THE EFFECTS OF ZOLPIDEM ON SPATIAL MEMORY, CELLULAR AND IONIC CO-TRANSPORTERS (KCC2/NKCC1) IN THE HIPPOCAMPUS OF LITHIUM-PILOCARPINE RAT MODEL

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UNIVERSITI SAINS MALAYSIA

2022

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by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

June 2022

ACKNOWLEDGEMENT

First and foremost, I would like to praise God for giving me strength and motivation to complete this project. I am grateful for having a family that has been very supportive and understanding to me in completing this thesis. I would like to express my greatest appreciation to my supervisor, Dr Ahmad Tarmizi Che Has for giving me support, motivation, guidance, and encouragement in completing this research and thesis. I am also thankful to my co-supervisors Associate Prof. Dr Zurina Hassan and Dr Mohd Nor Azim for their guidance and advice in this research project. My appreciation also goes to all my colleagues from Department of Neurosciences, Centre for Drug Research, and Animal Research and Services Centre that have supported me in completing this project. My acknowledgment on the Ministry of Higher Education, Malaysia for its financial support through the research grant incentive (Fundamental Research Grant Scheme 203/PPSP/6171233). May efforts and knowledge in this thesis can benefit those who are gratified to seeking understanding related to the field.

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LIST OF SYMBOLS

%	Percentage
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
θ	Theta
π	Pi
ρ	Rho
~	Approximate
±	Plus-minus
mM	Millimolar
g	Gram
mg	Milligram
m	Metre
cm	Centimetre
µm	Micrometre
ml	Millilitre
s	Second
min	Minute
h	Hour
ms	Millisecond

a.u.	Arbitrary unit
mm^2	square millimetre
μm^2	Square micrometre
ms^{-1}	Meter per second
mg/kg	Milligram per kilogram
kPA	Kilopascal
°C	Degree Celsius
a.m.	Ante meridiem
p.m.	Post meridiem
pH	Potential of hydrogen

LIST OF ABBREVIATIONS

nTBI	non-Traumatic Brain Injury
SE	Status Epilepticus
TBI	Traumatic Brain Injury
SPECT	Single-Photon Emission Computed Tomography
PET	Positron Emission Tomography
EEG	Electroencephalography
$GABA_ARs$	γ -Amino-Butyric Acid type-A Receptors
Na^+	Sodium ion
K^+	Potassium ion
Cl^-	Chloride ion
Ca^{2+}	Calcium ion
$[Cl^-]_i$	Intracellular Chloride Ion Concentration
$[Cl^-]_o$	Extracellular Chloride Ion Concentration
CCCs	Chloride-Cation Cotransporters
NKCC1	Sodium-Potassium-Chloride Cotransporter 1
KCC2	Potassium-Chloride Cotransporter 2
ABI	Acquired Brain Injury
PTSs	Post-Traumatic Seizures
SAH	Subarachnoid Haemorrhage
GCS	Glasgow Coma Scale
MCS	Minimally Conscious State
TLE	Temporal Lobe Epilepsy

SRSs	Spontaneous Recurrent Seizures
DG	Dentate Gyrus
DG_{sPB}	Dentate Gyrus supra-Pyramidal Blade
DG_{iPB}	Dentate Gyrus infra-Pyramidal Blade
CA 1 – 3	Cornu Ammonis 1 – 3
EC	Entorhinal Cortex
GCL	Granule Cell Layer
PCL	Pyramidal Cell Layer
PP	Perforant Path
MF	Mossy Fibre
SC	Schaffer Collateral
PTZ	Pentylenetetrazol
KA	Kainic Acid
ECT	Electric Convulsive Therapy
EPM	Elevated Plus Maze
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ED	Effective Dose
MFS	Mossy Fibre Sprouting
i.p.	Intraperitoneal
M1P	D-*Myo*-inositol-1-Phosphate
IP_3	Inositol 1, 4, 5 trisphosphates
BBB	Blood Brain Barrier
IL-1β	Interleukin-1β
SHH	Sonic Hedgehog

TNFα	Tumour Necrosis Factor α
IL-1ra	Interleukin-1 Receptor Antagonist
MK-801	(+)-5-methyl-10,11dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine
MPEP	2-Methyl-6-(phenylethynyl)pyridine
NMDARs	*N*-methyl-D-aspartate Receptors
M1R – M5R	Muscarinic type 1 – 5 Receptors
GPCRs	G-protein Coupled Receptors
PLC	Phospholipase C
PI3K	class I Phosphoinositide 3-kinases
PIP_2	Phosphoinositol-4,5-bisphosphate
DAG	Diacylglycerol
PKC	Protein Kinase C
ER	Endoplasmic Reticulum
IP_3Rs	IP_3 Receptors
PP-1	Protein phosphatase 1
WNK	with-no-lysine [K]
SPAK	STE20/SPS1-related proline–alanine-rich kinase
OSR1	Oxidative Stress-Responsive kinase 1
GABA	γ-Aminobutyric Acid
CNS	Central Nervous System
$GABA_BRs$	γ-Amino-Butyric Acid type-B Receptors
LGICs	Ligand Gated Ion Channels
nAChRs	nicotinic Acetylcholine Receptors
$5HT_3Rs$	5-Hydrotryptamine type-3 Receptors

GlyRs	Glycine Receptors
ECD	Extracellular Domain
SLC12	Solute Carrier 12
E_{Cl}	Chloride ion Equilibrium Potential
RMP	Resting Membrane Potential

KESAN ZOLPIDEM TERHADAP MEMORI SPASIAL, SELULAR DAN PENGANGKUT IONIK (KCC2/NKCC1) DALAM HIPOKAMPUS MODEL TIKUS LITIUM-PILOKARPIN

ABSTRAK

Pesakit yang mengalami *status epilepticus* terdedah kepada gangguan kognitif susulan daripada kecederaan pada hipokampus, struktur yang dikaitkan dengan kognitif. Kerentanan tinggi hipokampus terhadap permulaan gejala sawan dan yang berkaitan dengan epilepsi adalah punca utama kecederaan. Kekejangan akibat sawan seperti yang ditunjukkan dalam *status epilepticus* boleh mengakibatkan pelbagai kerosakan patologi pada hipokampus seperti keradangan, *neurogenesis* yang tidak normal, dan kemusnahan sel neuron. Penemuan awal mencadangkan potensi sifat terapeutik zolpidem iaitu bahan sedatif/hipnotik dalam pemulihan tingkah laku dan fisiologi pesakit yang mengalami kecederaan otak, semenjak penemuan kesan paradoks. Dalam kajian ini, model tikus litium-pilokarpin *isomorphic* yang ideal bagi *status epilepticus* dengan morbiditi tinggi dan kadar kematian rendah telah dihasilkan. Selepas itu, dengan menggunakan teknik labirin air Morris, model yang ideal telah digunakan untuk mengkaji potensi kesan terapeutik zolpidem sebagai pengantara pemulihan pembelajaran dan ingatan pada model tikus tadi. Selain itu, penilaian histologi secara kuantitatif dengan menggunakan kaedah *haematoxylin* dan *eosin* dijalankan untuk menilai kesan zolpidem pada morfologi selular dalam hipokampus model tikus. Akhir sekali, kaedah *immunohistochemistry* telah dijalankan untuk menilai kesan potensi zolpidem pada ekspresi protein KCC2 dan NKCC1 dalam beberapa kawasan hipokampus model tikus. Kami telah berjaya menghasilkan model

tikus litium-pilokarpin yang ideal bagi *status epilepticus* dengan kadar morbiditi dan kematian masing-masing sebanyak 78 % dan 22 %, yang menunjukkan kemerosotan ketara pada pembelajaran dan ingatan ($p < 0.0001$), selari dengan tingkah laku yang terhasil seperti kebimbangan yang berlebihan ($p < 0.0001$). Selain itu, melalui labirin air Morris, penggunaan zolpidem tidak menambahbaikkan fungsi ingatan pada tikus yang mengalami *status epilepticus* ($p > 0.05$). Walau bagaimanapun, penemuan kami menunjukkan bahawa kesan sedatif/hipnotik zolpidem tidak dapat dilihat dalam keadaan *status epilepticus* ($p > 0.05$). Tambahan pula, analisis histologi tidak menemui kesan zolpidem pada perubahan morfologi hipokampus ($p > 0.05$). Walau bagaimanapun, penemuan *immunohistochemistry* kami menunjukkan potensi zolpidem untuk memulihkan ekspresi KCC2 (DG, $p < 0.05$; CA3, $p > 0.05$; CA1, $p > 0.05$; SUB, $p > 0.05$) dan NKCC1 (DG, $p < 0.001$; CA3, $p < 0.001$, CA1, $p < 0.01$; SUB, $p < 0.001$) dalam pelbagai kawasan hipokampus, sesetengahnya menyamai dengan yang diperhatikan pada tikus normal. Penemuan ini menunjukkan bahawa walaupun kesan pemulihan yang tidak dapat dilihat secara jelas dalam proses pembelajaran dan gangguan ingatan serta perubahan histopatologi, zolpidem berkemungkinan terlibat dalam pemulihan secara molekular terutamanya melalui ekspresi protein KCC2 dan NKCC1 dalam hipokampus, yang penting untuk proses neurotransmisi perencatan yang berkesan di dalam otak.

THE EFFECTS OF ZOLPIDEM ON SPATIAL MEMORY, CELLULAR AND IONIC CO-TRANSPORTERS (KCC2/NKCC1) IN THE HIPPOCAMPUS OF LITHIUM-PILOCARPINE RAT MODEL

ABSTRACT

Patients with status epilepticus are susceptible to cognitive impairment due to injuries on the hippocampus, a structure associated with cognition. Hippocampus high susceptibility to initiation of convulsive seizure and epilepsy-related damages is the primary cause mediating the impairment. Convulsive seizure as per se exhibited in status epilepticus can result in various hippocampal pathologies such as inflammation, aberrant neurogenesis, and neuronal death. Promisingly, vast evidence has been suggesting the potential therapeutic hallmark of sedative/hypnotic zolpidem in mediating behavioural and physiological recovery in patients with brain injury, since serendipitous discovery about paradoxical awakening effect of zolpidem. In this study, an ideally isomorphic lithium-pilocarpine rat model of status epilepticus with high morbidity and low mortality rate was developed. Subsequently, using the Morris Water Maze task, the rat model was utilised to investigate the potential therapeutic effect of zolpidem in mediating recovery of learning and memory. Moreover, quantitative haematoxylin and eosin histological evaluation was performed to evaluate the effect of zolpidem on cellular morphology in the hippocampus of the rat model. Finally, fluorescence immunohistochemistry was conducted to assess the potential effect of zolpidem on the dysregulated KCC2 and NKCC1 protein expression in multiple hippocampal subregions. We successfully developed an ideal lithium-pilocarpine rat model of status epilepticus with respective morbidity and mortality rate of 78 % and

22 %, that showed significant impairments ($p < 0.0001$) on learning and memory, consistent with the exhibition of abnormal anxiety-like behaviour ($p < 0.0001$). Besides, in Morris Water Maze, zolpidem administration did not enhance memory function in the rats with status epilepticus ($p > 0.05$). Nonetheless, our results indicated that zolpidem sedative/hypnotic effect was unnoticeable in the status epilepticus condition ($p > 0.05$). Furthermore, histological analysis discovered insignificant zolpidem effect on the hippocampal morphological changes ($p > 0.05$). However, interestingly, our immunohistochemical findings indicated the potential of zolpidem to restore altered KCC2 (DG, $p < 0.05$; CA3, $p > 0.05$; CA1, $p > 0.05$; SUB, $p > 0.05$) and NKCC1 (DG, $p < 0.001$; CA3, $p < 0.001$, CA1, $p < 0.01$; SUB, $p < 0.001$) expression levels in certain hippocampal subregions, comparable to those observed in the normal rats. These results suggest that despite unobservable overt recovery in learning and memory impairment and histopathological changes, zolpidem may partly involve in molecular restoration especially through KCC2 and NKCC1 protein expression in the hippocampus, which is vital for an efficient inhibitory neurotransmission in the brain.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Non-traumatic brain injury (nTBI) is a non-congenital injury caused by internal factors including stroke, brain infection, aneurysm, tumour, and neurotoxic poisoning (Chen, Bushmeneva, *et al.*, 2012; Giustini, Pistarini and Pisoni, 2013; Chan *et al.*, 2016). The consequences of nTBI may also result in secondary brain injury events including neuronal excitotoxity which eventually leads to cell death (Giustini, Pistarini and Pisoni, 2013). This excitotoxity and neuronal death circumstances are also translatable from the occurrence of prolonged status epilepticus (SE) which may eventually result in the exacerbation of primary brain injury via cascades of cellular, biochemical and blood vessel changes (Castro *et al.*, 2017; Fontaine *et al.*, 2020). Clinically, patients suffering from SE may be highly susceptible to cognitive decline, due to damages on multiple brain regions including the hippocampus, a region that is associated with cognitive function such as learning and memory (Helmstaedter, 2007; Martinos *et al.*, 2013; Scott, 2014; Lisman *et al.*, 2017; Mutis, Rodríguez and Nava-Mesa, 2017). The adverse SE-associated insults on the hippocampus may be acknowledged by the exhibition of various neuropathological alterations including neuronal loss, astrogliosis, neuroinflammation, and aberrant neurogenesis and synaptic re-organisation (Scott, 2014; Danzer, 2017; Cavarsan *et al.*, 2018).

Nonetheless, treatments against these pathological outcomes have always been baffling. Therefore, various clinical treatments including physical rehabilitations and drug interventions have been addressed to combat further cognitive decline, worsening

of pathological insults and psychiatric comorbidities such as aggression, agitation, and insomnia which may further compromise the recovery process (Viola-Saltzman and Watson, 2012; Torbic *et al.*, 2013; Galgano *et al.*, 2017; Zhou and Greenwald, 2018; Edlow *et al.*, 2021). However, growing evidence have been reporting the potential therapeutic hallmark possessed by sedative/hypnotic drug zolpidem to mediate recovery in brain injury ever since serendipitous discovery about its paradoxical effect back in 2000 (Clauss *et al.*, 2000; Arnts *et al.*, 2020). Up to now, vast findings have consistently reported the ability of zolpidem to recover various physiological functions, such as brain functional connectivity, cerebral blood flow and activation of dormant brain areas, as well as enhancement of behavioural impairments and cognitive functions which is one of the focuses of the current study (Clauss and Nel, 2006; Kim *et al.*, 2016; Sutton and Clauss, 2017; Hahm and Woo, 2019; Arnts *et al.*, 2020).

Nonetheless, worth to consider that there are too inconsistent outcomes and even irreproducibility of the zolpidem paradox among patients with brain injury such reported by certain studies (Whyte and Myers, 2009; Thonnard *et al.*, 2013; Whyte *et al.*, 2014). Besides, decrease in drug-effect time reflecting reduced efficacy after chronic zolpidem administration has also been reported (Hahm and Woo, 2019). The inability to recapitulate these inconsistencies indicates incomplete understanding among researchers about mechanisms underlying zolpidem paradox, which therefore signify the need to further extend the related studies.

In term of mechanisms of action, as a sedative/hypnotic agent, zolpidem exerts its action on the γ-aminobutyric acid type-A receptors ($GABA_ARs$) by positively modulating the receptor function with the presence of GABA. In the normal physiology, after $GABA_AR$ activation, Cl^- flows into the neuron down its electrochemical gradient due to higher Cl^- concentration at the extracellular space

compared to the intracellular region, which therefore results in the neuronal hyperpolarisation. Cl^- electrochemical gradient is mainly maintained by the synergistic role of both cation-Cl^- co-transporters (CCCs), known as Na^+-K^+-Cl^- co-transporter 1 (NKCC1) and K^+-Cl^- co-transporter 2 (KCC2), which respectively intrude and extrude Cl^- (Ben-Ari *et al.*, 2012; Liu *et al.*, 2020). In normal neurons, KCC2 is vastly expressed but not for NKCC1. KCC2 vast expression results in the extrusion of Cl^- from the neurons, which results in the lower intracellular Cl^- concentration and mediate net Cl^- influx when $GABA_A$Rs are activated.

However, in the pathological events such as in epilepsy, KCC2 and NKCC1 are respectively downregulated and upregulated, resulting in the shift of Cl^- electrochemical gradient (higher Cl^- concentration in the neurons compared to the extracellular space) (Liu *et al.*, 2020). Due to this ramification, activation of $GABA_A$Rs causes net outflux of Cl^- which then depolarises the neurons (shifting the role of $GABA_A$Rs from inhibitory to excitatory). This $GABA_A$R-mediated depolarisation may be modulated or enhanced by number of the receptor modulatory drugs, and zolpidem is one of those substances. This hypothesis may underlie the zolpidem paradoxical effects in mediating awakening and behavioural improvements especially in the brain injury occasions. To answer this, Ben-Ari (2021) suggested that zolpidem-mediated awakening effects in the brain injury may be resulted from the shift of inhibitory GABAergic transmission to excitatory, which is mainly determined by the intracellular Cl^- concentration and synergistic role of both NKCC1 and KCC2. Nonetheless, with limited pre-clinical *in vivo* studies, this hypothetical mechanism is still far from comprehension and will remain elusive, due to inability to test the hypothesis in different experimental approaches that offer diverse comprehensive interpretations encompassing behavioural, cellular, and molecular analyses. On that

account, further fundamental and pre-clinical animal studies are essential to elucidate and recapitulate this bewildering paradox which may offer valuable outcomes, worthful for clinical purposes and fundamental scientific understanding. To comprehend the association between brain injury and zolpidem paradoxical effect, selection of the appropriate animal models mimicking brain injury needs to be carefully chosen. Animal model exploiting SE-induced brain injury, such as by the systemic administration of the combination of lithium chloride and pilocarpine, can be utilised to translate neuronal injury akin to such exhibited in clinical neuropathology (Müller, Bankstahl, *et al.*, 2009; Reddy and Kuruba, 2013; Castro *et al.*, 2017).

For that reason, in this study, an ideally isomorphic lithium-pilocarpine rat model of epilepsy with high SE morbidity and low mortality was developed using lithium and optimum dose of pilocarpine along with the application of xylazine and drug cocktail containing diazepam and MK-801. Once the ideal animal model has been developed, the potential of zolpidem in mediating recovery of learning and memory in the brain injury occasion was investigated using the Morris Water Maze (MWM) test. Additionally, using appropriate behavioural parameters in the MWM test, the effect of zolpidem on anxiety-like behaviour and motor function were also examined. Besides that, to determine the possible effect of zolpidem on the hippocampus of the lithium-pilocarpine-mediated SE-induced injury, quantitative histological analysis of the cell layer thickness, absolute cell number, and cellular/morphological changes in the principal cell layer of various hippocampal subregions, including in the dentate gyrus (DG), *Cornu Ammonis* 3 (CA3), CA1, and subiculum were also evaluated using haematoxylin and eosin. As the last part of the study, immunohistochemical staining and its fluorescent quantification were also analysed to assess the likely effect of zolpidem on the dysregulated KCC2 and NKCC1 protein expression in multiple

selected hippocampal subregions (DG, CA3, CA1, and subiculum) of the animals that were induced with SE.

1.2 Problem statement

The use of zolpidem for non-insomnia treatment, such as for brain injury conditions, has remained outside of the clinical practice. Given its potential in aiding paradoxical recovery for neurological disorders including disorders of consciousness, brain injury and neurodegenerative diseases, the prescription of zolpidem on these patients may offer substantial scientific evidence and profound clinical value.

However, due to lack of zolpidem pre-clinical investigations on non-human subjects, elucidation on cellular and physiological mechanisms of zolpidem underlying this paradox has remained inadequate. On top of that, insufficient scientific evidence and understanding about these mechanisms may fail to address ambiguities and inconsistent findings regarding contrasting patients' responses to zolpidem. Also, lacking evidence may be unable to address cellular mechanisms underlying decrease in zolpidem drug-effect time over chronic drug prescription which it will remain elusive and undetermined.

Therefore, fundamental investigations on animal studies, such offered in this study may further recapitulate behavioural and cellular fundamentals mediated by zolpidem in brain injury. Furthermore, findings from this study may partly explain the possible behavioural and molecular interactions of paradoxical action of zolpidem in brain injury after its administration which underlies varied clinical outcomes across studies.

1.3 Hypothesis and rationale of the study

Substantial clinical findings have reported the potential of zolpidem in mediating paradoxical, awakening, and recovery effect in patients with brain injury, neurodegenerative diseases, and disorders of consciousness. Additionally, acute zolpidem prescription has been widely reported to mediate transient recovery in communication, locomotion, and cognitive function in patients with brain injury. This paradoxical effect may potentially be mediated by dysregulated neuronal Cl^- homeostasis, and synergistic KCC2 and NKCC1 balance, such exhibited in the brain injury consequences. In the pathological conditions, alteration of protein expression, specifically upregulation of co-transporters NKCC1 and concomitant downregulation of KCC2 have been generally and consistently discovered in various brain regions, including in the hippocampus.

The general hypothesis of this study is that cellular changes in brain pathology after brain injury leads to changes in the expression levels of both NKCC1 and KCC2 co-transporters, which can potentially be restored by zolpidem. In the pathological condition, the cellular alteration of both NKCC1 and KCC2, and dysregulated Cl^- homeostasis determine the paradoxical action of zolpidem. Currently, understanding about zolpidem involvement in the NKCC1 and KCC2 dysregulation and its association with recovery of cognitive function have been limited which thereby require further evidence. Therefore, this study offered extended and additional *in vivo* findings of how zolpidem may potentially involve in such cellular changes, specifically in the hippocampus, and how those changes may mediate behavioural recovery, especially in cognitive function.

1.4 General objective

The lithium-pilocarpine rat model was developed to investigate the potential effect of zolpidem in restoring behavioural function, hippocampal histopathology and cation-Cl^- co-transporters' expressions in the hippocampus following SE-induced neuronal injury.

1.5 Specific objectives

1. To develop a clinically isomorphic lithium-pilocarpine induced SE model
2. To evaluate the effect of zolpidem on spatial learning and memory in the developed model using Morris Water Maze
3. To study the effect of SE-induced injury on anxiety-like behaviour and motor function in the developed model
4. To quantitatively compare the cellular and morphological differences on hippocampal principal cell layer in the developed model
5. To analyse the effect of zolpidem on the expression of KCC2 and NKCC1 in the hippocampal principal cell layer of the developed model

CHAPTER 2

LITERATURE REVIEW

2.1 Brain injuries: Traumatic and non-traumatic

According to the Brain Injury Association of America (BIAA), brain injury or also known as acquired brain injury (ABI) is defined as an insult to brain that occurs after birth and is not considered congenital, degenerative or hereditary (Chan *et al.*, 2016; Donker-Cools, Wind and Frings-Dresen, 2016). ABI is one of the leading cause of mortality and disability worldwide, and this non-congenital brain injury can be caused by either a traumatic brain injury (TBI) or a non-traumatic brain injury (nTBI) (Chen, Bushmeneva, *et al.*, 2012; Chan *et al.*, 2013; Giustini, Pistarini and Pisoni, 2013).

TBI is an injury that is caused by impact of external force (e.g. falls, vehicle accidents, sport injuries and head trauma) to the brain and is estimated to affect 64 – 74 million individuals worldwide every year, which is also considered as one of the most leading causes of death and disability (Giustini, Pistarini and Pisoni, 2013; Huang, 2013; Dewan *et al.*, 2018). It has been found that 15 % of patients suffering from TBI has the predisposition to develop post-traumatic seizures (PTSs) (Kruer *et al.*, 2013). PTSs are defined as a recurrent seizure disorder occurred as the consequence of brain injury. The disorder can be further classified into three classes which are immediate seizures (< 24 hours after TBI), early seizures (within 7 days) and late seizure onset (> 7 days) (Agrawal *et al.*, 2006; Pitkänen *et al.*, 2009; Wilson *et al.*, 2018).

Not as TBI, nTBI is brain injury that is caused by internal factors such as stroke (e.g. haemorrhage, ischaemia), infectious diseases (e.g. meningitis), aneurysm, tumour and

neurotoxic poisoning, which are not considered hereditary or congenital, rather, are caused due to the occurrences after birth (Wasterlain *et al.*, 1993; Yamaura, Ono and Hirai, 2000; Chen, Bushmeneva, *et al.*, 2012; Giustini, Pistarini and Pisoni, 2013; Chan *et al.*, 2016).

2.1.1 Features and medical emergencies of non-traumatic brain injury

Even though the effects of nTBI are akin to those of TBI, there are certain variabilities distinguishing between the effects of both injuries, where the injuries associated with nTBI have the ability to spread throughout the global region of the brain, whereas those associated with TBI are usually focal and non-spreading (Giustini, Pistarini and Pisoni, 2013). It has been found that the rate of children and youth suffering from the nTBI episode was 82.3 per 100,000 population (Chan *et al.*, 2016). Additionally, in average, paediatric nTBI patients stay in acute care hospitalisation for more than 13 days, of which 35 % of them are hospitalised under rigorous surveillance in intensive care units (ICU) (Chan *et al.*, 2016). In term of treatment cost for the patients, it has been found that patients suffering from nTBI are more expensive compared to those from TBI, suggesting that patients with nTBI experience more lasting health deficits which require more rigorous healthcare assistance than those with TBI (Chen, Bushmeneva, *et al.*, 2012; Chan *et al.*, 2016). Furthermore, in term of functional improvement, nTBI patients show worse recovery pattern than TBI patients, suggesting that those with nTBI should gain more intensive individual care to accelerate the recovery pattern, similar to that of patients with TBI (Cullen, Park and Bayley, 2008).

2.1.2 Pathological and psychiatric morbidities of non-traumatic brain injury

The worrisome concerns are also clinically considerable, where nTBI may also significantly predispose patients to diverse pathological and psychiatric morbidities such as impairments of speech, social behaviours (e.g. anxiety, depression, autism, schizophrenia, and bipolar disorder) as well as cognitive performance (Chan *et al.*, 2016; Lindberg, 2021). In term of pathology, nTBI may be associated with the extensive defect of the cortical ribbon, white matter tracts and lesion to the deep brain structures (Giustini, Pistarini and Pisoni, 2013). Besides pathological changes, it has been shown that patients suffering with nTBI may be commonly susceptible to the psychiatric comorbidities including depression and bipolar disorders (Colantonio *et al.*, 2011; Chen, Zagorski, *et al.*, 2012). Apart from that, patients suffering with nTBI are also associated with cognitive and memory decline (Giustini, Pistarini and Pisoni, 2013; Tölli *et al.*, 2018).

2.2 Effects of the non-traumatic brain injury on cognitive function

One of the examples of nTBI is subarachnoid haemorrhage (SAH) which has been shown to affect cognitive performance of the patients which profoundly affects their daily lifestyle (Mayer *et al.*, 2002; Rinkel and Algra, 2011; Wong *et al.*, 2014; Tölli *et al.*, 2018). Other than SAH, stroke is also a leading cause of nTBI where patients suffering from stroke-mediated brain injury are predisposed to many psychological deficits including attention, language as well as memory and cognitive function (Watson *et al.*, 2020). In a clinical study, it has been found that white matter hyperintensity is associated with the increased risk of stroke which precedes the exhibition of dementia and amnesic cognitive impairment (Debette *et al.*, 2010). Also,

patients suffering from stroke are vulnerable to insomniac episodes – which due to insomnia, patients may display deliberate self-harming acts including suicide – such reported in number of clinical cases (Bassetti, 2005; Tang *et al.*, 2011; Sterr *et al.*, 2018; Hahm and Woo, 2019). Additionally, insomnia after stroke may potentially result in the exacerbation of the brain injury due to the formation of reactive oxidative stress species, which eventually contributes to further neuronal injury and damage (Gopalakrishnan, Ji and Cirelli, 2004). Other than insomnia, the patients may also exhibit agitated behaviour (Kim *et al.*, 2016) and restlessness (Clauss *et al.*, 2000). In this situation, clinicians may administer sedative/hypnotic zolpidem to alleviate those perturbations, therefore allowing them to recover from the episodes hence avoiding further health exacerbations. Plus, with zolpidem intervention, it is also thought to promote neuro-recovery process, including alleviating the effect of brain injury (Cohen, Chaaban and Habert, 2004; Shames and Ring, 2008; Hall *et al.*, 2010; Hiu *et al.*, 2016).

However, attempts of using zolpidem to alleviate insomnia, restlessness and agitated behaviours have unintentionally leads to the discovery of zolpidem paradoxical effect in patients with brain injury. Following zolpidem administration, patients were reported to regain consciousness, exhibit enhanced motor, speech and cognitive functions (Clauss *et al.*, 2000; Clauss and Nel, 2006; Kim *et al.*, 2016; Hahm and Woo, 2019). However, interaction between zolpidem and brain injury and their potential molecular mechanisms underlying this paradoxical recovery remain elusive.

2.3 Paradoxical effect of zolpidem in brain injury

Sedative/hypnotic zolpidem was first reported to exhibit paradoxical arousal effect in patients with brain injury back in 2000, as reported by Clauss *et al.* (2000). In the case report, a 28-year-old male patient suffered from intra-cerebral haemorrhage in the left lentiform nucleus and thalamic area due to vehicle accident. The mental state of the patient was in semi-comatose condition. He also exhibited deficits in muscle control (poor sphincter control) as well as impairment of speech comprehension (e.g., no verbal response to commands and questions). After several months, the patients appeared to be intensely restless. Therefore zolpidem (10 mg) was prescribed to calm him. Unexpectedly, 15 minutes after zolpidem ingestion, the patient appeared to transiently regain consciousness and verbally able to greet his mother with the words "Hello Mom" (Sutton and Clauss, 2017). Following that remarkable improvement, zolpidem was administered in the subsequent mornings and the patient's consciousness seemed to be intact in the morning yet went back into semi-comatose condition in the afternoon and night. During his conscious phase, he was able to verbally respond to simple questions besides exhibiting intact cognitive and memory function. Disturbance of physical sensation and muscle control such as the exhibition of hyperaesthesia, spasticity and chorea were notably decreased during the conscious phase. Since then, Clauss and his colleagues have been extensively studied the effect of zolpidem on the cerebral perfusion flow using single-photon emission computed tomography (SPECT) scans in primate models (Clauss *et al.*, 2001, 2002) as well as in human studies (Clauss *et al.*, 2000; Clauss and Nel, 2004).

In another clinical report by Clauss and Nel (2006), – three patients suffering from brain injury which two of them were due to vehicle accident whereas another patient was due to near drowning – zolpidem (10 mg) prescription was shown resulted in

arousal effect in all three patients. Prior to zolpidem prescription, the patients were reported to display impaired muscle coordination, poor speech comprehension, disturbed visual and auditory functions. Zolpidem administration significantly improved the Glasgow Coma Scale (GCS) in all patients and from the reports, the arousal effect lasted for approximately four hours.

Other than that, Kim and colleagues (2016) also consistently reported the paradoxical effect of zolpidem in a 48-year-old male patient suffering from brain injury due to myocardial infarction (Kim *et al.*, 2016). Due to the brain injury, the patient exhibited disturbed consciousness (the patient was in semi-comatose phase), motor and speech function besides displaying agitated behaviour. Therefore, 10 mg zolpidem was given to ameliorate the agitation. Consistent with series of clinical cases such reported by Clauss *et al.* (2000) and Clauss and Nel (2006), the patient exhibited improved motor function and communication which lasted between two to three hours subsequent the zolpidem administration.

Besides that, in a more recent clinical case reported by Hahm and Woo (2019), a woman was admitted to the emergency room due to the attempt of suicide, which upon her arrival, she was in comatose condition apart exhibiting cardiac arrest. Emergency procedures were conducted including resuscitating the cardiopulmonary cerebral function to recover the spontaneous circulation. After she gained recovery, she was referred to the general ward for hospitalisation. Throughout this period, she exhibited drowsy mentality, disturbed motor movement including dysphagia and quadriparesis, impaired cognitive function, as well as sleep disturbance. Zolpidem (5 mg) was administered as the hypnotic therapy for the insomniac episode. Paradoxically, the patient regained a decent level of consciousness, improved verbal comprehension, and motor function (e.g., absence of dysphagia and quadriparesis). Due to this paradoxical

effect, the patient was experimentally given zolpidem twice a day. Throughout the treatment regimen, she exhibited and performed near-normal activities including exercises and normally communicating with other people.

Another study by Chatelle *et al.* (2014) who used positron emission tomography (PET) to evaluate the change of the regional brain metabolism after 10 mg zolpidem administration on three patients suffering with post-anoxic minimally conscious state (MCS) also discovered the consistent results. Congruent with other studies, the patients manifested improved auditory, visual, motor, and verbal functions after zolpidem administration. Additionally, the PET finding indicated that all patients who were given zolpidem exhibited increased brain metabolism in particular brain areas including in the frontal (e.g., Brodmann area 8, 9, 10 and 47) and parietal lobes (Brodmann area 40), reflecting enhanced metabolism effect induced by zolpidem in the post-anoxic MCS brain. Other PET studies also corroborated similar pattern of increased cerebral perfusion flow in the anterior cingulate gyrus, orbitofrontal cortices, striatum and thalamus in the patients with post-anoxic encephalopathy after the zolpidem administration (Cohen, Chaaban and Habert, 2004; Brefel-Courbon *et al.*, 2007).

However, other clinical cases have reported contrasting findings where the frequency of the zolpidem-induced paradoxical consciousness in patients with brain injury is rare and minor – only 4.8 - 6.7 % of participants with brain injury exhibited clinically significant responses to zolpidem (Whyte and Myers, 2009; Whyte *et al.*, 2014). Especially, the paradoxical action was not reproducible in a study by Thonnard *et al.* (2013), where all of the participants were reported failed to display significant clinical recovery following the zolpidem treatment. In addition to its inconsistent clinical

reproducibility, the zolpidem-effect time has been shown to be diminished after chronic administration (18 months to 3 years) (Hahm and Woo, 2019).

In this essence, clinical bedside behavioural assessment (e.g., clinical assessment for recovery of consciousness, cognitive, verbal, auditory and motor functions) alone may not be sufficient in answering the discrepancy in the recovery pattern and the drug reduced efficacy in patients exhibiting this bewildering paradox. These contrasting findings hinder attempts in understanding the paradoxical action mediated by zolpidem in brain injury, therefore providing a new perspective regarding the nature of injury contributing to this paradox. Plus, limited pre-clinical animal studies investigating the zolpidem paradoxical action signifies the need to further extend this investigation to untangle this ambiguity. Therefore, the unknown mechanism underlying discrepancy in clinical recoveries may require intricate and detailed scientific understanding and evidence.

These puzzling phenomena are less studied and needed to be discovered about this poorly understood mechanism. Plus, this awakening-effect rare occasion or even its irreproducibility (Whyte and Myers, 2009; Thonnard *et al.*, 2013; Whyte *et al.*, 2014) need detailed scientific elucidation. Therefore, further studies utilising various experimental models including *in vitro* and *in vivo* studies are needed in elucidating the zolpidem-mediated paradoxical effects in the brain injury. In this essence, scientific findings may give insights into possible neuroprotective features possessed by zolpidem and its potential neurotherapeutic hallmark on patients with brain injury which may shed a light in clinical neurology and neuroscience studies.

2.4 $GABA_A$ receptors

$GABA_A$Rs are the major inhibitory receptors, belonging to the ligand gated ion channels (LGICs) superfamily, alongside with the nicotinic acetylcholine receptors (nAChRs), 5-hydrotryptamine type-3 receptors ($5HT_3$Rs) and glycine receptors (GlyRs) (Olsen, 2018). In the central nervous system (CNS), $GABA_A$Rs are activated by GABA which is the principle inhibitory neurotransmitter (Farrant and Nusser, 2005; Farrant and Kaila, 2007; Olsen and Sieghart, 2008; Glykys et al., 2017). During development, the action of GABA through $GABA_A$Rs mediates various neuronal developmental processes including, synaptic formation, neuronal migration and growth, as well as neuronal proliferation (Ben-Ari, 2021). Neurons that synthesise and release GABA neurotransmitter as their output are characterised as the GABAergic neurons. GABA released into the synaptic cleft is then bound to the post-synaptic receptors, specifically to the ionotropic $GABA_A$ receptors ($GABA_A$Rs) and metabotropic $GABA_B$Rs. $GABA_B$Rs are members of GPCRs which are slow-acting whereas $GABA_A$Rs are part of fast-acting pentameric ligand gated ion channels (LGICs) receptor superfamily (Olsen, 2018; Smart & Stephenson, 2019).

2.4.1 The structure of $GABA_A$ receptors

$GABA_A$Rs are pentameric transmembrane protein complexes, comprising of five from different combination of 19 subunits that have been identified as α1 – 6, β1 – 3, γ1 – 3, δ, ε, θ, π and ρ1 – 3 (Simon et al., 2004; Farrant and Nusser, 2005; Olsen and Sieghart, 2008; Uusi-oukari and Korpi, 2010; Comenencia-Ortiz, Moss and Davies, 2014). The possible combinatorial co-assembly of these receptor subunit proteins allows a potentially enormous molecular heterogeneity of $GABA_A$R subtypes (Farrant and Nusser, 2005). Each receptor subunit from the same class exhibit up to 70 %

sequence homogeneity, and approximately 30 % homogeneity between the subunit classes (Olsen, 2018).

In term of the receptor subunit composition or known as receptor subtype, the most abundant $GABA_AR$ subtype found in the CNS is α1β2γ2, which exhibits a 2:2:1 stoichiometry – two α1, two β2 and one γ2, arranged in an anti-clockwise configuration of γ2β2α1β2α1 **(Figure 2.1A)** (Farrant and Nusser, 2005; Mortensen, Patel and Smart, 2012; Sigel and Steinmann, 2012), meanwhile minor subunits such as δ or ε are mostly found instead of γ in the extra-synaptic $GABA_ARs$ **(Figure 2.1B)** (Greenfield Jr, 2013). This subunit complex pseudo-symmetrically encompasses a central pore channel in the γ-β-α-β-α sequence, anti-clockwise when viewed from the extracellular view (Farrant and Nusser, 2005; Puthenkalam *et al.*, 2016).

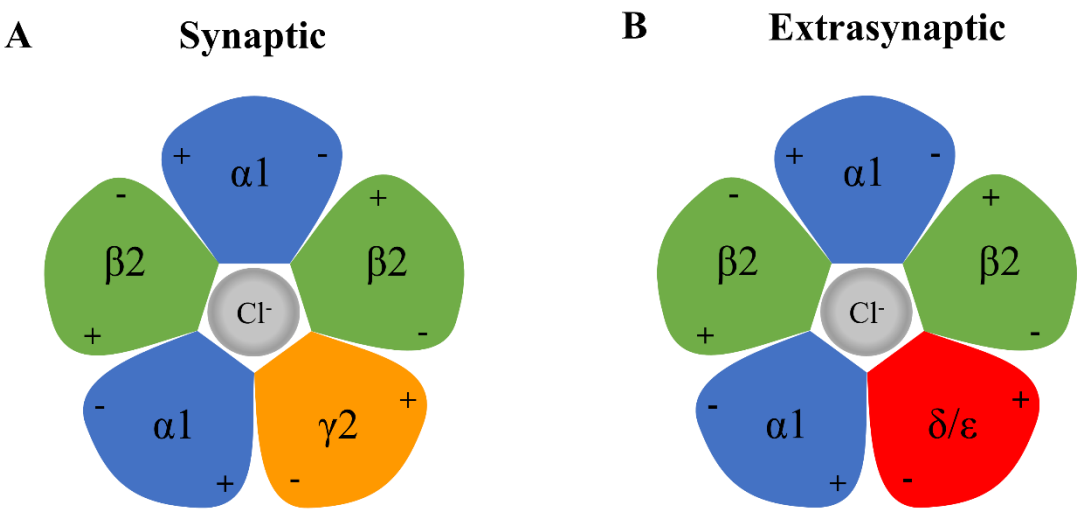

Figure 2.1 Depiction of different $GABA_AR$ subtypes. (A) The most abundant $GABA_AR$ subtype is α1β2γ2, which exhibits a 2:2:1 stoichiometry – two α1 two β2 and one γ2, arranged in an anti-clockwise configuration of γ2β2α1β2α1. **(B)** δ- or ε-containing $GABA_AR$ subtype is mostly found at the extra-synaptic region.

2.4.2 The activation and modulation of $GABA_A$ receptor

$GABA_A$Rs are activated by GABA which binds at the receptors' orthosteric sites, specifically located at the extracellular domain (ECD) of the receptor between the β(+) and α(-) subunits interface **(Figure 2.2)** (Puthenkalam *et al.*, 2016). The action of GABA on the $GABA_A$Rs leads to the receptor activation and trigger the opening of the channel, which is permeable to Cl^-. In the physiological event of mature neurons, this results in the influx of Cl^- from the extracellular region into the neurons via the $GABA_A$R ion channel. The $GABA_A$R-mediated Cl^- influx results in the rapid neuronal hyperpolarisation which is within milliseconds (ms) time range (Sigel and Steinmann, 2012). However, in immature neurons, the Cl^- concentration is relatively higher in the intracellular region as compared to that in the extracellular (Liu et al., 2020). This results in the net outflux of Cl^- from the cells causing subsequent cellular depolarisation when GABA acts on the $GABA_A$Rs.

$GABA_A$Rs are targets for many pharmaceutical drugs including benzodiazepine-based sedatives/hypnotics and anxiolytics (Vega Alanis *et al.*, 2020). Benzodiazepines bind at the classical α/γ subunit interface and also at α/β interface (due to its high homology to that of α/γ) (Sigel and Ernst, 2018; Vega Alanis *et al.*, 2020). Classical benzodiazepines bind at the receptor binding site(s) and positively modulate the receptor function by increasing the opening frequency of the receptor (Bianchi *et al.*, 2009; Sigel and Ernst, 2018). However, the canonical benzodiazepine binding sites, such as α/γ interface, can also be bound by non-benzodiazepine including imidazopyridines like zolpidem (Vega Alanis *et al.*, 2020).

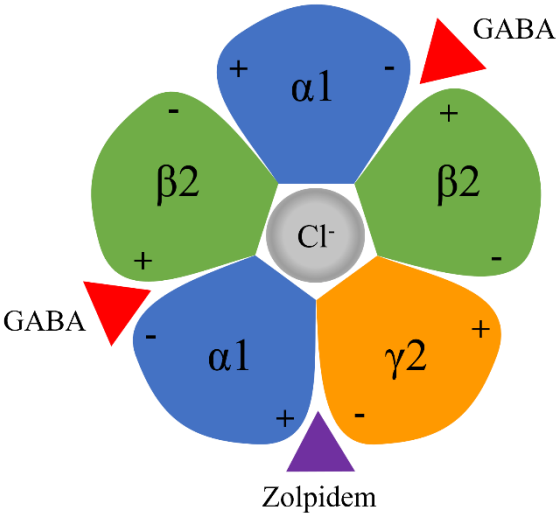

Figure 2.2 Depiction of binding sites on the GABA$_A$Rs. GABA binds at the orthosteric site β/α interface, whereas zolpidem at allosteric site α1/γ2.

2.4.3 Zolpidem: A positive allosteric modulator for GABA$_A$ receptors

Introduced in 1988, along with other Z-drugs such as zaleplon and eszopiclone, zolpidem ($C_{19}H_{21}N_3O$) belongs to imidazopyridine class **(Figure 2.3)**. In common clinical practice, zolpidem is used as a sedative/hypnotic agent to treat patients with insomnia (Hanson and Czajkowski, 2008). In term of pharmacokinetic, zolpidem achieves peak plasma concentration between 0.75 to 2.6 hours, and its elimination half-life ranges between 1.5 to 3.2 hours (Salvà and Costa, 1995). Through GABA$_A$Rs, zolpidem exerts its action on the same site with benzodiazepines at α(1, 2, 3)/γ2 subunit interfaces and positively modulates the receptor function by increasing the receptor channel opening frequency, thereby enhancing the Cl^- influx (Hanson and Czajkowski, 2008; Sieghart, 2015; Richter *et al.*, 2020). Zolpidem exhibits high affinity to α1-containing GABA$_A$Rs, intermediate affinity to both α2-, and α3-containing GABA$_A$Rs and insensitive to α5-containing GABA$_A$Rs (Crestani *et al.*, 2000; Ali and Thomson, 2008). Recently it has been shown that zolpidem also binds at the α1-α1 GABA$_A$R

interface, mimicking its binding profile at the classical α1/γ2 interface (Che Has *et al.*, 2016).

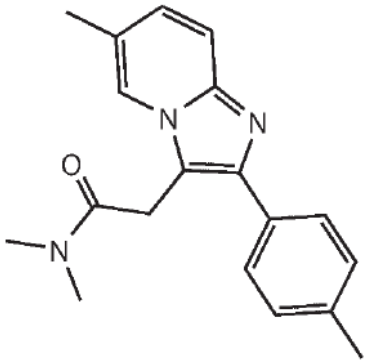

Figure 2.3 Depiction of zolpidem chemical structure. Figure adapted from Halasz and Dinnebier (2010).

Physiologically, the inhibitory effect of $GABA_ARs$ is mainly rendered by neuronal Cl^- homeostasis, and by the Cl^- current directionality and magnitude. In the physiological event, the binding of GABA at β/α subunit interface of $GABA_ARs$ results in the net influx of Cl^-. With the presence of zolpidem, it potentiates the net influx of Cl^-, hence enhancing the inhibitory GABA-mediated Cl^- current. The influx of Cl^- down the electrochemical gradient in physiological event is mainly due to the difference in the Cl^- electrochemical gradient between the intra- and extracellular region - lower intracellular Cl^- concentration, $[Cl^-]_i$ relative to the extracellular Cl^- concentration, $[Cl^-]_o$. This phenomenon is primarily established and maintained by the action of neuronal cation-Cl^- co-transporter (CCC) proteins. Besides, the physiological Cl^- homeostasis, maintained by CCCs, significantly underpins the inhibitory nature of the GABAergic transmission in the CNS (Schulte, Wierenga and Bruining, 2018). Plus, the $[Cl^-]_i$ determines the polarity and strength of the GABAergic transmission, mediated by the $GABA_ARs$, in the nervous system (Kahle *et al.*, 2008).

2.5 CCCs: KCC2 and NKCC1

Nonetheless, latterly, a promising theory rendering the pathophysiological basis of brain injury, primarily rooting from the seminal role of neuronal CCC proteins on the GABAergic inhibitory effect, may give an insight into further explaining this illness. NKCC1 and KCC2 are the main co-transporters regulating the intracellular Cl^- homeostasis which is critical for the GABAergic inhibitory effect. The synergistic role between the GABAergic transmission and the neuronal Cl^- homeostasis, regulated by NKCC1 and KCC2, may entail explanation on the zolpidem-mediated awakening effect. The solute carrier 12 (SLC12) family of the CCCs constitutes of the four KCCs (KCC1 encoded by *SLC12A4*, KCC2 encoded by *SLC12A5*, KCC3 encoded by *SLC12A6* and KCC4 encoded by *SLC12A7*), two NKCCs (NKCC1 encoded by *SLC12A2* and NKCC2 encoded by *SLC12A1*) and one Na^+-Cl^- co-transporter (NCC encoded by *SLC12A3*) (Kaila *et al.*, 2014).

2.5.1 Distinct types of the KCCs and NKCCs

KCCs and NKCCs are comprised of distinct types that are specifically expressed throughout different organs and cell types (Blaesse *et al.*, 2009; Kaila *et al.*, 2014). KCC4 is expressed in the kidney, liver, lung, heart and gastric luminal membrane (Fujii *et al.*, 2011; Arroyo, Kahle and Gamba, 2013). KCC3 is composed of two variants which are KCC3a and KCC3b. KCC3a is expressed in heart, lung, muscle and brain whereas KCC3b is mostly in kidney (Arroyo, Kahle and Gamba, 2013). KCC2 is specifically expressed in neurons of the CNS, such as in the hippocampus, cerebellum, cortex and brain stem (Payne, Stevenson and Donaldson, 1996; Rivera *et al.*, 1999). KCC2 is also neuronally specific and absent from glial cells (Williams *et al.*, 1999). In the neurons, KCC2 is vastly expressed in the soma and dendrites of

pyramidal neurons in the hippocampus and neocortex (Liu *et al.*, 2020). KCC2 was found to exhibit 67 % amino acid sequence similarity to KCC1, that were found in the rat brain and rabbit kidney (Payne, Stevenson and Donaldson, 1996).

NCC and NKCC2 are not expressed in the brain, yet predominantly expressed in the kidney, especially in the renal medullary and cortical ascending limbs (Itoh *et al.*, 2014; Kaila *et al.*, 2014; Liu *et al.*, 2020). NKCC1 is expressed in the peripheral and central neurons, including glial cells (Javdani *et al.*, 2020; Liu *et al.*, 2020; Virtanen *et al.*, 2020). NKCC1 has two splice variants which are NKCC1a and NKCC1b, with NKCC1a variant is highly expressed in the brain (Cutler and Cramb, 2002; Liu *et al.*, 2020).

2.5.2 Structures of KCC2 and NKCC1

In term of secondary protein structure, KCC2 **(Figure 2.4)** and NKCC1 **(Figure 2.5)** possess similar structures in which are composed of twelve transmembrane protein domains, six extracellular loops, and intracellular N- and C-terminals (Agez *et al.*, 2017; Chew *et al.*, 2019; Virtanen *et al.*, 2020). KCC2 forms monomeric and dimeric structures, not trimeric or any other higher oligomeric structures **(Figure 2.6)** (Agez *et al.*, 2017; S. Zhang *et al.*, 2021). Meanwhile, NKCC1 is found to form dimeric structures in order to be functional, despite of its potential monomeric structure expression **(Figure 2.7)** (Parvin, Gerelsaikhan and Turner, 2007; Chew *et al.*, 2019; Virtanen *et al.*, 2020; S. Zhang *et al.*, 2021). Both of them exhibit different phosphorylation sites, with the most studied KCC2 phosphorylation sites are S940, T1007 and S1022 (Agez *et al.*, 2017), whereas for NKCC1 are T197, T201, T206, T211 and T224 (Virtanen *et al.*, 2020).

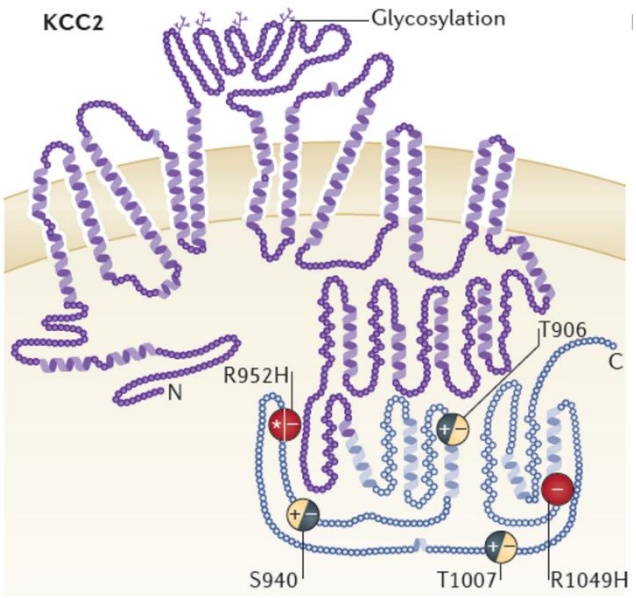

Figure 2.4 Illustration of KCC2 secondary structure comprising of 12 transmembrane segments. Figure adapted from Kaila *et al.* (2014).

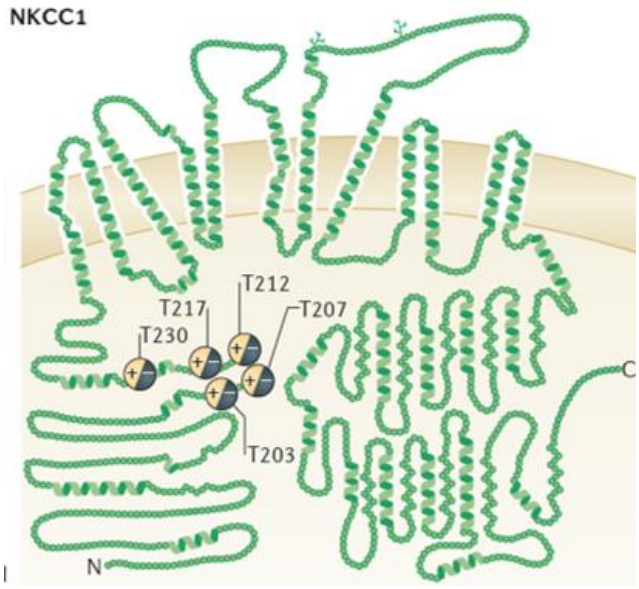

Figure 2.5 Illustration of NKCC1 secondary structure comprising of 12 transmembrane segments. Figure adapted from Kaila *et al.* (2014).