease and Epilepsy: mechanistic insights and treatment opportunities

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Abstract

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by progressive cognitive deficits compromising patients’ execution of daily activities. The incidence of unprovoked seizures has been reported to be higher in AD compared to healthy age matched population and is considered a risk factor for the development of acquired epilepsy. Abnormal electrical activity has also been correlated with increased cognitive decline in these patients and may be associated with a faster progression of AD symptoms. Thus, understanding how seizures develop in AD and how to treat them would be of extreme relevance for developing more effective therapies. The work presented in this thesis used a bidirectional approach to investigate the relationship between epilepsy and AD and potential treatment options.

First, three antiepileptic drugs were assessed in their ability to delay the development of acquired epileptogenesis in the Tg2576 mice. These widely studied AD mice overexpress the human form of the amyloid precursor protein (APP) with a mutation that results in increased deposition of amyloid plaques with age. The amygdala kindling model was used to induce epileptogenesis and previous studies have shown that these mice have increased susceptibility to kindling induced seizures. The antiepileptic drugs brivaracetam (BRV), levetiracetam (LEV) and lacosamide (LAC) were administered via subcutaneously implanted osmotic pumps. BRV and LEV act through ligand binding to the SV2A synaptic vesicle protein and LAC through Na\(^+\) channels. After 28 days of continuous treatment with either one of the drugs or vehicle (VEH) animals were submitted to daily electrical stimulations, also under treatment, and the only drug able to slow down the epileptogenic process was BRV.

Therefore, in the second study, the effect of BRV was further investigated. Mice were then pre-treated for 28 days, but pumps were removed, and drug allowed to wash-out for one week before kindling. BRV demonstrated a long-lasting effect and treated Tg2576 mice required significantly more stimulations to experience the first class 5 seizure. Investigations of the potential mechanisms underlying this effect revealed that BRV is not acting through changes in the expression levels of APP protein or SV2 mRNA.

Lastly, with an approach in the opposite direction, a histopathological evaluation of resected tissue from patients with temporal lobe epilepsy (TLE) aimed at correlating the
cognitive deficits often found in these patients with the presence of AD lesion hallmarks (tau and amyloid pathology). However, no significant tau deposition was found and although amyloid plaques were present in a higher proportion of patients compared to other similar studies, there was no strong evidence to suggest it is the mechanism responsible for cognitive impairments.

In conclusion, network treatment strategies, such as antiepileptic drugs, could be useful in the treatment of seizures in AD, and in this study a potential antiepileptogenic effect was demonstrated for BRV in an AD-acquired epilepsy model. We have also shown that amyloid and tau pathology may not be the main underlying cause of cognitive deficits in TLE, but understanding these mechanisms might also advance our understanding of cognitive decline in AD.
**Declaration**

This is to certify that:

i. the thesis comprises only my original work except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

Juliana de Castro e Silva

March 2019
Preface

Chapters 2 and 3 of this thesis will be combined into one manuscript that is currently being refined for future publication. These studies have been performed thanks to a Pre-clinical Investigator Initiated Study funded by UCB Pharma. The company funded the costs of the Chapter 2 and provided the drugs tested in both studies. I have carried out all in vivo experiments described, analyzed the data and wrote the initial manuscript draft with the assistance and guidance from Dr Jianxiong Chan. The western blotting experiments were carried out by Dr Shijie Liu and I was trained and assisted in the qPCR technique by Dr Ezgi Ozturk. Prof Patrick Kwan and A/Prof Nigel Jones jointly conceived this proposal and were involved in the analysis and interpretation of data.

Chapter 4 describes work that has been jointly created by Prof Patrick Kwan, Prof Terence O’Brien, Dr Lucy Vivash and Dr Charles Malpas. The manuscript containing the results for this chapter is also being drafted by me and prepared for submission. I independently carried out the selection of the best specimen tissue blocks for subsequent sectioning by the Pathology staff from The Royal Melbourne Hospital, and amyloid plaques immunostaining after training by Dr Ian Birchall from the Florey Institute of Neurosciences. The immunostaining for tau was performed by Dr Catriona McLean at Anatomical Pathology laboratory facilities at the Alfred Hospital. Dr Catriona has also assisted with analysis and interpretation of the pathology results. Dr Lucy Vivash and Dr Charles Malpas have participated in the assembly, analysis and interpretation of data. I also would like to acknowledge the contribution of Dr Marian Todaro that arranged approval of the project by the Melbourne Health Human Research Ethics Committee and assisted me with the application to obtain the control tissue specimens from the Victoria Brain Bank.

My contribution to experiments described in each chapter involved collection and assembly of data, statistical analysis, interpretation of results and manuscript writing.
Acknowledgement

First and foremost, to my supervisors, Professor Patrick Kwan and Associate Professor Nigel Jones for the incredible opportunity to complete my PhD degree under your supervision. Thank you for trusting me with this project and for always believing in my capacity, for all the challenging opportunities that made me grow and go beyond my comfort zone. You guided me through this degree with great support and patience and I value all your constructive feedback. It was an absolute honor to be supervised by you.

I would also like to sincerely thank all members of the Department of Medicine/Neuroscience that somehow helped me during the last years. Without your support this PhD would not be possible: Prof Terence O’Brien, Dr Jianxiong Chan, Dr Shijie Liu, Dr Ezgi Ozturk, Crystal Li, Dr Shôbi Sivathamboo, Dr Pablo Casillas-Espinosa, Dr Emma Braine, Dr Charles Malpas, Dr Lucy Vivash.

I would like to specially thank my husband, my best companion and friend, that supported me in unprecedent ways and accepted the adventure of moving to another country with me. Without your love and care I would not be able to accomplish this.

Also, I want to thank my family, my mum and dad, and my friends for supporting my decision to pursue this dream.

This thesis could not have happened without any of you.
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Bibliographical references
Publications and scientific presentations

Manuscripts in preparation

Published articles

Scientific presentations


Contributed talk: Epileptogenesis in animal models of Alzheimer’s disease – role of amyloid. Epilepsy Melbourne @ MBC. Melbourne, Australia, March 2018.


Scholarships and Awards

2018: Epilepsy Society of Australia Travel Scholarship

2015: Nick Christopher PhD Top-Up Scholarship

2015: Melbourne International Research Scholarship

2015: Melbourne International Fee Remission Scholarship
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<td>AD</td>
<td>Alzheimer's disease</td>
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<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
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<tr>
<td>NIA-AA</td>
<td>National Institute on Aging and the Alzheimer’s Association</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>PSEN 1</td>
<td>Presenilin 1 gene</td>
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<tr>
<td>PSEN 2</td>
<td>Presenilin 2 gene</td>
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<td>APOE</td>
<td>Apolipoprotein E</td>
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<td>Aß</td>
<td>Amyloid-ß peptide</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<td>pTau</td>
<td>Phosphorylated tau</td>
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<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>FDG</td>
<td>$^{18}$Fluorodeoxyglucose</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>AChE</td>
<td>Acetyl cholinesterase</td>
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<td>ACh</td>
<td>Acetyl choline</td>
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<tr>
<td>NMDAR</td>
<td>$N$-methyl-D-aspartate receptor</td>
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<tr>
<td>hAPP</td>
<td>Human amyloid precursor protein</td>
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<tr>
<td>FAD</td>
<td>Familial Alzheimer's disease</td>
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<td>ILAE</td>
<td>International League Against Epilepsy</td>
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<td>TBI</td>
<td>Traumatic brain injury</td>
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<td>AED</td>
<td>Antiepileptic drug</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>LEV</td>
<td>Levetiracetam</td>
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<tr>
<td>BRV</td>
<td>Brivaracetam</td>
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<td>SV2A</td>
<td>Synaptic vesicle glycoprotein 2A</td>
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<tr>
<td>SV2B</td>
<td>Synaptic vesicle glycoprotein 2B</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SV2C</td>
<td>Synaptic vesicle glycoprotein 2C</td>
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<tr>
<td>MES</td>
<td>Maximal electroshock seizure test</td>
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<td>TLE</td>
<td>Temporal lobe epilepsy</td>
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<td>EEG</td>
<td>Electroencephalographic recording</td>
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<td>LTM-EEG</td>
<td>Long-term EEG monitoring</td>
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<tr>
<td>MEG</td>
<td>Magnetoencephalography</td>
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<td>AβO</td>
<td>Aβ oligomers</td>
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<tr>
<td>LAC</td>
<td>Lacosamide</td>
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<td>ADT</td>
<td>Afterdischarge threshold current</td>
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<td>WT</td>
<td>wild-type</td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<td>RAVLT</td>
<td>Rey Auditory Verbal Learning Test</td>
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<td>VPA</td>
<td>Verbal Pair Associates test</td>
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<td>Rey Complex Figure Test</td>
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<td>AT8</td>
<td>Antibody against hyperphosphorylated tau</td>
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<td>1E8</td>
<td>Antibody against amyloid-β plaques</td>
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<tr>
<td>GTCS</td>
<td>Generalized tonic clonic seizures</td>
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<td>HS</td>
<td>Hippocampal sclerosis</td>
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Chapter 1

Alzheimer’s disease and Epilepsy: a review of the literature on this bidirectional relationship

1.1. Alzheimer’s disease

1.1.1. Incidence and prevalence

Alzheimer’s disease (AD) is a neurodegenerative disease that accounts for 60 to 80% of the cases of dementia (Alzheimer's Association, 2016). This condition is characterised by cognitive deficits that compromise the execution of daily activities, like memory loss and difficulty in evoking recent events, comprehension and speaking deficits, reduced capacity of planning and object recognition (Scheltens et al., 2016). Dementia can be divided into three stages: preclinical, mild cognitive impairment (MCI) and dementia, and the chronic profile leads to symptoms that become progressively severe, hampering patients of their independence, autonomy and freedom (Albert et al., 2011; Sperling et al., 2011). As recommended by the National Institute on Aging and the Alzheimer’s Association (NIA-AA), a diagnosis of dementia, in general, is given when the cognitive or neuropsychiatric (behavioural) symptoms start to interfere with the ability to work or perform everyday activities, when it is noted as a decline from previous levels of functioning and cannot be explained by a major psychiatric disorder (McKhann et al., 2011). The cognitive or behavioural symptoms were defined as impairments involving at least two of the following domains: ability to acquire and recall new information, poor judgement and handling of complex tasks, visuospatial abilities, language functions, and changes in personality and behaviour. These impairments can be diagnosed combining neuropsychological testing and patient history taken from a knowledgeable companion and the patient itself. These individuals then clinically diagnosed with dementia can be classified as “probable or possible AD”, but a definite diagnosis can only be given after a post-mortem assessment of the brain (Hyman and Trojanowski, 1997). However, the word “AD” has been used over the years to refer to both, the dementia syndrome and the underlying pathology. Thus, in 2018, a new criterion has determined that in the context of research the “AD” term denotes the pathology (amyloid-β deposition, tau pathology, and neurodegeneration), and the clinical consequence is then termed “Alzheimer’s dementia” or “Alzheimer’s clinical syndrome” (Jack et al., 2018). Therefore,
throughout this thesis the term “AD” is used to refer to the pathophysiological processes that result in a clinical dementia syndrome.

Dementia affects around 50 million people worldwide with almost 10 million new cases every year (World Health Organization, 2017). It comprises a group of neurocognitive disorders, i.e. dementia with Lewy bodies, vascular dementia, behaviour variant frontotemporal dementia, and Alzheimer’s dementia contributes to almost the same number of deaths caused by cancer in the U.S.A (Roman et al., 1993; McKeith et al., 2005; Rascovsky et al., 2007; James et al., 2014; Alzheimer's Association, 2015). The risk of developing Alzheimer’s dementia increases with age and its prevalence reaches around 30% in the population ageing over 85 years old (Ballard et al., 2011). With the progressive increase in life expectancy, it is estimated that the case frequency will triple in the next 30 years, bringing Alzheimer’s dementia to the level of an important case of public health (World Health Organization, 2017).

Alzheimer’s dementia can be of genetic origin (autosomal dominantly inherited AD) or unknown cause (sporadic forms). Mutations have been characterized in three genes that account for the autosomal dominant cases, in the amyloid precursor protein (APP) and in the presenilin 1 and 2 genes (PSEN1 and PSEN2) (Tanzi, 2012). These mutations are associated with an early onset of the disease in most cases (<65 years old), and have been initially characterized in large families with Alzheimer’s dementia, although they only refer to 0.5% of all Alzheimer’s cases (Cacace et al., 2016). The late-onset forms can be associated or not with a genetic risk factor, which can be a family history of Alzheimer’s dementia or the apolipoprotein E (APOE) genotype (Liu et al., 2013). The presence of one or two copies of the APOE e4 allele is associated with a 3 to 15-fold increased risk of developing Alzheimer’s dementia compared to the other allele, e3 and e2 (Rhinne et al., 2013). APOE plays a large role in Aβ metabolism, aggregation and clearance, but its main function is as a cholesterol carrier, supporting lipid transport in the bloodstream and injury repair in the brain (Kim et al., 2009; Liu et al., 2013).

1.1.2. Pathological hallmarks of Alzheimer’s disease

The primary histopathological hallmarks of AD are the extracellular plaques of β-amyloid peptide (Aβ) and the intracellular neurofibrillary tangles (NFT) formed by hyperphosphorylated tau protein (Galimberti and Scarpini, 2011). The Aβ peptide is naturally
found in neurons as an integral part of the membrane-bound amyloid precursor protein (APP), and is secreted to the extracellular compartment as a product of the enzymatic degradation of APP. The imbalance between the production and clearance of this peptide leads to its accumulation and aggregation (Querfurth and LaFerla, 2010). Additionally, the tau protein is also found in neurons but associated with microtubules. When tau becomes hyperphosphorylated it aggregates creating the tangles. Due to these neuropathological alterations, the neurotransmission becomes abnormal in different regions of the Central Nervous System (CNS).

In non-pathological situations, these proteins have important physiological functions. Some research has shown that endogenous APP protein plays a role in multiple stages of cortical development, including neuronal migration, synaptic formation and electrical activity (Priller et al., 2006; Young-Pearse et al., 2007; Zheng and Koo, 2011; Hoe et al., 2012). The APP processing occurs naturally in two ways, the amyloidogenic and the non-amyloidogenic pathway (Figure 1.1). In the first cleavage processing, the enzymes β- and γ-secretases can generate the Aβ peptide in different isoforms Aβ40, Aβ42, Aβ43 (Nhan et al., 2015; Muller et al., 2017). Mutations might be present in the APP gene around the β- and γ-secretases cleavage sites and in these cases, an increase in the Aβ42/Aβ40 ratio is observed (Citron et al., 1992; Suzuki et al., 1994). The longer forms of Aβ have additional hydrophobic amino acid residues that make the peptide more susceptible to aggregation, resulting in the formation of dimers, trimmers, large oligomers, protofibrils and ultimately amyloid plaques (Jarrett et al., 1993; Selkoe and Podlisny, 2002). In contrast, the products of the non-amyloidogenic pathway have been shown to have a neuroprotective effect and to increase neurite outgrowth and enhance learning and memory (Gakhar-Koppole et al., 2008; Young-Pearse et al., 2008). In this processing pathway, APP is cleaved by α- and γ-secretase generating the secreted APPα and P3 fragments.
The Aβ peptide role in the CNS has been attributed to synaptic plasticity and memory modulation (Kamenetz et al., 2003). After APP expression inhibition or after administration of an Aβ antibody, animals showed deficits on long-term potentiation (LTP) and memory tests (Puzzo et al., 2011). Nonetheless, the synaptic plasticity and the cognitive decline were restored after Aβ42 introduction at physiologic levels. These studies show that the peptide plays a significant role in the maintenance of cognitive function homeostasis. However, high levels of Aβ peptide are harmful to normal execution of animal behaviours, leading to a complete change in the neuronal activity pattern (for review see Walsh and Selkoe, 2007; Morley and Farr, 2012).

The neurotoxicity of Aβ was first demonstrated by Yankner et al. (1990) using different synthetic fragments of Aβ in hippocampal cell cultures. The biochemical mechanism of this process involves the rapid production of hydrogen peroxide and lipid peroxidation after Aβ addition to the neuronal cells, suggesting that damage caused by free radicals and oxidative stress are involved in the neurotoxicity process (Behl et al., 1994; Yankner, 1996; Velez-Pardo et al., 1998). Furthermore, there is also evidence that Aβ peptide alters ionic cellular activity by interacting with channels and affecting cerebral ionic homeostasis (Fraser et al., 1997). Over time, the super production or the insufficient clearance of Aβ peptides favours oligomer generation and its accumulation as insoluble extracellular Aβ plaques (Selkoe and Podlisny, 2002).
The amyloid hypothesis has been the dominant model of AD pathogenesis for decades (Selkoe and Hardy, 2016). However, major critiques have arisen from biomarker studies including failing of Aβ-directed therapies to improve clinical outcomes in AD patients and the fact that there is a group of people with extensive amyloid pathology and are cognitively normal (Bennett et al., 2006; Salloway et al., 2014). Therefore, a growing body of evidence has indicated that amyloid alone, while considerably relevant, is not sufficient to cause Alzheimer’s dementia and that the joint effect of other neurodegeneration markers is more likely to induce the complex symptoms of AD. Considering this, in the staging of preclinical AD the deposition of Aβ alone represents the earliest stage only, followed by subsequent neurodegeneration and changes in brain function, and the third stage is reflected by subtle, asymptomatic cognitive decline (Sperling et al., 2011). In addition, studies suggest that Aβ fragments may also exert its detrimental effects through interactions with the tau protein leading to its abnormal phosphorylation and aggregation and to the next phase of the disease (Roberson et al., 2007; Stancu et al., 2014).

Under normal physiological conditions, tau protein maintains a balance of coupling and uncoupling with the microtubules. This balance is primarily driven by phosphorylated tau, regulated by a variety of kinases and phosphatases that allow microtubules stabilisation (Mazanetz and Fischer, 2007; Morris et al., 2011). On the other hand, under pathological conditions this balance is affected, a super production of phosphorylated tau (pTau) reduces its microtubule affinity and, consequently, disrupts cells’ cytoskeleton and cause neuronal death (Ballatore et al., 2007; Gendron and Petrucelli, 2009; Martin et al., 2011). Pathologically, hyperphosphorylated tau aggregates as neurofibrillary tangles (NFTs), accumulates on neurons, astrocytes and oligodendroglia, leading to a synaptic and axonal dysfunction and, consequently, neurodegeneration (Mazanetz and Fischer, 2007). The pattern of tau aggregation and accumulation throughout the brain regions in AD develops in a predictable way and a staging system was proposed in 1991 by Braak and Braak (Braak and Braak, 1991). The classification into Braak stages from I to VI are now part of the post-mortem histopathological diagnosis of AD.

In the new NIA-AA research framework, a descriptive classification scheme was then proposed based on the three general groups of biomarkers used in AD research, Aβ plaques, tau tangles and neurodegeneration or neuronal injury [termed AT(N)] (Jack et al., 2018). The amyloid biomarkers (A) comprise low cerebrospinal fluid (CSF) Aβ42 and positive cortical amyloid positron emission tomography (PET) ligand binding (Villain et al., 2012). The tau biomarkers (T) encompass elevated CSF pTau and positive tau PET ligand binding (Buerger
et al., 2006; Brier et al., 2016). The neurodegeneration biomarkers (N), comprise elevated total tau (tTau), decreased $^{18}$fluorodeoxyglucose (FDG) uptake on PET (representative of brain hypometabolism), and atrophy on structural magnetic resonance imaging (MRI), but because they can be attributed to many other different causes, they are not considered the main features of AD like the other two (Fox et al., 2001; Landau et al., 2011; Besson et al., 2015).

### 1.1.3. Current treatment of Alzheimer’s disease

Although significant advances on the characterization of AD pathology have been made in the last years, no disease-modifying or preventable treatment is available yet. Currently, five drugs are approved by the U.S. Food and Drug Administration (FDA) for AD symptomatic treatment, all of those acting through neurotransmitter increase (Alzheimer's Association, 2015). The main targeted system is the cholinergic system and available drugs act as acetyl cholinesterase (AChE) inhibitors (for review see Pepeu and Giovannini, 2009; Aisen et al., 2012). An impairment on this neurotransmission has been shown since the first reports on AD neurochemistry as a decline in AChE activity, acetyl choline (ACh) and ACh receptors (AChR), and both APP and Aβ have already been linked to this (Nalivaeva and Turner, 2016). The anticholinergic therapy has proved its efficacy on patient’s reported outcomes such as cognition, memory, communication and the ability to perform daily activities (Zemek et al., 2014). Drugs are, in general, well tolerated and the most common side effects are typical of cholinergic agents like nausea, diarrhoea, vomiting, constipation, dizziness and weight loss (Deardorff and Grossberg, 2016).

The other possibility of treatment that is usually prescript in combination with AChE inhibitors is memantine, used for moderate to severe AD (Lo and Grossberg, 2011). It is clinically well-tolerated with only mild side effects reported over the years (Dominguez et al., 2011). Memantine’s main mechanism of action consists in reduce the effects of glutamatergic excitotoxicity through non-competitive NMDA receptor (NMDAR) antagonism. Besides that, a non-specific neurotransmitter modulation is reported for memantine, as it can act on serotonergic, dopaminergic and cholinergic systems with similar potency as that for the NMDAR (Rammes et al., 2001; Aracava et al., 2005; Dominguez et al., 2011; Deardorff and Grossberg, 2016). Although, no new drug for AD treatment has been introduced in the last 13 years, a combination of memantine and AChE inhibitors is frequently used due to its complementary activity that may provide additive benefits compared with inhibitors monotherapy (Deardorff and Grossberg, 2016).
The challenge of developing treatments for Alzheimer’s dementia also relies on the fact that it is a neurodegenerative syndrome, and thus is characterized by multiple dimensions, defined by clinical outcomes of one or more diseases. In fact, post-mortem studies have shown multiple associated pathologies in confirmed cases of AD that also impact cognition, including vascular pathologies, hippocampal sclerosis and white matter changes (Schneider et al., 2007; Matthews et al., 2009). Moreover, comorbidities such as depression, psychosis, bipolar disorder and epilepsy are often diagnosed during the course of the disease and are also thought to be major contributors to the overall burden of Alzheimer’s dementia (Novais and Starkstein, 2015; Haaksma et al., 2017; DeMichele-Sweet et al., 2018). Therefore, there is not one strategy, but rather a combination of therapies and a multi-target approach that should be driving AD research.

1.1.4. Animal models in Alzheimer’s research

Considering the mere symptomatic effectiveness of the available treatments, the search for new compounds becomes more urgent every year. In this context, animal models play a crucial role in Alzheimer’s research as an important tool to help understand the disease pathophysiological aspects and in the screening of new drugs. The creation of transgenic lines became possible with the discovery of genes associated with AD, and despite some divided opinion regarding their advantages, they are extensively used (Kokjohn and Roher, 2009; Zahs and Ashe, 2010; Webster et al., 2014). The wide variety of models can be separated into two main kinds of mutations, the autosomal dominant mutations, associated to the familial forms of AD; and the polymorphisms on risk factor genes, predominantly related to sporadic and late onset cases (for review see Hall and Roberson, 2012).

Although the autosomal dominant forms of AD are quite rare, the most widely used mouse models of AD are based on transgenic expression of human APP (hAPP), with many different transgenic lines that develop robust amyloid pathology and memory deficits. Other important models of familial AD target PSEN1 and 2 genes that encode the catalytic subunit of γ-secretase and the mutations result mainly in an increase in the Aβ42/Aβ40 ratio (Shen, 2014; Stiller et al., 2014). Despite exhibiting a massive and often rapid accumulation of APP, Aβ peptides or amyloid plaques, none of these familial AD (FAD) transgenic mice can replicate the full spectrum of the pathology, in special neuronal loss and NFT-like lesions (Kokjohn and Roher, 2009). New animal models were then generated to express amyloid plaques and NFT-
like lesions, through a combination of mutations in the tau protein isolated from the spectrum of frontotemporal dementias and FAD mutations (Haugarvoll et al., 2007; Elder et al., 2010).

Finally, it is important to bear in mind that Alzheimer’s and the diseases that comprise the dementia continuum are a group of complex intricate features that cannot be reduced to an overexpression and accumulation of malfunctioning proteins. Associated comorbidities like anxiety, depression, mood disorders and even epilepsy, that join the diagnosis somewhere in the course of the disease, increase the complexity of this condition and hinders treatment options. However, one might consider these downstream features of AD as a treatable component and therefore as a window of opportunity to improve patients’ quality of life.

1.2. Epilepsy

1.2.1. Epidemiology of epilepsy

The International League Against Epilepsy (ILAE) has defined epileptic seizure as a “transient occurrence of signs and/or symptoms due to an abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). Epilepsy then is characterized by a persisting predisposition of the brain to generate epileptic seizures and can perpetuate so that the patient may require medical care throughout life (Fisher et al., 2005; Liu et al., 2010; Fisher et al., 2014). The epileptic activity has a significant and lasting impact in the brain and a few spontaneous seizures may increase the risk for subsequent episodes, of greater severity or not, indicating that the hypersynchronous activity generates continuous changes in neuronal network excitability (Hauser et al., 1998; Cornejo et al., 2007). This lasting structural and functional reorganization of the neuronal circuitry is accompanied by a decline in cognitive and behavioural functions (Sayin et al., 2003; Sutula, 2004). Moreover, epileptic seizures can affect consciousness, emotional state, memory, sensory, motor and autonomic functions (Fisher et al., 2005). Not all seizures affect all these factors, but all of them influence at least one, and although cognitive deficits in epileptic patients are very common, the pattern and extension of these deficits are widely variable (Avanzini et al., 2013).

Epilepsy affects individuals of all ages and is one of the most common neurological diseases along with stroke, Alzheimer’s and Parkinson’s disease, affecting 50 million people worldwide according to the World Health Organisation (2016). The prevalence of active epilepsy (people with continuing seizures or with the need for antiepileptic treatment) in the general population is between 4 and 10 per 1000 people, and between 7 and 14 in low- and –
middle income countries. Every year diagnosis of 2.4 million new cases of epilepsy are estimated, being two times higher in low-income countries (between 30 and 50 per 100 000 people). However, incidence and prevalence data may be underestimated in some parts of the world as epilepsy is still a disease with a great stigma and so leads to the concealing of the symptoms and diagnosis (Banerjee et al., 2009).

1.2.2. Classification and causes of epilepsy

According to the terms defined by the ILAE Commission on Classification and Terminology, with respect to aetiology epilepsy may be of genetic origin, unknown cause or due to some structural damage or metabolic dysfunction (Berg and Scheffer, 2011). About the latter, it can be said that they are associated with a substantial increased risk of developing epilepsy. Structural lesions responsible for that include acquired disorders, such as stroke, traumatic brain injury (TBI), infections, tumours, neurodegenerative diseases, intracerebral haemorrhage among others, and are then referred to as acquired epilepsies (Berkovic et al., 2006; Loscher and Brandt, 2010). While people at risk can be identified, there is currently no approved intervention to prevent epilepsy development (Dichter, 2009a; Loscher and Brandt, 2010).

The epilepsies of genetic origin, also called idiopathic epilepsies, comprise a group of complex disorders often determined by multiple genes and non-inheritable factors (for review see Berkovic and Scheffer, 2001; Glazier et al., 2002). The majority of these genetic defects affect voltage-gated ion channels, in particular K⁺ and Na⁺ channels, and ligand-gated channels, including GABA_A and nicotinic ACh receptors (Steinlein, 2004; Mulley et al., 2005a). Various missense mutations have been described for the most important gene associated with epilepsy discovered so far, SCN1A, which encodes a subunit of Na⁺ channels (Mulley et al., 2005b). These different types of mutations in SCN1A can result in a wide variety of epileptic phenotypes, highlighting the complexity of the genetics of epilepsy (Scheffer and Berkovic, 1997; Claes et al., 2001). Moreover, increasing knowledge in the physiology of ion channels and the discovery of such defects, in conjunction with a better understanding of the genetics of the epilepsies, have paved the way to the development of most of the anti-epileptic drugs currently available (Meldrum and Rogawski, 2007).

About 40% of all cases of epilepsy have acquired causes (Banerjee et al., 2009). Acquired insults promote a cascade of changes in the neuronal circuits that ultimately lead to the occurrence of spontaneous recurrent epileptic seizures (Loscher, 1993; Dichter, 2009a).
This insult-induced process in which a normal brain ultimately becomes epileptic, is called epileptogenesis (Pitkanen and Lukasiuk, 2011). The biochemical, morphological, neurophysiological changes associated to this increased neuronal excitability are still poorly understood but can include inflammatory processes, neuronal loss, neurogenesis, gliosis, blood-brain barrier changes, molecular reorganization and many more (Dichter, 2009b; Engel et al., 2013; Shultz et al., 2013; Webster et al., 2017). Similarly, acquired insults might also permanently affect the structure of circuits, and hippocampal sclerosis and mossy fibre sprouting are the main features (Parent et al., 1997; Malmgren and Thom, 2012). In acquired epilepsies, a characteristic latent period exists between the precipitating injury and the appearance of the first epileptic seizure (for review see Loscher et al., 2015). This concept has motivated numerous studies, both experimental and clinical, to design and test therapies that can prophylactically prevent or, at least, modify development of epilepsy following a brain injury. Although the goal of drug development in epilepsy over the years has been to find an antiepileptogenic compound, no truly disease modifying therapy has been found despite all efforts (Loscher and Brandt, 2010; Pitkanen and Lukasiuk, 2011).

1.2.3. Antiepileptic drugs in the treatment of epilepsy

Historically, antiepileptic drugs (AED) can be classified in 3 generations. The first includes drugs commercialized between 1857 and 1958, like potassium bromide, phenobarbital and a number of drugs derived from the barbiturates chemical structure (Loscher and Schmidt, 2011). The second generation encompasses drugs introduced between 1960 and 1975 and chemically different from the barbiturates, like carbamazepine, valproate and the benzodiazepines (Shorvon, 2009). After valproate was added in 1960, no new AED was introduced for almost two decades, except for a few benzodiazepines (Loscher and Schmidt, 2011).

Thereafter, an increase in the knowledge about the pathophysiology of neurological diseases and in drug pharmacology, more rational strategies for drug development were enabled (Figure 1.2). 1980 was the beginning of the third generation of AEDs, including compounds selectively designed to target the basic molecular mechanisms of epilepsy. Experimental findings started to reinforce the idea that an impairment on the GABAergic inhibition and/or on the glutamateergic excitation systems could be critically involved in the generation and propagation of seizures (Meldrum, 1992; Porter and Rogawski, 1992; Loscher, 1993). Besides that, evidences started to suggest that abnormalities on ion channels or calcium...
regulated enzymatic processes could be the basis of altered neuronal excitability resulting in epileptic activity (DeLorenzo, 1988). Thus, drugs that acted increasing GABA mediated neuronal inhibition (e.g. tiagabine, progabide, vigabatrin), reducing glutamate mediated excitation (e.g. felbamate, topiramate), modulating sodium channels (e.g. carbamazepine, lamotrigine, lacosamide) and specially calcium channels (e.g. pregabalin, gabapentin) became objects of study for epilepsy treatment (Porter and Rogawski, 1992; Loscher and Schmidt, 1994).

![Diagram](image.png)

**Figure 1.2.** Representation of the molecular targets and mechanisms of action of the main antiepileptic drugs used in the treatment of epilepsy. Image obtained from (Löscher et al., 2016), reproduced with permission of Springer Nature.

A new possibility in the AED development, in terms of molecular target, arose with the development of levetiracetam (LEV) and later, brivaracetam (BRV). These drugs shifted the paradigm in the AED discovery and represent an example of a successful shared effort between academia and industry (Löscher et al., 2016). The two drugs act through ligand binding to the synaptic vesicle glycoprotein 2A (SV2A) and hinder neuronal overexcitation. The SV2A proteins can be found integrating the synaptic vesicle membranes of presynaptic terminals and
have been proven to be involved in vesicle trafficking and exocytosis (Janz et al., 1999; Bartholome et al., 2017). Notably, when tested in the amygdala kindling model, LEV had a notorious effect not only in retarding kindling acquisition but in the fact that this outlasted the period of drug treatment indicating a potential antiepileptogenic activity, a finding that was also confirmed later by other groups (Loscher et al., 1998). On the other hand, BRV has proven to have a higher affinity for the receptor, higher brain-blood barrier permeability, and also a distinctly greater potency in models of both generalized and partial seizures (Bialer et al., 2009; Nicolas et al., 2016; Wood et al., 2018). Studies on the LEV capacity to reduce neurotransmission using hippocampal slices from non-epileptic rats, showed that it decreased vesicular release in a dose-, time-, and stimulation-dependent manner, therefore requiring repetitive stimulation for it to be able to entre recycling synaptic vesicles (Yang et al., 2007; Yang and Rothman, 2009). For BRV, it has been shown to decrease synaptic transmission in concentrations 100-fold lower than LEV (Yang et al., 2015). For a complete history on how these drugs came to the approval by the FDA for the adjunctive treatment of complex partial seizures, LEV in 1999 and BRV in 2016, see reviews by Klitgaard and Verdru (2007) and Klitgaard et al. (2016), respectively.

However, despite the advantages over the first and second-generation drugs, because of a reduced risk of idiosyncratic and hyper sensibility reactions and more specific targets, studies have been showing that the new generation AEDs are no more effective than the older ones, and only a few have similar efficacy (Kwan and Brodie, 2000; Chen et al., 2018b). Around 50% of the acquired epilepsy cases are still resistant to treatment and about 30% of all epilepsy patients do not respond to any of the available drugs (Perucca, 1997; McIntosh et al., 2004; Patsalos, 2013). This is due to the fact that these drugs have been designed for seizure control and not as disease-modifying therapy. None of the commercially available AEDs are able to stop or reverse the process that turns a healthy brain into an epileptic one, named epileptogenesis (Loscher and Brandt, 2010). However, understanding the mechanisms that relate acquired epilepsies to other disorders and neurodegeneration may provide important information about the molecular and cellular bases of this epileptogenic process. In acquired epilepsies, there is a latent period that can vary from weeks to years between the initial insult and the onset of epileptic seizures. This period is an important temporal window as it provides an opportunity for interventions that can prevent or stop the epileptogenic process (Loscher et al., 2015).
1.2.4. Animal models used in the study of epilepsy

Nonetheless, to identify these neuroprotective or antiepileptogenic compounds, confirm its efficacy and safety is challenging and also depends on the selection of appropriate animal models (Denayer et al., 2014; Loscher, 2016). All the current antiseizure drugs clinically used in the treatment of epilepsy were mostly identified by either the maximal electroshock seizure (MES) test, subcutaneous pentylenetetrazole (sc PTZ) seizure test and the kindling model for TLE (Loscher, 2017). The MES test is a predictive model for tonic-clonic generalized seizures, induced by a transcorneal or transauricular suprathreshold electrical stimulus in normal mice or rats that led to the discovery of phenytoin (Putnam and Merritt, 1937; Bialer and White, 2010). The sc PTZ is also a seizure model, useful on tests of drugs for generalized nonconvulsive seizures (absence, myoclonic) and when injected into normal animals induces a clonic seizure through an antagonistic effect at the GABA_A receptor (Loscher et al., 1991).

Different from the previous two, the kindling model of epilepsy has a chronic profile. A depth electrode is implanted on the limbic system and used to deliver repeated electrical stimulations (McIntyre et al., 2002). The seizures progressively increase in severity, until a stage where the animal is considered to be fully kindled and starts to present spontaneous recurrent seizures. Kindling can also be used as a model of TLE because it induces long-lasting alterations in the brain similar to those occurring in human TLE (Sato et al., 1990). A number of other different models are available in epilepsy research, including genetic models, but similar to the transgenic models of AD they do not reflect all features of the disease (Tan et al., 2007; Wimmer et al., 2010; for review see Maljevic et al., 2017). Therefore, an appropriate screening for new therapies in fact needs to use a combination of such models.

1.3. Epilepsy in Alzheimer’s Disease

AD is considered a risk factor for acquired epilepsy and 10-22% of AD patients experience at least one unprovoked seizure during the course of the disease, with higher rates in familial and early-onset cases (Mendez and Lim, 2003; Amatniek et al., 2006). The incidence of unprovoked seizures is high in AD patients and may lead to a faster progression of AD symptoms, more severe neuronal loss and consequently greater cognitive impairments compared to those patients without seizures (Forstl et al., 1992; McAreavey et al., 1992; Volicer et al., 1995). In FAD, mutations of APP, PSEN1 and PSEN2 genes are associated with
increased seizure risk, with rates being estimated at 28% (ranging from 15-67) (Shea et al., 2016). Around 180 mutations have been described for the PSEN1 gene and more than 30 were found to be positively associated with epileptic seizures (Larner and Doran, 2006).

A multicentre study performed in autosomal dominant early-onset AD patients, linked seizure frequency with the causative mutations and showed that for those with APP duplications the risk of having epileptic seizures was significantly higher than for those with pathogenic mutations with PSEN1, PSEN2 or APP genes (Zarea et al., 2016). Corroborating the findings of a previous 8-year prospective study with both Down’s syndrome and AD patients that reported seizures in 41 of 49 patients (Lai and Williams, 1989). Seizures are more common in autosomal dominant early-onset AD (>45% of cases affected) compared to the sporadic late-onset cases (Zarea et al., 2016). They are mainly focal and localized in the temporal and frontal lobes in sporadic AD, but can be generalized in the familial forms and in the context of Down syndrome, especially in the more advanced stage of dementia (Hommet et al., 2007; Vossel et al., 2013; Zarea et al., 2016; Lam et al., 2017). However, non-motor complex partial seizures are considered the predominant subtype so far and can often overlap with cognitive features of AD, perhaps being interpreted as confusion or delirium, frequently going unnoticed to the untrained eyes (Rao et al., 2009; Cretin et al., 2016; Sarkis et al., 2016).

However, there are major caveats in this relatively new area of research that investigates this intersection between Alzheimer’s and epilepsy. First, scalp electroencephalographic recording (EEG) is the main tool used in the diagnosis of epilepsy and can also be used as a diagnostic instrument in Alzheimer’s dementia (for review see Hegerl and Möller, 1997; Noachtar and Remi, 2009). However, a study by Vossel et al. (2016) has argued that a routine 20-minute electroencephalographic monitoring protocol in awake patients is not sensitive enough to detect epileptiform activity in Alzheimer’s dementia patients. Authors have shown that long-term monitoring (LTM-EEG) would be preferred for these patients, since most of the subclinical epileptiform activity happened during stage 2 sleep, but even though prolonged EEG might not be sufficient. A combination of LTM-EEG with concomitant 1-hour monitoring using resting magnetoencephalography (MEG) offered better sensitivity, since there were epileptiform spike waves detected in the temporal lobe through MEG that did not have a correlate in the EEG and vice versa. Furthermore, it has been previously shown that focal seizures located deep on the mesial temporal lobes not always produce a detectable sign of ictal activity on the scalp EEG (Ebersole and Pacia, 1996; Pacia and Ebersole, 1997). Therefore, the questions on how and for how long Alzheimer’s dementia patients should be monitored so there can be a confident affirmation that these patients do or do not have epilepsy remain open.
A recent study using intracranial EEG monitoring detected clinically silent hippocampal seizures and epileptiform discharges in two patients with AD without substantial scalp EEG abnormalities (Lam et al., 2017). This reinforces the limitation of scalp EEG in the detection of seizures in Alzheimer’s dementia, but also raises the debate on the ethics of doing depth EEG recordings in these patients. As outlined by the review of Noachtar and Remi (2009), invasive EEG-recordings are currently only used when imaging and scalp-EEG show evidence that the epileptogenic foci is potentially resectable, and there is a clear hypothesis on the epileptogenic zone that can be tested with the invasive electrodes, plus there is a low risk of complications. This type of EEG recording leads to complications in 1-4% of patients and can include haemorrhages or infections with consecutive hemiparesis, hemianopsia, or aphasia but this can vary between different types of implants (Arya et al., 2013; Taussig et al., 2015). The use of invasive electrodes, like foramen ovale electrodes, could yes provide a better picture of the incidence of silent seizures in Alzheimer’s dementia patient, but first we cannot discard the benefits and acknowledge the lack of more thorough investigations using LTM-EEG combined with MEG, MRI and/or PET imaging in this patient population.

Nevertheless, this study by Lam et al. (2017) alongside others are challenging the view that occult seizures and epilepsy occur only as a late sequela of neurodegeneration in AD, but instead play an important role in disease progression (Volicer et al., 1995; Vossel et al., 2013). A ‘seizure theory’ of AD has been proposed recently, postulating that abnormal protein development in AD leads to the death of inhibitory interneurons, increases network hyperexcitability which lowers the seizure threshold and leads to chronic subclinical seizures that might be an underlying cause of cognitive impairment (Lam et al., 2017). Supporting this idea, Vossel and colleagues proposed a hypothetical model for this temporal association of network dysfunction and the accumulation of Aβ and tau deposition (Figure 1.3) (Vossel et al., 2017). The authors suggest that a critical period exists during MCI stages, in which an increase in NFT deposition is associated with hippocampal hyperactivation while amyloid begins to plateau (Huijbers et al., 2015; Vossel et al., 2017).
Clinicopathological and neuroimaging studies have also highlighted some common features shared between TLE and AD in the last years. An increased incidence of amyloid plaques was found in 101 TLE patients (aged 30-61 years) compared to 406 autopsy control patients (aged 30-92 years) without epilepsy (Mackenzie and Miller, 1994). Another study also on resected temporal lobe tissue from drug-resistant epilepsy patients (aged 50-65 years) reported the presence of hyperphosphorylated tau in 94% of the patients, and the more severe cases correlated with a decline in verbal learning (Tai et al., 2016). Similarly, a post-mortem study revealed an association of chronic epilepsy and increased tau NFT in a cohort of 138 patients (Thom et al., 2011). Hippocampal sclerosis and a decrease in the expression of the calcium binding protein calbindin-D28k in the dentate gyrus, representative of increased excitation in the area, are also a common feature between TLE and AD (Minkeviciene et al., 2009; Palop and Mucke, 2010; You et al., 2017). From the neuroimaging perspective, a reduced resting-state activity and functional connectivity within the default mode network was found in functional MRI of patients with either AD, TLE or absence epilepsy, suggesting that these diseases share common regions of network dysfunction (Greicius et al., 2004; Luo et al., 2011).

Considering that amyloid plaques can begin to accumulate more than a decade before the first clinical signs of the disease, seizures detected in these early stages before the onset of cognitive decline might then reflect the epileptogenic potential of Aß peptide (Villemagne et al., 2013; Vossel et al., 2013; Bakker et al., 2015). In fact, the link between amyloid plaques and cognitive decline has been challenged for decades although it still remains as the main
focus of therapeutic development. Cumulative data of 20 years of research, pioneered by the work of Lambert et al. (1998), have been showing that soluble Aβ oligomers (AβO), not plaques, can impair learning and memory and prompt major aspects of AD pathology, including inflammation, oxidative stress, tau pathology, synapse loss and epileptiform activity (Lambert et al., 1998; Hartley et al., 1999; Lei et al., 2016; for review see Cline et al., 2018). Data from transgenic mice overexpressing hAPP indicate that high levels of Aβ are sufficient to elicit epileptiform activity, seizures and induce remodelling of inhibitory circuits (Palop et al., 2007). In support of this notion, Minkeviciene et al. (2009) could replicate the increased neuronal excitability detected in the APdE9 transgenic mice by incubating wild-type mice brain slices with soluble fibrillar Aβ species.

Moreover, Cirrito et al. (2005) using acute brain slices have shown that interstitial fluid levels of Aβ are directly influenced by synaptic activity and synaptic vesicle exocytosis, therefore not only Aβ oligomers can induce neuronal excitability but abnormal excitatory activity can also increase the release of soluble Aβ in the extracellular space. Another study using hippocampal slices from APP mice have reported reduced levels of Aβ in media after a 96-hour period of reduced neuronal activity (Kamenetz et al., 2003). Thus, silent seizures and abnormal electrical activity might be early signs of AD as a consequence of this initial phase of amyloid accumulation before plaques can be detected and the appearance of the first cognitive symptoms.

Interestingly, experimental manipulations that prevent seizures in these transgenic animals also reduce cognitive deficits (Roberson et al., 2007; Sanchez et al., 2012; Wang et al., 2016). An increasing number of studies have been investigating antiepileptic treatments in AD mouse models and have reported varied levels of efficacy in suppressing the abnormal spikes and seizures often observed in these animals (Ziyatdinova et al., 2011; Sanchez et al., 2012; Shi et al., 2013). Both experimentally and in clinical trials, the use of the AEDs lamotrigine and levetiracetam have been supported by the strongest evidence (Tekin et al., 1998; Belcastro et al., 2007; Cumbo and Ligori, 2010; Zhang et al., 2014). A recent review on antiepileptic drug treatments in Alzheimer’s have suggested a classification of AEDs for AD-related epilepsy (Cretin, 2018). The authors point out that the challenge in this patient population is not only the AED effectiveness in controlling the seizures, but their cognitive tolerance and ability to not worsen behavioural impairments (Vossel et al., 2013; Vossel et al., 2017; Cretin, 2018). In this sense, the group of AEDs that seem to be ideal for these patients are the ones proven to be cognitively neutral or stimulating drugs.
1.4. Research aims

While the debate on whether to treat subclinical epileptiform activity and silent seizures is still open, there is strong evidence to suggest that AD patients have a higher incidence of seizures compared to the general population and therefore a better understanding of how AEDs act in the Alzheimer’s context is needed. Thus, the first aim of this PhD thesis was to examine the efficacy of antiepileptic drug treatment on preventing or delaying epileptogenesis in the Tg2576 mouse model of AD, in particular levetiracetam and brivaracetam.

Moreover, considering the neurobiological similarities between AD and TLE, a better understanding of these shared mechanisms could bring new insights into the development of therapeutic alternatives to both conditions. The second aim of this PhD was then to investigate whether Alzheimer’s lesion hallmarks, amyloid plaques and tau tangles, could be an underlying cause of the cognitive impairment often observed in patients with drug refractory temporal lobe epilepsy. Overall, these studies explore the commonality of pathways and the bidirectional relationship between these two disorders.

Aim 1: To investigate the effect of chronic treatment with three new generation AEDs, brivaracetam, levetiracetam and lacosamide, on acquired epileptogenesis and amyloid pathology in aged Tg2576 mice.

Hypothesis 1: Chronic treatment with brivaracetam and levetiracetam, but not lacosamide, can reduce the innate susceptibility to epileptogenesis induced by amygdala kindling observed in aged Tg2576 mice.

Aim 2: To investigate a potential long-lasting, antiepileptogenic effect of brivaracetam treatment on the development of amygdala kindling and on the brain expression levels of APP and SV2 mRNA in young Tg2576 mice.

Hypothesis 2: Brivaracetam is able to attenuate the epileptogenesis development induced by amygdala kindling in young Tg2576 mice even after drug wash-out.

Aim 3: To correlate the presence of the clinico-pathological hallmarks of dementia (hyperphosphorylated tau and amyloid plaques) in the resected temporal lobe tissues with pre-
operative cognitive test scores and to identify potential clinical risk factors that mediate the interaction between pathology and cognitive decline.

**Hypothesis 3:** The severity of tau and amyloid pathology in resection specimens is associated with pre-operative cognitive dysfunction in patients with drug-resistant temporal lobe epilepsy.
Chapter 2

Exploring the effects of three antiepileptic drugs on a novel Alzheimer’s disease-acquired epilepsy model

2.1. Introduction

Unprovoked seizures occur at rates 8-10-fold higher in Alzheimer’s dementia patients compared with the general population, and at even higher rates in autosomal-dominant and early onset cases (Hauser et al., 1986; Mendez and Lim, 2003; Amatniek et al., 2006; Cabrejo et al., 2006; Scarmeas et al., 2009). In these familial forms of AD, mutations on APP, PSEN1 and PSEN2 genes are associated with increased seizure risk, with rates being estimated at 28% (ranging from 15-67) (Shea et al., 2016). The different transgenic mouse models of AD currently available were developed using these mutations characterised in humans and have largely contributed to our understanding of the function of these proteins as well as various aspects of the AD pathophysiology. Over the years, studies have been showing that these transgenic mice have abnormal electroencephalographic activity, and some models of more severe mutations also present spontaneous seizures and a considerably high mortality rate possibly due to these seizures (Palop et al., 2007; Westmark et al., 2008; Minkeviciene et al., 2009; Busche et al., 2012; Verret et al., 2012).

The Tg2576 mice is an AD model that has been extensively characterised. These mice overexpress the human isoform of the amyloid precursor protein (APP695) with a double mutation found in a Swedish family with early onset AD (K670N and M671L) and this mutation increases the rate of APP production by almost 6-fold compared to nontransgenic littermates mice (Hsiao et al., 1996). Although the APP expression is elevated and constant throughout the animals’ life, dense amyloid deposits that relate to the plaques observed in humans can only be found at a later stage, over a year of age, and that also correlates with the appearance of memory and learning deficits in these mice (Hsiao et al., 1996; Corcoran et al., 2002; Jacobsen et al., 2006). Long-term EEG recordings of Tg2576 mice have found inter-ictal spikes (IIS) at a surprisingly young age, 5-weeks old, that occurred primarily during rapid-eye movement sleep and IIS frequencies increased with age (Bezzina et al., 2015; Kam et al., 2016). Spontaneous seizures were also detected, although in only 2 out of 5 mice monitored and after 7 months of age. A recent study showed that the Tg2576 mouse model of AD has an...
increased susceptibility to amygdala kindling induced seizures (Chan et al., 2015). This chronic model of TLE consists of repeated induction of focal seizures by electrical stimulations to the amygdala that progressively evolve to generalized seizures (for review see Morimoto et al., 2004). A development of chronic epileptogenesis is then observed and once the generalized, bilateral clonic seizures (class V seizures) are established, the persistent molecular and structural alterations can be observed after months (Racine, 1972). The transgenic animals required significantly fewer stimulations to experience the first class V seizure, had a greater seizure-associated mortality and showed evidence of aberrant axonal connectivity in the form of mossy fiber sprouting. Findings from this study represent a potentially novel model of epilepsy in AD, whereby the epileptogenic process induced by amygdala kindling may be used to study the pharmacologic and cognitive response to AD medications and antiepileptic drugs.

Interventions that suppress network excitability have been shown to improve synaptic and cognitive function both in patients and transgenic mouse models of AD (Sanchez et al., 2012; Shi et al., 2013; Zhang et al., 2014; Bakker et al., 2015; Nygaard et al., 2015). In particular, levetiracetam (LEV), a third-generation antiepileptic drug widely used in the treatment of partial-onset seizures have also shown promising results in several AD clinical trials (Loscher and Honack, 1993; Klitgaard, 2001; Klitgaard and Verdu, 2007). In an open-label, observational study, 72% of the 25 patients with advanced AD and epilepsy under treatment with LEV for 1 year or longer (1000-1500 mg per day) were seizure-free for at least 1 year (Belcastro et al., 2007). A randomized, three-group parallel case-control study of LEV (500-2000 mg per day), lamotrigine (LAM; 25-100 mg per day) and phenobarbital (PHE; 50-100 mg per day) in 95 patients with AD and epilepsy and 68 age-matched control patients with AD but without epilepsy also showed positive results for LEV (Cumbo and Ligori, 2010). Despite the equivalent effect on seizure reduction (response rates: LEV 71%, LAM 59% and PHE 64%), LEV and LAM treatment caused fewer adverse events and resulted in better cognitive outcomes than PHE. Patients with AD and epilepsy under LEV treatment also had improved performance on the Mini-Mental State Examination (MMSE) and on the AD Assessment Scale, similar to AD patients without epilepsy. In another study, patients with MCI seemed to improve hippocampal-based memory performance with a low-dose of LEV treatment twice a day (125 mg each), however whether low-dose is also effective at suppressing AD-associated seizures and epileptiform activity is still unclear (Bakker et al., 2015; Vossel et al., 2017). Finally, there is a phase 2 clinical trial investigating LEV in AD patients in recruitment phase (clinicaltrials.gov, NCT03489044) and a phase 3 clinical trial of LEV to treat
amnestic MCI is already collecting data from patient cohorts (clinicaltrials.gov, NCT01044758).

The discovery of LEV opened up a new path for antiepileptic drugs development. LEV has a mechanism of action that was not typically associated with the previous antiseizure drugs available at the time (Klitgaard and Verdru, 2007). It acts mainly through binding selectively at the synaptic vesicle protein 2A of hypersynchronous neurons and has no activity in normal neuronal response (Margineanu and Klitgaard, 2000). Subsequent to the development of LEV, another ligand with a higher affinity for the SV2A receptor, namely brivaracetam (BRV) (Matagne et al., 2008; Klitgaard et al., 2016), has also been approved by the FDA for the treatment of refractory partial-onset seizures (Mula, 2014). Pre-clinical studies have shown the superior efficacy of BRV over LEV treatment, however there is still a need for clinical trials comparing the two drugs in different epilepsy populations (Matagne et al., 2008; Gillard et al., 2011; Nicolas et al., 2016). So far, the beneficial effects of the treatment with SV2A ligands in AD has mostly been investigated with LEV and not BRV. Therefore, we aimed to investigate the effects of brivaracetam, levetiracetam and lacosamide treatments on epileptogenesis development, amyloid pathology and behavioural outcomes in the Tg2576 mice. Lacosamide was chosen as a control medication because it has a different mechanism of action from levetiracetam and brivaracetam via modulation of neuronal sodium channels.

2.2. **Hypothesis and aims**

**Hypothesis:** Chronic treatment with brivaracetam and levetiracetam, but not lacosamide, can reduce the innate susceptibility to epileptogenesis induced by amygdala kindling observed in aged Tg2576 mice.

**Aims:** To investigate:

1. The effect of chronic treatment with three new generation AEDs, brivaracetam, levetiracetam and lacosamide, on acquired epileptogenesis in aged Tg2576 mice.

2. The effect of the referred AEDs on the amyloid pathology of aged Tg2576 mice after kindling.
2.3. Experimental design

The amygdala kindling model of acquired epileptogenesis was used to test the effect of antiepileptic drug treatments in the Tg2576 mouse model of AD. Aged Tg2576 mice were treated continuously with either, BRV, LEV, LAC or vehicle for 30 days (n = 5-7 per group). Two open field sessions were performed during this period, one before the implantation of the osmotic pumps containing the drugs and another at the end of 23 days, to investigate any potential anxiety-like behaviour induced by the chronic treatment. After this, animals had the pumps removed and new ones were reimplanted to ensure the treatment would continue during the kindling period. A stereotaxic surgery was performed to implant the EEG recording electrodes and the bipolar stimulating electrode in the left amygdala and animals were allowed 7 days to recover. Following this, the afterdischarge threshold current was tested and then mice electrically stimulated once a day for 14 days at their respective threshold current value. Animals were then euthanised at the end of experiment and had the brains collected for quantification of APP expression levels.

![Figure 2.1](image_url)

**Figure 2.1.** Experimental timeline for the treatment, kindling and post-mortem assessments of young wild-type and Tg2576 mice. OF$_1$, open field baseline session; Pi, pump implantation; S, surgery for electrodes implantation; OF$_2$, open field session 2; Pr, pump replacement; ADT, afterdischarge threshold test; K, kindling period; Pm, post-mortem assessments.

2.4. Methods

2.4.1. Animals

Male and female littermates Tg2576 mice, of C57/B6-SJL background, aged between 13-25 months were maintained under controlled temperature (20°C) and lighting conditions (12 h light/dark cycle with lights on at 6:00 am) in the animal facility of the Department of Medicine at the Royal Melbourne Hospital, with *ad libitum* access to food and water. Mice
were housed in groups of 3 or 4 per cage and individually housed after surgical procedures. The aged animals required an extra level of care because of the high mortality rate observed in this age group, and therefore were weighed, health checked and fed with sunflower seeds and mash daily. All procedures herein described had approval from the University of Melbourne Animal Ethics Committee (AEC #13-070 and #15-003 UM) and were conducted according to the guidelines of the National Health & Medical Research Council of Australia Code of Practice for the Care and use of Animals for Experimental Purposes in Australia. The experiments also included randomisation of treatments, blinding during data collection and appropriate control groups.

2.4.2. Antiepileptic drug treatment of aged Tg2576 mice

The drugs used in this study, levetiracetam (LEV), brivaracetam (BRV) and lacosamide (LAC) were provided by UCB Pharma SA (Belgium) in the context of an Investigator Initiated Study. The administration was via mini-osmotic pumps (model 2004; Alzet, Durect, U.S.A) implanted subcutaneously in the interscapular region of Tg2576 mice (Li et al., 2018). This pump model can deliver solutions at a mean pumping rate of 0.23 µL/hour for approximately 30 days. The animals were treated continuously for 30 days prior to and during the whole amygdala kindling stimulation period. The osmotic pumps had to be replaced after 28 days to ensure animals continued receiving treatment until the end of the experiments.

The drugs concentration was calculated for an infusion of 5 mg/kg/day for LAC, 150 mg/kg/day for LEV or 10 mg/kg/day for BRV, all dissolved in sterile saline solution (0.9% NaCl) that was also the treatment for the vehicle group (VEH). Doses were chosen based on previous studies that investigated the efficacy of these drugs in the kindling model, however for LAC a lower dose had to be used because of solubility issues (Brandt et al., 2006; Matagne et al., 2008). Drugs were diluted one day before preparing the pumps that after filled, were incubated at 37 °C in sterile saline for 24 h. The subcutaneous implantation was under isoflurane anaesthesia and all the procedures conducted with the pumps, including the surgery, were performed according to the manufacturer’s instruction.

2.4.3. Open field test

Possible behavioural impairments induced by the antiepileptic drugs were investigated in the open field test. Mice were tested for the occurrence of anxiety-like behaviours in two
time points, the first being the baseline measurement, performed before the pumps were implanted, and the second session was performed after 23 days of continuous treatment. The open field was the test of choice as it has been validated and widely used to assess anxiety-like behaviour and also has the advantage of allowing the detection of abnormalities in general mobility of the animals (Prut and Belzung, 2003; Jones et al., 2008).

The open field is a circular arena of 1 m diameter enclosed by 20 cm walls, with an inner circle of 66 cm diameter, placed in a light-controlled room (Figure 2.1). For each session, mice were individually positioned close to the border of the arena and allowed to explore freely for 10 min. Sessions were video-recorded by a camera placed directly on top of the open field and mice movements were tracked using Ethovision tracking software (v3.1.16 Noldus Information Technology, Netherlands). The parameters evaluated in this test were total distance travelled, latency for the first entry in the inner circle, frequency of entries and time spent in the centre area. A total of 32 mice (including 22 female and 10 male) were tested in the first session then randomised based on sex, age and on their test performance into four groups named according to the treatment to be further administered (BRV, LAC, LEV and VEH; n=5-7 per group). The median age for each experimental group was 19 months for BRV, 14 for LAC, 16 for LEV and 15.5 for VEH. The second session of the open field was performed right before the surgery for pump replacement and kindling electrodes implantation.

2.4.4. Amygdala kindling induced epileptogenesis

2.4.4.1. Electrode surgical implantation

For the amygdala kindling test, mice underwent electrode implantation through a stereotaxic surgery (Tan et al., 2012). For this procedure, under complete anaesthesia by isoflurane, the head region was shaved and disinfected with iso-betadine (10% povidone-iodine) and 4% chlorhexidine gluconate. With the skull exposed, stereotaxic measures were made relative to the bregma point, based on the atlas of Paxinos and Franklin (2001). The stimulating bipolar electrode was implanted into the left amygdala (anteroposterior [AP]: -0.8, mediolateral [ML]: 3.1, dorsoventral [DV]: -5.0), together with two extradural EEG recording electrodes (Figure 2.2). However, the stereotaxic coordinates had to be adjusted for this batch of mice as we used -0.8 mm posterior to bregma, but it actually corresponded to -1.70 mm on the atlas. The bipolar electrode consists of two individually insulated stainless steel twisted wires, with 6 mm height and 0.28 mm diameter (Plastics one, USA), and the recording electrodes of a gold pin (Ginder Scientific, Canada) manually soldered to a screw. Dental
Cement was used to cover the exposed region and animals received a subcutaneous injection of the anti-inflammatory and analgesic carprofen (5 mg/kg with an injection volume of 1 ml/kg). After surgery mice were weighed and health checked twice a day for five days.

**Figure 2.2.** Coronal section of a mouse brain showing the position where the bipolar stimulating electrode (yellow cylinder) was targeted is represented on the left (figure modified from Paxinos and Franklin, 2001). An illustration of the position of the electrodes implanted on mice brain for amygdala kindling and cortical EEG recording. 1: ground electrode; 2: recording electrode; 3: reference electrode; 4: kindling bipolar electrode; Black dot: bregma point.

### 2.4.4.2. Amygdala kindling procedure

After a 1-week of postsurgical recovery, kindling started by testing animal’s after discharge threshold (ADT) current with stimulations of the bipolar electrode using an Accupulser pulse generator/stimulator connected to a battery-operated constant stimulus isolator (WPI, U.S.A.). The stimulation consisted of a 1-second train of biphasic square wave pulses of 4.5-millisecond duration and 5 V. Consecutive stimulations were given to the amygdala electrode, starting at 0.04 mA up to 0.4 mA, with 0.02 mA increments every 1 minute until an after discharge, a local seizure triggered by the electrical current, was evoked. The
ADT was defined as the lowest intensity stimulation producing an after discharge of at least 5 sec duration. To evaluate the amygdala kindling development in the wild-type and Tg2576 mice, a constant current stimulation at the respective ADT value for each animal, was given once a day every day for 2 weeks. Seizure length was measured from EEG recordings obtained with LabChart software (ADInstruments, Australia) and defined as the time between the end of the electrical stimulation and the after-discharge cessation. Racine scale was used to assess seizure severity and is described in table 2.1 (Racine, 1972; Durmuller and Porsolt, 2003).

Table 2.1. Stages of motor seizure development after electrical amygdala kindling, according to the Racine scale (Racine, 1972).

<table>
<thead>
<tr>
<th>Class</th>
<th>Animal behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No seizure</td>
</tr>
<tr>
<td>1</td>
<td>Mouth and facial automatism</td>
</tr>
<tr>
<td>2</td>
<td>Head nodding and jerks</td>
</tr>
<tr>
<td>3</td>
<td>Score 2 plus forelimb clonus</td>
</tr>
<tr>
<td>4</td>
<td>Score 3 plus rearing, Straub tail (nearly vertical)</td>
</tr>
<tr>
<td>5</td>
<td>Score 4 plus animal falling over</td>
</tr>
</tbody>
</table>

2.4.5. **Tissue collection and APP quantification by western blot**

At the end of the stimulation period, animals that did not die during the amygdala kindling period were euthanized through CO₂ inhalation and had the brains excised. The left hemisphere was collected and used for histological assessment of the bipolar electrode placement. The contralateral hemisphere was micro dissected into amygdala, hippocampus and cortex for protein analysis and should reflect changes in APP expression as a result of recurrent generalized seizures. Previous studies have shown that once generalized motor seizures are evoked in amygdala kindling, molecular changes including protein and gene expression correlate between the two hemispheres (Hosford et al., 1995; Christensen et al., 2010). The brain samples were frozen in liquid nitrogen and stored at -80 °C for subsequent western blotting quantification of amyloid precursor protein (APP).

The samples were aliquoted, ground on dry ice and dissolved in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 0.5% sodium
deoxycholate and 1% NP-40] with protease inhibitors cocktail and phosphatase inhibitors cocktail. The brain extracts were centrifuged at 12 000 g for 10 min at 24°C and the supernatant was used for western blotting. The protein concentration was determined with a BCA kit, the supernatant was mixed with 4X SDS sample buffer [300 mM Tris-HCl (pH 6.8), 300 mM dithiothreitol, 12% SDS, 0.6% bromophenol blue, and 60% glycerol [5:1 (v/v) ratio], boiled for 5 min at 95°C, then centrifuged at 12 000 g for 10 min, and the supernatant was stored at −80°C for western blotting analysis. Proteins were separated with SDS-PAGE, and the bands of proteins were electro-blotted onto polyvinyl difluoride (PVDF) membranes. These membranes were stripped and blotted with anti-mouse APP WO-2 (1:10 000). After stripping a second time, the membranes were rebotted by anti-ß-actin (1:750 000). All protein blots were visualized by enhanced chemiluminescent substrate kit and exposed to X-ray film. Blots were scanned in high-resolution and the mean intensity of the blots was quantified using NIH ImageJ software (Abramoff et al., 2004). The ratio of immunoreactivity of APP in relation to the loading control (ß-actin) was calculated and the results were expressed for each treatment and brain region.

2.4.6. Data analysis

Statistical significance was assessed using GraphPad Prism software (v7.0a; La Jolla, CA, U.S.A.) and data is presented as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) and Bonferroni’s multiple comparison test were used to analyse the open field data (total distance travelled, latency for the first entry in the inner circle, frequency of entries and time spent in centre area). Two-way ANOVA was used to analyse seizure severity and duration in the amygdala kindling. The independent variable assessed with ANOVA was treatment. In this study, it wasn’t possible to use repeated measures for the kindling data because animals died of seizures in different stages of the kindling and therefore not all animals completed the 2 weeks of stimulations. Differences in the ADT and number of stimulations for the first class V seizure were analysed with one-way ANOVA based on a Gaussian distribution and Tukey’s post hoc assessment. The latter was the primary endpoint of the kindling experiments and behavioural seizure progression and ADT were secondary. Statistical significance of protein expression levels was determined with unpaired t-test. The significance level was set at p < 0.05.
2.5. Results

2.5.1. Epileptogenic profile of aged Tg2576 mice in the amygdala kindling model of epilepsy after treatment with different AEDs

The kindling model can be used to assess effects of drug on epileptogenesis acquisition. As seizures induced by repeated stimulations become progressively more severe, drugs can be compared in their capacities of delaying or preventing this progression. In our study, vehicle (VEH), lacosamide (LAC), brivaracetam (BRV) or levetiracetam (LEV) were given for a period of 30 days prior to the stimulations, and also during the kindling acquisition period via an osmotic-pump subcutaneously implanted. Results of the second trial of the open field test, after 23 days of continuous treatment, showed no significant differences between the Tg2576 mice treated with VEH and the antiepileptic drugs in all four parameters analysed (Figure 2.3): total distance travelled ($p = 0.3347; F_{(3,26)} = 1.1850$), time spent in the inner circle $p = 0.5514; F_{(3,25)} = 0.7165$), frequency of entry ($p = 0.8811; F_{(3,25)} = 0.2206$) and latency for the first entry in the centre region ($p = 0.2178; F_{(3,25)} = 1.5850$).

**Figure 2.3.** Effect of 23-days continuous treatment with VEH, LEV, LAC or BRV on four parameters related to anxiety-like behaviour in aged Tg2576 mice tested on the open field. Data represent mean ± SEM; n = 5-7 per group.
In the kindling, although no difference was observed in the afterdischarge threshold (ADT) value between VEH and the drug treated groups (F(3,18) = 0.40, p = 0.75; Figure 2.4A), the afterdischarge duration was shorter in the BRV treated animals (F(3,18) = 3.17, p < 0.05; Figure 2.4B). A representative afterdischarge from an animal in the VEH and in the BRV group is shown in figure 2.4C and D respectively, highlighting the significant reduction in the seizure duration.

When assessing the potential effects of the drugs on epileptogenesis, the BRV treatment was also the only one able to retard the acquisition process (Figure 2.5A) promoting a reduction in the severity of seizures especially in the first 5 days of stimulation (F(3,128) = 10.23; p < 0.001; Figure 2.5B) and also reducing the length of these seizures (F(3,128) = 1.031; p < 0.001; Figure 2.5C and D). Because of the innate susceptibility of the Tg2576 mice to amygdala kindling, the VEH treated group presented seizures of scores 3 and 4 on day 1 of stimulation. However, this effect was attenuated with BRV treatment, and animals required significantly more stimulations to reach the first class V seizure compared to the VEH group (p = 0.046; Figure 2.5E).

The effect of BRV on aged Tg2576 mice was also reflected in the mortality rate. On the VEH treated group, 50% of the animals died during kindling immediately after a class 5 seizure, 33% on the LEV group, 20% on the LAC but there was no death in the group treated with BRV.
**Figure 2.4.** After-discharge threshold current (ADT) necessary to elicit electrographic seizures (A; p = 0.72) and duration of this respective afterdischarge in aged Tg2576 mice treated with either VEH, LAC, LEV or BRV (B; p < 0.05). Representative afterdischarge from a Tg2576 mouse treated with VEH (C) and from a mouse treated with BRV (D). Electrographic artefacts from the electrical stimulus are highlighted with the red line and the blue arrow indicates the end of the seizure. The y axis shows the signal amplitude in mV and the x axis the duration in seconds. Data represent mean ± S.E.M; n = 5-7 per group.
Figure 2.5. Progression of behavioural seizure severity during electrical amygdala kindling in Tg2576 mice (A). Pre-treatment with BRV delayed the epileptogenic process and reduced seizure severity in comparison to VEH group significantly in the first 5 days of stimulation as highlighted in B (p < 0.001). Treatment with BRV also reduced the seizure duration (C) and the first five days of kindling in which the effect was more pronounced is highlighted in D (p < 0.001). Tg2576 mice treated with BRV required significantly more stimulations to present the first class V seizure (E; p = 0.046). Data represent mean ± S.E.M; n = 5-7 per group.
2.5.2. Antiepileptic drug treatment did not affect APP expression levels in aged Tg2576 mice

To investigate whether the results found in the amygdala kindling test reflected an altered expression of APP, western blot analysis were performed in limbic regions of Tg2576 mice post-kindling. Results revealed that in regard to APP expression none of the treatments significantly affected the ratio of APP to β-actin as they were similar to VEH in both cortex, amygdala and hippocampus samples (p = 0.736, p = 0.965, p = 0.115, respectively; Figure 2.6). Wild-type kindled mice of the same age were used as controls and, since mouse APP is expressed in physiological levels in these mice, no APP band was detected compared to the marked band of human APP seen for the Tg2576 mice. Lastly, since WO-2 antibody is also used to recognize Aβ residues, through our western blotting protocol an Aβ band at 4 kDa could not be detected in the brain sections of aged Tg2576 mice analysed in this study and therefore could not be compared between treatment groups (data not showed).
Figure 2.6. Treatments with LAC, LEV or BRV were similar to VEH and had no effect on the ratio of APP (anti-mouse WO-2 antibody; 1:10000) to β-actin immunoreactivity in the amygdala, hippocampus and cortex of kindled Tg2576 mice (p = 0.736, p = 0.965, p = 0.115, respectively). Data represent mean ± S.E.M; n = 4-5 per group. IR = immunoreactivity; WT = wild-type kindled; Tg = Tg2576 kindled with no treatment.
2.6. Discussion

The main finding of the present study was that chronic treatment with brivaracetam was effective in slowing down the epileptogenesis in the seizure-prone Tg2576 mice and that this effect was not mediated by modulation of APP expression. The Tg2576 mice is one of the first AD models to be created based on APP overexpression, it expresses the Swedish mutation (K670N/M671L) and exhibits an age-dependent amyloid deposition (Hsiao et al., 1996). It is widely used in AD research and also one of the most well characterized animal models. However, considering what the recent literature have been showing that seizures and epilepsy might play an important role in the development and progression of the Alzheimer’s pathology, there is still a lack of studies characterising the seizure profile of transgenic mice (Lam et al., 2017; Vossel et al., 2017). Abnormal epileptiform activity has been reported for a few animal models of AD, such as the hAPPJ20, APP/PS1, APdE9, 3xTg and the Tg2576 mice, with spikes and clusters of spike wave discharges being the most common electrographic features (Palop et al., 2007; Minkeviciene et al., 2009; Nygaard et al., 2015; Kam et al., 2016). When detected, the electrographic seizures in these models have little or no motor manifestations, although generalized seizures have been reported, especially for the animals expressing both APP and PSEN1 mutations. In regard to testing these transgenic mice in models of epilepsy most studies have only investigated susceptibility to acute seizures chemically induced by kainate or PTZ (Del Vecchio et al., 2004; Palop et al., 2007; Roberson et al., 2007; Bezzina et al., 2015). Our group was the first to demonstrate in a chronic model that epileptogenesis was significantly facilitated in 12-month-old Tg2576 mice compared to WT littermates, resulting in faster increments in seizure progression and increased rates of seizure-induced mortality in the amygdala kindling (Chan et al., 2015). Post-mortem analysis of brain tissue showed that these mice have increased levels of mossy fiber sprouting in the supragranular layer of the dentate gyrus independent of kindling when compared to WT animals. This aberrant hippocampal circuitry has also been reported for other AD animal models and it might play a role in the kindling susceptibility of these mice (Palop et al., 2007). Considering the innate seizure-prone profile of the Tg2576 mice we believe this could be used as a novel model to study acquired epilepsy in AD. This model then allows us to screen drug treatments that would be relevant for epileptic seizure control in the context of Alzheimer’s pathology as well as to explore different AD-like pathologies on epilepsy development.

In our study, we used this model to evaluate the efficacy of three new generation AEDs, lacosamide (LAC), levetiracetam (LEV) and brivaracetam (BRV). These drugs have diverse
mechanisms of action and in between LEV and BRV we could test different potencies as they have a common target but BRV has a higher affinity for it (SV2A receptor) (Gillard et al., 2011). The choice of treating the animals for 30 days prior to the commencement of stimulations in addition to the treatment during kindling allowed us to investigate possible disease modifying effects of a long-term continuous administration of the drugs. The doses were chosen based on previous studies that validated these AEDs on amygdala kindling model, except for LAC in which the dose had to be lowered because of solubility issues and the capacity of the osmotic pumps (Loscher and Honack, 1993; Brandt et al., 2006; Matagne et al., 2008). As expected with these doses, none of the drugs induced anxiety-like behaviours in the animals as shown by the open field results.

The superior anti-seizure and anti-epileptogenic effect of BRV over LEV found in preclinical studies was corroborated in our study as the BRV dose was 15 times lower than the LEV and it still showed a pronounced effect on kindling (Matagne et al., 2008). ADT values were not different between the groups, and a similar current intensity produced significantly shorter afterdischarges in animals treated with BRV compared to VEH treated animals (p<0.05). BRV was also the only drug that significantly slowed the epileptogenic process by reducing seizure severity and duration and increasing the number of stimulations required to reach the first generalized tonic-clonic seizure. Both BRV and LEV act by reducing neurotransmitter release in a synaptic activity-dependent manner, suggesting that repetitive stimulation is required to allow drug action (Klitgaard et al., 2016). Animals then pre-treated with BRV might have benefitted from a reduction in the intrinsic abnormal excitatory activity induced by amyloid, and the higher specificity of BRV for the SV2A target may have conferred it an advantage. Moreover, the most prominent effect of BRV was observed on the first 5 days of kindling. During this period, Tg2576 mice treated with VEH progress very quickly from focal to convulsive responses due to their seizure prone phenotype, but this was reversed by BRV that hampered generalization and reduced seizure length.

Recently, a work compared different AEDs, including LEV but not BRV, on a transgenic mouse model of AD (hAPPJ20) and only LEV was able to reduce abnormal spike activity, reverse behavioural abnormalities, cognitive impairments, remodelling of hippocampal circuits, and synaptic deficits (Sanchez et al., 2012). Likewise, Shi et al. (2013) suggested similar protective effects on behaviour deficits and neuropathology in APPswe/PS1dE9 transgenic mice. Another study showed that spike wave discharges in APP/PS1 transgenic mice is associated with poor cognitive performance on spatial memory tasks, and this can be reversed with BRV but not ethosuximide (Nygaard et al., 2015). These
data in conjunction with ours suggest that the mechanisms targeted by BRV and LEV may be critically involved in AD epileptogenesis, opening an array of possibilities to be further investigated. In contrast, LAC did not have an effect in the kindling induced seizures and it might also not be the best antiepileptic alternative for the treatment of seizures in AD patients since it has been reported that it can worsen cognition or behaviour abnormalities in the older epileptic subjects, even though it has been proven to be cognitively safe in younger patients (Javed et al., 2015; Meador et al., 2016; Sarkis et al., 2017).

Our experiments showed that BRV is not acting through the reduction of APP expression to slow the kindling rate as the protein expression levels were not affected by the drug treatment in neither of the three limbic regions investigated, cortex, amygdala and hippocampus. Interestingly, a number of studies in the past decade have been trying to understand the relationship between APP and the proteins involved in neurotransmitter release (Yang et al., 2005; Priller et al., 2006; Lassek et al., 2014). These works resulted in APP being recently included in the proteome of the presynaptic active zone and it is also thought to structurally and functionally modulate neuronal communication and signalling (Lassek et al., 2013; Lassek et al., 2016). The mechanism through which Aβ and APP act to increase neurotransmitter release in the presynaptic terminal was described by Fogel et al. (2014) and we believe this might explain the positive effects we observe with the SV2A modulators in animal models of AD. The authors showed that an increase in the extracellular concentration of Aβ_{40} results in an increase in synaptic vesicle exocytosis and glutamate release, both in mouse neuronal hippocampal cell culture and slices. This happens because APP can act as a cell-surface receptor that responds to extracellular Aβ_{40} fragments. The binding of the two molecules results in the formation of APP homodimers in the neuron membrane that when activated forms a signalling complex with G_o proteins, promoting an increase in Ca^{2+} influx in the presynaptic bouton and subsequently glutamate release. Under pathological conditions, a deficit in the feedback mechanism that controls Aβ production and clearance leads to an increase in synaptic activity. More specifically, in transgenic mouse models of AD the overexpression of APP and consequently high levels of Aβ oligomers in the extracellular space could create an environment of constant excitability and therefore these animals could be more susceptible to the development of epilepsy. Hence why young Tg2576 mice, even months before the age in which amyloid plaques are detected, are as susceptible to epileptogenesis induced by amygdala kindling as the aged Tg2576 mice (data not shown). However, future studies will need to investigate whether the increased APP expression in this AD mouse model is responsible for increased neurotransmission and excitability that reflects in increased seizure
susceptibility. Furthermore, patients with MCI and high Aβ levels have decreased hippocampal activity, smaller hippocampal volumes and slightly greater functional impairment, and longitudinally showed persistent greater fMRI activity, higher rates of hippocampal atrophy, greater cognitive decline and faster clinical progression, when compared to MCI patients with low Aβ (Huijbers et al., 2015). Notably, soluble Aβ oligomers have been demonstrated to impair hippocampal LTP in a way similar to the glutamate uptake inhibitor DL-threo-β-benzylxyaspartic acid (TBOA) (Lei et al., 2016). This study performed in wild-type mouse hippocampal slices, showed that the disruption in synaptic function by soluble Aβ oligomers is mediated by an increase in NMDAR-dependent neuronal excitability and a simultaneous dysfunction of GABAergic interneuron activity. Thus, considering that the main mechanism of LEV and BRV is to reduce neurotransmitter release in hyperactive neuronal circuits, these drugs, in particular BRV, might be the perfect candidates to disrupt this vicious cycle caused by Aβ fragments considering that increased synaptic activity is also associated with increased Aβ release in the extracellular space (Cirrito et al., 2005).

In conclusion, the findings from this study show that, compared with LEV and LAC, BRV had a significant effect in reducing this innate seizure prone profile of the Tg2576 mice in aged animals. Eventually, all animals reached the stage of class 5 seizures, so this suggests that the process is delayed, and is not a result of the anti-seizure actions but rather suggest an anti-epileptogenic effect. However, the continuous treatment protocol was a limitation of this study in a way that it does not allow us to completely distinguish the anti-seizure from the anti-epileptogenic effect, thus the next chapter of this thesis will use a different treatment paradigm to cover this. For future studies, quantification of Aβ oligomers in the Tg2576 mice before and after treatment with BRV, as well as in other AD models, would also provide a better understanding of the results observed in this study. If synaptic activity and Aβ oligomer levels are actually linked, then interventions with AEDs like BRV and LEV might be an interesting approach for AD and MCI patients, but for this more studies investigating the effects of AEDs in animal models of AD are needed. Furthermore, understanding how epilepsy, subclinical seizures and abnormal spike activity impact the AD brain as well as developing pharmacological treatments that specifically target hyperexcitability in AD should be a critical priority.
Chapter 3

Potential antiepileptogenic effects of brivaracetam in an animal model of Alzheimer’s disease

3.1. Introduction

SV2A targeting drugs seems to be the most promising drugs for the treatment of epileptic seizures in the Alzheimer’s context and this have been supported by numerous studies in animal models of AD. As reported in Chapter 2 of this thesis, BRV unveiled an effect in delaying epileptogenesis acquisition in aged Tg2576 mice worth being investigated. Thus, after identifying the limitation of the treatment protocol in the previous study, i.e. drugs continued during the kindling stimulation phase, we changed the experimental design in order to evaluate the potential disease modifying effect of BRV. Mice were pre-treated with BRV and the kindling development was tested after a period of drug wash out. In addition, this time Tg2576 mice were tested at a young age (4-6 months). Also, as mentioned in the previous chapter, APP overexpression itself might be able to promote changes in neurotransmission, synaptic activity and neuronal excitability that can affect seizure susceptibility even months before the appearance of insoluble amyloid plaques. To further investigate the relationship between kindling susceptibility and synaptic transmission, SV2 mRNA expression was quantified between the experimental groups. Moreover, a recent study in our group have found that the Tg2576 mice at this young age, when submitted to the amygdala kindling test, display a similar susceptibility to induced epileptogenesis as the aged mice (manuscript in preparation). The animals showed a faster progression in the seizure class curve compared to the wild-type, requiring significant less stimulations to reach the first generalized tonic clonic seizure and also displayed longer seizure duration, therefore justifying its use in this study.

3.2. Hypothesis and aims

Hypothesis: Brivaracetam is able to attenuate the epileptogenesis development induced by amygdala kindling in young Tg2576 mice even after drug wash-out.

Specific aims: To investigate:
1. A potential long-lasting, antiepileptogenic effect of brivaracetam treatment on the development of amygdala kindling in young Tg2576 mice.

2. The effects of brivaracetam treatment on the brain expression levels of APP and SV2 mRNA after kindling in young Tg2576 mice.

### 3.3. Experimental design

The amygdala kindling model was used to induce epileptogenesis in wild-type and young Tg2576 mice and the surgical procedures, kindling protocol and post-mortem assessments were carried out as described in Chapter 2.

In order to determine the potential antiepileptogenic effects of BRV, young Tg2576 mice were treated continuously for a period of 4 weeks after which the osmotic pumps containing the drug were removed (Figure 3.1). Two control groups, one of wild-type animals and another of Tg2576, were also implanted with osmotic pumps and treated with vehicle for 4 weeks. All animals were allowed 7 days of to recover from the surgical procedure to implant the cortical and amygdala electrodes and remove the pump. The kindling then started after this period of recovery and drug wash-out by testing the ADT, followed by consecutive stimulations at the ADT value twice a day for 5 days. To investigate the potential long-lasting effects of BRV treatment on APP and SV2 mRNA expression levels, animals were euthanised after kindling and brain tissue was collected.

![Figure 3.1](image-url)

**Figure 3.1.** Experimental timeline for the treatment, kindling and post-mortem assessments of young wild-type and Tg2576 mice. Pi, pump implantation; S, surgery for electrodes implantation; Pr, pump removal; ADT, afterdischarge threshold test; K, kindling period; Pm, post-mortem assessments.
3.4. Methods

3.4.1. Animals

For this study, young male and female littermates of Tg2576 and wild-type mice aged between 4-6 months were used. Detailed housing conditions are described in Section 2.4.1. Briefly, animals were maintained under controlled temperature (20°C), on a 12 h light/dark cycle, in the animal facility of the Department of Medicine at the Royal Melbourne Hospital, with free access to food and water. A total of 30 mice were used for this study separated into the following groups listed in table 3.1.

Table 3.1. List of experimental groups used in this study.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>n</th>
<th>Females (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (kindled)</td>
<td>Vehicle</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Wild-type (naïve)</td>
<td>-</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Tg2576 (kindled)</td>
<td>Vehicle</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Tg2576 (kindled)</td>
<td>Brivaracetam</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Tg2576 (naïve)</td>
<td>-</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

3.4.2. Brivaracetam pre-treatment before kindling

The BRV drug used in this study was provided by UCB Pharma SA (Belgium) together with the other antiepileptic drugs used in the previous chapter. As described in detail on Section 2.4.2, the drug treatment (BRV, 10 mg/kg/day) or vehicle (0.9% NaCl) was continuously delivered via mini-osmotic pumps implanted subcutaneously in the interscapular region of the animals. After 28 days, pumps were removed, and the drug allowed to wash-out.

3.4.3. Amygdala kindling test in young Tg2576 mice

A full description of the electrode implantation surgery and the amygdala kindling protocol can be found in Section 2.4.4.1. However, different from the aged group, young Tg2576 mice were electrically stimulated twice a day for 5 consecutive days at the ADT value. Similarly, the seizure length was measured from the EEG recordings and the behavioural seizure severity was assessed according to the Racine scale described in table 2.1 (Racine, 1972; Durmuller and Porsolt, 2003).
3.4.4. Brain tissue extraction

At the end of the kindling stimulation period, animals were euthanized through CO\textsubscript{2} euthanasia and had the brains excised and dissected into hippocampus and cortex. Brain samples were frozen in liquid nitrogen and stored at -80 °C for subsequent western blotting quantification of APP and quantitative polymerase chain reaction (qPCR) for SV2 mRNA expression levels (Liu et al., 2016).

3.4.5. Subcellular fractionation and western blotting quantification of amyloid precursor protein

For this study, instead of quantifying only the total amount of APP protein, the collected brain tissue was separated into extracellular and plasma membrane fractions according to a modified version of the protocol described in Lesne et al. (2006). The samples were aliquoted, ground on dry ice and dissolved in extracellular (EC) buffer [50 mM Tris (pH 7.6), 150 mM NaCl, 2mM EDTA] then manually homogenised. The brain extracts were centrifuged at 3,000 g for 5 min at 4°C and the supernatant containing the EC fraction was aliquoted for western blotting. The remaining extract were again manually homogenised after addition of intracellular (IC) buffer [50 mM Tris (pH 7.6), 150 mM NaCl, 2mM EDTA, 0.1% Triton X-100], incubated on ice for 10 minutes and then centrifuged at 15,000 g for 30 min at 4°C to obtain the IC fraction. The supernatant was collected, 40 uL of 20% sodium dodecyl sulphate (SDS) was added and samples were heated at 100°C before being frozen and stored. The remaining pellets were washed with EC buffer and centrifuged 15,000 g for 30 minutes at 4°C. The supernatant was then collected and corresponded to the plasma membrane (ME) compartment.

The protein concentration was determined with a BCA kit, the EC and ME samples were mixed with 4X SDS sample buffer [300 mM Tris-HCl (pH 6.8), 300 mM dithiothreitol, 12% SDS, 0.6% bromophenol blue, and 60% glycerol [5:1 (v/v) ratio], boiled for 5 min at 100°C and stored at -80°C for western blotting analysis. SDS-PAGE was performed to separate the bands of proteins that were then electro-blotted onto polyvinyl difluoride (PVDF) membranes. These membranes were blocked in 10% skim milk dissolved in Tris pH 7.6/0.1% Tween 20 (TBST) and blotted with anti-mouse APP W02 (1:500,000) overnight at 4°C. The membranes were again washed with TBST and incubated with the appropriate conjugated-HRP mouse secondary antibody for 1 hour (1:10,000; Dako). All protein blots were visualized by enhanced chemiluminescent substrate kit and exposed to X-ray film. After high-resolution
scanning, the mean intensity of the blots was quantified using NIH ImageJ software (Abramoff et al., 2004). The ratio of EC and ME APP immunoreactivity was calculated in relation to the total APP band and the results were expressed for each treatment and brain region.

### 3.4.6. Quantitative polymerase chain reaction (qPCR) for SV2 mRNA expression

To investigate whether BRV treatment would exert its effect on epileptogenesis through modulation of its molecular target, mRNA expression of SV2A and also SV2B and C were quantified using qPCR. RNA was extracted and purified from hippocampal tissue using RNeasy plus mini kit and the automated QIAcube platform (QIAGEN). To determine RNA concentration and purity readings were taken with the QIAxpert spectrophotometer (QIAGEN). Samples with less than 98 \( \mu \text{g/\mu L} \) were excluded from the experiment. The Omniscript Reverse Transcription kit (QIAGEN) was used to reverse transcribe 1000 \( \mu \text{g} \) of RNA into cDNA that was then stored at -20°C. Quantitative real time PCR (qPCR) was performed on 25 \( \mu \text{g} \) of cDNA using TaqMan gene expression assays for SV2A, SV2B and SV2C using the QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems). SV2 mRNA gene expression levels are expressed relative to the geometric means of the reference genes ACTB, GAPDH, TBP and YWHAZ. All the assay IDs are listed in table 3.2. The \( \Delta \Delta CT \) method was used for analysis (Livak and Schmittgen, 2001). No significant differences were found in the expression levels of the reference genes between the test groups.

**Table 3.2. List of gene expression assays used in the qPCR analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV2A</td>
<td>Mm00491537_m</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>SV2B</td>
<td>Mm00463805_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>SV2C</td>
<td>Mm01282622_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Beta-actin (ACTB)</td>
<td>Mm02619580_g1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>Mm99999915_g1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>TATA-binding protein (TBP)</td>
<td>Mm01277042_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Tyrosine 3-monooxygenase/tryptophan 5-</td>
<td>Mm03950126_s1</td>
<td>Applied Biosystems</td>
</tr>
</tbody>
</table>
3.4.7. Data analysis

Data was analysed as described in section 2.4.6. However, in this study repeated measures two-way ANOVA was used to analyse seizure progression and duration. A statistical difference of p < 0.05 was considered significant.

3.5. Results

3.5.1. Sustained effect of brivaracetam in the kindling phenotype of Tg2576 mice

To test whether BRV would have a persistent and disease modifying effect in kindling epileptogenesis in Tg2576 mice, the animals were treated continuously for 28 days after which time treatment was stopped and the drug allowed to ‘wash-out’ before starting the electrical stimulations. Measures of the ADT current showed no differences between the genotypes or treatment groups (F(2,21) = 0.273; p = 0.763; Figure 3.2). However, pre-treatment with BRV produced a significant increase in the number of stimulations necessary to elicit the first generalized class V seizure (F(2,21) = 20.23; p < 0.001; Figure 3.3A) compared to the Tg2576 mice treated with VEH only (p<0.05), thereby producing an anti-epileptogenic effect in these AD mice. A difference between the WT mice and the BRV treated animals was also observed for the number of stimulations for the first generalized seizure (p<0.01), therefore the drug treatment was not able to reverse the genotype effect completely. The progression of behavioural seizure severity (F(2,17) = 3.165; p = 0.067) and duration (F(2,17) = 2.732; p = 0.093) over the days of stimulation are shown in figures 3.3B and C respectively, and statistical differences were only observed between the WT group and the Tg2576 mice treated with VEH (p<0.05). There was also no seizure associated death in this study compared to the high mortality rate observed in the aged cohort reported in the previous chapter and also by Chan et al. (2015). This shows that the Tg2576 mice at 4 to 6 months of age have a milder pathology and kindling phenotype.
Figure 3.2. Afterdischarge threshold current (mA) necessary to elicit an electrographic seizure in young Tg2576 mice pre-treated with vehicle (VEH) or brivaracetam (BRV) and wild-type mice. Data represent mean ± S.E.M; n = 7-10 per group.
Figure 3.3 Number of electrical stimulations necessary for animals to reach the first class V seizure compared between genotypes and treatment groups (A). Progression of behavioural seizure severity (B) and duration (C) during electrical amygdala kindling in young Tg2576 pretreated with brivaracetam (BRV) or vehicle (VEH) compared to wild type mice (WT). Data
represent mean ± S.E.M; n = 7-10 per group; *p<0.05, **p<0.01 and ***p<0.001 when compared to WT - VEH; #p<0.05 when compared to Tg-VEH.

3.5.2. Quantification of APP protein and SV2 isoforms mRNA expression in the Tg2576 mice after kindling

APP expression was quantified by western blot analysis on cortical and hippocampal plasma membrane and cytosolic fractions from Tg2576 mice after kindling. The pre-treatment with BRV did not affect the APP expression neither in the extracellular compartment of cortex (p = 0.717; Figure 3.4A) and hippocampus (p = 0.358; Figure 3.4A) nor in the membrane compartment of the cortex (p = 0.891; Figure 3.4B) and hippocampus as well (p = 0.809; Figure 3.4B).

![Figure 3.4](image-url)

**Figure 3.4.** Quantification by western blot of APP expression in the membrane (ME) and extracellular space (EC) of the cortex and hippocampus of young Tg2576 mice pre-treated with brivaracetam or vehicle after amygdala kindling. Data represent mean ± S.E.M; n = 8 per group.

The results for the mRNA expression of the SV2 family members in the hippocampus of wild type and Tg2576 mice are summarized in figure 3.5. Tg2576 naïve animals have a significantly lower expression of SV2A mRNA in the hippocampus compared to the wild type naïve mice (t0 = 3.688; p = 0.01; Figure 3.5A). Similarly, wild type kindled animals also had reduced levels of SV2A mRNA expression compared to the naïve (t10 = 2.456; p = 0.03). The same kindling effect was observed for the SV2C mRNA expression (t10 = 3.442; p = 0.006;
Figure 3.5C) even though there was a great variability in the naïve group. No differences were observed between the groups in the SV2B mRNA expression levels (Figure 3.5B).

**Figure 3.5.** Relative expression of SV2A (A), SV2B (B) and SV2C (C) mRNA in the hippocampus of wild type and Tg2576 mice, naïve or after amygdala kindling. Data represent mean ± S.E.M and has been normalised to geometric mean of ACTB, GAPDH, TBP and
YWHAZ mRNA expression levels; n = 4-8 per group; *p<0.05 when compared to the WT naïve group.

3.6. Discussion

In this study we have shown that brivaracetam treatment had a long-lasting effect in the kindling phenotype of young Tg2576 mice by increasing the number of stimulations to the first generalized seizure even after 7 days of drug washout. This effect was not mediated by changes in APP expression pattern or modulation of the SV2 genes.

The Tg2576 mice is one of the many APP mutant mice that models an AD autosomal dominant like-phenotype (Hsiao et al., 1996). It has been widely used in AD pre-clinical research, and its pathology that progresses with age allows researchers to investigate therapeutic interventions at different time points as well as understand the time course of this amyloid driven pathology. At the age of 4-6 months, Tg2576 mice do not have detectable levels of insoluble amyloid plaques, but increased levels of Aß monomers and oligomers have been reported (Jacobsen et al., 2006). Aß oligomers can induce GABAergic interneuron dysfunction, thus impairing long-term potentiation and causing atrophy that leads to hippocampal hyperactivation and even tau deposition (Palop et al., 2007; Verret et al., 2012; Stancu et al., 2014). Thus, as emerging evidence indicates that increased neuronal activity enhances the secretion of both Aß and tau, recurrent epileptic activity in AD augment the anomalous aggregation of these proteins establishing a vicious cycle (Yamamoto et al., 2015; Wu et al., 2016). Hence why not only detecting abnormal electrical activity early in the course of AD is important, but also interrupting this cycle through antiepileptic treatment in AD might give patients a better prognosis.

In AD animal models, epileptiform discharges have been detected in some strains of mice, including the hAPPJ20, APP/PS1, APdE9 and the Tg2576 mice (Palop et al., 2007; Minkeviciene et al., 2009; Nygaard et al., 2015; Kam et al., 2016). Experimentally, the antiepileptic drugs that have been reported to be effective in suppressing epileptiform activity in these animals were levetiracetam, brivaracetam, ethosuximide, lamotrigine and valproate (Ziyatdinova et al., 2011; Sanchez et al., 2012; Zhang et al., 2014; Nygaard et al., 2015). However, the SV2A ligands are the ones that have the most consistent results between studies. In the study of Nygaard et al. (2015), an interesting finding was reported, that although both ethosuximide and brivaracetam significantly reduced abnormal epileptic activity in the
APP/PS1 mice, only brivaracetam was able to reverse the impairments in spatial memory performance. Therefore, the ability to suppress network hyperexcitability might not be sufficient to improve memory function in AD, suggesting that targeting SV2A might provide additional benefits in alleviating AD symptoms.

In our study, BRV treatment had an antiepileptic effect in young Tg2576 mice that lasted even after drug wash-out. It increased the number of stimulations necessary to elicit the first generalized tonic clonic seizure one week after drug delivery was suspended (Racine class V; Figure 3.3A). To test whether this effect reflected a reduction in APP expression, western blot quantification of APP was performed in brain samples collected after kindling. The effect of SV2A ligands in amyloid pathology have been reported for some AD models, but results vary between studies. In the APdE9 mice, treatment with daily injections of LEV for 30 days reduced amyloid plaques, increased Aβ clearance, up-regulated Aβ transport and suppressed γ-secretase activity, reducing Aβ generation (Shi et al., 2013). In contrast, 20 days of treatment with LEV did not alter Aβ_1−x and Aβ_1−42 levels in the hippocampus of hAPPJ20 mice, neither affected levels of APP and C-terminal fragments in cortex homogenates (Sanchez et al., 2012).

In the study of Nygaard et al. (2015), the only that has investigated BRV treatment in an AD model, showed that the effect of reducing spike wave discharges and reversing memory impairments in the APP/PS1 model was not due to a change in the concentrations of soluble Aβ and insoluble plaques.

The SV2 proteins are found in synaptic vesicles of neuronal and endocrine cells (for review see Bartholome et al., 2017). The SV2A isoform is ubiquitously expressed in the glutamatergic and GABAergic synapses in all regions of the adult brain, whereas SV2B follows almost the same pattern with the exception of a few areas like the dentate gyrus and seems to be restricted to glutamatergic synapses (Bajjalieh et al., 1994; Crevecoeur et al., 2013). Finally, the SV2C is encountered in certain GABAergic cell types and has a more variable expression throughout the brain (Dardou et al., 2011). Increased SV2A expression has been reported for the amygdala kindling model in the ipsilateral hippocampus (Matveeva et al., 2007; Matveeva et al., 2008; Ohno et al., 2012). This asymmetric accumulation of SV2A, specific to the hippocampus, was observed 4 weeks after the last electrical stimulation and persisted for at least 1 year (Matveeva et al., 2007; Matveeva et al., 2008). Interestingly, this could be prevented by treating the rats with LEV during kindling acquisition. However, in our study there was a reduction in the SV2 mRNA expression, in particular SV2A and SV2C, after amygdala kindling in the wild-type mice and treatment with BRV did not affect SV2 levels. Notably, the Tg2576 naïve mice had reduced levels of SV2A in the hippocampus which might
help explain their susceptibility to kindling induced seizures. It has been reported that SV2A-deficient mice display increased kindling acquisition, either via amygdala or corneal electrical stimulation (Kaminski et al., 2009). Similarly, SV2A (+/-) heterozygous mice also have a pro-epileptic phenotype when tested in the pilocarpine, kainate, pentylentetrazol and 6 Hz models (Kaminski et al., 2009). Corroborating the findings of these animal studies, SV2A expression was reported to be reduced in TLE patients in the anterior temporal neocortex and in the hippocampus of the patients with hippocampal sclerosis (Gorter et al., 2006; Feng et al., 2009). A decrease in SV2A was also observed within the dysplastic cortex and cortical tubers, malformations responsible for focal cortical dysplasia and tuberous sclerosis, intractable forms of epilepsy (Toering et al., 2009). These studies collectively suggest a role for SV2A in epilepsy, in which its decreased expression and/or subsequent loss of function may contribute to the instability of neuronal networks, through a dysfunction of synaptic exocytosis and altered neurotransmission, therefore promoting epileptogenesis (Feng et al., 2009; Toering et al., 2009; de Groot et al., 2010).

Currently, analysis of the expression of SV2 proteins have not been reported for other transgenic mouse models of AD, but in clinical research, an SV2A PET tracer have been recently developed. The first study investigating patients with AD and amnestic mild cognitive impairments (aMCI) was published in 2018 (Nabulsi et al., 2016; Finnema et al., 2018). Synaptic density was then quantitatively assessed using $^{11}$C-UCB-J, a highly selective SV2A radiotracer, in 10 patients with AD (stages varying from aMCI to mild dementia) and 11 cognitively normal controls. As hypothesized, AD patients had decreased levels of SV2A specific binding in the hippocampus, the reduction remained significant after measures were corrected for atrophy and it also correlated with episodic memory scores (Chen et al., 2018a). One patient with TLE was also evaluated during the validation of the radiotracer and a reduction of $^{11}$C-UCB-J binding was reported for the right mesial temporal lobe, which colocalized with the sclerosis observed on MRI (Finnema et al., 2016). Although there have been numerous studies investigating and characterizing synaptic loss in AD, there is only one study that measured SV2A in post-mortem brain samples from AD individuals and a reduction in the hippocampus was also reported (Robinson et al., 2014).

Increasing number of studies have been showing that early-onset AD and young age at dementia onset are associated with greater risk of developing epilepsy (Scarmeas et al., 2009; Jayadev et al., 2010; Pandis and Scarmeas, 2012; Zelano et al., 2019). These data combined with the studies that reported worsening of cognitive decline associated with epileptiform activity, reinforce the idea that early detection of seizures and early treatment may have an
impact in disease progression. In conclusion, our study points out in the direction of SV2 ligand drugs as interesting therapeutic alternatives to treat epileptic seizures in AD. Targeting SV2A might be an interesting antiepileptogenic strategy, in special with BRV treatment, even though its underlying mechanistic effect is not fully elucidated yet. Considering that the drug treatment did not reverse the SV2A phenotype in the Tg2576 mice, one can hypothesize that it might be exerting its persistent effect through a potentiation or optimization of SV2A function, instead of altering its expression. However, there is still an urge for further investigations on the role and pattern of SV2A distribution, both in transgenic mouse models and in larger AD patient cohorts spanning more disease stages, to clarify its correlation with cognitive outcomes and epileptic seizures.
Chapter 4

Amyloid and tau pathology in drug-resistant temporal lobe epilepsy

4.1. Introduction

Temporal lobe epilepsy (TLE) is classified under the group of focal epilepsies and its seizure foci resides in the temporal lobe structures, such as hippocampus, amygdala, entorhinal cortex and subiculum (Engel, 1996). A variety of pathological substrates are recognised in TLE, in particular hippocampal sclerosis (Sharma et al., 2007), which is associated with a high rate of pharmacoresistance (Semah et al., 1998). In selected patients, anterior temporal lobectomy will render around 70% seizure-free (Wiebe and Derry, 2000; Ramey et al., 2013). The procedure involves resection of the anterior temporal lobe and mesial temporal structures (amygdalohippocampectomy) with the aim to effectively remove the epileptogenic zone and preserve as much neocortex as possible (Al-Otaibi et al., 2012).

Because the temporal lobe is the main structure involved in cognitive processes, such as memory and learning, frequent seizures and hippocampal sclerosis may result in deficits in cognition (for revision see Tai et al., 2018; Vrinda et al., 2018). Moreover, these neurobehavioural comorbidities may increase the chance of dementia development (Hermann et al., 2006; Hermann et al., 2008). Although progressive cognitive deterioration over time has been known to be a feature of TLE, particularly following temporal lobectomy, the mechanisms contributing to this decline are still unknown (Oyegbile et al., 2004; Marques et al., 2007). The studies of Thom et al. (2011) and Tai et al. (2016) have reported increased deposition of tangles of hyperphosphorylated tau in brain tissue of patients with epilepsy and that correlated with a decline in cognition, in particular 1 year after temporal lobe resection.

Given that TLE and AD have shared pathological and neuroimaging features, this study aimed to investigate whether tau burden and/or amyloid pathology are associated with cognitive impairments in TLE, by examining temporal lobe tissues resected from patients during epilepsy surgery.
4.2. Hypothesis and aims

**Hypothesis:** The severity of tau and amyloid pathology in resection specimens is associated with pre-operative cognitive dysfunction in patients with drug-resistant temporal lobe epilepsy.

**Aims:**
1. To correlate the presence of the clinico-pathological hallmarks of dementia (hyperphosphorylated tau and amyloid plaques) in the resected temporal lobe tissues with pre-operative cognitive test scores.
2. To identify potential clinical risk factors that mediate the interaction between pathology and cognitive decline.

4.3. Methods

4.3.1. Patient selection and clinical data

Eligible patients were retrospectively selected from the archives of the Royal Melbourne Hospital (RMH), from 1993 to 2017. All patients with pharmaco-resistant TLE who underwent anterior temporal lobectomy with hippocampal sclerosis or no identified pathology, and who had pre-surgical neurocognitive evaluation, were eligible for inclusion. Patients without available paraffin blocks containing the brain tissue, or cognitive testing results, or who had a pre-operative diagnosis of Alzheimer’s disease, were excluded. All patients provided written informed consent. The study was approved by The Melbourne Health Human Research Ethics Committee (Project No 2005.243).

The clinical history and demographic data of each patient was retrieved from medical records. Recorded information relevant to this study included age at onset of epilepsy, duration of epilepsy, side of surgery and handedness, pre-operative cognitive assessment, seizure type and frequency, history of head injury and seizure control post-surgery.

4.3.2. Cognitive test data

Patients with drug-resistant TLE who are referred to resective surgery routinely undergo cognitive testing before surgery by a trained neuropsychologist at our centre. Different cognitive tests are commonly used in our centre for this pre-operative assessment, but for the
purposes of this study 3 tests were selected based on sensitivity to temporal lobe pathology. Verbal learning was measured by the Rey Auditory Verbal Learning Test (RAVLT), with a maximum score of 75 and 50 considered normal. The RAVLT A7 score represents delayed component of verbal learning and patients are asked to recall the list of words 30 min after it has been read to them (McMinn et al., 1988). To assess verbal memory, the Verbal Pair Associates test (VPA) was applied in the ‘easy’ (pairs of related words) and ‘hard’ versions (pairs of unrelated words) (Elwood, 1991). The scoring is between 0 to 12 and between 9 to 11 points is considered normal for the VPA ‘easy’ and 4 to 6 for the ‘hard’. The VPA ‘hard’ measure has been shown to be sensitive to memory processes encoded in the perirhinal and entorhinal cortices (Cameron et al., 2001). The Rey Complex Figure Test (RCFT) provided measures of visual-spatial memory and executive function and involves copying a complex figure and then reproducing it from memory after a delay of 30 min (Shin et al., 2006). Different scores given to location, accuracy and organization of the figure traces are combined to form the final score.

4.3.3. **Immunohistochemistry for tau and amyloid**

Formalin-fixed, paraffin-embedded tissue blocks from the recruited patients were retrieved from the pathology archives. Blocks containing temporal lobe and hippocampus were selected for immunohistochemical analysis according to anatomical position, best preserved tissue, and representative pathology (Figure 4.1). The selection was carried out by reviewing previously prepared hematoxylin & eosin stained sections representative of each block.

Immunohistochemistry for phosphorylated tau protein and Aβ peptide was carried out on 7-µm thick paraffin-embedded sections mounted on charged slides. The staining for tau was performed using the automated immunostainer DAKO Autostainer with the primary antibody anti-AT8 (1:1000, DAKO), following established protocol used at the Pathology laboratories of The Alfred Hospital. The Aβ staining was performed manually and the 1E8 primary antibody (1:4000) used was kindly provided by Dr. Qiao-Xin Li from the Florey Institute of Neurosciences. Briefly for amyloid staining, sections were deparaffinized with xylene, rehydrated in graded solutions of ethanol (100%, 95% and 50%) and washed in distilled water. Hydrogen peroxide was used to quench endogenous peroxidase activity and then slides were immersed in a solution of 90% formic acid for antigen retrieval. Sections were incubated with primary antibody in Tris-HCl buffer for 1 hour at room temperature in a humidified chamber.
Followed by incubation with biotinylated anti-mouse/rabbit immunoglobulins for 10 min and peroxidase conjugated streptavidin also for 10 min (DAKO LSAB+ kit). 3,3’-diaminobenzidine tetrahydrochloride (DAB, DAKO) was used as the chromogen substrate and sections were counterstained with hematoxylin (DAKO). After dehydration through sequential washes with increasing ethanol concentrations and finally in xylene, slides were cover slipped with DPX mounting medium.

Slides from a confirmed case of Alzheimer’s disease were obtained from the Victorian Brain Bank (Application No 18.18) and used as a positive control for each staining run. All sections were analyzed using a light microscope.

Figure 4.1. Illustration of the two blocks of tissue used for the staining of tau tangles (AT8 labelling) and Aβ plaques (1E8 labelling). Block 1 (in blue) contains the hippocampus and block 2 (in red) represents the temporal neocortex. Modified with permission from Braak et al. (2006).
4.3.4. Tau and amyloid pathology analysis

Tau burden and Aβ immunohistochemistry were assessed semi-quantitatively with scores of none, sparse (<3 plaques/100x microscopic field), moderate (3-6 plaques) or frequent (>6 plaques), as defined in the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) (Mirra et al., 1991). Our brain samples did not have significant and widespread tau pathology that justified the use of Braak staging or even a modified tau score as described by Tai et al. (2016) for TLE sections. Scoring of sections were carried out by one observer and reviewed by an experienced neuropathologist. All patients that had evidence of amyloid pathology, regardless of the score, were collectively termed “amyloid positive group” (Amyloid +), and the patients with no pathological evidence were termed “amyloid negative group” (No amyloid).

4.3.5. Data analysis

Statistical analysis was performed using GraphPad Prism 7. Spearman correlation analysis was used to correlate the presence of amyloid plaques, tau tangles or both with the clinical data, such as age at surgery, epilepsy onset and duration, side of resection, history of head injury and occurrence of generalized tonic clonic seizures (GTCS). Mann-Whitney U test was used to compare the cognitive test scores, clinical data and MRI volumetric measurements, between patients with amyloid plaques and without. For all analysis p value of <0.05 was considered significant.

4.4. Results

4.4.1. Demographics of the patient cohort

A total of 56 patients were included for analysis. Their clinical characteristics are summarized in table 4.1. Their age at surgery ranged from 20 to 68 years, with the majority under 50 (85%). Most had a pathological diagnosis of hippocampal sclerosis (51/56, 91%), of these 10 were classified as ILAE type I, 5 were type II and 1 was type III, while the other 35 were not subclassified (Blumcke et al., 2013). HS type I refers to severe neuronal loss and gliosis mainly in the CA1 and CA4 regions, while HS type II is predominant in CA1, and type III in CA4. From the 56 patients included in this study, the paraffin blocks containing hippocampal tissue were available for all patients (100%) and the temporal lobe blocks for 40
patients (71%). History of secondarily generalized seizures was identified for 31 patients of 35 patients (88%), and previous head injury was reported for 7 patients (12%).

**Table 4.1.** Clinical characteristics of the TLE patient population studied (total n=56).

<table>
<thead>
<tr>
<th>Number of patients for whom data was available</th>
<th>Mean (range) or proportion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex - female, n (%)</td>
<td>56 (31 (55%))</td>
</tr>
<tr>
<td>Age at surgery, mean (range)</td>
<td>56 (37 (20 - 68))</td>
</tr>
<tr>
<td>Age of onset, mean (range)</td>
<td>54 (17 (0 - 56))</td>
</tr>
<tr>
<td>Duration of epilepsy in years, mean (range)</td>
<td>54 (20 (1 - 56))</td>
</tr>
<tr>
<td>Side of resection - right, n (%)</td>
<td>56 (28 (50%))</td>
</tr>
<tr>
<td>Handedness - right, n (%)</td>
<td>49 (39 (79%))</td>
</tr>
<tr>
<td>HS</td>
<td>56 (51 (91%))</td>
</tr>
<tr>
<td>History of secondary generalized seizures (GTCS), n (%)</td>
<td>35 (31 (88%))</td>
</tr>
<tr>
<td>History of head injury, n (%)</td>
<td>56 (7 (12%))</td>
</tr>
<tr>
<td>RAVLT total</td>
<td>31 (44 (21 - 62))</td>
</tr>
<tr>
<td>RAVLT A7</td>
<td>31 (8 (0 - 15))</td>
</tr>
<tr>
<td>VPA easy</td>
<td>48 (9.1 (2 - 12))</td>
</tr>
<tr>
<td>VPA hard</td>
<td>48 (3.7 (0 - 10))</td>
</tr>
<tr>
<td>RCFT delay</td>
<td>52 (12 (0 - 27))</td>
</tr>
</tbody>
</table>

4.4.2. Amyloid-β plaques and hyperphosphorylated tau

Paraffin blocks containing hippocampal tissue were available for all 56 patients while temporal lobe blocks were available for 40. Amyloid plaques were present in 12 of the 56 patients (21%). Of these, 3 had a ‘sparse’ score, 5 had ‘moderate’ and 4 had plaques considered ‘frequent’. From the 4 patients with frequent plaques, the ages at the time of surgery for each one was 36, 38, 55 and 61 years old and the plaques were present mainly in the temporal lobe cortex section, with only the eldest patient having widespread plaques in the hippocampus section as well (Figure 4.3C and E). In the moderate score group, 3 patients had plaques only
in the hippocampus and 2 patients only in the temporal lobe section. Of the patients with sparse plaques, 2 were in the hippocampus and 1 in the temporal lobe only. Overall, the higher incidence of amyloid plaques was found in the temporal neocortex section compared to the hippocampus. In general, patients in the amyloid positive group were older than the negative group (43 vs. 35, \( p = 0.014 \); Mann-Whitney \( U = 146.5 \)). Older age also correlated with severity of amyloid pathology, which was not associated with age of onset or duration of epilepsy (Figure 4.2).

Scattered neurofibrillary tangles and neuropil threads of hyperphosphorylated tau were identified in the temporal lobe sections of 2 patients (Figure 4.3E and H). Both patients were over 50 years of age (55 and 61 years old) with an amyloid plaque score of ‘frequent’. AT8 labelling was not found in the hippocampus of any of the two patients. Representative photos of the positive control of a confirmed case of Alzheimer’s disease are shown in figure 4.4.

Combining the presence of amyloid plaques and/or tau tangles did not show any association with age of onset of epilepsy, duration of epilepsy, side of resection, handedness, history of head injury or history generalized tonic-clonic seizures (Table 4.2).

![Figure 4.2](image)

**Figure 4.2.** Distribution of age at time of surgery (A), age of onset of epilepsy (B) and epilepsy duration (C) between the groups of TLE patients with an amyloid positive or negative pathology, and correlation these parameters with the severity of amyloid pathology (0, none; 1, sparse; 2, moderate; 3, frequent). Data represent mean ± S.D.; *\( p<0.05 \); Spearman correlation.
Table 4.2. Spearman’s coefficient and $p$ value for correlation analysis between clinical characteristics and the presence of amyloid plaques among different scores. Significant $p$ values are indicated with an asterisk (*).

<table>
<thead>
<tr>
<th></th>
<th>Number of patients for whom data was available</th>
<th>Mean (range) for each amyloid plaque score</th>
<th>Spearman's rho</th>
<th>Spearman correlation significance test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56</td>
<td>37 (20 - 68)</td>
<td>37 (20 - 68)</td>
<td>39 (30 - 46)</td>
</tr>
<tr>
<td>Age of onset</td>
<td>54</td>
<td>17 (0 - 56)</td>
<td>17 (0 - 56)</td>
<td>5 (1 - 12)</td>
</tr>
<tr>
<td>Epilepsy duration</td>
<td>54</td>
<td>20 (1 - 56)</td>
<td>20 (1 - 56)</td>
<td>34 (28 - 45)</td>
</tr>
<tr>
<td>Side of resection - right</td>
<td>56</td>
<td>28 (50%)</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>History of injury</td>
<td>56</td>
<td>7 (12%)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>GTCS</td>
<td>35</td>
<td>31 (88%)</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>44</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>
**Figure 4.3.** Photos of AT8 and 1E8 labelling for tau tangles and amyloid-β plaques in the temporal neocortex (red frame) and hippocampus (blue frame) sections from 3 patients that had resection surgery for TLE and were classified as having frequent amyloid plaques. Patient 1 is a 38-year old man with 36 years of epilepsy (A and B). Amyloid-β plaques were found in temporal neocortex (A), but not in the hippocampus (B) and no tau tangles in neither area analysed. Patient 2 is a 61-year old man with 11 years of epilepsy (C-F), showing frequent amyloid-β plaques in the temporal neocortex (C) and hippocampus (D) and scattered neurofibrillary tangles of tau in the temporal neocortex section (E) but not in the hippocampus (F). Patient 3 is a 55-year-old woman with 32 years of epilepsy (G and H). Amyloid-β plaques were found in the cortex sections, but only scattered tau tangles were detected. Scale bars = 100 µm in all photos and 50 µm in the inset. Arrows indicate amyloid plaques and arrowheads tau tangles.

**Figure 4.4.** Sections of temporal neocortex (red frame) and hippocampus (blue frame) of a control patient with Alzheimer’s disease stained with 1E8 for amyloid- β plaques (arrows; A-B) and AT8 for tau tangles (arrowheads; C-D). Scale bars = 100 µm.
4.4.3. Cognitive decline in relation to amyloid pathology

To explore a possible association between the presence of amyloid pathology and a cognitive phenotype, preoperative neuropsychometric scores relative to verbal learning, verbal and visuo-spatial memory and executive function were compared between groups. The amyloid positive patients had significant lower scores for the visual memory test RCFT delay (Figure 4.5; \( p = 0.016 \); Mann-Whitney \( U = 108 \)), and although it failed to reach significance, scores in the VPA hard test was also lower for this group. Correlation analysis revealed that the patients with frequent amyloid plaques scored less in both, VPA hard and RCFT delay test, compared to the patients with sparse plaques.

![Figure 4.5. Comparison of scores for the cognitive tests RAVLT total (A), RAVLT A7 (B), VPA easy (C), VPA hard (D) and RCFT delay (E) between the groups of TLE patients with and without amyloid plaques, and correlation analysis of the VPA hard (F) and RCFT delay](image-url)
(G) scores with severity of amyloid pathology. Data represent mean ± S.D.; *p<0.05; Spearman correlation.

4.5. Discussion

This study had 3 main findings. First, in the epilepsy cohort investigated we did not find widespread tau pathology as it was hypothesized. Second, the incidence of amyloid plaques was higher compared to previous studies of temporal lobectomy samples and it correlated with old age of patients. Third, the severity of amyloid pathology correlated with lower scores in the VPA hard and RCFT delay tests. Overall, when compared to healthy older people, older adults with epilepsy have greater cognitive deficits across different domains, including executive functions, attention and psychomotor, short and long-term visual and verbal memory (Griffith et al., 2006; Piazzini et al., 2006; Witt et al., 2014; Miller et al., 2016). The basis for cognitive deficits and in particular cognitive decline following temporal resection is still unknown. Up to 25% of patients experience a further decline in memory following temporal lobe resective surgery and older patients are particularly vulnerable (Hermann et al., 2006; Thompson et al., 2015).

Considering that the Alzheimer’s lesion hallmarks, amyloid deposition and tau burden, have been the main characters underlying cognitive impairments in AD patients over decades, studies have been investigating whether this could also be the case in TLE. Findings from a few studies with human epileptogenic tissue have reported varied levels of amyloid plaques and tau tangles and the pathology might predispose to a decline in memory following epilepsy surgery (Mackenzie and Miller, 1994; Thom et al., 2011; Tai et al., 2016). Notably, amyloid-β pathology is not often detected and tau hyperphosphorylation seems to be an important component of the underlying neurodegeneration and cognitive decline associated with TLE, yet we did not find support for this in our study.

Our results appear to differ from what was reported previously by Tai et al. (2016) and Thom et al. (2011) that also investigated the presence of hallmark lesions in cohorts of epilepsy patients (Table 4.3). In both studies, the incidence of AT8 labelling was considerably higher compared to that of amyloid plaques, and the severity of tau pathology correlated with cognitive decline. In our study we found the opposite, higher incidence of amyloid lesions compared to tau and lower performance in cognitive tests for patients with increasing levels of Aβ plaques. The study of Tai et al. (2016) investigated a patient population between 50 and 65
years old that, similar to ours, underwent resective surgery for TLE. They have reported the presence of tau pathology in 93% of the cases, with more than a half receiving a Braak stage of III or more (57%), while Aβ plaques were found in only 12% and most cases were classified as ‘sparse’. Most of our cohort of patients is under 50 years old (78%), but if we compare only the older group of patients (>50), our incidence of amyloid plaques is still higher (66%) and of tau tangles is still lower (25%) than the rates reported by Tai et al. (2016) (12% and 93%, respectively).

Table 4.3. Comparison between findings from the present study and other two other that investigated the incidence of amyloid and tau pathology in epilepsy patients (Thom et al., 2011; Tai et al., 2016).

<table>
<thead>
<tr>
<th>Amyloid plaques</th>
<th>Present study</th>
<th>Tai 2016</th>
<th>Thom 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>44</td>
<td>28</td>
<td>91</td>
</tr>
<tr>
<td>Sparse</td>
<td>3</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Frequent</td>
<td>4</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Total pts</td>
<td>56</td>
<td>33</td>
<td>138</td>
</tr>
<tr>
<td>Age range</td>
<td>20-68</td>
<td>50-65</td>
<td>15-96</td>
</tr>
<tr>
<td>Amyloid %</td>
<td>21%</td>
<td>12%</td>
<td>34%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tau pathology</th>
<th>Present study</th>
<th>Tai 2016</th>
<th>Thom 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>54</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>Sparse (I + II)</td>
<td>2</td>
<td>12</td>
<td>50</td>
</tr>
<tr>
<td>Moderate (III + IV)</td>
<td>0</td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>Frequent (V)</td>
<td>0</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Total pts</td>
<td>56</td>
<td>33</td>
<td>138</td>
</tr>
<tr>
<td>Age range</td>
<td>20-68</td>
<td>50-65</td>
<td>15-96</td>
</tr>
<tr>
<td>Tau %</td>
<td>3%</td>
<td>93%</td>
<td>69%</td>
</tr>
</tbody>
</table>

Because there is a lack of lifespan studies investigating amyloid deposition in normal adults, our understanding of what is a ‘normal’ amyloid deposition in the general population is
still very limited. However, it has been reported that 20 to 30% of healthy older controls between 65- and 90-years old show significant amyloid deposition and can still be cognitively normal (Nelissen et al., 2007; Rowe et al., 2007). In our study, the presence and severity of amyloid pathology correlated with older ages at the time of surgery, but not with younger age of onset or longer duration of epilepsy. Amyloid deposits have been found in other epilepsy cohorts. A study with 101 TLE patients (30-61 years old) that also underwent temporal lobe resection surgery found plaques of amyloid in 10 patients that also correlated with age, and when compared with temporal lobe tissue obtained from 406 autopsy control patients (30-92 years old) without dementia or epilepsy, the age-related incidence of amyloid deposits was higher in the epilepsy patients (Mackenzie and Miller, 1994). A more recent report from the cohort of 245 patients with childhood epilepsy that have been followed up for over 50 years by the Turku University Hospital in Finland, found evidence of amyloid deposition on PET imaging with a higher frequency than healthy controls (Joutsa et al., 2017). Therefore, plaques may not be considered characteristic of the epilepsy phenotype, but taken together, these data from human studies suggest that a history of epilepsy might increase the chances of amyloid deposition compared to healthy controls.

In our study, the severity of amyloid pathology correlated with lower scores in the VPA hard and RCFT delay tests preoperative. A study that recorded single neurons in the mesial temporal lobe of patients while they were being tested for remembering pairs of words reported that the activity of the entorhinal cortex reflected the capacity of successfully recalling the pairs (Cameron et al., 2001). The significant correlation found for the VPA test scores might be a result of the amyloid accumulation in this region, although we haven’t looked beyond the level of presence/absence and number of plaques in this study and a more in-depth analysis of plaque localization within the mesial temporal lobe would give us a better insight into this correlation. We have also found a significantly lower score in the RCFT delay test in the amyloid positive patients. The RCFT is one of the most common tests to evaluate visuospatial skills in neuropsychological assessments of elderly people and has been considered effective in discriminating Alzheimer’s disease (Fujimori et al., 1998; Camara et al., 2000). Although it has failed to discriminate cognitively normal patients with low and high Aß deposits, it might be a test sensitive enough in the context of TLE (Harrington et al., 2017).

Nevertheless, other pathology factors might predispose TLE patients to memory decline. Reduced calbindin expression in granule cells is often observed in TLE and has been shown to correlate with verbal memory dysfunction in these patients and in Alzheimer’s as well (Abraham et al., 2011; Karadi et al., 2012; Stefanits et al., 2014). Overall, the dentate
gyrus is marked by several other changes in TLE, including astroglial abnormalities, reorganization of the mossy fiber pathway, altered zing signalling, neuronal loss, and all can potentially influence memory dysfunction (Henneberger et al., 2010; Karadi et al., 2012; Takeda et al., 2014; Thom, 2014). Hippocampal volume loss and atrophy have also been reported to be an important feature of cognitive decline, although measures of hippocampal volume did not correlate with neuropsychometric test scores or amyloid pathology in our study (Jack et al., 2000). However, some individuals seem to be better equipped to handle these physiological abnormalities that occur with aging and disease processes because of their cognitive reserve. Higher levels of education, baseline IQ, occupational complexity or socioeconomic status may mitigate some of these secondary processes that lead to cognitive impairment in epilepsy (Stern, 2002; Hermann et al., 2006).

In conclusion, although there seem to be a commonality of pathways that may underpin epilepsy and Alzheimer’s disease, the histopathological and molecular basis for this is yet to be defined. The raising debate on whether TLE have an Alzheimer-like pathology as an underlying cause for cognitive impairments still needs further investigation, especially in larger cohorts. In our study, tau burden was not the main pathology found in the temporal lobe resected tissue and therefore other factors might be involved in cognitive decline in this cohort of patients. For future studies we plan to also correlate the pathology findings with postoperative seizure and cognitive outcomes, i.e. seizure freedom and neuropsychometric scores 1 year after surgery. Long-term follow up, in particular of the patients with high levels of amyloid plaques, would also be necessary to further understand the effect of this pathology.
Chapter 5

Summary and conclusions

5.1. Summary

In this thesis, the bidirectional relationship between epilepsy and Alzheimer’s disease was investigated in both ways. First, a novel model of AD-acquired epilepsy was used to evaluate the efficacy of the new generation antiepileptic drugs, LEV, BRV and LAC. In this two-hit model, mutant APP overexpression acts as the first hit in promoting epileptogenesis and the amygdala kindling electrical stimulations as the second hit. The seizure-prone phenotype of the Tg2576 AD mice was then attenuated by chronic treatment with BRV. This outcome was further explored, and a different protocol was tested as an attempt to identify whether the drug had an anti-epileptogenic, in addition to anti-seizure, effect. Young Tg2576 mice treated with BRV required significantly less stimulations to reach the first tonic clonic generalized seizure compared to the vehicle treated animals. Besides, the unique mechanism of action that BRV and LEV have of preferentially targeting hyperexcitable neurons, might confer these drugs an advantage in the treatment of the silent epileptic seizures that have been reported in AD patients. Therefore, future studies will need to investigate the molecular basis of this effect of SV2 ligands in the epilepsy phenotype associated with Alzheimer’s pathology.

Second, this thesis also focused on the other way of the bidirectional pathway, by exploring the potential mechanisms associated with cognitive impairment in TLE. A new debate has emerged based on the recent findings that patients with chronic epilepsy, in particular TLE, have abnormal deposition of tau tangles. However, in our cohort of patients we could not find the same results and no significant tau pathology was detected in the temporal lobe of patients with drug-refractory epilepsy. We have then shown that this is not an assumption that can be generalized, and we also reinforce the need for more studies with larger cohorts to either confirm or contradict this idea.
5.2. Methodological considerations and future directions

5.2.1. Acquired epilepsy studies in AD animal model

The Tg2576 mice is a well characterized model of Alzheimer’s disease and it is thought to have increasing levels of amyloid deposition with age, however in our study we could not detect and quantify the Aß levels. It might be that the western blot protocol was not appropriate or that we need to use a different method. Immunohistochemistry analysis could also have been performed to confirm the presence of amyloid plaques in the aged versus young mice to differentiate their phenotypes.

For the study presented in Chapter 2, wild-type controls were not used, although vehicle treated transgenic mice served well as the control group. In addition, because of the advanced age, the mortality rate of the Tg2576 mice in the kindling was high, with a considerable number of animals dying during tonic clonic seizures. This impacted the statistical analysis of the kindling progression data and also reduced the number of brain samples available for post mortem assessments. A few animals were also found dead in their cages hours after kindling stimulation and it might have been a consequence of spontaneous seizures happening in the interval of stimulations. Long-term EEG monitoring of these mice during kindling could have confirmed this. Also, reports of spontaneous seizures and abnormal spikes in the Tg2576 mice are still variable and future studies could provide a better characterization of the epilepsy phenotype of these animals. It would also be worth investigating the effect of BRV treatment in the cognitive profile of these mice.

Moreover, if we consider these AD transgenic mice as a model of epilepsy that display recurrent spontaneous seizures, they could even be used in the screening of new antiepileptic drugs. On the other hand, this novel AD-acquired epilepsy model (transgenic AD mice + amygdala kindling) can also be used to investigate other treatments, including drugs that are on trial for Alzheimer’s disease and their ability to reduce seizure susceptibility.

5.2.2. Investigation of cognitive deficits in TLE patients

In Chapter 3, a limitation of the study was that clinical data was not consistently available for all patients such as history of head injury and generalized tonic clonic seizures, and both factors have been previously correlated with histopathology severity in other studies. Also, because the pre-operative assessment of the patients is not identical but based on individual clinical needs, the number of patients scored in each cognitive test was variable.
Future studies will need to correlate the histopathological findings with post-operative outcomes, such as cognitive test scores and seizure freedom. This might indicate whether the underlying pathology can be a predictor of seizure recurrence or susceptibility to cognitive decline following epilepsy surgery. Other histological markers, i.e. calbindin, neuropeptide Y expression, could also provide some insights on the mechanisms behind this cognitive decline. It would also be worth correlating the findings of this study with APOE genotyping. It has been reported that APOE ε4 allele is associated with reduced memory capacities, cognitive impairments, earlier age of TLE onset and reduced hippocampal volume (Busch et al., 2007; Aboud et al., 2013). APOE ε4 genotype is also related to elevated amyloid deposition and it could be the case of the young patients that displayed ‘frequent’ amyloid plaques in our study.

5.3. Conclusions

In conclusion, although the benefits of reducing abnormal electrical activity and seizures in AD have been extensively reported over the last years, we need to better understand how the anti-seizure drugs perform in the context of the Alzheimer’s pathology. Brivaracetam and levetiracetam have been found as potential candidates, with evidence of antiepileptogenic effect of brivaracetam in an AD-acquired epilepsy model, although the mechanisms underlying this effect require further investigation. We have also shown that amyloid and tau are not good predictors of cognitive decline in TLE, therefore the reason why some TLE patients experience cognitive deficits is still unknown and understanding this might be an important step to better comprehend the mechanisms that contribute to cognitive decline in AD.
Bibliographical references


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De Castro E Silva, Juliana

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