EXPLORING THE EFFECTS OF ZOLPIDEM ON COGNITIVE IMPAIRMENT AND CELLULAR CHANGES USING A LITHIUM-PILOCARPINE RAT MODEL

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

TBI	Traumatic brain injury
PTS	Post traumatic seizures
PTE	Post traumatic epilepsy
AED	Anti-epileptic drug
VGSC	Voltage-gated sodium channel
VGPC	Voltage-gated potassium channel
VGCC	Voltage-gated calcium channel
$GABA_A$	Gamma-aminobutyric acid type A
BZD	Benzodiazepines
SE	Status Epilepticus
PRE	Pharmacoresistant Epilepsy
CA	Cornu Ammonis
DG	Dentate Gyrus
H.M.	Henry Gustav Molaison
EPSP	Excitatory Postsynaptic Potential
Cl^-	Chloride Ion
Na^+	Sodium Ion
K^+	Potassium Ion
NKCC1	Sodium-Potassium-Chloride Cotransporter 1
KCC2	Potassium-Chloride Cotransporter 2
SRS	Spontaneous Recurrent Seizures

ARASC	Animal Research And Service Centre
i.p.	Intraperitoneal
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
NaOH	Sodium Hydroxide
H&E	Haematoxylin And Eosin
SEM	Standard Error of the Mean
ANOVA	One-Way Analysis of Variance
p	Probability
BBB	Blood Brain Barrier
NMDA	N-Methyl-D-Aspartate
EEG	Electroencephalogram
MPEP	2-Methyl-6-(phenylethynyl)pyridine

ABSTRAK

Epilepsi pasca trauma berlaku apabila serangan sawan berterusan selama > 1 minggu susulan kecederaan otak secara traumatik. Lobus temporal yang menempatkan hippocampal adalah bahagian bertanggungjawab yang mencetuskan serangan sawan, yang mengakibatkan kerosakan kognitif dan juga ingatan. Lebih daripada 30% pesakit epilepsi pasca trauma rintang terhadap ubat-ubatan anti-epilepsi konvensional seperti diazepam, membuatkan perlunya untuk mendapatkan pilihan terapi yang baharu. Terdapat kelemahan bagi sesuatu model penyakit yang diaplikasi secara klinikal yang melibatkan epilepsi pasca trauma, terutamanya kadar kematian haiwan kajian yang tinggi dan kadar induksi epilepsi yang rendah, menjadikan ujikaji secara terapi mengalami kesukaran. Oleh itu dalam kajian ini, kami menggunakan protokol induksi status epileptikus menggunakan lithium-pilocarpine yang melibatkan jangka masa selama 4 hari dan menggunakan dos pilocarpine secara pembahagian. Status epileptikus dinilai berdasarkan skala Racine, diikuti dengan penggunaan koktail ubat yang sesuai (diazepam atau zolpidem) yang diformulakan untuk mengurangkan kadar kematian haiwan kajian. Kami juga menguji kesan zolpidem pada fungsi kognitif pada haiwan ini menggunakan labirin air Morris, juga menggunakan teknik pewarnaan Haematoxylin dan Eosin untuk mengkaji perubahan morfologi pembentukan hippocampal susulan epilepsi pasca trauma, sebelum dan selepas perawatan dengan zolpidem. Hasil kajian kami menunjukkan kadar induksi status epileptikus adalah 100% dengan protokol induksi selama 4 hari. Kami juga mendapati kadar kematian adalah 0% dengan koktail diazepam yang berjaya menghentikan status epileptikus pada haiwan kajian berbanding dengan koktail zolpidem. Waktu pemulihan yang diambil untuk haiwan

yang dirawat dengan koktail diazepam juga relatifnya lebih singkat dibandingkan dengan koktail zolpidem, membuatkan kami meneruskan kajian lanjut dengan menggunakan koktail diazepam. Ujian tingkah laku yang dijalankan merumuskan kebanyakan haiwan menunjukkan tingkah laku eksplorasi, dengan haiwan dengan epilepsi pasca trauma yang diberikan zolpidem 30 minit sebelum ujian menunjukkan corak renang secara thigmotaxic di labirin air Morris, fenomena yang biasanya terjadi pada haiwan yang mengalami ketakutan dan kegelisahan, menunjukkan zolpidem mempunyai kesan anti-kegelisahan secara paradoks. Analisis histologi ke atas sampel haiwan dengan epilepsi pasca trauma menunjukkan sejumlah besar kehilangan sel dan kerosakan pada lapisan CA1, CA3, gyrus dentate bahagian atas, dan subiculum. Secara perbandingan, analisis gambar otak dari haiwan yang sebelumnya diberikan zolpidem semasa ujian tingkah laku menunjukkan pertambahan dalam jumlah sel dan ketebalan lapisan sel di kawasan CA1, CA3, gyrus dentate bahagian atas dan subiculum, menunjukkan zolpidem memiliki potensi untuk proses pemulihan.

ABSTRACT

Post traumatic epilepsy occurs when seizures persist for > 1 week following a traumatic brain injury. The temporal lobe that houses the hippocampal formation is a common site for seizure initiation in these patients, resulting in cognitive and memory deficits. More than 30% of patients with post traumatic epilepsy are resistant to conventional anti-epileptic drugs such as diazepam, prompting the need to find new therapeutic options. There is also a lack of clinically translatable disease models of post traumatic epilepsy due to high animal mortality rates and low status epilepticus induction rates, making it difficult to test potential therapeutics. Therefore, in our study we utilized a new lithium-pilocarpine status epilepticus induction protocol that spanned a period of 4 days and comprised of fractionated doses of pilocarpine. Status epilepticus was graded using the Racine scale, completion of which was followed by the application of a suitable drug cocktail (diazepam- or zolpidem-cocktail) designed to reduce mortality rates. We also tested the effects of zolpidem on cognitive function in these animals using Morris water maze, while also utilizing Haematoxylin and Eosin staining to investigate morphological changes in the hippocampal formation following post traumatic epilepsy before and after zolpidem application. Our results showed a 100% status epilepticus induction rate with the 4-day induction protocol. We also observed a 0% mortality rate with the diazepam-cocktail which was successfully able to stop status epilepticus in the animals compared to the zolpidem-cocktail. The recovery time for animals treated with the diazepam-cocktail was also relatively shorter as compared to that of the zolpidem-cocktail, compelling us to proceed with diazepam-cocktail treated animals for further tests. Our behavioural test revealed that while most

animals displayed exploratory behaviour, animals with post traumatic epilepsy that were administered zolpidem 30 minutes prior to the test exhibited thigmotaxic swimming pattern in the Morris water maze, a phenomenon common in models of fear and anxiety, suggesting zolpidem having a paradoxical anti-anxiolytic effect. Histological analysis of animals with post traumatic epilepsy showed a significant amount of cell loss and damage to the layers of the CA1, CA3, upper dentate gyrus, and subiculum. Comparatively, analysis of brain images from animals that were previously administered zolpidem during behavioural testing revealed an improvement in both the cell count and thickness of cell layers in the CA1, CA3, upper dentate gyrus, and subiculum regions, suggesting zolpidem to have a potential restorative effect.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Traumatic brain injury (TBI) is the damage of healthy brain tissue in response to a direct blow to the head resulting in bruising, bleeding and destruction of neuronal tissue which can result in an altered state of consciousness, and impaired cognitive and motor function (Okidi et al., 2020). Often dubbed "the silent epidemic" it has been estimated to affect nearly 69 million people worldwide every year and is known to vary in origin, severity, pathogenesis, and clinical outcome (Corps et al., 2015; Dewan et al., 2018). TBI affects people from all socio-economic backgrounds including athletes, soldiers, and individuals with accidental injuries such as falling from heights or road traffic collisions (Feigin et al., 2013; Jordan et al., 2013; Logan et al., 2013). The primary mechanical injury often leads to secondary neurological damage such as increased intracranial pressure, increased metabolic demands and excessive neurotransmitter release resulting in unfortunate complications notably post traumatic seizures (PTS) which can occur in 10-50% of patients with TBI (Englander et al., 2014). A seizure is a pathological condition that occurs when there is aberrant or elevated levels of electrical activity in the brain resulting in recurrent paroxysmal attacks causing an altered state of consciousness, confusion, and uncontrolled jerking movements (Panayiotopoulos, 2010). Approximately 86% of patients who have suffered their first PTS are at a risk of having their second PTS within the next 2 years and developing post traumatic epilepsy (PTE) (Verellen and

Cavazos, 2010). Anti-epileptic drugs (AEDs) are customarily used for treatment of PTE and their mechanisms of action can be divided into 4 basic categories: (a) modulation of voltage-gated sodium channels (VGSCs; e.g., phenytoin, carbamazepine), voltage-gated potassium channels (VGPCs; e.g., retigabine), or voltage-gated calcium channels (VGCCs; e.g., ethosuximide); (b) enhancing inhibitory neurotransmissions through gamma-aminobutyric acid type A ($GABA_A$) receptors (e.g., benzodiazepines, tiagabine); (c) diminishing excitatory neurotransmissions through glutamate receptors (e.g., perampanel); and (d) controlling the levels of neurotransmitter release at the presynaptic terminal (e.g., levetiracetam, gabapentin; Sills and Rogawski, 2020). However, AEDs are not always efficient in supressing seizure activity. In fact, studies have reported that these drugs often fail to curb seizures in more than 30% of the diagnosed cases, resulting in seizures becoming refractory or drug-resistant (Weaver and Pohlmann-Eden, 2013). For example, benzodiazepines (BZD) are the most popular class of AEDs used for seizure treatment, and they mediate their function by binding to the α+(1/2/3/5)/γ2- interface of the $GABA_A$ receptors (Juvale and Che Has, 2020). Unfortunately, several studies have reported alterations in the composition of $GABA_A$ receptor subunits in PTE (Gibson et al., 2010; Kharlamov et al., 2011; Drexel et al., 2015) These alterations might disrupt the formation of BZD-binding sites which subsequently influence the ineffectiveness of these drugs. The search for newer pharmaceutical options is further impeded due to a lack of good animal disease models that can replicate clinical cases. This is because the process of creating an animal model of status epilepticus (SE) is often circumscribed by high mortality rates, resistance to seizure induction, and animal wastage (Juvale and Che Has, 2020). Therefore, pharmacoresistance in PTE is a challenging problem and

there is an ongoing search for better disease models that can help researchers understand the underlying pathophysiology of this condition while also aiding in the development of newer and more effective therapeutics that can combat this resistance and alleviate the symptoms of patients with PTE. One such promising agent is the hypnotic/sedative drug, zolpidem, which is more commonly known by its commercial name Ambien or Stilnox. Zolpidem carries out its function through the $GABA_A$ receptors at the α1+/γ2- interface, which is similar to the binding site of BZD (Hanson and Czajkowski, 2008). However, studies have also reported positive modulation by zolpidem at the α1+/α1- interface of the $GABA_A$ receptors (Che Has et al., 2016) suggesting that unlike BZD, this drug can also act at other alternative sites besides the α1+/γ2- interface. Used previously to treat patients with insomnia, recent studies have highlighted a new potentially therapeutic role for zolpidem in patients with brain injury, showing promising recovery of cognitive and motor function (Chen, Sy and Wu, 2008; Williams et al., 2013; Chang and Weirich, 2014; Frisardi et al., 2016). Therefore, in our current study we investigate this possible therapeutic role of zolpidem on cognitive function in PTE, while also developing a more effectual lithium-pilocarpine animal model of SE.

1.2 Hypothesis

Zolpidem, a sedative-hypnotic agent, has potential therapeutic effects which can lead to an improvement in cognitive function and structural architectures of hippocampal sub-regions in PTE.

1.3 General Objective

The focus of this study revolves around the therapeutic potential of zolpidem and its resulting impact on cognitive impairment and cellular changes in the aftermath of PTE using a rat model.

1.4 Specific Objectives

1. To develop a clinically isomorphic lithium-pilocarpine seizure model in rats with a lower mortality rate.
2. To investigate the therapeutic effect of zolpidem on cognitive impairment in a rat model of PTE using Morris water maze.
3. To compare the histopathological and morphological differences in the hippocampal formation (focusing on Cornu Ammonis 1 (CA1), CA3, dentate gyrus (DG), subiculum) between the control and PTE groups.

CHAPTER 2

LITERATURE REVIEW

2.1 Post traumatic epilepsy

Every year more than 50 million people throughout the world experience a TBI (Dadas and Janigro, 2019). A sizeable number of these patients proceed to develop PTS; 11% with serious non-perforating TBI, and about 35-50% individuals with perforating TBI (Teasell et al., 2009 Ding et al., 2016). The risk factor for developing PTS is higher for individuals with extended periods of amnesia or who have been in extended lengths of coma, with a score <10 on the Glasgow Coma Scale, and/or suffer severe perforating brain injuries (e.g. depressed skull fracture, intracerebral haematoma etc) (Teasell et al., 2009). An increase in PTS frequency further increases the incidence rate of PTE. According to the International League Against Epilepsy (ILAE), an individual is to be diagnosed with epilepsy when he/she develops ≥2 unprovoked seizures >24 hours apart, an isolated unprovoked seizure with a high recurrence rate, or an already established epilepsy syndrome (Fisher et al., 2014).

There is a slight variation in this definition in terms of TBI. Unlike PTS which refers to the immediate or early stages of seizures following TBI (within 24 hours to one week), PTE is defined as recurrent seizures that occur >1 week following TBI (Ding et al., 2016). Factors that can increase the probability of developing PTE include severity of TBI, age, genetic factors/history of epilepsy in the family, PTS,

drug/alcohol abuse, and depression (Semple et al., 2019). The temporal lobe is a common site for PTE origination, from where it can spread to other regions of the brain causing secondary neurological damage (Semple et al., 2019). This disorder is often associated with cognitive deficits, anxiety, personality changes, as well as social deficits thus hampering an individual's quality of life (Semple et al., 2019). While it is more likely for an individual to develop PTE within the first few months following TBI, patients with moderate TBI have been shown to develop PTE 5 years following the injury (Fordington and Manford, 2020). In comparison, soldiers who have incurred serious perforating TBI can develop PTE even 15 years after the initial injury (McKee and Robinson, 2014). The incidence rate of PTE was shown to be 9.1% within the first 3 years following TBI, with more serious forms of TBI increasing the risk to 53% (Verellen and Cavazos, 2010). In the general population, TBI encompasses 20% of symptomatic epilepsy cases and 5% of all epileptic cases, while also being the major cause of epilepsy in young adults (Teasell et al., 2009).

Although AEDs are prescribed to these individuals as the first line of defence against seizures, these drugs have only shown to be useful in curbing initial PTS (Garga and Lowenstein, 2006); >30% of patients develop pharmacoresistance to these drugs (Weaver & Pohlmann-Eden, 2013). The ILAE defines pharmacoresistant epilepsy (PRE) as a condition where ≥2 AEDs fail to control an individual's seizures even after a thorough examination of a patient's history and prescribing tailored drugs based on this history for a sufficient period of time (Kwan et al., 2010). Based on clinical observations, PRE can be segregated into 3 categories: (i) *de novo* drug resistance, in which the patients show symptoms of pharmacoresistance from the first seizure itself, even before appropriate AED treatment; (ii) progressive drug

resistance, where pharmacoresistance manifests in patients over a period of time; and (iii) waxing and waning drug resistance, wherein a patient's reception to the drug frequently fluctuates between response and resistance (Pati and Alexopoulos, 2010). Studies have reported that if the initially prescribed AED fails to curb seizure activity in recently diagnosed epilepsy patients, there is a 1.73-times greater risk of the consecutive AEDs failing to do the same (Chen et al., 2018). This further increases the burden on patients with PTE as well as their care takers. There are currently several theories of PRE, the most famous being the gene variant hypothesis, the multidrug hypothesis, the network hypothesis, and the target hypothesis. However, no single theory has been able to account for the drug resistance seen in all patients, making PRE an enigma (Juvale and Che Has, 2021). Thus, there is an ongoing search to comprehend the underlying mechanisms of this condition in order to improve patient welfare.

2.2 The hippocampal formation and cognition

The temporal lobe is one of the most common sites for seizure origination in patients with PTE (Gupta et al., 2014; Semple et al., 2019). These lobes are essential for processing complex information, language, and emotions in healthy individuals, and an injury to this region can result in cognitive impairment, attention- and memory-deficits (both short- and long-term memory), difficulties in word comprehension, and altered emotional states (aggressiveness and/or anxiety disorders) (Antonis, 2020). Within the temporal lobes, lies the crucial structure that actually orchestrates all these vital functions, the hippocampal formation (**Figure 2.2.1**). This important three-layered, C-shaped structure comprises of the hippocampal proper which is made up of the four CA subfield forms (CA1-CA4), the DG, the subiculum, and the entorhinal

cortex (EC) (Schultz and Engelhardt, 2014). Transmission of information through this structure is largely unidirectional and begins with the lateral and medial EC (layer II) sending sensory information to the granule cells of DG through the perforant pathway (Strange et al., 2014), whereas layer III EC neurons project information to the CA1 via the temporoammonic pathway (Bartsch and Wulff, 2015; Temido-Ferreira et al., 2019).

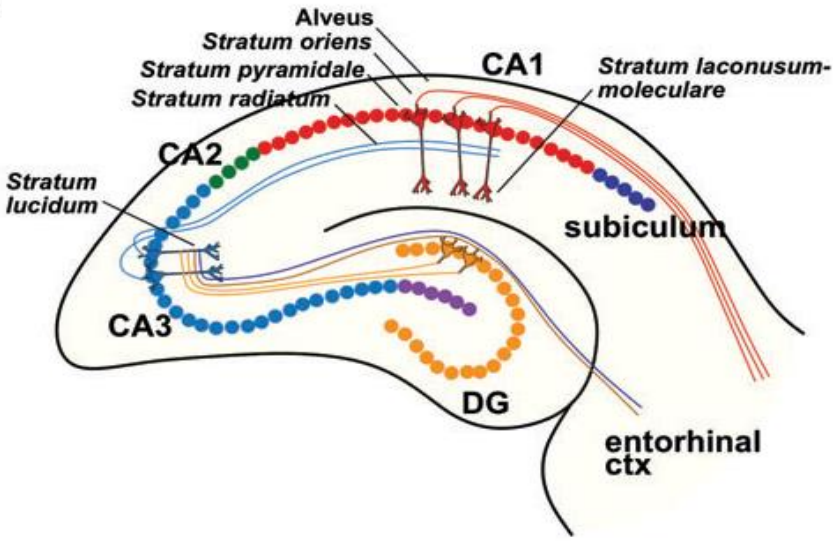

Figure 2.2.1: The hippocampal formation (Adapted from Temido-Ferreira et al., 2019; Cornu Ammonis, CA; Dentate gyrus, DG; cortex, ctx).

In the DG, granule cell axons create mossy fiber systems which send information to the CA3 and back to these granule cells (Bartsch and Wulff, 2015; Temido-Ferreira et al., 2019). The contralateral hippocampus also sends afferent information to the DG cells through commissural projections (Bartsch and Wulff, 2015; Temido-Ferreira et al., 2019). Furthermore, the pyramidal neurons of the CA3 synapse with other CA3 neurons via axon collaterals, while also transmitting information back to the DG, and also projecting information to the CA1 neurons via Schaffer collaterals

(Bartsch and Wulff, 2015; Temido-Ferreira et al., 2019). Since the pyramidal cells of the CA1 are the primary efferent neurons, these cells transmit information via the subiculum back into the EC, as well as other cortical and subcortical brain regions, thus completing the loop (Temido-Ferreira et al., 2019). Due to the various direct and indirect sensory inputs received by the CA1, it has been suggested that this region plays a critical role in scanning information and detecting errors, as it possesses the ability to compare information received from the EC, with the already accumulated information from the DG and the CA3 (Bartsch and Wulff, 2015; Temido-Ferreira et al., 2019). Therefore, this circuit plays a prominent role in learning and memory formation.

The significant role of the hippocampus in memory formation has been well documented by various surgeons who have resected the mesial temporal structures of patients with temporal lobe epilepsy (Bell et al., 2011). It was reported that patients following surgeries such as unilateral temporal lobectomy exhibited global amnesia (Milner and Penfield, 1955; Barr et al., 1990; Loring et al., 1994; Kapur and Prevett, 2003; Bell et al., 2011). Studies have also detected memory loss in patients who underwent anterior temporal lobectomy (Meador, 2006; Loring, 2007; Bell et al., 2011). Perhaps the most famous reported case was of Henry Gustav Molaison (H.M.), who after undergoing bilateral temporal lobe resection developed severe anterograde amnesia and temporally graded retrograde amnesia (Shah et al., 2014). H.M. could speak, perform tasks, and had intact intellectual functioning due to intact short-term and procedural memory function (Shah et al., 2014). He could also access his long-term memories such as those from his childhood (Shah et al., 2014). However, he had difficulty accessing memories from around the time of his surgery

and was unable to create new long-term episodic memories (Shah et al., 2014), thus highlighting the essential role of the hippocampus in the encoding, consolidation, and retrieval of information. Furthermore, the hippocampus has also been known to play a pivotal role in executive function, spatial processing, flexible cognition and social behaviour, processing speed, intelligence, autonoetic consciousness, and path integration (Bartsch et al., 2011; Reuben et al., 2011; Papp et al., 2013; Rubin et al., 2014; Yamamoto et al., 2014). Due to its prominent role in normal cognitive function, injuries such as TBI to the hippocampal formation can result in learning and memory impairments. Previous studies have linked shrunken hippocampal volume and reduced white matter with memory problems (Antonis, 2020). Both *in vivo* and *in vitro* studies have reported senescence of new-born immature neurons, as well as, dendritic and synaptic degeneration in the hippocampal DG upon induction of moderate TBI (Gao et al., 2008; Gao et al., 2011). These dendrites also exhibited signs of swelling along with a diminished density of dendritic spines and synapses.

Furthermore, hippocampal TBI in paediatric patients have been shown to result in lifelong deficits in cognitive function (Zhang et al., 2020). Recurrent induction of moderate TBIs in rats were shown to result in significant neuronal loss and cognitive deficits (Aungst et al., 2014). Chronic neuroinflammation was also observed with elevated levels of activated microglia in the ipsilateral and contralateral hippocampus. The study also reported altered synaptic plasticity, with increased long-term potentiation reported in the ipsilateral hippocampus as compared to the contralateral hippocampus. Electrophysiological studies in TBI rat models have also shown interneuronal loss and reduced long-term depression causing an elevation in excitatory postsynaptic potentials (EPSPs) in hippocampal DG resulting in cognitive

impairment (Zhang et al., 2018). In patients with TBI, this augmentation of EPSPs has been shown to hinder the inhibitory system in the DG due to a depolarizing switch in the reversal potential of GABA currents (Bonislawski et al., 2007), turning the inhibitory $GABA_A$ receptors into excitatory receptors thus generating seizures, and resulting in PTE. Since the temporal lobe is a frequent site for the emergence of seizures in PTE (Semple et al., 2019), damage to the hippocampal formation is unavoidable. Several studies have noted detrimental neurological damage in patients with PTE such as hyperexcitability in hippocampal formation, hippocampal sclerosis, unilateral/bilateral neuronal loss in hippocampal hilus and CA3, and aberrant mossy fiber sprouting in the inner molecular layer of DG (Sharma et al., 2021). Studies have suggested PTE to result in hippocampal atrophy (Lamar et al., 2014; Szaflarski et al., 2014). 94% of the PTE cases have also reported neocortical gliosis, mossy fibre reorganization, and hippocampal neuronal loss (Hunt et al., 2009; Lamar et al., 2014). The higher the degree of PTE, greater is the extent of hippocampal mossy fibre sprouting and SRS (Hunt et al., 2013). Studies have also shown that temporal lobe injuries can increase the frequency of early PTS and result in chronic temporal lobe atrophy upon PTE (Tubi et al., 2019).

Although the initial TBI-induced PTE may occur in one region of the brain, for example frontal parietal seizures, they often tend to migrate to the temporal lobe as temporal lobe epilepsy in later stages of the disorder (D'Ambrosio et al., 2005; Garga and Lowenstein, 2006; Hunt et al., 2009). Hippocampal degeneration has been shown to increase with time in patients with PTE (Lamar et al., 2014). Studies have also reported neuronal loss in the hippocampus of patients with PTE (Swartz et al., 2006; Blume, 2007). Studies focusing on the DG of PTE brains have also noted

structural deformities in dendrites along with spine remodeling (Sharma et al., 2021). Altered hippocampal neurogenesis due to PTE has also been implicated in impaired cognitive function (Ngwenya and Danzer, 2019); a single seizure alone is adequate enough to increase granule cell neurogenesis in the DG (Bengzon et al., 1997). Reorganization of pyramidal cell dendrites were also observed in the DG of PTE brains with either reduced spine density or spine loss (Sharma et al., 2021). Recurrent seizures due to PTE can also impede cellular migration and integration in the hippocampal formation thus altering normal synapse function, and creating aberrant networks (Scharfman et al., 2003; Parent et al., 2006; Jessberger et al., 2007; Hunt et al., 2010; Jackson et al., 2012; Murphy et al., 2012; Danzer, 2018; Ngwenya and Danzer, 2019). Furthermore, the augmentation of granule cell neurogenesis has been suggested to increase neuronal excitability and seizure susceptibility (Neuberger et al., 2017), thus functioning as a positive-feedback loop and causing additional damage to the hippocampal formation and impairing cognitive function.

2.3 The animal models of SE

In order to create a suitable animal model that can replicate the cognitive dysfunction observed in patients with PTE, researchers may utilize a variety of methods to induce SE. In clinical settings, a generalized tonic-clonic seizure usually persists for 3 minutes, with any lengthening in duration resulting in the seizures becoming self-sustaining or refractory (Wray and Knupp, 2011; Juvale and Che Has, 2020). Therefore, the ILAE describes SE as a recurrent seizure that persists for ≥5 minutes, with seizure activity ≥30 minutes resulting in the creation of aberrant circuits and long-lasting neuronal damage (Trinka et al., 2015). A competent animal model is one

that can replicate most of the characteristics of clinical cases while giving scientists a better understanding of the underlying disease pathophysiology, as well as elucidating the pharmacokinetics, adverse effects, potency, efficacy, and tolerance of the prospective AEDs under investigation, thus allowing an effortless transition from preclinical laboratory testing to the later stages of human clinical trials (Juvale and Che Has, 2020).

Similarly, in the case of PTE, a good animal model is one that can replicate the prominent symptoms seen in patients with PTE such as seizures persisting ≥30 minutes (for replicating brain damage seen in severe PTE cases), loss of memory, muscle spasms, falling, and confusion (Fujikawa, 1996; Varelas and Claassen, 2017; Juvale and Che Has, 2020). Since clinical cases observe recurrent seizures and neurological changes in PTE brains after a silent time period (days/weeks) following the initial injury, animal models must also serve a "latency period" (period of no seizures) following the primary injury, which should then be accompanied by spontaneous recurrent seizures (SRS) at the end of the "latency period" (Cavalheiro, 1995; Juvale and Che Has, 2020). Lastly, since numerous patients with PTE exhibit PRE, a successful animal model must also be able to replicate this observed pharmacoresistance to conventional AEDs in order to facilitate the testing of new prospective AEDs (Kapur and Macdonald, 1997; Mazarati et al., 1998b; Jones et al., 2002; Chakir et al., 2006; Juvale and Che Has, 2020).

In order to create such a successful animal model, a researcher must take several factors into consideration such as the time and effort needed to develop the model,

expense, laboratory skills of the investigators, accessibility of materials/research environment, pharmacokinetics, animal strain/susceptibility of animals to seizure induction, and the mortality rate of animals (Juvale and Che Has, 2020). For instance, the kindling model of SE which utilizes implanted depth electrodes to produce repetitive electrical stimulation in the brain is a useful technique, but at the same time is also costly, laborious, and time consuming, unlike chemoconvulsants (Song et al., 2018; Juvale and Che Has, 2020). The electrodes used in the kindling model are delicate, expensive, and cannot be used for prolonged experimentation periods. This procedure also requires the investigator to be highly skilled and precise to avoid damaging the electrodes (Song et al., 2018; Juvale and Che Has, 2020). Studies have also shown that kindling models generate a lower frequency of SRS as compared to chemoconvulsant models of SE (Brandt et al., 2004; Juvale and Che Has, 2020). There are several other SE animal models that utilize electrical stimulation, like perforant path stimulation, however they have all been shown to cause lesser neuronal damage when compared to chemoconvulsant models such as kainic acid and pilocarpine models (Reddy and Kuruba, 2013; Juvale and Che Has, 2020). If the brain damage created in the animal model is not significant enough, it fails to replicate the PTE symptoms from clinical cases, thus making electrical stimulation models like the perforant path stimulation undesirable (Juvale and Che Has, 2020). Compared to other animal models of SE, the laboratory skills and equipment required for using chemoconvulsants are relatively simple (Juvale and Che Has, 2020).

Between the two common chemoconvulsants used, intracerebral kainic acid injections result in intense seizures, extreme neurological damage, and high mortality

rates as compared to pilocarpine (Kienzler-Norwood et al., 2017). Unlike pilocarpine, that gradually results in SE and can utilize the Racine scale (Racine, 1972) to determine the stages/severity of seizures, kainic acid-generated seizures cannot be standardized using this scale due to the rapid and indistinguishable nature of its seizure progression (Lévesque et al., 2016; Juvale and Che Has, 2020). Previous studies in mice models that used systemic administration of kainic acid have also reported difficulties with seizure induction (McKhann et al., 2003; Juvale and Che Has, 2020). Animals administered with kainic acid were also shown to exhibit higher levels of anxiety and depression (Ratté and Lacaille, 2006; Gröticke et al., 2008; Juvale and Che Has, 2020). Animal models also show varying degrees of resistance to kainic acid; C57BL/6, C57BL/10, and F1 C57BL/6*CBA/J mice strains have been reported to exhibit resistance to seizure induction by kainic acid, resulting in animal wastage (McLin and Steward, 2006; Juvale and Che Has, 2020).

Kainic acid animal models also have inconsistent reactions to conventional AEDs as compared to pilocarpine models, making them unsuitable for pharmacoresistance studies (Reddy and Kuruba, 2013; Juvale and Che Has, 2020). Studies have also reported difficulties in distinguishing kainic acid-mediated neuronal damage from secondary neuronal damage caused by seizure activity (Reddy and Kuruba, 2013; Juvale and Che Has, 2020). This makes the kainic acid model unreliable as a true SE animal model should only cause secondary damage through seizure activity and not through the chemoconvulsant itself. It was also reported that animals induced with kainic acid showed varying degrees of neuronal damage even though the kainic acid dose used and the SRS observed were identical for all animals; 77% showed moderate bilateral hippocampal damage, while 23% showed substantial damage

throughout the hippocampal region (Rao et al., 2006; Juvale and Che Has, 2020). This makes it difficult to standardize the neurodegeneration caused by the kainic acid model. Another factor to consider is the expense; pilocarpine is much cheaper to purchase than kainic acid (Lévesque et al., 2016; Juvale and Che Has, 2020). Furthermore, kainic acid-induced SRS disappear within 22-46 days (Cavalheiro et al., 1982; Cronin and Dudek, 1988; Juvale and Che Has, 2020), whereas pilocarpine-induced SRS have been reported to last for 120-325 days (Cavalheiro et al., 1991; Mello et al., 1993; Juvale and Che Has, 2020). The time period reported for the pilocarpine model is considered to be the "entire observation period", suggesting that if the investigators wished to continue their studies with these animal models, the SRS would be observed for a longer period of time, making it more reliable than the kainic acid model (Juvale and Che Has, 2020). Just like the clinical cases, animals injected with pilocarpine also exhibit a "latency period" following the primary injury before proceeding to SRS (Turski et al., 1989; Lévesque et al., 2016; Juvale and Che Has, 2020).

Numerous electrophysiological and morphological studies have observed similar neurological changes in the hippocampal region of pilocarpine animal models of SE and patients with SE (Scheibel et al., 1974; Babb, 1986; Masukawa et al., 1989; Isokawa et al., 1991; Isokawa and Mello, 1991; Mello et al., 1992; Juvale and Che Has, 2020). A common similarity observed is the dispersion of DG granule cells along with supragranular and intragranular mossy fibre sprouting (Houser et al., 1990; Babb et al., 1991; Mello et al., 1993; Juvale and Che Has, 2020). Additionally, since PTE induced damage is common in the temporal lobe, the pilocarpine model is well suited for replicating this condition as pilocarpine is an M1 muscarinic

acetylcholine receptor agonist (Juvale and Che Has, 2020). These receptors are highly expressed in the hippocampus, and their prolonged activation through pilocarpine generates recurrent excitatory signaling, eventually resulting in brain damage in the hippocampal formation (Dennis et al., 2015; Juvale and Che Has, 2020), thus making the pilocarpine model of SE a suitable model for studying pharmacoresistance and cognitive dysfunction in PTE.

2.4 Challenges with creating a lithium-pilocarpine model of SE

Although its initial discovery in the early 1980s lead to a remarkable breakthrough in understanding the underlying mechanisms of SE, there are currently several areas of the pilocarpine model that need improvement. The first pilocarpine model was developed in rats using a single dose of 300-400 mg/kg of pilocarpine (Turski et al., 1983). This resulted in SE generation in 83% of the animals, but a mortality rate of 100% (Jope et al., 1986; Juvale and Che Has, 2020). Since the pilocarpine dose used in this method was relatively high, it was difficult to induce seizures in the resistant animals using extra doses without causing toxicity. This problem was somewhat tackled by the discovery of the lithium-pilocarpine model which utilizes 127 mg/kg of lithium chloride (LiCl), with subsequent administration of 30 mg/kg of pilocarpine administration 18 hours later (Fan et al., 2020). Unlike the original pilocarpine model, the use of LiCl made animals more sensitive to pilocarpine causing a 20-fold shift in the dose-response curve for SE generation, therefore reducing SE induction time while also reducing the pilocarpine dose required (Clifford et al., 1987; Juvale and Che Has, 2020).

Both models were reported to exhibit similar seizure patterns with matching behavioural, metabolic, electrographic, and neurological symptoms (Clifford et al., 1987; Müller et al., 2009a; Juvale and Che Has, 2020). Some studies even reported this newer model increasing the SE induction rate in animals as compared to the original model (Goffin et al., 2007; Ahmad, 2013; Juvale and Che Has, 2020). However, one concerning factor with the lithium-pilocarpine model is the high mortality rate (92-95%) (Jope et al., 1986; Morrisett et al., 1987; Huang et al., 2018; Juvale and Che Has, 2020). Like its predecessor, this model was also unable to reduce the mortality rates in the induced animals without compromising the development of the model itself; reducing the pilocarpine dose resulted in lower SE induction rates (Jope et al., 1986; Huang et al., 2018; Juvale and Che Has, 2020). Furthermore, compared to electrical stimulation models, chemoconvulsant-induced SE is more difficult to terminate (Bankstahl and Löscher, 2008; Juvale and Che Has, 2020). Similar to patients with PTE, diazepam, a common BZD is not able to terminate seizures in these animals (Walton and Treiman, 1988; Kapur and Macdonald, 1997; Treiman et al., 1998; Jones et al., 2002; Goodkin et al., 2003; Nardou et al., 2011; Apland et al., 2014; Zhao et al., 2016; Juvale and Che Has, 2020). Other conventional AEDs such as phenobarbital, phenytoin, valproate, and carbamazepine also fail to curb seizures in lithium-pilocarpine models (Morrisett et al., 1987; Biagini et al., 2001; Kubová et al., 2005; Juvale and Che Has, 2020). While this pharmacoresistance provides a good opportunity to study PTE and test newer potential AEDs, it is a futile as ≥95% of the animals die upon SE induction.

Furthermore, with more research being conducted worldwide it was soon discovered that several rodent strains had developed resistance to pilocarpine over time (Chen et

al., 2005; Winawer et al., 2007a, 2007b; Juvale and Che Has, 2020). This varied sensitivity was observed between: (i) same strain of rodents from the same batch purchased from the same breeder, (ii) same strain of rodents from a different batch but from the same breeder, and (iii) same strain of rodents purchased from a different breeder (Borges et al., 2003; Bankstahl et al., 2009; Portelli et al., 2009; Müller et al., 2009b; Schauwecker, 2012; Bankstahl et al., 2012; Juvale and Che Has, 2020). These recent substrain differences have made SE induction even more difficult causing discrepancies in the results within the same laboratory. Therefore, it is imperative for researchers to modify the current lithium-pilocarpine model such that the animals can replicate all aspects of PTE, while also having low mortality rates, but high SE induction rates.

2.5 The paradoxical effects of zolpidem in brain injury

One of the most arduous tasks when treating patients with PTE is battling pharmacoresistance. Since the conventional AEDs such as the BZD class have little to no effect on these patients, there is an ongoing search for better and more effective pharmaceutical options. One such drug of interest is Zolpidem, more commonly known as Ambien or Stilnox (**Figure 2.5.1**). Introduced in Europe in 1988 and in the USA in 1998 as Z-drugs along with eszopiclone and zaleplon, zolpidem belongs to the imidazopyridine class (Kirkwood et al., 2007; Brandt and Leong, 2017). Used traditionally to treat patients with insomnia, this drug mediates its sedative/hypnotic effects through the $GABA_A$ receptors (Hanson and Czajkowski, 2008).

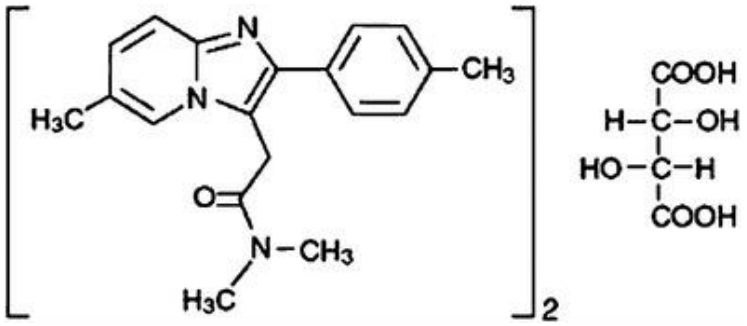

Figure 2.5.1: The structure of zolpidem (Mahapatra et al., 2015).

The $GABA_A$ receptors are pentameric, cys-loop ligand-gated ion channels that encircle a chloride ion (Cl^-) pore and are modulated by the chief inhibitory neurotransmitter of the central nervous system, GABA. These receptors can exist in both homomeric and heteromeric forms and have 19 known subunits: α1-6, β1-3, γ1-3, ρ1-3, δ, ε, θ, and π, with the most common composition being two α1, two β2, and one γ2 subunit (**Figure 2.5.2A**; Olsen and Sieghart, 2009). Based on the subunits that they co-assemble with, these receptors may differ in their pharmacological profile, location (synaptic or extrasynaptic), biophysical properties, and neurotransmitter affinity (Juvale and Che Has, 2021). In healthy adult neurons, there is a higher extracellular concentration of Cl^-, therefore binding of 2 molecules of GABA at the β+α- interface causes a conformational change in the receptor structure, opening the channel and resulting in an influx of Cl^-, thus making the intracellular environment more negative leading to hyperpolarisation (**Figure 2.5.2B**; Akk and Steinbach, 2011). In $GABA_A$ α1β2γ2 receptors, Zolpidem binds at the α1+/γ2- interface, similar to the binding site of BDZs (Hanson and Cjazkowski, 2008). Although it exhibits similar sedative properties to that of BZDs, its other effects of anxiolysis, and muscle

relaxation are negligible (Mahapatra et al., 2015). Also, compared to BZDs, it has fewer side effects as it has a shorter half-life (2.5 hours) in circulation and is also highly selective for the α1-containing $GABA_A$ receptors (Mahapatra et al., 2015; Che Has et al., 2016).

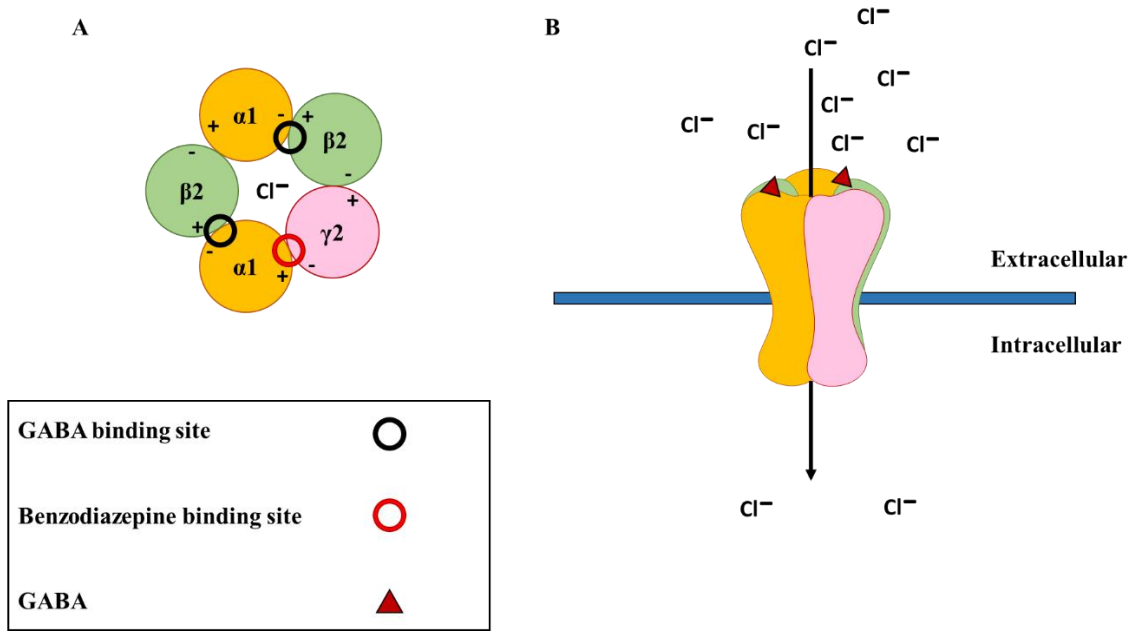

Figure 2.5.2: The physiological function of $GABA_A$ receptors. (**A**) The most common subunit configuration of $GABA_A$ receptors along with the binding sites for GABA (shown with black circle), and benzodiazepines (shown with red circle); (**B**) The binding of GABA (shown with red triangle) results in the opening of the channel, causing an influx of chloride ions (Cl^-) into the cell.

Unlike its usual sedative function, several recent studies have suggested zolpidem to have a more peculiar role in patients with TBI (Cohen and Duong, 2008; Du et al., 2014; Khalili et al., 2020; Sripad et al., 2020). Perhaps, one of the most famous cases is of Sam Goddard (I am Sam - Australian Story, aired by ABC channel in June 2017). At the young age of 23, Sam had a series of eight strokes that put him in a coma (Warren, 2021). While the doctors believed that there was no hope for him and that he would remain in a permanent vegetative state for the rest of his life, his fiancé

convinced the doctors to administer him with zolpidem to aid with his sleep (Warren, 2021). Within a few days, Sam who was previously only able to create unintelligible sounds was able to speak (Warren, 2021). Up until 2017, there have been 23 published clinical reports and 6 studies that have detected zolpidem mediating a paradoxical awakening effect from previous vegetative states in patients who have suffered TBI (Sutton and Clauss, 2017). Like Sam, several patients were shown to revert back to having their TBI symptoms when the drug left their system and were shown to improve again upon re-administration of zolpidem. It was reported that zolpidem not only improved perfusion around the site of injury but was also shown to boost their metabolic and neuronal activity (Sutton and Clauss, 2017). Moreover, a recent study noted a spectacular improvement in speech, cognition, and motor function (including the ability to walk) in brain injury patients administered with zolpidem (Arnts et al., 2020). Furthermore, similar improvements have also been observed in patients with Parkinson's disease and cerebral palsy (Chen, Sy and Wu, 2008; Williams et al., 2013; Chang and Weirich, 2014; Frisardi et al., 2016). Treatment with a placebo or an analogous sedative such as zopiclone is not shown to have a similar awakening effect (Sutton and Clauss, 2017). Interestingly, this effect of zolpidem is only detected after several months following TBI (Sutton and Clauss, 2017), which correlates with the observed changes in the $GABA_A$ receptor subunit composition in PTE and TBI-induced animal models and patients with PTE. Recently, zolpidem has also been shown to act on an alternative site, the α1+/α1- interface of the $GABA_A$ receptors with higher potency than diazepam, a classical BZD (Che Has et al., 2016). Therefore, unlike BZD, this drug could potentially bind to other sites besides the α1+/γ2- interface which could prove beneficial in cases of PTE that have altered $GABA_A$ receptor subunits.

However, alterations in the $GABA_A$ receptor subunits may not be the sole reason for this observed "awakening effect" of zolpidem. In healthy individuals, the $GABA_A$ receptors are able to mediate hyperpolarisation and exhibit their inhibitory effects because GABA has a negative reverse potential. This reversal potential depends on the intracellular levels of Cl^- that are monitored by two essential cation-chloride-cotransporters, sodium-potassium-chloride cotransporter 1 (NKCC1), and potassium-chloride cotransporter 2 (KCC2). The NKCC1 actively pumps sodium ions (Na^+), potassium ions (K^+), and Cl^- into the cell, whereas the KCC2 actively pumps K^+ and Cl^- out of the cell, thus establishing a higher extracellular concentration of Cl^- in healthy adult brains (Liu et al., 2020). Encoded by *SLC12A2*, NKCC1 is highly expressed in glial cells, as well as, both central and peripheral neurons, while KCC2, which is encoded by *SLC12A5* is expressed in the hippocampal and neocortical pyramidal neurons and interneurons (Benarroch, 2013; Rahmati et al., 2018). Interestingly, studies in neonatal brains have shown GABA to play an excitatory role in immature neurons and has been reported to generate giant depolarising potentials (Ben-Ari, 2002; Valeeva, Valiullina and Khazipov, 2013; Kirmse et al., 2015), suggesting that these receptors are not necessarily restricted to serving an inhibitory role alone. This excitatory function of GABA is further reiterated in cases of TBI (Shulga et al., 2008; Guerriero et al., 2015), proposing an alteration in the inhibitory system. These observed changes in the reverse potential of GABA can be attributed to the expression level of the NKCC1 and KCC2. In immature neurons, NKCC1 is highly expressed as compared to KCC2, causing an influx of Cl^- ions into the cell and resulting in a depolarized Cl^- equilibrium potential (**Figure 2.5.3**; Dzhala et al., 2005; Sipilä et al., 2006; Chen et al., 2014; Kolbaev et al., 2020). Compared to

mature neurons that can recover in a few seconds, the recovery time for the Cl^- gradient to go back to its pre-stimulatory levels is longer in immature neurons (several minutes); similar recovery time has also been observed in epilepsy models (Nardou et al., 2011). Several epilepsy studies have also reported a downregulation in KCC2 expression levels and an upregulation of NKCC1 expression levels (Wang et al., 2016; Liang and Huang, 2017; Moore et al., 2017; Kelley et al., 2018; Luo et al., 2018; Tillman and Zhang, 2019; Liu et al., 2020; Hampel et al., 2021). Since both these cotransporters are essential for the maintenance of Cl^- homeostasis, alterations in either of their expression levels can cause an imbalance in the Cl^- gradient, resulting in seizures and eventually PTE.

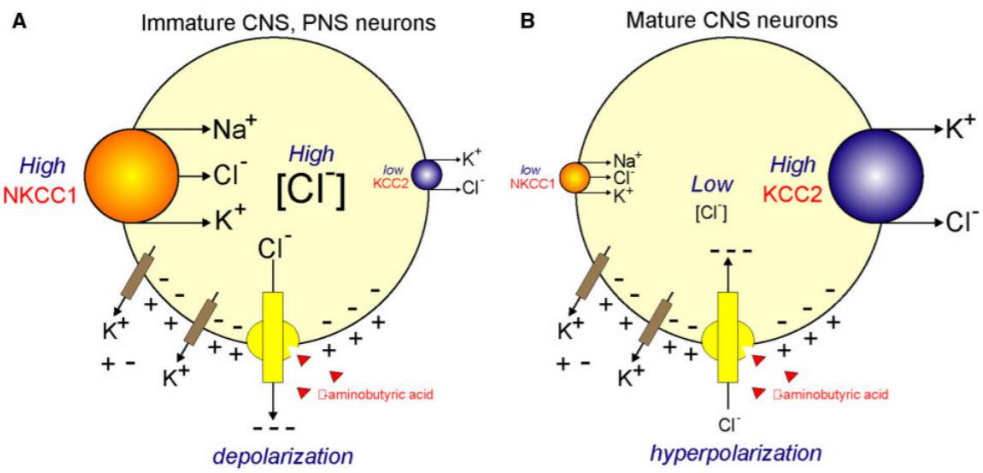

Figure 2.5.3: Differences in the functional and expressional levels of NKCC1 and KCC2. (**A**) Immature neurons exhibit higher expression levels of NKCC1, as compared to KCC2, resulting in depolarisation. (**B**) Mature neurons express lower levels of NKCC1 in contrast to KCC2, resulting in hyperpolarisation. The yellow structure represents the $GABA_A$ receptors, with the red triangles representing the neurotransmitter GABA (Adapted from Koumangoye et al., 2021).