NOVEL BINDING SITES ON GABA (A) RECEPTORS FOR THE ALTERNATIVE EFFECTS OF ZOLPIDEM: *IN VIVO* AND *IN SILICO* STUDIES

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	Percentage
/	Per
<	Less than
≤	Less than and equal to
*	Asterisk
=	Equal
≈	Approximate
-	Minus
+	Addition
±	Addition and minus
x	Multiplication
α	Alpha
β	Beta
γ	Gamma
δ	Delta
π	Pi
ρ	Rho
θ	Theta
ε	Epsilon
ψ	Psi
ϕ	Phi
π-π	pi-pi bond
Å	Angstrom
ºC	Celsius
atm	Atmospheric pressure
a.u	Raw integrated density
bar	Pressure
cm	Centimetre

dt	Step size
g	Gram
GB	Gigabyte
GHz	Gigahertz
H	Hour
i.p	Intraperitoneal
K	Kelvin
kDa	Kilodalton
kcal/mol	Kilocalorie per mole
Ki	Inhibitory constant
$kJmol^{-1}$	Kilojoule per mole
M	Molar
Mb	Megabase
mg	Miligram
mg/kg	Miligram per kilogram
ml	Mililiter
n	number of samples
nM	Nanomolar
nm	Nanometre
ns	Nanosecond
ps	Picosecond
r	Radius
s	Second
TB	Terabyte
μM	Micromolar
μm	Micrometre

LIST OF ABBREVIATIONS

2D	Two dimension
2-VO	Two-vessels occlusion
3D	Three dimension
4-VO	Four-vessels occlusion
ABU	Gamma-amino-butanoic acid
AD	Alzheimer's Disease
ADHD	Attention Deficit Hyperactivity Disorder
Ala	Alanine
AMBER	Assisted Model Building Energy Refinement
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of Variance
ARASC	Animal Research and Service Center
Arg	Arginine
ASD	Autism Spectrum Disorder
Asn	Asparagine
Asp	Aspartic acid
BBB	Blood-brain barrier
CA1	Cornus ammonis 1
CA2	Cornus ammonis 2
CA3	Cornus ammonis 3
CA4	Cornus ammonis 4
CADD	Computer-aided drug design
CBF	Cerebral blood flow
CCH	Chronic cerebral hypoperfusion
CHARMM	Chemistry at Harvard Macromolecular Mechanics
CHARMM-GUI	CHARMM with Graphic User Interface
Cl^-	Chloride ion
CNS	Central nervous system

CPU	Central processing unit
cRNA	Complementary ribonucleic acid
Cryo-EM	Cryo – Electron Microscopy
CSF	Cerebrospinal fluid
Cys	Cysteine
DAPI	Fluoroshield^{TM} containing 4',6'-diamidino-2-phenylindole
DG	Dentate gyrus
DOC	Disorders of consciousness
DZP	Diazepam
E	East
ECA	External carotid artery
ECD	Extracellular domain
EEG	Electroencephalogram
E/I	Excitatory/Inhibitory
EPM	Elevated plus maze
EPSC	Excitatory postsynaptic current
EST	Expressed sequence tag
GABA	γ-aminobutyric acid
GABA (A)	γ-aminobutyric acid type A
GABA (C)	γ-aminobutyric acid type C
GABA – T	GABA transaminase
GABRα1	γ-aminobutyric acid receptor α1
GABRε	γ-aminobutyric acid receptor ε
GAD	Glutamic acid decarboxylase
GAT	GABA transporters
GAT-1	GABA transporters type 1
GAT-2	GABA transporters type 2
GAT-3	GABA transporters type 3
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GNU	GNU's not UNIX

GPi	Globus pallidus interna
GPU	Graphic processing unit
GROMACS	Groningen Machines for Computer Simulations
H-bond	Hydrogen bond
HDD	Hard disk drive
His	Histidine
ICA	Internal carotid artery
ICD	Intracellular domain
IHC	Immunohistochemistry
Ile	Isoleucine
IPS	Institute Postgraduate Studies
IPSC	Inhibitory postsynaptic current
IUPHAR	International Union of Basic and Clinical Pharmacology
K^+	Potassium ion
LAMMPS	Large-scale Atomic/Molecular Massively Parallel Simulator
LC	Locus coeruleus
Leu	Leucine
LINCS	Linear constraint solver
Lys	Lysine
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MD	Molecular dynamics
Met	Methionine
mRNA	Messenger ribonucleic acid
MRX3	X-linked mental retardation
MSNs	Median spiny neurons
MWM	Morris water maze
N	North
Na^+	Sodium ion
NAMD	Nanoscale Molecular Dynamics
NAMs	Negative allosteric modulators
NCBI	National Center for Biotechnology Information

NGS	Normal goat serum
NIH	National Institute of Health, USA
NMDA	N-methyl-D-aspartic acid
NMR	Nuclear magnetic resonance
NPT	Constant pressure and temperature
NVT	Constant volume and temperature
NW	North-west
OFT	Open-field test
P	Pressure
PAMs	Positive allosteric modulators
PBC	Periodic boundary conditions
PBOCCA	Permanent bilateral occlusion of common carotid arteries
PBS	Phosphate buffered saline
Pcoupl	Pressure coupling
PCR	Polymerase chain reaction
PD	Parkinson’s disease
PDB	Protein Data Bank
PFA	Paraformaldehyde
pH	Potential of hydrogen
Phe	Phenylalanine
PIO	Picrotoxin
pK	Negative of acid dissociation constant (Ka)
PME	Particle mesh Ewald
PNS	Peripheral nervous system
POPC	1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine
Pro	Proline
PTSD	Post-traumatic stress disorder
RCSB	Research Collaboratory for Structural Bioinformatics
RAM	Random access memory
res	Residue
Rg	Radius of gyration
RMSD	Root-mean square deviation

RMSF	Root-mean square fluctuation
S	South
SD	Sprague Dawley
Ser	Serine
SPECT	Single photon emission computed tomography
SSD	Solid state drive
sub	Subunit
SW	South west
T	Temperature
TBI	Traumatic brain injury
TCA	The citric acid cycle
tcoupl	Temperature coupling
Thr	Threonine
TIP3P	Transferable intermolecular potential 3P
TLE	Temporal lobe epilepsy
TMD	Transmembrane domain
Trp	Tryptophan
Tyr	Tyrosine
V	Volume
VaD	Vascular dementia
Val	Valine
VMD	Visual molecular dynamics
vs	Versus
W	West
WHO	World Health Organization
Zn^{2+}	Zinc ion
Zol	Zolpidem

LIST OF APPENDICES

TAPAK PENGIKATAN YANG BAHARU PADA RESEPTOR GABA (A) PADA KESAN ALTERNATIF UNTUK ZOLPIDEM: KAJIAN *IN VIVO* DAN *IN SILICO*

ABSTRAK

Pesakit yang mengalami kecederaan otak lazimnya menghadapi kemerosotan fungsi motor dan kognitif yang merencatkan proses penyembuhan. Untuk meningkatkan kualiti hidup pesakit-pesakit ini, intervensi farmakologi yang mensasarkan fungsi motor dan kognitif selalunya dijalankan. Terdapat beberapa laporan saintifik yang menyebut kesan kebangkitan ubat tidur, zolpidem ke atas pesakit-pesakit yang mengalami kecederaan otak yang berbeza. Memandangkan kesan sedatif dan hipnotik ubat ini adalah dari tapak pengikatan α(1/2/3/5)-γ dari reseptor GABA (A), adalah berkemungkinan kesan kebangkitan ini berasal dari tapak pengikatan lain yang tidak pernah diketahui. Oleh itu, tesis ini cuba untuk mengkaji sebab farmakologi kesan kebangkitan zolpidem ini melalui kajian tingkah laku haiwan (*in vivo*) dan disokong dengan analisis kompleks protein-ligan melalui kaedah pengkomputeran (*in silico*). Dalam kajian ini, tikus-tikus yang mengalami kerosakan fungsi kognitif daripada kecederaan iskemia diberikan dos zolpidem yang berbeza (kawalan, saline, 1.0, 2.0 dan 4.0 mg/kg). Apabila dibandingkan dengan kumpulan yang tidak dirawat dengan kumpulan yang menerima dos yang lebih tinggi, pemberian dos pada 1.0 mg/kg zolpidem menunjukkan perkembangan yang lebih baik. Selanjutnya, kajian kimia-imunohisto menunjukkan kesan yang jelas ke atas ekspresi subunit α1 dan ε reseptor GABA (A) yang diperoleh daripada

hippokampus. Kecederaan iskemia menyebabkan kekurangan ekspresi subunit α1, manakala ekspresi subunit ε menunjukkan peningkatan hampir dua kali ganda. Sementara itu, protein analisis ke atas hippokampus yang menerima rawatan zolpidem menghasilkan peningkatan ekspresi subunit α1 dan mengurangkan ekspresi subunit ε kepada tahap yang hampir sama dengan kumpulan kawalan yang normal. Pemerhatian ini mencadangkan akibat kesan kecederaan iskemia, kedua-dua subunit mempunyai tindak balas untuk melengkapkan satu sama lain dan kedua-dua subunit mempunyai tindak balas modulasi ke atas dos 1.0 mg/kg zolpidem. Sebagai bukti, reseptor GABA (A) binari α1β3α1 dan yang mempunyai subunit ε dihasilkan menggunakan teknik pemodelan homologi, diikuti dengan proses pemadanan molekular zolpidem pada tapak-tapak pengikatan reseptor-reseptor tersebut. Kemudiannya, simulasi dinamik molekular dijalankan ke atas kombinasi protein-ligan itu. Perbandingan kuasa ikatan, interaksi protein-ligan dan kestabilan struktur tapak pengikatan $\alpha1^{+}$-$\alpha1^{-}$ daripada binari α1β3α1 dan tapak pengikatan $\alpha1^{+}$-ε^{-} daripada α1β3εε reseptor GABA (A) dengan tapak pengikatan kawalan $\alpha1^{+}$-$\gamma2^{-}$ daripada α1β3γ2 berjaya menunjukkan potensi tindak balas daripada ikatan-ikatan ini. Walaupun mekanisma kesan kebangkitan zolpidem daripada tapak-tapak pengikatan ini tidak dapat diketahui secara mendalam, kajian ini masih dapat menunjukkan tarikan pengikatan terhadap tapak pengikatan yang baharu pada $\alpha1^{+}$-$\alpha1^{-}$ dan $\alpha1^{+}$-ε^{-}. Sehubungan dengan itu, ini membuktikan kewujudan tapak pengikatan lain bagi zolpidem pada reseptor GABA (A) yang berkemungkinan dihasilkan susulan daripada penyusunan subunit dalam kecederaan otak, yang boleh menyebabkan kesan kebangkitan zolpidem.

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ABSTRACT

Patients with brain injuries commonly suffered significant motor and cognitive decline which interfere with their recovery process. To improve the quality of life in these patients, pharmacological intervention targeting the motor and cognitive functions are usually administered. There have been reports on the awakening effects of the sleeping pill, zolpidem when administered to patients with different types of brain injury. Since the sedative and hypnotic effect of the drug is elicited from the α(1/2/3/5)-γ interface of GABA (A) receptor, it is possible that this effect is contributed from different binding sites which have not been explored before. Hence, this thesis aimed to investigate the molecular and pharmacological reason behind this paradoxical effect through zolpidem administration in animal behavioural studies (*in vivo*) supported with analysis on protein-ligand complex through computational methods (*in silico*). These rats with induced ischaemic injury and impaired cognitive functions were given different doses of zolpidem (control, saline, 1.0, 2.0 and 4.0 mg/kg) and underwent open-field test and Morris water maze tests. Zolpidem administration on these animals showed improvement from the groups receiving 1.0 mg/kg zolpidem in comparison to the non-treated and those receiving higher doses of zolpidem. Further, immunohistochemistry study showed significant effects on the expression of hippocampal α1 and ε subunits of GABA (A)

receptors. The observations suggested; both subunits have complementary action during ischaemic injury and both subunits have modulatory action towards 1.0 mg/kg of zolpidem. To prove this, binary α1β3α1 and GABA (A) ε-containing receptors were created using homology modelling technique and molecular docking of zolpidem were performed at several targeted interfaces of these receptors, followed by molecular dynamics simulation. Comparison of energy binding, protein-ligand interaction and structural stability to the control $\alpha1^{+}$-$\gamma2^{-}$ interface of α1β3γ2 GABA (A) receptor, showed potential binding action of zolpidem towards $\alpha1^{+}$-$\alpha1^{-}$ interface from binary α1β3α1 and $\alpha1^{+}$-ε^{-} interface of α1β3εε GABA (A) receptors. Even though the mechanism on how exactly these interfaces contribute to the awakening action of zolpidem is not revealed, these data are still able to demonstrate the binding affinity of zolpidem towards the novel binding sites of $\alpha1^{+}$-$\alpha1^{-}$ and $\alpha1^{+}$-ε^{-} interfaces. As a result, proving the existence of other binding sites of zolpidem within GABA (A) receptors possibly created due to subunit rearrangement in post-brain injuries, which could have caused the awakening action of zolpidem.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Zolpidem ($C_{19}H_{21}N_3O$) or systematic name N,N,6-trimethyl-2[4-methyl-phenyl]imidazo[1,2-a]pyridine-3-acetamide hemitartrate was first synthesized in France by Syntelábo Recherche in the 1980s as one of the Z-drugs including zopiclone, eszopiclone and zaleplon (Monti et al., 2017). Commercially known as Ambien® or Stilnox®, this drug is a sedative-hypnotic agent which usually prescribed at 5 – 10 mg to address sleeping difficulties in people experiencing insomnia (Holm et al., 2000). Unlike the classical benzodiazepines such as diazepam, flurazepam and lorazepam, zolpidem appears devoid of the side effects that commonly associated with these drugs, such as rebound insomnia, amnesia and development of tolerance (Dang et al., 2011). Therefore, zolpidem is fast becoming the preferred choice over benzodiazepines in inducing sleep for patients with insomnia (Dang et al., 2011).

Zolpidem modulates its action through GABA (A) receptors, at the same binding site with benzodiazepines which located at the interface of α-γ protein subunits of the receptors. Although the binding site is constituted by both α and γ subunits, it is the type of α that determines the pharmacological properties of the receptors. Previous research discovered the sedative-hypnotic properties of zolpidem is mediated through the α1 while α2 and α3 subunits demonstrated anxiolytic effects (Monti et al., 2017). However among all, zolpidem displays highest affinity towards α1 subunit (Monti et

al., 2017; Vlainić et al., 2012) and this subunit selectivity explains its lesser side effects in comparison with the classical benzodiazepines.

But contradictorily to its actual action as sleeping pill, zolpidem has been consistently associated to awaken patients with brain injuries such as traumas, stroke and disorders of consciousness (DOC). This could also be expanded to hypoxic damage, cerebrovascular ischaemic injury, infection of the central nervous system (CNS), toxins and poisoning, degenerative diseases, tumours and also congenital disorders (Georgiopoulos et al., 2010; Thonnard et al., 2014). The awakening effect of zolpidem was first described in South Africa when a patient (male, 30 year-old) suffering from traumatic brain injury in road accident, was given zolpidem and uttered “hello mom” to his mother not 20 minutes later (Clauss et al., 2000; Sutton & Clauss, 2017). Intriguingly, the awakening effect has also been replicated and reported by few other researchers in different brain injury studies (Brefel-Courbon et al., 2007; Du et al., 2014; Miyazaki et al., 2012; Nyakale et al., 2011; Shames & Ring, 2008; Sripad et al., 2020; Sutton & Clauss, 2017; Whyte et al., 2014). Considering the modulatory action of zolpidem is exerted through the inhibitory action of GABA (A) receptors, this awakening effect sometimes referred as the paradoxical action of the drug, as a manifestation of the opposite effect to its original action. Hence, both ‘awakening’ and ‘paradoxical’ will be used interchangeably to describe this phenomenon in this thesis.

Furthermore, this awakening effect has also been observed in patients with movement disorders such as Parkinson’s disease (PD) and dystonia to neurological deficits and anaphylactic hypoxia (Daniele et al., 2016; Miyazaki et al., 2012; Nyakale et al., 2011). Indisputably, the wide spectrum of the injuries reported among these cases provides a challenge in investigating the exact mechanism of action

behind the awakening effect. However, it is speculated that zolpidem may influence the brain wave activities related to brain injuries which cause both motor and cognitive declined in these patients (Arnts et al., 2020). For instance, as a result of abrupt restriction or blockage of the cerebral blood flow (CBF), cognitive impairments including memory loss, language and attention deficits are commonly observed in post-stroke patients (Al-Qazzaz et al., 2014). In the brain, the cognitive functions are associated with the S-shaped structure or hippocampus which is highly vulnerable to brain injuries. This structure has been extensively studied for its association to learning and memory mechanisms as well as emotional network (Cherubini & Miles, 2015), which makes this region and the related functions as an ideal focus for studying the potential therapeutic effect of zolpidem in brain injury.

Based on the information that has been mentioned above, the question here is very obvious – how is it possible for a substance originally intended as sedative-hypnotic agent, can be associated with awakening action? This question indeed leads to the next question – what is the target receptor for zolpidem to elicit this paradoxical action? Unequivocally, these questions suggest the involvement of different subtype (or subtypes) of GABA (A) receptors with novel binding site (or sites), other than the conventional sites for zolpidem at α-γ of subunit interfaces. Regarding the binding sites for drugs/substances on GABA (A) receptors, most of these sites are located at the interfaces of adjacent receptor subunits. Thus the position or arrangement of these subunits plays critical role in forming the structure of the sites, which eventually determines the pharmacological properties of the receptors. Previous studies have indicated the arrangement is depending on the expression levels of the subunits which can be potentially altered in brain injuries and traumas, not to mention other factors such as neurological disorders. For instance, Kharlamov et al.,

(2008) have reported an increase in mRNA and protein level of α1 subunit following an ischaemic insult. Meanwhile, Drexel et al., (2015) observed down regulation of α1, α2, α5, β2, β3, γ2 and δ in induced TBI of animal model whilst Fatemi et al., (2013) showed up regulation of α2 and ε subunits in brain samples with induced schizophrenia, bipolar disorder and major depression. This is further supported by Tossell & Ungless, (2021) who reported the association of ε subunit with temporal lobe epilepsy (TLE). While, Mtchedlishvili et al., (2010) demonstrated that benzodiazepines sensitivity of synaptic receptors in hippocampal dentate granule cells was absence following a TBI induction, suggesting an interruption at the binding site for the substances, which is most likely due to the alteration in subunit expression.

In a more comprehensive *in vitro* study, Che Has et al., (2016) has proposed novel zolpidem binding site at α1-α1 subunit interface of the binary GABA (A) α1β3 receptor, at the same time raising speculation about the target for paradoxical effect of zolpidem. Further, the same study also tried to investigate more on the mysterious inhibitory action of the ε subunit and its pharmacological action. By manipulating relatively higher ratio of ε to α1 and β3 subunits, the team conducted an *in vitro* study on GABA (A) α1β3ε receptors, before the expressed receptors were tested with zolpidem at different concentrations. From this study, the findings showed zolpidem at picomolar and nanomolar concentrations modulated the inhibitory action of GABA but the modulation has not been observed at higher concentration (at micromolar). However, similar results failed to be repeated with lower ratio of ε subunit. To validate the data, the team also built a concatamer of α1-β3 and injected along with ε subunit to affirm the existence of ternary α1β3ε receptor and managed to replicate the similar results. Thus, it can be concluded that the formation of α1β3ε

subtype of GABA (A) receptors could potentially hold novel binding pocket for zolpidem. However, unlike the binary α1β3 subtype which possess the α1-α1 interface, the pentameric arrangement of the subunits within the α1β3ε subtype of GABA (A) receptors are yet to be known. These findings have strongly showed subunit manipulations and rearrangement could create new binding sites which potentially could answer the awakening effect of zolpidem.

Taken together, it is believed that brain injuries could have affected the expression and arrangements of GABA (A) receptor subunits. However, it is still undetermined how these alterations able to build potential binding sites, in this case for zolpidem and explain its intriguing effects through the binding process at the site. For that reason, with the aim of connecting the link between brain injury and its effect on GABA (A) receptors to the potential awakening effect of zolpidem, we have designed a series of experiments that combine two techniques; *in vivo* and *in silico*. In this study, as for *in vivo* part, an ischaemic injury that affect the cognitive functions was induced in Sprague Dawley (SD) rats through chronic cerebral hypoperfusion (CCH) using permanent bilateral occlusion of the common carotid arteries (PBOCCA) method or two-vessels occlusion (2-VO). This model has been known to cause less severe but progressive long term effects on the cognitive functions of the animal with insignificant motor dysfunctions (Farkas et al., 2007). Then, the animals were administered with different doses of zolpidem and the locomotor along with learning and memory functions of the animals were assessed through open-field test and Morris water maze. Subsequently, the brains of the animal were extracted to analyse the hippocampal expression of GABA (A) receptor α1 and ε subunits, to investigate the effects of ischaemic injury and zolpidem administration on the expression of these subunits. Further, since it is challenging to

singly create and manipulate GABA (A) receptors with varying subunit arrangement, *in silico* methods were applied. Different pentameric arrangements/positions possessing different interfaces of α1 and ε subunit interfaces were created using homology modelling techniques and these modelled receptors were docked with zolpidem at different positions. Conserved amino acids residues, protein-ligand interactions and energy binding affinity were then analysed using molecular dynamics method. As the last part of this study, the structural stabilities of these interactions were then validated and supported through molecular dynamics simulation in membrane lipid bilayer subjected under force fields, to prove the ability of zolpidem to bind to these novel sites which could potentially be the target for the awakening effect of the drug. By combining several approaches together, this thesis has presented a comprehensive study regarding the relationship between brain injury, behavioural, cellular and also the interaction between zolpidem and GABA (A) receptors through computational methods.

1.2 Problem statement

The phenomenon of awakening effect by sedative-hypnotic agent zolpidem on patients with neurological problems including disorders of consciousness, brain injuries and neurodegenerative diseases is still shrouded in mystery. Although this drug is widely prescribed as sleeping pill, given its potential in aiding recovery for the disorders, the understanding related to the mechanism may offer substantial scientific value especially in rehabilitation process. However, the insufficient of related research works that have been conducted, specifically in behavioral and cellular studies makes this situation to be difficult to understand. Moreover, the available data and evidence is not expanded into further studies, especially the application of *in silico* method and binding studies in drug-receptor interactions.

In relation to that, fundamental studies focusing on the role of zolpidem in the awakening effect should be conducted and explored to offer a necessary understanding for zolpidem as therapeutic agent for brain injury. Back to this study, here we offer a link between *in vivo* and *in silico* studies in supporting the relationship between brain injury, GABA (A) receptors and the binding profiles of zolpidem at novel binding sites on the new receptor subtypes. Our *in vivo* study includes three parts; animal model development for ischaemic injury, behavioral tests (open-field and Morris water maze) and protein subunit expression of GABA (A) receptors. While *in silico* focuses on modeling of the receptors, docking of zolpidem at the modelled receptors and molecular dynamics simulation on the receptors. With the combination of our *in vivo* and *in silico* data, added to the existing *in vitro* data we believe that some questions related to the main issues studied – the awakening effect of zolpidem, have been answered in this thesis.

1.3 Hypothesis and rationale of the study

For more than 20 years zolpidem has been reported to have an awakening effect on patients with different types of brain injury as well as neurological deficits. However, this effect still cannot be explained especially the mechanism involved and the type of targeted receptors. As a sedative-hypnotic agent, zolpidem acts through GABA (A) receptors with the highest affinity at GABA (A) α1-containing receptors. This awakening effect may potentially be mediated by different type of GABA (A) receptors which is likely to form after the occurrence of brain injury. In the pathological conditions, alteration of protein subunit of GABA (A) receptors have been generally and consistently discovered in various brain regions, including in the hippocampus which is crucial in the cognitive process.

The general hypothesis of this study is that cellular changes in brain pathology after brain injury leads to alteration in the expression levels of GABA (A) receptor α1 and ε subunits. The alteration in subunit expression then interferes with the formation of the original receptors and further forms new type of receptors that can be modulated by zolpidem, leading to the awakening effect. Currently, understanding about the potential of zolpidem modulation on the new type of GABA (A) receptors and its association with the awakening effect or in this study our focus is the recovery of cognitive function have been very limited which thereby require further evidence. Therefore, this study offered extended and additional *in vivo* and *in silico* findings of how zolpidem may potentially mediate cognitive recovery in ischaemic injury which speculated through the modulation of novel type of GABA (A) receptors, which involves participation of α1 and ε subunits.

1.4 General objective

To investigate the potential binding sites of GABA (A) receptors as the target for the awakening effect of zolpidem through animal behavioural and cellular studies on impaired cognitive functions in ischaemic rat model (*in vivo)* and analysis on the ligand-receptor interactions using *in silico* methods.

1.5 Specific objectives

1. To investigate the effects of zolpidem on locomotion in the developed model through open-field test.
2. To study the effects of zolpidem on learning and memory in the developed model using Morris water maze test.
3. To analyse the expression level of GABA (A) receptor α1 and ε subunits in the hippocampus of the developed model.
4. To manipulate the arrangement of GABA (A) receptors, analyse and screen possible new targets of the modelled receptors built through homology modelling for zolpidem using molecular dockings technique.
5. To validate the postulate novel binding sites of zolpidem using molecular dynamics simulation of the GABA (A) receptors in membrane lipid bilayer through analysis on the energy bindings, molecular and amino acids interactions, structural compatibility and protein-ligand interactions.

CHAPTER 2

LITERATURE REVIEW

2.1 Synaptic transmission

Synaptic transmission is a communication between a neuron with other neuron or muscle cell (effectors). There are two types of synapse; the chemical and electrical synapse. The latter uses gap junctions to allow exchange of signalling while chemical synapse utilizes signalling through chemicals called neurotransmitters within the CNS. The chemical synapse is more extensively studied as it requires more intricate and complex molecular machinery of presynaptic, postsynaptic and neurotransmitters. The neurotransmitters are released in small vesicles from presynaptic to postsynaptic terminals. These neurotransmitters will travel across the synaptic cleft and bind to their respective receptors at the postsynaptic membrane and initiate subsequent reactions in the form of signalling (Pereda, 2014). Signal resulted from this will travel from the dendritic ends from the previous neuron (presynaptic) along the axonal length to the axon terminal and continue relaying signals towards another neuron (postsynaptic). The types of neurotransmitters will determine whether the generated postsynaptic current is excitatory or inhibitory. The major excitatory neurotransmitter in the CNS is glutamate while GABA (γ-aminobutyric acid) acts as the most dominant inhibitory neurotransmitter.

Action of these neurotransmitters are specific to their respective receptors which are permeable to the movement of K^+, Na^+, Ca^{2+} and Cl^- across the plasma membrane, which will determine the resulting excitatory or inhibitory action. These ions are unequally distributed and the homeostasis of these ions usually regulated by ionic

pumps and exchangers through active or passive transport. The action of neurotransmitters initiates and triggers the opening of channels responsible for these ionic flow down their concentration gradients, resulting in changes within the membrane potential (McCormick, 2014; Pereda, 2014), which is one of the most important elements in the CNS.

2.2 γ-aminobutyric acid or GABA

GABA or γ-aminobutyric acid ($C_4H_9NO_2$) is first discovered in the human brain in 1950 (Johnston, 2017; Olsen & Sieghart, 2009). This major inhibitory neurotransmitter exhibits its action mainly through GABA (A) receptors. Characterized by an amino group on the γ-carbon, GABA joins glutamate and glycine as part of the amino acid cluster of neurotransmitters (Shelp et al., 1999). It carries both positive and negative charge which can also be known zwitterionic and is highly soluble in water with physiological pH values; pK values 4.03 (positive charge) and 10.56 (negative charge) (Shelp et al., 1999). **Figure 2.1** showed the chemical structure of GABA molecule consisting of the amino group.

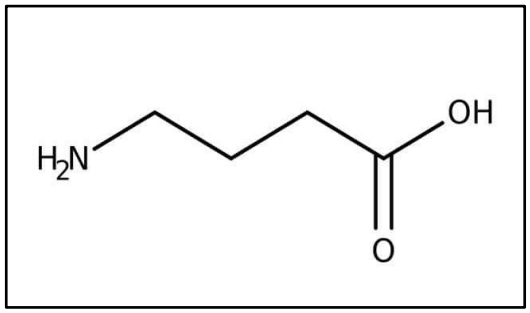


Figure 2.1: The chemical structure of GABA molecule

This neurotransmitter is initially synthesized from glutamate through the action of glutamic acid decarboxylase (GAD) in a pathway called GABA shunt (D'Hulst et al., 2009). Glutamate is obtained through Krebs cycle where α-ketoglutarate is converted to glutamate by transaminase reaction by glutamate dehydrogenase. There are two isoforms of GAD; one with molecular size 65 kDa while another with 67 kDa which are termed GAD_{65} and GAD_{67} respectively (Liguori et al., 2015). Released GABA is removed from the synaptic cleft to prevent excessive GABA build-up in the extracellular space by GABA transporters or GATs (Jin et al., 2011; Zhou & Danbolt, 2013) and broken down by α-ketoglutarate transaminase (GABA-T) into succinic semi-aldehyde and eventually succinic and glutamic acid (D'Hulst et al., 2009; Lanctot et al., 2004; Shelp et al., 1999). It is estimated that 20 – 30 % of GABA are synthesized in the neurons of the CNS and positioned as the primary inhibitory neurotransmitter in nearly 25 – 50 % of synapses in the mammalian brain (Hinton & Johnston, 2008). **Figure 2.2** describes the synthesis of GABA molecule from GABA shunt.

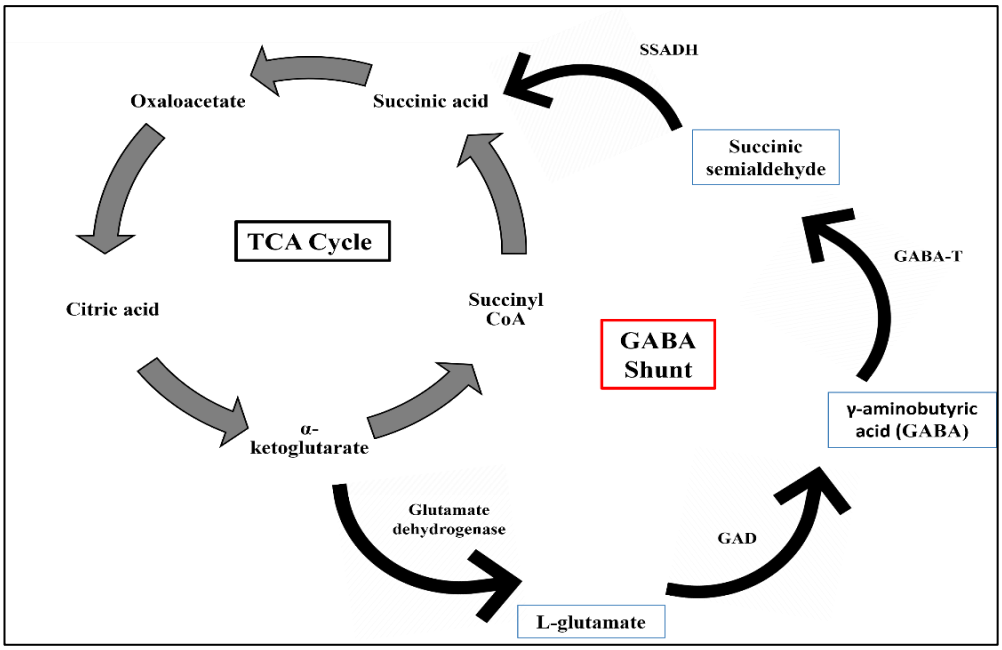


Figure 2.2: The synthesis of GABA neurotransmitter in GABA shunt. The cycle started with α-ketoglutarate from the citric acid (TCA) cycle and involving transaminase process by glutamate dehydrogenase before producing GABA. Adapted from D'Hulst et al. (2009).

2.3 GABA (A) receptors

GABA exhibits its action mainly through GABA (A) receptors. The ionotropic GABA (A) receptor is the major inhibitory receptors in the CNS (Olsen & Sieghart, 2008). It is postulated that almost 50 % of all central synapse is made of GABA (A) receptors (Bowery & Smart, 2006; Nutt, 2006). These receptors are permeable to Cl^- where in mature neurons, the influx of the ion results in strong inhibitory hyperpolarization (Goetz et al., 2007). GABA (A) receptors belong to the ligand-gated ion channels and categorized under the 'cys-loop' family along with nicotinic acetylcholine receptors (nAChRs), glycine receptors (GlyRs) and 5-hydroxytryptamine 3 receptors (5-HT_3Rs, or serotonin type 3 receptors).

GABA (A) receptors are pentameric, built by five subunits to create an ionic pore in the middle (Nuss, 2015). The five different subunits could interchangeably formed from different subunit class identified as α1-6, β1-3, γ1-3, ρ1-3 and one each for δ, ε, θ and π which totalled to 19 different subunits (Weir, 2016). Subunits of the same class such as α1 and α2 shared 60 – 80 % sequence similarities while those of 30 – 40 % are grouped to different classes (Darlison et al., 2005). Alternative splicing of primary gene transcripts such as in β2 and γ2 subunits gene further added to these selection of subunit pools (Darlison et al., 2005; Luscher et al., 2011). Further, the subunits may exists as short (S) and long (L) splices variants, such as γ2S and γ2L which identified by their absence or presence of certain amino acid residues (Luscher et al., 2011). As such, an estimation of more than 151,887 possible pentameric receptors assembly and arrangements could be formed from the available 19 GABA (A) receptor subunits (Bollan et al., 2008; Sieghart et al., 1999). However to date, only few dozen subtypes have been discovered in the human brain with some unique possible arrangement successfully created through *in vitro* methods (Olsen & Sieghart, 2008).

The pentameric arrangement may consist of only homomeric subunit such as β3 homomeric receptors (Miller & Aricescu, 2014) or heteromeric with more than one subunits such as; binary (α-β-α-β-β) (Sieghart & Sperk, 2002), ternary (α-β-α-β-γ) (Baumann et al., 2002) or up to 4 subunits (α**x**-α**x**-α**y**-β-γ) (Ralvenius et al., 2015; Sigel et al., 2006). Co-expression of only αβ (α-β-α-β-β) subunits is sufficient to produce functional Cl^- channels and it is thought that the fifth subunit which usually consist of γ, δ, ε, θ and π is a requirement to provide additional binding site for pharmacologically and clinically relevant drugs for GABA (A) receptors (Zheleznova et al., 2009). **Figure 2.3** illustrates an example of pentameric subunit

arrangement represented by homomeric and heteromeric GABA (A) receptor subtypes with ionic pore in the middle.

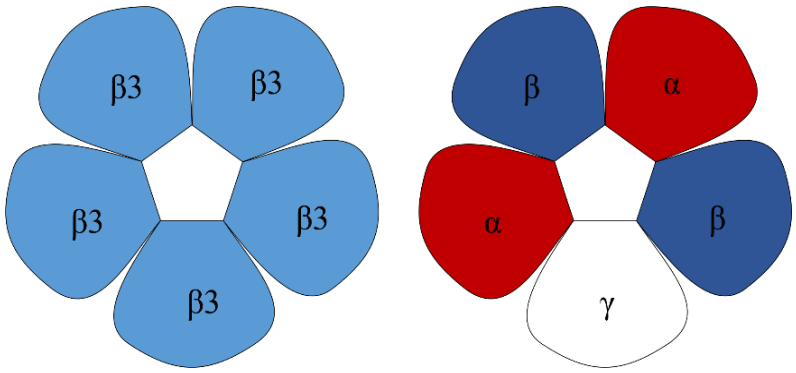
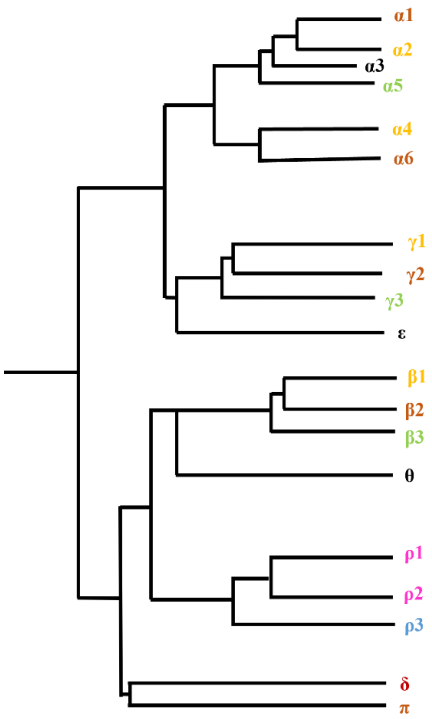


Figure 2.3: Pentameric arrangement of GABA (A) receptor subtype. On the **left** is homomeric β3 GABA (A) receptor subtype and on the **right** the heteromeric αβγ GABA (A) receptor subtype.

The most widely dispersed and major subtype of GABA (A) receptor is the α1β2γ2 which has been suggested to constitute more than 80 % of all mammalian GABA (A) receptors and can be found throughout the CNS (Bollan et al., 2008; Olsen & Sieghart, 2008). This is followed by α2β3γ2 (approximately 15 – 20 %), α3βnγ2 (10 – 15 %), α4βnγ2 (5 %), α5β2γ2 (less than 5 %) and α6β2/3γ2 (less than 5 %) (Rudolph & Knoflach, 2011). The most common arrangement for ternary GABA (A) receptor αβγ is 2α, 2β and 1γ while the binary αβ could either be composed of 3α:2β or 2α:3β (Baumann et al., 2002; Tretter et al., 1997). Interestingly, it is also possible for some GABA (A) receptors to have more than two different α subunits (Puthenkalam et al., 2016).

Remarkably, the ρ subunit (1 – 3) can form homo-oligomeric channels which are insensitive to both bicuculine and baclofen (Johnston, 2013). As a result, these groups of subunits are initially classified into a different GABA (C) receptors (Bormann, 2000). However, the International Union of Basic and Clinical Pharmacology (IUPHAR) has recommended that GABA (C) receptors are to be classified as a 'special' subtype under GABA (A) receptors as they are both structurally ionotropic (Johnston, 2013; Olsen & Sieghart, 2008). Nevertheless, the term is still be used interchangeably till today.

Most of these GABA (A) receptor subtypes can be found localized or expressed higher in specific region of the CNS such as α5 in the hippocampus (Caraiscos et al., 2004), α4 concentrated in the thalamus, hippocampus and basal ganglia (Nutt, 2006) and ρ in the retina (Olsen & Sieghart, 2008). Gene mapping studies have revealed that 14 out of the 19 subunits are gathered on four chromosomes; 4p12-p13 (α2, α4, β1, γ1), 5q31-q35 (α1, α6, β2, γ2), 15q11-q13 (α5, β3, γ3) and Xq28 (α3, ε and θ) while π subunit is located further on chromosome 5q34-q35 and δ subunit is encoded in the shorter arm of chromosome 1 (Darlison et al., 2005; Sigel & Steinmann, 2012; Simon et al., 2004). Meanwhile, ρ1 and ρ2 subunits have been mapped to chromosome 6q14-q21 whereas ρ3 has been suggested to be located in 3q11-q13.3 (Bailey et al., 1999). Following these genetic studies, it has been assumed that most GABA (A) subunits have evolved from common ancestral lineage with 'α-like', 'β-like' and 'γ-like' subunits (Sigel & Steinmann, 2012). This would most likely explain why most of the functional GABA (A) receptors discovered in the human CNS constitute of these subunits (Olsen & Sieghart, 2008). **Figure 2.4** summarized the genetic phylogeny of subunit of GABA (A) receptors.



Chromosome	Location	Subunit	Gene
1	1p36.3	δ	GRBRD
3	3q11.2	ρ3	GRBRR3
4	4p12	α2	GRBRA2
		α4	GRBRA4
		β1	GRBRB1
		γ1	GRBRG1
5	5q34-q35	α1	GRBRA1
	5q34	α6	GRBRA6
	5q34	β2	GRBRB2
	5q31.1-q33.1	γ2	GRBRG2
	5q33-q34	π	GRBRP
6	6q13-q16.3	ρ1	GRBRR1
		ρ2	GRBRR2
15	15q-q13	α5	GRBRA5
	15q11.2-q12	β3	GRBRB3
	15q11-q13	γ3	GRBRG3
X	Xq28	α3	GRBRA3
		ε	GRBRE
		θ	GRBRQ

Figure 2.4: Genetic phylogeny of human GABA (A) receptor subunits. The dendrogram illustrates the 19 subunits genes highlighted according to their respective genes and chromosomal clusters as demonstrated in the table. Adapted from Chuang & Reddy, (2018).

2.4 The structure of GABA (A) receptors

In general, the structure of GABA (A) receptor can be fragmented into three; the extracellular domain (ECD), transmembrane domain (TMD) and intracellular domain (ICD) (**Figure 2.5A**) (Smith & Olsen, 1995). The ECD houses the orthosteric and allosteric binding cavities and becomes the target for various drug binding studies (Amundarain, Viso, et al., 2019). The -N and -C terminals of each subunits are also located in ECD (Sigel & Steinmann, 2012). The TMD consists of four transmembrane sequences (M1 – M4) within each subunit (**Figure 2.5B**). The M2 of each subunit located along the ion channel, faces the centre and creating an ionic pore of 11 nm and a diameter of 8 nm (**Figure 2.5C**) (Nuss, 2015; Olsen & Sieghart,

2008; Sigel & Steinmann, 2012). A large intracellular loop involved in modulation by phosphorylation is located between each M3 and M4 (**Figure 2.5D**) (Sigel & Steinmann, 2012). However, not much is known on the ICD except to be partially formed by alpha helix and aids in ion permeation, gating properties, speed of desensitization and supporting synapse formation (Amundarain, Viso, et al., 2019; Crnjar et al., 2019; O'Toole & Jenkins, 2011). **Figure 2.5** illustrates the structure of GABA (A) receptor, the arrangement of M1 – M4 and the positions of the positive (+) and negative (-) interfaces along with the loops.

Structurally, the ECD of GABA (A) receptors contains a pair of disulphide-bonded cysteine (C) residues, which enclosed the signature 13 amino acid loops giving the 'cys-loop' identity of the receptor (Amundarain, Ribeiro, et al., 2019). The binding pockets at the extracellular domain are located between the interfaces of the subunits. Hence, the arrangement of each subunit within the pentamer is extremely crucial and strongly influences the pharmacological response of each type of GABA (A) receptors. As shown in **Figure 2.5C**, the interface are enclosed by (+) and (-) determined by six loops usually labelled as A, B, C, D, E and F (Thompson et al., 2010). The principal binding face of the interface (+) is made of A, B and C from a subunit while the adjacent subunit with loops D to F builds the complementary face of the pocket (-) (Amundarain, Viso, et al., 2019).

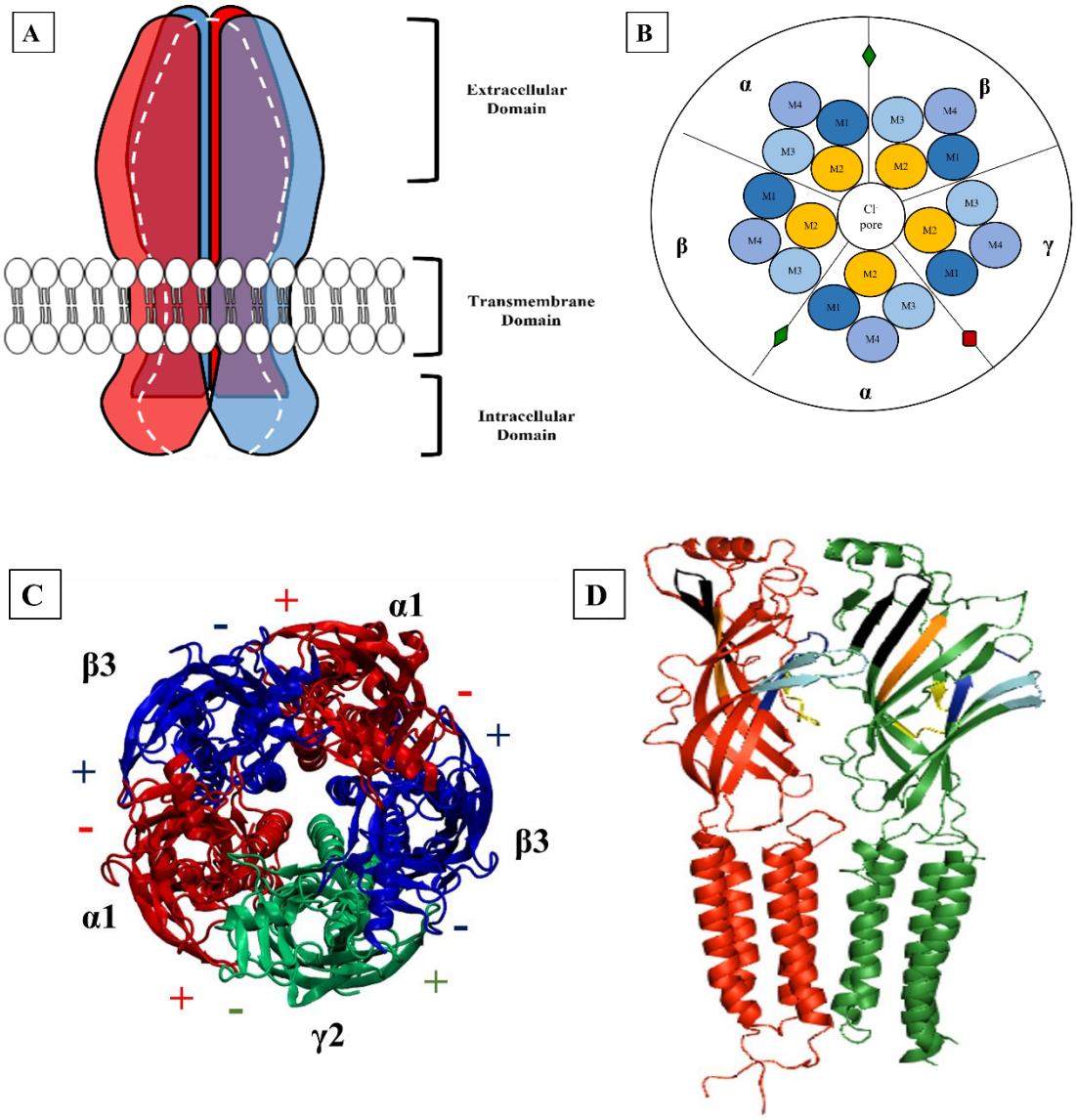


Figure 2.5: Structure of GABA (A) receptor. A) The side view of GABA (A) receptor embedded in lipid bilayer with all three domains (ECD, TMD and ICD) **B**) Top view of the receptor showing M1 – M4 in each subunit, with M2 facing the Cl^- pore. Also shown, GABA (in **green** diamond) between α and β subunits and benzodiazepine (in **red** square) between α and γ subunits **C**) Each subunit represented by different colours possess positive and negative interfaces in between two subunits **D**) Closer view of the interface where each loop (A, B, C, D, E and F) is coloured differently. Figures C and D were generated and illustrated using Pymol Visualization Software.

2.5 GABA (A) receptors: Activation and modulation

The pharmacological properties of GABA (A) receptors are manipulated from the structural arrangement of its subunits. Consequently, these further add to the diversity and wide pharmacological possibilities of GABA (A) receptors. For instance, the ternary α1β2γ2 is arranged in counter clockwise γ2-β2-α1-β2-α1 as seen from the synaptic cleft, creating two β^{+}-α^{-} interfaces which accommodate two GABA and one α^{+}-γ^{-} benzodiazepine interface (Mohamad & Has, 2019; Sieghart & Sperk, 2002). Altering the position of one of α or β subunits would reduce the number of GABA binding pocket, reducing the potency of GABA and influencing the inhibitory current. Also, substituting the third subunit in the subtype (γ) would also change benzodiazepine sensitivity towards the receptor. Hence, structural studies of GABA (A) receptors have widely aided in understanding the consequences of subunits alterations on the pharmacological responses of the receptors, which allowing researchers to manipulate the characteristics in their studies.

The GABA-binding site is also known as the active site or the orthosteric site which accommodates agonists and antagonists ligands. The orthosteric agonists such as GABA, gaboxadol, isoguvacine, muscimol and progabide cause increased in Cl^{-} conductance. Meanwhile, orthosteric antagonists can be divided into competitive and non-competitive. Competitive antagonist such as bicuculline and gabazine, compete with the agonist at the same site and lower the Cl^{-} conductance (Ghit et al., 2021). Whereas, the non-competitive antagonist (or channel blocker) such as picrotoxin binds to or near the pore, blocking the ion conductance (Alanis et al., 2020; Ghit et al., 2021).

On the other hand, there are also substances which can bind elsewhere on the receptor, causing conformational changes to the receptor by positively enhancing (positive allosteric modulators) or negatively reducing (negative allosteric modulators) the inhibitory effect of the receptor (Czajkowski & Sancar, 2011). These modulators require the presence of the endogenous ligand (GABA) to exert their own effects. Positive allosteric modulators or PAMs may include barbiturates, benzodiazepines, Z-drugs (non-benzodiazepines), alcohol such as ethanol, etomidate, glutethimide, anaesthetics such as halothane and isoflurane and certain neurosteroids. Examples of negative allosteric modulators (NAMs) includes pregnenolone sulfate and zinc (Ghit et al., 2021; Olsen, 2018). Meanwhile, null or silent allosteric modulators such as flumazenil does not influence the effects of the endogenous ligands but could compete with PAMs and NAMs at the binding site (Alanis et al., 2020).

2.6 GABA (A) receptors: Synaptic and extrasynaptic

Once released, GABA molecules travel from presynaptic neurons to their receptors located at the dendritic spines of postsynaptic neurons which will further initiate the inhibitory neurotransmission. This inhibitory action can be divided into two types; the synaptic phasic and the extrasynaptic tonic inhibitions. Both inhibitory mechanisms are regulated by GABA (A) receptors but with different receptor subtypes (with distinct subunit compositions) which is prominently affected by the localization of the subunits along the synapse, as shown in **Figure 2.6**. Phasic inhibitory action of GABA (A) receptors is mostly composed by α1-3, β2-3 and γ2 subunits which can be abundantly found along the synapse (Wu et al., 2013). These

receptors are low-affinity and produce transient, rapidly desensitizing postsynaptic responses (Lee & Maguire, 2014). According to Chuang & Reddy, (2018), receptors containing γ2 subunit with combination of α1, α2 and α3 subunits are the major synaptic receptors eliciting phasic inhibition whereas those with δ subunit in combination with α4, α5 and α6 are the primary extrasynaptic receptors generating tonic inhibition.

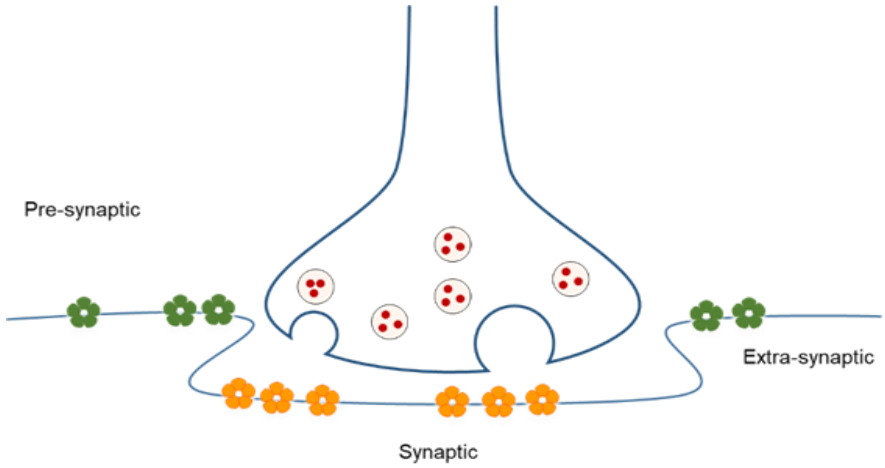


Figure 2.6: The synaptic and extrasynaptic GABA (A) receptors along the postsynaptic region. The synaptic receptors are situated directly across the synaptic cleft, receiving the most GABA released from the presynaptic receptor while the extrasynaptic receptors are located further down the membrane. Adapted from Chuang and Reddy (2018).

Initially described by Salin & Prince, (1996), extrasynaptic GABA (A) receptors can be specifically found in brain regions such as the hippocampus, amygdala, neocortex, thalamus, hypothalamus and cerebellum (Chuang & Reddy, 2018; Mortensen et al., 2012). These receptors are expressed a little further down the synapse and are activated by; low levels of GABA around the neurons, increased extracellular GABA concentrations due to disruption on the functions of GABA transporters, GABA spill

over from synapses or non-vesicular release or newly induced or sensitized extrasynaptic receptors by extracellular factors such as insulin which can interrupt the ambient GABA level (Farrant & Nusser, 2005; Li et al., 2013). In general, these factors results in presence of ambient GABA in the extracellular space, resulting in activation of the extrasynaptic GABA (A) receptors (Davies, 2014).

Since these receptors are activated by ambient GABA in the extrasynaptic area, the determination, changes and dysregulation of tonic inhibition should stem from the regulation of concentration of ambient GABA. GABA transporters are crucial in regulating tonic inhibition and is responsible in the 'cleaning' of GABA in the extrasynaptic space (Richerson, 2004; Scimemi, 2014). GABA transporters can be found widely expressed throughout the brain and belongs to a large family of neurotransmitters; the sodium symporters which is known as solute carrier 6 (SLC6) family in human (Scimemi, 2014). Three GABA transporters have been discovered so far; SLC6A1 (GAT-1), SLC6A13 (GAT-2) and SLC6A11 (GAT-3) with GAT-1 and GAT-3 accounting for highest density around the presynaptic neurons and astrocytes respectively (Egawa & Fukuda, 2013).

Meanwhile, the extrasynaptic receptors can be found localized at the extrasynaptic membrane and are majorly composed of subunits such as α4-6, β, δ, and γ2 subunits (Wu et al., 2013). Although, other subunit combinations have also been reported to show tonic inhibitory characteristics such as the αβ receptors and the debatable ε-containing receptors (Davies, 2014). As mentioned by Glykys & Mody, (2007), only four types of hetero-pentameric GABA (A) receptors containing either δ, α5, ε or αβ have been shown to mediate high affinity GABA with little desensitization making them the perfect receptors for tonic inhibition (Mortensen & Smart, 2006). However, as these receptors are also able to assemble with other α, β and γ subunits, it is

possible that no more than a dozen GABA (A) receptor subunit combinations can mediate the tonic conductance in the brain.

The α5 is another subunit which plays a crucial role in tonic inhibition in the CA1 and CA3 hippocampal pyramidal neurons (Caraiscos et al., 2004; Semyanov et al., 2004) and layer 5 cortical neurons (Brickley & Mody, 2012). The subunit is more commonly found assembled with β2/3 and γ2 forming α5β2γ2 and α5β3γ2 with the latter being more abundant (Mohamad & Has, 2019). Fascinatingly, the majority of the GABA (A) α5-containing receptors are found to be concentrated in the hippocampus, suggesting its role in the hippocampal-dependent learning and memory mechanisms which inadvertently also proposing the role of tonic inhibition in learning and memory (Möhler & Rudolph, 2017).

Meanwhile, the α4 and α6 subunits are potent extrasynaptic subunits present in thalamus and cerebellar granule cells respectively and are insensitive to diazepam and zolpidem (Farrant & Nusser, 2005). Subunits such as ε and θ which are less abundant, are not yet fully established as extrasynaptic receptors but possess high potency for GABA and being located at extrasynaptic sites suggesting potential contributors for tonic inhibition (Mortensen et al., 2012). These subunits have been proposed to assemble as α3βε and α3βθ due to co-localization of α3, ε and θ on the same chromosomes (Davies, 2014; Mortensen et al., 2012). However to date, the full characterization on the exact inhibitory action of GABA (A) ε-containing receptors is still undetermined due to the limited studies on the subunit.