EXPLORING NEW ZOLPIDEM BINDING SITE ON ε-CONTAINING GABA (A) RECEPTOR VIA COMPUTATIONAL METHODS

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2025

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Neuroscience

May 2025

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude to my main supervisor, Dr. Ahmad Tarmizi Che Has, for his unwavering guidance, trust, and mentorship throughout this research project. It has been a privilege working with him as his passion, dedication, and expertise in the field have been a constant source of inspiration, motivating me to strive for excellence in my work. I am also deeply grateful to my co-supervisors, Dr. Azzmer Azzar Abdul Hamid, and Dr. Khairul Bariyyah Abdul Halim, whose wisdom and insights have been invaluable in shaping the course of my research.

Next, I would like to extend my sincere thanks to Syarifah Maisarah for her consistent support and patience in teaching me the foundational skills needed for this research. A special mention also goes to my course mates, Sutharshinnii Murugan, and Faris bin Razak, whose care and camaraderie have made this journey not only rewarding but also fun and exciting. This research would also not be possible without the financial support from the Fundamental Research Grant Scheme (FRGS) provided by the Ministry of Higher Education, Malaysia.

Above all else, I am eternally grateful to my family who have been my pillars of support, believing in me and encouraging me to embark on this journey and see it through to completion. I also thank the Lord God Almighty for opening doors to this incredible opportunity and for guiding me to where I am today.

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LIST OF SYMBOLS

Å	Angstrom
α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
K	Kelvin
kJ	kilojoules
M	molarity
ms	millisecond
N	particles
ns	nanosecond
π	pi
P	pressure
ρ	rho
T	temperature
θ	theta
V	volume

LIST OF ABBREVIATIONS

BZD	benzodiazepine
Cl^-	chloride ions
CNS	central nervous system
DMCM	methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
DOC	disorders of consciousness
DOPE	Discrete Optimized Protein Energy
ECD	extracellular domain
FMZ	flumazenil
GABA	gamma-aminobutyric acid
$GABA_AR$	gamma-aminobutyric acid type A receptor
GROMACS	Groningen Machine for Chemical Simulations
i-BZD	imidazobenzodiazepine
ICD	intracellular domain
MD	molecular dynamics
NAM	negative allosteric modulator
PAM	positive allosteric modulator
PLIP	Protein-Ligand Interaction Profiler
POPC	1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine
RCSB PDB	Research Collaboratory for Structural Bioinformatics Protein Data Bank
Rg	radius of gyration
RMSD	root-mean square deviation
RMSF	root-mean square fluctuation
TBI	traumatic brain injury
TMD	transmembrane domain
ZPM	zolpidem
2D	two-dimensional
3D	three-dimensional

EKSPLORASI TAPAK PENGIKATAN ZOLPIDEM BAHARU PADA RESEPTOR GABA (A) YANG MENGANDUNGI SUBUNIT ε MELALUI KAEDAH PENGKOMPUTERAN

ABSTRAK

Reseptor GABA (A) ($GABA_AR$) yang mengandungi subunit ε, salah satu subjenis yang kurang dikaji dalam keluarga $GABA_AR$, semakin mendapat perhatian disebabkan ciri farmakologi uniknya dan potensi keterlibatannya dalam kecederaan otak. Zolpidem (ZPM), sejenis ubat kategori Z yang digunakan secara meluas, telah dilaporkan mencetuskan kesan paradoks pada pesakit yang mengalami kecederaan otak, walaupun asas molekular fenomena ini masih belum jelas. Kajian ini bertujuan untuk menjelaskan mekanisme struktur dan molekul di sebalik fenomena tersebut melalui pendekatan *in silico* secara menyeluruh. Satu model homologi bagi $GABA_AR$ α1-β2-ε-α1-β2 berjaya dihasilkan dan disahkan melalui proses peminimuman tenaga dan keseimbangan menggunakan CHARMM-GUI dan GROMACS. Pengedokan molekul menggunakan AutoDock 4.2 telah menilai perkaitan pengikatan dan profil interaksi bagi ZPM, flumazenil (FMZ), dan DMCM - yang mewakili modulator allosterik positif, neutral, dan negatif. ZPM menunjukkan perkaitan pengikatan tertinggi pada antara muka $\alpha1^+/\varepsilon^-$, diikuti oleh FMZ dan DMCM. Analisis struktur mencadangkan bahawa penggantian γ2F77I dan γ2A79S pada subunit ε mungkin mengurangkan perkaitan ligan, terutamanya bagi ligan yang bersaiz lebih besar seperti FMZ dan DMCM. Tambahan pula, pemuliharaan yang rendah bagi sebatian asid amino dalam Gelung F (Loop F) yang merangkumi γ2Glu182–Arg197 mungkin mempengaruhi keberkesanan modulator allosterik positif (PAM) seperti ZPM. Simulasi dinamik molekul (100 ns) secara umumnya menyokong profil farmakologi ligan-ligan tersebut. Walaupun ZPM menunjukkan aktiviti terhad di tapak α/ε, model allosterik Monod-Wyman-Changeux mencadangkan bahawa PAM boleh meningkatkan fungsi $GABA_AR$ yang aktif secara spontan dengan hanya satu molekul GABA

terikat. Ini menyokong kesan modulasi positif khusus ZPM terhadap reseptor yang mengandungi ε dan menekankan potensinya sebagai sasaran terapeutik dalam kecederaan otak.

EXPLORING NEW ZOLPIDEM BINDING SITE ON ε-CONTAINING GABA (A) RECEPTOR VIA COMPUTATIONAL METHODS

ABSTRACT

The ε-containing GABA (A) receptors ($GABA_ARs$), a lesser-studied subtype within the $GABA_AR$ family, have gained attention due to their distinct pharmacological properties and potential involvement in brain injury. Zolpidem (ZPM), a widely used Z-drug, has been reported to induce paradoxical effects in brain-injured patients, although the molecular basis remains unclear. This study aimed to elucidate the structural and molecular mechanisms underlying this phenomenon through a comprehensive *in silico* approach. A homology model of the α1-β2-ε-α1-β2 $GABA_AR$ was successfully constructed and validated following energy minimization and equilibration using CHARMM-GUI and GROMACS. Molecular docking with AutoDock 4.2 assessed the binding affinities and interaction profiles of ZPM, flumazenil (FMZ), and methyl-6,7-dimethoxy-4-ethyl-β-carboline-2-carboxylate (DMCM) — representing positive, null, and negative allosteric modulators, respectively. ZPM exhibited the highest binding affinity at the $\alpha1^+/\varepsilon^-$ interface, followed by FMZ and DMCM. Structural analysis suggested that the γ2F77I and γ2A79S substitutions in the ε subunit may reduce ligand affinity, especially for bulkier ligands like FMZ and DMCM. Additionally, poor conservation of Loop F residues spanning γ2Glu182–Arg197 may influence modulatory efficacy of positive allosteric modulators (PAMs) like ZPM. Molecular dynamics simulations (100 ns) largely supported the pharmacological profiles of the ligands. Although ZPM showed limited activity at the α/ε site, the Monod-Wyman-Changeux allosteric model suggests that PAMs can enhance function in spontaneously active $GABA_ARs$ with single GABA occupancy. This supports ZPM's specific positive modulatory effects on ε-containing receptors and highlights their potential as therapeutic targets in brain injury.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Zolpidem (ZPM) is a potent sedative-hypnotic agent primarily prescribed for the management of insomnia-related sleep disturbances (Monti et al., 2017). Its chemical structure, as an imidazopyridine, plays a critical role in its selective binding affinity and subsequent pharmacological effects (Hasegawa et al., 2018; Kumar et al., 2024; Monti et al., 2017; Sun et al., 2023; Zhu et al., 2022). ZPM exhibits a high affinity for the α1 subunit of GABA (A) receptors ($GABA_ARs$), an intermediate affinity for the α2 and α3 subunits, and minimal affinity for the α5 subunit (Baur & Sigel, 2007; Zhu et al., 2022). This unique selectivity for the α1 subunit makes ZPM therapeutically effective in promoting sleep while minimizing the risk of adverse effects. As a result, ZPM has rapidly emerged as the drug of choice for treating sleep disorders (Anderson et al., 2023; Monti et al., 2017).

ZPM's action is modulated through ternary αβγ $GABA_ARs$ at the extracellular $\alpha(1,2,3,5)^+/\gamma2^-$ interface, a site shared with benzodiazepines (BZDs). Acting as a positive allosteric modulator (PAM), ZPM increases the frequency of channel opening of $GABA_ARs$ and enhances chloride ion (Cl^-) influx, resulting in hyperpolarization and sedative effects (Clauss & Nel, 2006; Hanson & Czajkowski, 2008; Richter et al., 2020; Sancar et al., 2007; Sieghart, 2015). Paradoxically, despite its primary function as a sleep aid, ZPM has been reported to induce arousal in patients with various brain injuries, including those with trauma, stroke, and disorders of consciousness (DOC) (Arnts et al., 2020; Bomalaski et al., 2017; Wu & Sun, 2015). This paradoxical effect extends to the recovery of cognitive and motor functions across a wide spectrum of neurological conditions (Arnts et al., 2024; Daniele et al., 2016; Georgiopoulos et al., 2010; Hahm & Woo, 2019; Miyazaki et al., 2012; Nyakale et al., 2011;

Thonnard et al., 2013). These observations raise several important questions: how can a drug originally designed as a sedative-hypnotic agent produce such awakening effects? What specific receptor targets are responsible for this paradoxical action?

These questions suggest the involvement of distinct $GABA_AR$ subtypes that may form novel binding sites beyond the classical $\alpha^+/\gamma2^-$ BZD interface. Previous studies have demonstrated alterations in $GABA_AR$ subunit composition under pathological conditions such as those mentioned above (Che Has et al., 2016; Drexel et al., 2015; Fatemi et al., 2013; Kharlamov et al., 2008; Mtchedlishvili et al., 2010; Tossell et al., 2021). *In vitro* studies have explored potential alternative binding sites for ZPM on $GABA_ARs$. For instance, Che Has et al. (2016) observed that ZPM modulates binary α1β3 $GABA_ARs$ expressed in a 3α1:2β3 stoichiometry, with its effect attenuated by flumazenil (FMZ), suggesting that ZPM binds at the $\alpha1^+/\alpha1^-$ subunit interface, which resembles the classical $\alpha1^+/\gamma2^-$ interface. Additionally, the study found that ZPM modulates the ε-containing $GABA_ARs$ at lower concentrations, with a dose-dependent response.

While $(\alpha1^+/\alpha1^-)$-containing $GABA_ARs$ are associated with sedative-hypnotic effects, the current study shifts focus to the potential role of $(\alpha1^+/\varepsilon^-)$-containing $GABA_ARs$ in mediating the paradoxical effects of ZPM. Research has shown that the ε subunit is capable of substituting other $GABA_AR$ subunits, forming functional Cl^- channels with distinct biophysical and pharmacological properties (Bollan et al., 2008; Che Has et al., 2020; Davies et al., 2001; Has & Chebib, 2018; Ranna et al., 2006). The unique pharmacological profile of these receptors could contribute to aberrant neuronal excitability and give rise to distinct effects related to sedation, stress, emotional regulation, and pain perception. This may provide a potential explanation for the paradoxical actions of ZPM (Markus et al., 2020; Ranna et al., 2006).

Previous studies have extensively characterized ZPM's selectivity in its binding sites, particularly at the classical α^+/γ^- BZD binding interface of $GABA_ARs$, which it shares with FMZ and methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM). As a null and negative allosteric modulator (NAM) of $GABA_ARs$ respectively, FMZ and DMCM block and attenuate the effects of ZPM (Evans & Lowry, 2007; Hood et al., 2014; Patat et al., 1994; Siebert et al., 2018; Zhu et al., 2022).

Therefore, this study aims to investigate the binding properties of all three compounds, ZPM, FMZ, and DMCM on $(\alpha1^+/\varepsilon^-)$-containing $GABA_ARs$ to gain a deeper understanding of the mechanistic action of ZPM. Using *in silico* methods, distinct pentameric arrangements featuring the $\alpha1^+/\varepsilon^-$ interface will be constructed via homology modelling techniques. These modelled receptors will then be subjected to docking studies with ZPM, FMZ, and DMCM - null and NAMs of $GABA_ARs$. Finally, the structural stability of these interactions will be assessed through molecular dynamics (MD) simulations, conducted within a lipid bilayer membrane and subjected to specific force fields.

1.2 Problem Statement

The paradoxical awakening effect of ZPM in patients with brain injuries, strokes, movement disorders, and neurological deficits challenges conventional understandings of GABAergic modulation. Despite numerous studies attempting to investigate and elucidate this phenomenon, most are largely descriptive in nature, and a comprehensive mechanistic explanation remains elusive. The pharmacological responses of ZPM suggest its interaction with distinct binding sites within $GABA_ARs$, which may be altered in pathological conditions.

Therefore, this study aims to investigate the underlying mechanisms of ZPM's awakening effect by exploring its interaction with ($\alpha1^+/\varepsilon^-$)-containing $GABA_AR$s, which have a unique pharmacological profile.

A significant challenge in this exploration lies in the ability to create and manipulate the composition of individual $GABA_AR$ subunits. Additionally, there is a lack of comparative studies on the binding of ZPM and other $GABA_AR$ allosteric modulators, making it difficult to fully understand the drug's mechanism of action. Furthermore, interactions between $GABA_AR$s and their modulators are highly dynamic, influenced by factors such as receptor conformational states and the surrounding membrane environment.

To address these challenges, this study will employ *in silico* methods to identify key residues and structural motifs within the ε subunit of $GABA_AR$s that influence ZPM binding. This approach will also raise important questions regarding whether this novel binding site is specific to ZPM or whether it also interacts with other $GABA_AR$ allosteric modulators, such as FMZ and DMCM. Computer-aided simulations will provide deeper insights into the binding interactions of these compounds, revealing structural nuances and ligand interactions that govern $GABA_AR$ modulation, and potentially offering a molecular explanation for the paradoxical action of ZPM.

1.3 Hypothesis and Rationale of the Study

Following brain injury, cellular alterations in brain pathology leads to significant modifications in the expression levels of specific subunits of $GABA_AR$s. Particularly, it is suggested that the expression of the ε subunit of $GABA_AR$s undergoes notable changes. These alterations in subunit expression are anticipated to result in the formation of a novel subtype of the receptor, one that exhibits distinct pharmacological properties. Importantly, this newly formed receptor

subtype, characterized by the inclusion of the ε subunit, is hypothesized to harbour a binding site to ZPM. The presence of this ε-containing binding site is crucial for mediating the paradoxical awakening effect, a phenomenon in which a patient or subject experiences an unexpected state of consciousness or wakefulness under circumstances where such an outcome would typically be considered improbable. This effect is thought to be driven by the altered pharmacological properties of the ε-containing receptor subtype, which may differ significantly from the conventional $GABA_AR$s forms in terms of their responsiveness to pharmacological agents and their physiological roles in neural signalling and brain function following injury.

1.4 General Objective

To postulate and validate novel binding site of ZPM, FMZ and DMCM on $GABA_AR$ and analysis on the substances-receptor interactions using *in silico* approaches.

1.5 Specific Objectives

1. To construct the 3D structure of the α1β2ε $GABA_AR$ through homology modelling.

2. To screen and analyse possible new targets for ZPM, FMZ and DMCM on the modelled receptor using molecular dockings.

3. To predict potential novel binding site for zolpidem using molecular dynamic simulation of the α1β2ε $GABA_AR$ in membrane lipid bilayer.

CHAPTER 2

LITERATURE REVIEW

2.1 $GABA_ARs$: Structural and Functional Properties

Gamma-aminobutyric acid type A receptors ($GABA_ARs$) are the principal inhibitory neurotransmitter receptors in the central nervous system (CNS) (Jewett & Sharma, 2023; Lynagh & Pless, 2014). These receptors are members of the "cys-loop" family of pentameric ligand-gated ion channels, consisting of five protein subunits that collectively form an ion channel primarily permeable to Cl^- ions (Sieghart, 2015). The subunits that make up $GABA_ARs$ are derived from a pool of 19 distinct subunits, which belong to eight receptor subunit families: α1–6, β1–3, γ1–3, ρ1–3, δ, ε, θ, and π (Ghit et al., 2021) (**Figure 2.1 A**). Each $GABA_AR$ subunit shares a common structural framework, typically consisting of approximately 450 amino acid residues (Ghit et al., 2021). Structurally, the subunits feature a large hydrophilic extracellular domain (ECD), which contains a substantial N-terminus and a smaller C-terminus, along with four hydrophobic transmembrane domains (TMD: M1–M4) (**Figure 2.1 B**). The M2 segment of the TMD is thought to form the Cl^- channel pore, while an intracellular domain (ICD) located between M3 and M4 serves as a crucial site for protein interactions and post-translational modifications that regulate receptor activity (Chen & Olsen, 2007; Jacob et al., 2008; Kasaragod et al., 2022; Xie et al., 2013) (**Figure 2.1 C**).

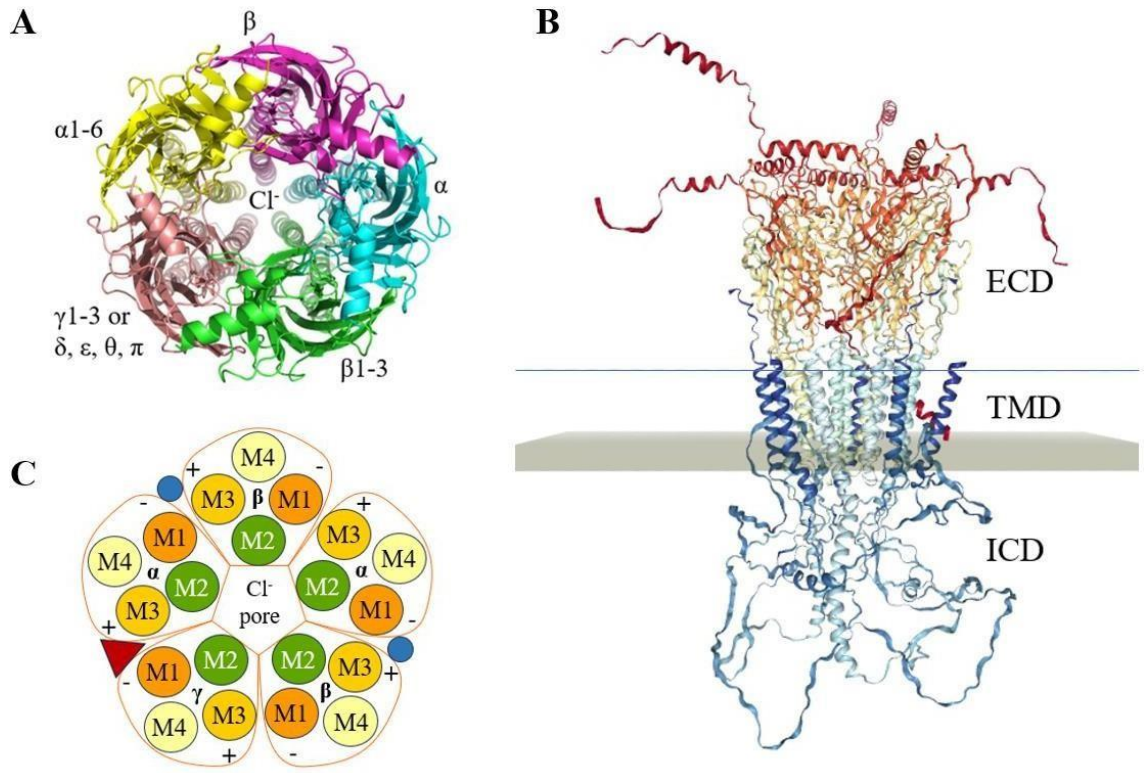

Figure 2.1 Structural Composition of $GABA_AR$. **(A)** Top view of $GABA_AR$ illustrating its pentameric structure. The receptor is composed of five subunits selected from a pool of 19 possible subunits: α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3. Together, these subunits form a central Cl^- pore. **(B)** Side view of $GABA_AR$, highlighting its tripartite architecture. The receptor consists of a large hydrophilic ECD, a hydrophobic TMD embedded within the lipid bilayer, and an ICD. (**C)** Schematic representation of $GABA_AR$. The TMD of each subunit contains four helical segments (M1–M4), with M2 lining the Cl^- pore. The principal (+) and complementary (-) interfaces are labelled, and the ligand binding sites are depicted, with the blue circle representing the GABA binding sites at the β^+/α^- interface, and the red triangle representing the BZD binding site at the α^+/γ^- interface.

The pentameric assembly of $GABA_ARs$ can be homomeric, such as the human β3 homopentamer, which consists of identical subunits (Miller & Aricescu, 2014). However, most $GABA_ARs$ are heteromeric, composed of different subunits that give rise to various binary (α-β-α-β-β), ternary (α-β-α-β-γ), and quaternary (αx-αx-αy-β-γ) subunit configurations (Baumann et al., 2003; Has & Chebib, 2018; Sieghart & Sperk, 2002; Zhou et al., 2025). While the co-expression of α and β subunits alone (α-β-α-β-β) is sufficient to form functional Cl^- channels, it is believed that the inclusion of a fifth subunit—typically γ, δ, ε, θ, or π - is essential for the

complete pharmacological profile of $GABA_AR$s. This fifth subunit provides an additional binding site for the attachment of pharmacologically and clinically relevant drugs (Blednov et al., 2014; Pan et al., 2022; Zheleznova et al., 2009).

2.2 Regional Distribution and Functional Roles of $GABA_AR$ Subtypes

The distribution of $GABA_AR$s with distinct subunit compositions varies across different regions of the mature brain. Receptors containing α1 and γ2 subunits are predominantly located at the synaptic cleft and are primarily responsible for mediating fast, phasic inhibition in the brain. In contrast, receptors containing α4, α5, α6, and δ subunits are typically found at extrasynaptic or perisynaptic sites and contribute to tonic inhibition (Lee & Maguire, 2014) (**Figure 2.2**).

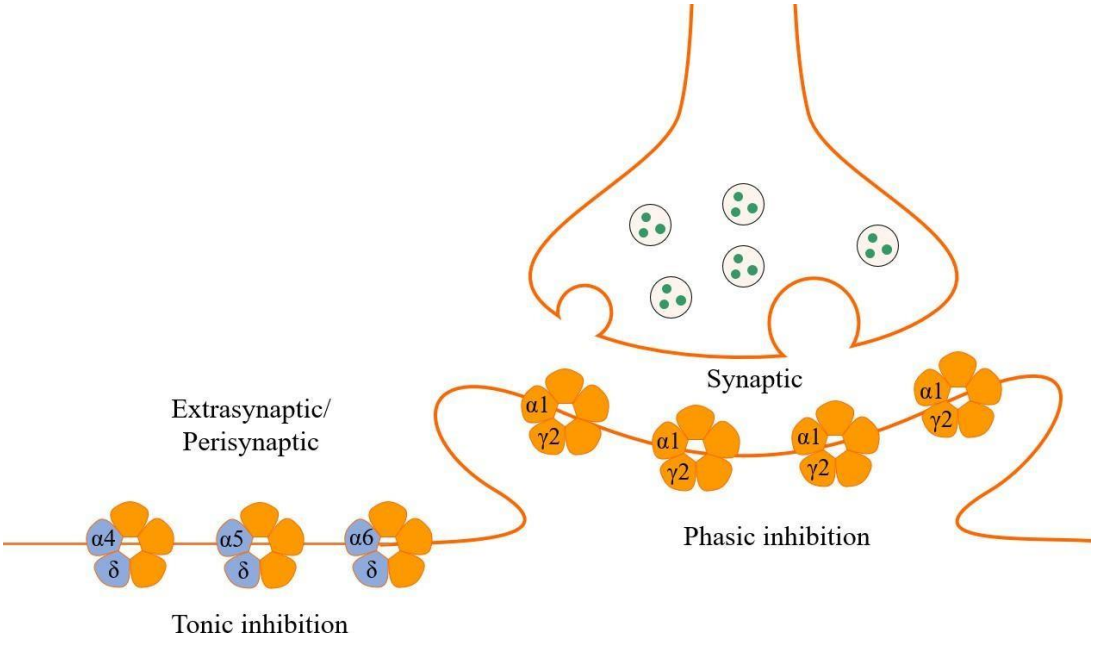

Figure 2.2 Distribution of $GABA_AR$ Subtypes in the Brain. α1- and γ2-containing receptors are mainly located at the synaptic cleft, mediating fast, phasic inhibition, whereas α4-, α6- and δ-containing receptors are found at extrasynaptic or perisynaptic sites, mediating tonic inhibition.

In situ hybridization and immunostaining studies have demonstrated that the γ2 subunit is present in the vast majority of $GABA_A$Rs in the CNS (Sieghart & Ernst, 2005). Among the α subunits, α1 is the most abundant and is widely distributed throughout the cortex (layers I-VI), hippocampus, amygdala, basal forebrain, substantia nigra pars reticulata, inferior colliculus, cerebellum, and brainstem (Rudolph & Mohler, 2014). The β subunit typically associates with only one type of subunit per pentamer, with β2 being more widely distributed than β1. The canonical $GABA_A$R subtype, α1β2γ2, which is arranged in a counterclockwise configuration as γ2-β2-α1-β2-α1 (**Figure 2.1 A**), is the most prevalent and widely distributed receptor type, estimated to comprise over 80% of all mammalian $GABA_A$Rs. It is found throughout the CNS (Olsen & Sieghart, 2009; Zhou et al., 2025). On the other hand, α5 subunit is relatively rare, being found predominantly in the olfactory bulb, hypothalamus, and hippocampus, where they are primarily located at extrasynaptic sites (Żarnowska et al., 2009). Similarly, α6 subunit is mainly expressed at extrasynaptic sites, with high expression levels in the forebrain and cerebellum. The δ subunit is located at extrasynaptic dendritic and somatic membranes, particularly in the forebrain and cerebellar granule cells. It is believed that the δ subunit may substitute for the γ subunit in the pentameric receptor (Nusser et al., 1998). The ε subunit is relatively rare but can replace γ or δ subunits in certain regions, such as the hypothalamus (Whiting et al., 1997). The functions of the θ and π subunits remain poorly understood and have yet to be fully characterized (Waldvogel et al., 2010).

2.3 Activation and Modulation of $GABA_A$Rs

$GABA_A$Rs are activated by the neurotransmitter gamma-aminobutyric acid (GABA), which binds to the N-terminal region at the β-α subunit interface (Sigel & Lüscher, 2011). Upon GABA binding at the postsynaptic site, the receptor undergoes a conformational change, leading to the opening of the ion channel. This allows Cl^- to enter the cell along their

concentration gradient, resulting in hyperpolarization of the postsynaptic neuron (Blednov et al., 2014). In the CNS, GABA functions synergistically with the excitatory neurotransmitter glutamate to regulate neuronal firing. This balance is essential for fine-tuning the excitability of neuronal networks and maintaining homeostasis in brain activity (Wu & Sun, 2015).

The diverse pentameric assemblies of $GABA_AR$s greatly expand the range of neuronal responses to external signals, thereby contributing to the modulation of the electrical activity of neurons and other cell types (Ghit et al., 2021; Jacob et al., 2008; Sente et al., 2022). In addition to GABA binding, $GABA_AR$s can also be modulated by various psychotropic drugs. For example, benzodiazepines (BZDs) bind to the α^+/γ^- subunit interface (Sigel & Lüscher, 2011), while neurosteroids and anaesthetics such as barbiturates interact with the transmembrane domains (TMD) of the α and β subunits (Rudolph & Antkowiak, 2004; Wang, 2011).

The specific subunit composition of $GABA_AR$s is critical to their physiological and pharmacological properties, influencing a wide array of processes such as sleep regulation, learning and memory, as well as neuronal development, including synaptic formation, neuronal growth, migration, and proliferation (Ben-Ari, 2021; Ghit et al., 2021; Jacob et al., 2008; Mohamad & Has, 2019; Sakimoto et al., 2021). Dysregulation of $GABA_AR$s has been implicated in a range of neuropsychiatric and neurodevelopmental disorders, including anxiety, depression, insomnia, autism, Down syndrome, epilepsy, schizophrenia, eating disorders, and substance use disorders (Bloss et al., 2011; Macdonald et al., 2010; Rudolph & Mohler, 2014; Thompson, 2024).

2.4 Zolpidem: Structure, Mechanism of Action, and Therapeutic Use

Zolpidem (ZPM), chemically known as N,N,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide hemitartrate ($C_{19}H_{21}N_3O$), is a sedative-hypnotic agent commercially available under the brand names Ambien® and Stilnox® (Monti et al., 2017). It is primarily prescribed at doses of 5 or 10 mg to treat sleep disturbances associated with insomnia (Holm & Goa, 2000; Monti et al., 2017). Commonly classified as a "Z-drug," ZPM, along with eszopiclone, zopiclone, and zaleplon, is a non-BZD derivative (**Figure 2.3**). These agents act as positive allosteric modulators (PAMs) at the BZD binding site located at the $\alpha(1,2,3,5)^{+}/\gamma2^{-}$ subunit interface on $GABA_ARs$ (Monti et al., 2017; Vlainić et al., 2012; Zhu et al., 2022) (**Figure 2.4**). ZPM is the first BZD-site agonist developed and its unique imidazopyridine structure is central to its selective binding affinity and pharmacodynamic properties (Hasegawa et al., 2018; Kumar et al., 2024; Monti et al., 2017; Sun et al., 2023; Zhu et al., 2022).

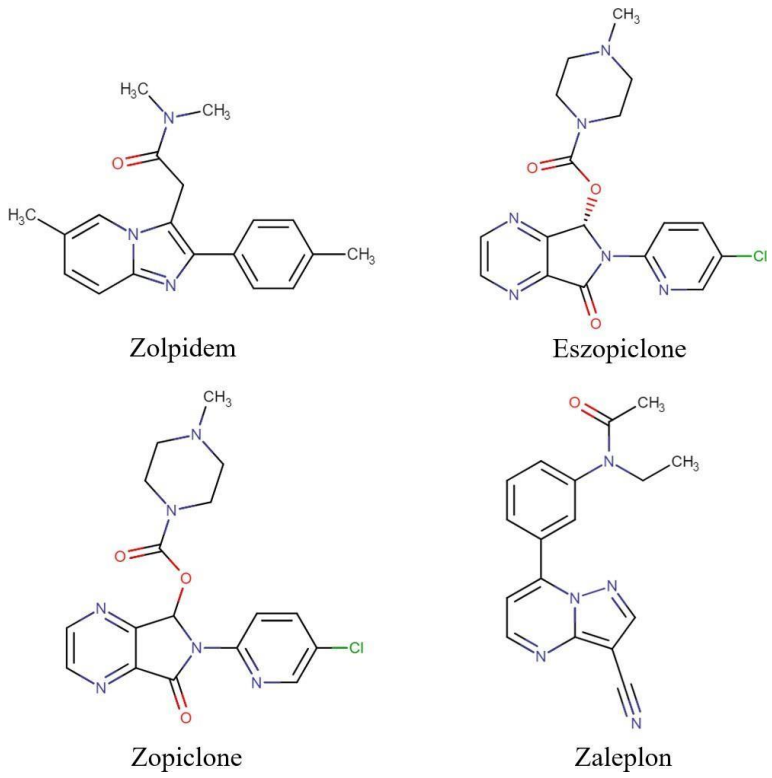

Figure 2.3 Z-drugs. ZPM along with eszopiclone, zopiclone, and zaleplon, are non-BZD derivatives acting as PAMs at the $\alpha1^{+}/\gamma2^{-}$ BZD binding site.

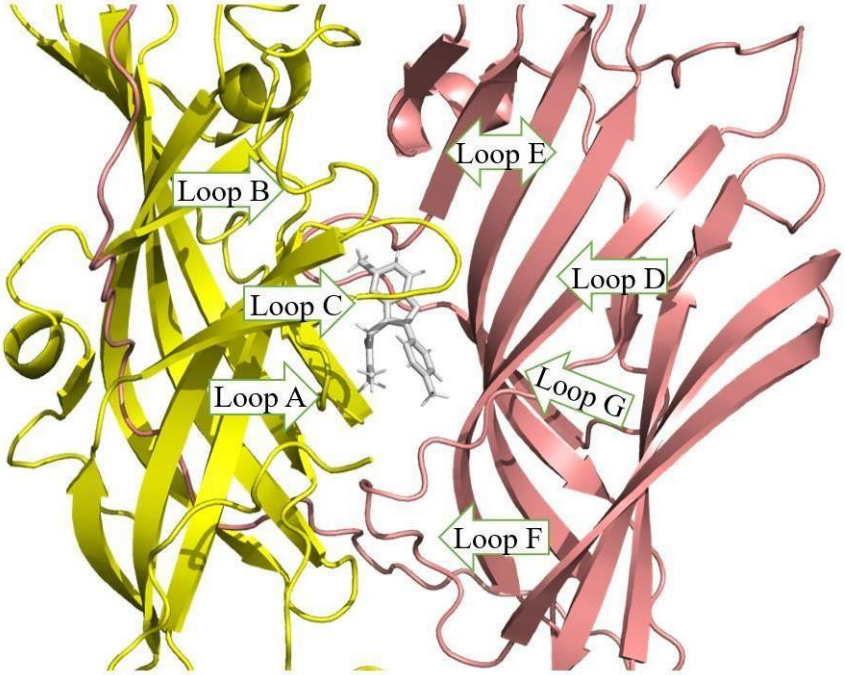

Figure 2.4 Homology Model of the GABA$_A$R α1$^+$/γ2$^-$ Interface. The α1 subunit is depicted in yellow, while the γ2 subunit is in peach. Binding site loops A to F are labelled to indicate key regions involved in ligand interactions.

ZPM exhibits a high affinity for GABA$_A$Rs containing the α1 subunit, with a 20-fold lower affinity for the α2 and α3 subunits, and minimal affinity for the α5 subunit (Baur & Sigel, 2007; Zhu et al., 2022). In contrast, other non-selective Z-drugs, such as eszopiclone and zopiclone, along with classical BZDs like diazepam and flurazepam, bind to the α1, α2, α3, and α5 subunits to a similar extent (Anderson et al., 2023; Baur & Sigel, 2007; Louzada et al., 2022; Pritchett & Seeburg, 1991; Sun et al., 2023).

The α1-containing GABA$_A$Rs are primarily responsible for mediating the sedative and hypnotic effects of ZPM, while the α2 and α3 subunits contribute to anxiolytic, anticonvulsant, myorelaxant, ataxic, and withdrawal effects (Monti et al., 2017). The selective binding of ZPM, particularly to the α1 subunit of GABA$_A$Rs, distinguishes it from other BZDs, making it highly effective for inducing sleep with a reduced risk of adverse side effects such as rebound insomnia, memory impairments, and a lower potential for dependence, tolerance, and abuse - issues commonly associated with traditional BZDs (Edinoff et al., 2021; Holm & Goa, 2000;

Möhler, 2010; Monti et al., 2017). Consequently, ZPM has rapidly become the preferred pharmacological treatment for sleep disorders (Anderson et al., 2023; Monti et al., 2017).

2.5 Paradoxical Awakening Effect of Zolpidem in Brain Injury and Neurological Conditions

Contrary to its primary function as a sedative-hypnotic agent, ZPM has consistently been observed to induce awakening in patients with various forms of brain injury, including trauma, stroke, and disorders of consciousness (DOC) (Arnts et al., 2020; Bomalaski et al., 2017; Wu & Sun, 2015). This paradoxical awakening extends to the recovery of brain function following conditions such as hypoxic damage, cerebrovascular ischemic injury, CNS infections, toxin exposure, degenerative diseases, tumours, and congenital disorders (Arnts et al., 2024; Georgiopoulos et al., 2010; Hahm & Woo, 2019; Thonnard et al., 2013). In addition to brain injuries, ZPM's awakening effect has been documented across a range of neurological conditions, including movement disorders like Parkinson's disease and dystonia, as well as neurological deficits and anaphylactic hypoxia (Daniele et al., 2016; Miyazaki et al., 2012; Nyakale et al., 2011).

This unexpected arousing effect is often referred to as the "paradoxical action" of ZPM, wherein the drug, instead of exerting its typical sedative-hypnotic effect through $GABA_AR$ modulation, induces wakefulness. The first documented case of ZPM's paradoxical awakening was in a male patient who had suffered an intracerebral haemorrhage following a vehicular accident (Sutton & Clauss, 2017). Despite being in a semi-comatose state, with impaired speech comprehension and poor sphincter control, the patient transiently regained consciousness and was able to greet his mother after receiving a 10 mg dose of ZPM. During this conscious phase, the patient exhibited intact cognitive and memory functions, as well as significantly improved motor control (Sutton & Clauss, 2017).

Another clinical report described three patients who had sustained brain injuries from vehicular accidents or near-drowning incidents. After administration of 10 mg ZPM, all patients demonstrated significant improvements in their Glasgow Coma Scale (GCS) scores, with the arousal effect lasting approximately 4 hours (Clauss & Nel, 2006). Additionally, a 48-year-old male patient with brain injury due to myocardial infarction and a female patient in a comatose state following cardiac arrest from a suicide attempt both showed marked improvements in consciousness, verbal communication, and motor function after receiving 10 mg and 5 mg ZPM, respectively, to alleviate agitation and insomnia (Hahm & Woo, 2019; Kim et al., 2016).

2.6 $GABA_ARs$ as Potential Targets for the Paradoxical Action of Zolpidem

ZPM acts on $GABA_ARs$ by binding to the same site as BZDs, specifically at the interface between the $\alpha(1,2,3)^+/\gamma2^-$ subunits. By binding to this site, ZPM positively modulates receptor function by increasing the frequency of channel opening, which, in turn, enhances Cl^- influx and leads to postsynaptic hyperpolarization (Hanson & Czajkowski, 2008; Sieghart, 2015; Richter et al., 2020). As previously noted, the subunit composition of $GABA_ARs$ is crucial in determining the structure of the binding site, which directly influences the receptor's physiological and pharmacological properties. Dynamic regulation of $GABA_AR$ expression and function occurs primarily through two mechanisms: (1) the assembly of subunits into transport-competent $GABA_ARs$, facilitating their trafficking to the plasma membrane, and (2) the endocytosis-mediated recycling and degradation of these receptors (Braat & Kooy, 2015; Chuang & Reddy, 2018; Ghit et al., 2021; Jacob et al., 2008; Ye et al., 2021). The distinct pharmacological responses observed with ZPM suggest its interaction with potentially different binding sites on $GABA_ARs$.

Previous research has indicated that subunit expression and structural arrangements within $GABA_ARs$ can be altered in response to conditions such as brain injury, trauma, and various

neurological disorders (Clauss, 2004; Cohen & Duong, 2008; Drexel et al., 2015; Fatemi et al., 2013; Kharlamov et al., 2008; Mtchedlishvili et al., 2010; Tossell et al., 2021). For example, ischemic injury has been shown to increase the mRNA and protein expression of the α1 subunit (Kharlamov et al., 2008). Similarly, upregulation of the α2 and ε subunits has been observed in the lateral cerebellum of individuals with psychiatric conditions such as schizophrenia, bipolar disorder, and major depression (Fatemi et al., 2013). Furthermore, Tossell et al. (2021) demonstrated an association between the ε subunit and temporal lobe epilepsy, a finding consistent with earlier *in vitro* work by Che Has et al. (2016), which identified modulation of GABA currents by ε-containing $GABA_A$Rs in *Xenopus* oocytes. Conversely, stroke results in a reduction of β subunit mRNA expression, which houses both GABA binding sites within $GABA_A$Rs (Che Has et al., 2016). Additionally, regional reductions in α1, α2, α3, α5, and γ2 subunits were noted in adult rats with focal cortical malformations induced by neonatal freeze lesions (Redecker et al., 2000). A similar downregulation of α1, α2, α5, β2, β3, γ2, and δ subunits has been observed in a traumatic brain injury (TBI) animal model (Drexel et al., 2015). These findings support earlier research by Mtchedlishvili et al. (2010), which reported reduced sensitivity of synaptic $GABA_A$Rs to BZDs in hippocampal dentate granule cells following TBI, suggesting disruptions at the binding sites for these compounds due to alterations in subunit expression.

In light of these findings, several *in vitro* studies have explored potential alternative binding sites for ZPM on $GABA_A$Rs. In an investigation by Che Has et al. (2016), the effects of various $GABA_A$R subunit arrangements were examined by manipulating the expression ratios of subunits using two-electrode voltage-clamp electrophysiology. ZPM was found to modulate binary $GABA_A$ α1β3 receptors expressed in a 3α1:2β3 stoichiometry, with its effects attenuated by flumazenil (FMZ). This suggests that within the 3α1:2β3 $GABA_A$R, a ZPM binding site exists at the $\alpha1^+/\alpha1^-$ subunit interface, which is analogous to the classical $\alpha1^+/\gamma2^-$ interface.

Notably, since classical BZDs show limited efficacy in improving speech and motor function, this observation raises the possibility of an unusual subtype of $GABA_A$Rs involved in these actions (Chang & Weirich, 2014). Furthermore, Che Has et al. (2016) reported that ZPM was able to modulate the recently characterized ε-containing of $GABA_A$Rs, particularly at lower concentrations, suggesting a dose-dependent response. Importantly, binary αβ receptors, known to be present extrasynaptically, could explain ZPM's ability to amplify tonic GABA currents, potentially influencing its motor effects after severe brain injury (Che Has et al., 2016). Although this specific arrangement has yet to be observed in *in vivo* models, these findings underscore the possibility that alterations in subunit expression, due to brain injury or trauma, could result in the formation of functional $GABA_A$Rs with novel binding interfaces for ZPM. Such changes could potentially explain the paradoxical awakening effect of ZPM in these conditions.

2.7 The ε-containing $GABA_A$Rs

The ε subunit of the $GABA_A$R belongs to a novel gene family first identified in 1997 (Whiting et al., 1997). Its mature mRNA transcript expression appears to be regulated through a neuron-specific RNA splicing mechanism (Whiting et al., 1997). Compared to other subunits, the ε subunit has a rather restricted distribution, with only the ρ and α6 subunits exhibiting an even more limited expression pattern (Whiting et al., 1997). Specifically, the ε subunit is found in distinct brain regions, including the amygdala, hippocampus, locus coeruleus, hypothalamus, and subthalamus (Davies et al., 1997; Whiting et al., 1997). Amino acid homology studies have shown that the ε subunit shares moderate homology with the γ subunits (38–47%) and lower homology with the α subunits (28–30%) as well as other $GABA_A$R subunits (less than 25%) (Bollan et al., 2008; Davies et al., 1997). The ε subunit can replace the γ2 subunit to form ternary αβε receptors (Davies et al., 2001; Saxena & Macdonald, 1994), and it has also been

shown to replace α at position 1 in αβγε receptors (ε–β2–α1–β2–γ2) and β at position 4 in αβε receptors (α1–β2–α1–ε–β2) (Bollan et al., 2008; Davies et al., 2001; Has & Chebib, 2018). In these configurations, the GABA binding site remains conserved, resulting in functional Cl^- channels with distinct biophysical and pharmacological properties (Bollan et al., 2008; Che Has et al., 2020; Davies et al., 1997; Saxena & Macdonald, 1994). Notably, functional binary β3ε $GABA_A$Rs have also been reported (Che Has et al., 2020; Neelands et al., 1999).

Unlike other $GABA_A$R subtypes, ε-containing receptors exhibit rapid desensitization, lower sensitivity to zinc ions (Zn^{2+}), and smaller GABA-mediated current amplitudes (Che Has et al., 2020; Davies et al., 1997; Davies et al., 2001; Neelands et al., 1999; Wagner et al., 2005; Whiting et al., 1997). These receptors have also been characterized as agonist-independent, demonstrating spontaneous channel activity, as well as slower deactivation and recovery kinetics (Neelands et al., 1999; Wagner et al., 2005). Markus et al. (2020) proposed that substituting α1 with a mutated ε subunit could contribute to epileptic phenotypes by altering receptor properties, thereby promoting aberrant neuronal excitability. Additionally, $GABA_A$ α3β1ε receptors exhibit differential responses to BZDs and anaesthetics compared to α3β1 and α3β1γ2 receptors, potentially leading to distinct effects on sedation, stress responses, emotional regulation, and pain perception (Ranna et al., 2006). Furthermore, the ε subunit imparts high sensitivity to neurotransmitters, highlighting its potential as a key site for physiological modulation, especially within extrasynaptic receptors (Ranna et al., 2006). Taken together, the unique pharmacological profile of ε-containing $GABA_A$Rs, in contrast to conventional $GABA_A$Rs, suggests that this subunit may be a critical site responsible for the paradoxical action of ZPM.

2.8 Allosteric Modulation of $GABA_ARs$ by BZD Site Ligands

The anxiolytic, anticonvulsant, myorelaxant, and sedative-hypnotic effects associated with BZD-site ligands are primarily mediated through the allosteric modulation of $GABA_ARs$ at the extracellular α^+/γ^- interface (Hanson et al., 2008; Sancar et al., 2007; Wang et al., 2022). These ligands, in isolation, do not directly induce significant channel opening; rather, they enhance the ability of endogenous agonists like GABA to facilitate pore opening. This modulation occurs without direct activation of the receptor's ion channel, but instead, it sensitizes the receptor to GABA, resulting in increased Cl^- influx upon GABA binding (Goldschen-Ohm, 2022).

When ZPM, as a PAM, binds to the BZD site, it amplifies GABA's inhibitory effects, primarily by enhancing the opening of Cl^- channels. This effect leads to greater membrane hyperpolarization, reinforcing the inhibitory action of GABA and contributing to the overall pharmacological effects of BZD site ligands (Clauss & Nel, 2006; Sancar et al., 2007). This allosteric enhancement of $GABA_AR$ activity is fundamental to the therapeutic actions of these ligands, as well as their potential for producing sedative, anticonvulsant, and anxiolytic outcomes.

However, not all BZD-site ligands act as PAMs like ZPM. Some are classified as null modulators, while others function as negative allosteric modulators (NAMs). The next section will explore one ligand from each category: flumazenil (FMZ), a null modulator, and DMCM, a NAM.

2.8.1 Flumazenil as a Null Modulator of $GABA_ARs$

The imidazobenzodiazepine (i-BZD) FMZ (or Ro 15-1788), chemically known as ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate,

is a high-affinity null modulator of most $GABA_AR$ subtypes (Hood et al., 2014; Siebert et al., 2018; Zhu et al., 2022). As a well-established BZD antagonist, FMZ competitively binds to the BZD binding site but does not elicit intrinsic receptor activity (Bohnen et al., 2023; Pitsikas & Tarantilis, 2020). It is commonly used to reverse BZD tolerance and mitigate postoperative sedation caused by BZD anaesthetics (Bohnen et al., 2023; Hood et al., 2014). Several studies have demonstrated FMZ's ability to block and attenuate the effects of ZPM due to their shared binding specificity at the classical BZD site (Evans & Lowry, 2007; Patat et al., 1994; Zhu et al., 2022). In healthy individuals, concurrent administration of ZPM and FMZ has been shown to decrease GABAergic receptor activity due to FMZ's antagonistic effects (Kim et al., 2016). However, in the context of hypoxic brain injury, the combined use of ZPM and FMZ resulted in an unexpected increase in GABAergic receptor activity, suggesting a modified receptor that is selective for ZPM, potentially inducing a paradoxical arousal effect (Kim et al., 2016).

Additionally, Che Has et al. (2016) identified ZPM as a potent allosteric modulator of the 3α1:2β3 $GABA_AR$ subunit combination, with its modulatory effects being attenuated by FMZ. Further supporting this, ZPM-induced loss-of-righting reflex (LORR) in mice was antagonized by FMZ, a response not observed with diazepam- or midazolam-induced LORR (Wang et al., 2022). Given the characteristics of FMZ when co-applied with ZPM, it is intriguing to explore whether FMZ can also occupy the alternative binding site for ZPM.

2.8.2 DMCM as a Negative Allosteric Modulator of $GABA_ARs$

DMCM, a β-carboline derivative, chemically known as methyl-6,7-dimethoxy-4-ethyl-β-carboline-2-carboxylate, acts as a NAM of $GABA_ARs$ and has been extensively studied for its effects on receptor function. As a NAM, DMCM inhibits GABAergic neurotransmission, leading to cognitive enhancement and learning improvement at low, sub- sedative concentrations (Zhu et al., 2022). The concentration-dependent actions of DMCM are

primarily attributed to its interaction with the ECD of the $GABA_AR$, where it induces a significant conformational change. When DMCM binds to the ECD, it causes a substantial rotation (~15°) of the receptor, shifting it into an inactive, twisted conformation. The bulky, rigid structure of DMCM, along with its close packing with the complementary subunit of the receptor, hinders the necessary contraction of the ECD for channel activation, thus limiting Cl^- conductance. This behaviour defines DMCM as a NAM, as it inhibits the receptor's activation by GABA (Zhu et al., 2022).

Recent studies have further elucidated the binding dynamics of DMCM at the classical BZD binding site, which it shares with other compounds like ZPM and FMZ. However, unlike ZPM, which can enhance receptor activity, DMCM's binding to the receptor stabilizes an inactive state, reducing GABAergic signalling. This negative modulation contrasts with other allosteric modulators and emphasizes DMCM's role in regulating $GABA_AR$ activity in a concentration-dependent manner. Considering that DMCM acts as a NAM, it is intriguing to investigate whether it can maintain its characteristics at a potential alternative binding site, distinct from the common BZD binding site at the α^+/γ^- subunit interface, which is typically occupied by BZD-site ligands like ZPM.

2.9 Advancements in *in silico* Methods: Revolutionizing Drug Discovery and Protein-Ligand Interaction Studies

In recent decades, significant advancements in computational techniques have transformed the study of life sciences, providing unprecedented tools to investigate biological phenomena. In particular, *in silico* methods have become indispensable for testing and validating hypotheses related to the mechanisms underlying cellular functions, disease pathogenesis, and pathophysiology. These computational approaches also play a crucial role in identifying novel drug targets and optimizing drug design (Prieto-Martínez et al., 2019). *In silico* methods

leverage the vast amounts of biological data obtained through high-throughput experimental and analytical techniques, enabling researchers to gain deeper insights into complex biological systems (Prieto-Martínez et al., 2019). By providing a computational framework, these methods enable the simulation and prediction of biological processes that would be challenging or impossible to study through traditional experimental means.

The primary goal of *in silico* models is to complement experimental research. Experimental data is essential for tasks such as the parameterization, calibration, and validation of computational models, ensuring their accuracy and relevance to real-world scenarios (Prieto-Martínez et al., 2019). These models become particularly powerful when they are able to replicate real-world biological phenomena and processes. Their effectiveness largely depends on how well they can align with experimental findings. Furthermore, *in silico* approaches allow for the exploration of interactions and phenomena that are difficult to measure directly in the laboratory, such as the quantification of residual interactions and energy binding events (Prieto-Martínez et al., 2019). This capability is especially useful in drug discovery, where the detailed analysis of molecular interactions can lead to more targeted and efficient therapeutic designs.

In the context of protein-ligand interactions, *in silico* methods such as molecular modelling, molecular docking, and molecular dynamics simulations have proven to be invaluable tools. These techniques are essential for elucidating the binding characteristics of ligands to specific binding sites on target proteins. Molecular docking, for example, can predict the optimal binding poses of ligands, providing insights into their binding affinity and potential for therapeutic efficacy. Molecular dynamics simulations further enhance this by allowing the study of ligand-receptor interactions over time, capturing the dynamic behaviour of the system under physiological conditions. Together, these computational techniques offer a comprehensive understanding of molecular interactions, enabling researchers to identify

promising drug candidates, optimize their structures, and predict their behaviour in biological systems. By combining *in silico* methods with experimental validation, researchers are able to accelerate the drug discovery process and improve the precision of therapeutic interventions. The following sub-sections will explore three computational topics; molecular modelling, molecular docking, and molecular dynamics simulation which highlighting their relevance as key *in silico* techniques for the research presented in this thesis.

2.9.1 Molecular Modelling

With the advent of high-performance computing, virtual experimentation *in silico* has become an invaluable tool, bridging the gap between laboratory experiments and theoretical models. Structure determination techniques, such as X-ray crystallography, NMR, and cryo-EM, have led to a rapid increase in the number of proteins with known 3D structures, which are made publicly available in databases like the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, www.rcsb.org) (Muhammed & Aki-Yalcin, 2019). However, due to variations in protein and receptor subtypes, the 3D structures of less common subtypes are not always available in the RCSB PDB. In these cases, the structures must be constructed using comparative homology modelling (Muhammed & Aki-Yalcin, 2024; Prieto-Martínez et al., 2019).

Homology modelling is widely regarded as the most accurate computational method for predicting a protein's 3D structure from its amino acid sequence (Muhammed & Aki-Yalcin, 2019). In comparative homology modelling, a protein from the RCSB PDB is used as a template to reliably construct a 3D model of a target protein, with minimal deviations from the alpha-carbon of the template, provided the target protein shares at least 50% sequence similarity with the template (Song et al., 2009). For example, most deposited $GABA_AR$

structures in databases are of the α(1/2/5)β(2/3)γ2 subtypes. In these cases, molecular modelling serves as the ideal approach for constructing models of other subtypes, enabling the visualization of their 3D structures. This is particularly useful for analysing and predicting interactions between proteins, or between proteins and ligands (such as hormones and drugs), providing valuable insights at the amino acid and atomic levels (Krause, 2021).

2.9.2 Molecular Docking

Since the development of its first algorithms in the 1980s, molecular docking has become an essential tool in the drug discovery process (Huang & Zou, 2010). In computational drug discovery, molecular docking typically involves interactions between macromolecules and small molecules, such as in protein-ligand docking (Raval & Ganatra, 2022). This technique explores the conformations that ligands adopt within the targeted binding sites of macromolecules, enabling the study of crucial molecular events such as ligand binding modes and the intermolecular interactions that stabilize ligand-receptor complexes (Ferreira et al., 2015; Huang & Zou, 2010; Raval & Ganatra, 2022). Additionally, molecular docking algorithms provide quantitative predictions of binding energetics and rank docked compounds based on the binding affinity of ligand-receptor complexes (Kapetanovic, 2008). This process involves assessing each ligand conformation with specific scoring functions until a solution with minimum energy is achieved (Ferreira et al., 2015; Huang & Zou, 2010). As a result, millions of compounds can be virtually screened in a time- and cost-efficient manner, reducing expenses related to hit identification and increasing the likelihood of discovering promising drug candidates (Pinzi & Rastelli, 2019; Prieto-Martínez et al., 2019).

However, a common limitation of molecular docking is the neglect of target binding site flexibility (Ferreira et al., 2015). During the molecular recognition process, enzymes and

receptors often undergo conformational changes. In some cases, these changes are minimal, allowing the ligand to fit into the binding site with limited mobility. In other cases, proteins may experience more significant conformational rearrangements, impacting their secondary and tertiary structures (Lin, 2011). Techniques such as molecular dynamics simulations can address these flexibility issues by capturing the dynamic behaviour of proteins and providing a more accurate representation of ligand binding in a biologically relevant context.

2.9.3 Molecular Dynamics Simulation

Based on Newton's laws of motion, molecular dynamics (MD) simulation is a computational technique used to predict and calculate the spatial positions and velocities of individual atoms within a system. This allows for the examination of the trajectory and temporal evolution of a ligand-receptor complex (Nichols et al., 2011). The forces acting on each atom are determined by molecular interaction potentials, typically parameterized using quantum chemical calculations or experimental data, collectively known as the force field. Among the various force fields commonly employed, AMBER, CHARMM, and GROMACS are frequently used in MD simulations (Ferreira et al., 2015; Nichols et al., 2011). The significance of MD simulations lies in their ability to capture the dynamic nature of biomolecules, such as proteins and nucleic acids, which is essential for understanding their function. MD provides detailed information about flexibility, fluctuation, and conformational changes at an atomistic level, information that is often difficult to obtain through experimental methods alone (Badar et al., 2022; Hollingsworth & Dror, 2018; Sinha et al., 2022). Moreover, MD simulations enable precise control and replication of *in vitro* and *in vivo* conditions, such as pH, temperature, the presence of water and ions, salt concentrations, and the presence of lipid bilayers and other cellular components (Hollingsworth & Dror, 2018; Sinha et al., 2022). Using MD, phenomena