



***In Silico* Exploration of Endocannabinoid Receptor–CB₁ and CB₂–Interactions
Comparing Cannabidiol and Cannabidiol Diacetate: A Comprehensive Computational
Study**

by

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26 September 2024

DECLARATION

University of the Witwatersrand, Johannesburg

School of **MOLECULAR AND CELL BIOLOGY**

SENATE PLAGIARISM POLICY

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ABSTRACT

In the rapidly evolving field of cannabinoid research, acetylated phytocannabinoids such as cannabidiol diacetate (CBDDA) have shown prominence due to its enhanced effects compared to its natural counterpart, cannabidiol (CBD). Despite the growing popularity in the consumption of acetylated phytocannabinoids, in-depth research on its pharmacological impact, especially on CB₁ and CB₂ receptors, remains scarce. With rising reports of adverse reactions to acetylated phytocannabinoids, a molecular understanding of their interaction with endocannabinoid receptors (CBRs) is imperative. This study aimed to fill this knowledge gap by analysing receptor interactions of CBDDA in comparison with receptor interactions of CBD. The study showed that CBDDA forms stronger interactions with CBRs than CBD. Recognised for its heightened potency, the potential of CBDDA as a biopharmaceutical product was examined. CBR interactions with known endocannabinoids, agonists and inverse agonists validated the computational models used to determine the difference in conformational dynamics upon ligand binding. In this work, bioinformatics, molecular docking, and molecular dynamics (MD) simulations were used to determine the structural differences of CBRs when bound to CBD/CBDDA. Simulations in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and water environment successfully mimicked physiological conditions. Subsequent high-throughput virtual screening (HTVS) was conducted using CBDDA as a reference where ligands 142730975 and 21568811 were identified as the top scoring hits for CB₁ and CB₂ receptors, respectively. The identification of these ligands via HTVS highlights the therapeutic potential of targeting CBRs and the biopharmaceutical potential of CBDDA. This study elucidates the specific interactions of CBD and CBDDA with CB₁ and CB₂ receptors, laying a foundation for assessing the safety and efficacy of acetylated phytocannabinoids. Overall, the differential interaction of CBDDA compared to CBD with CBRs suggests that acetylation changes the conformational dynamics of CBRs thereby potentially affecting signalling. The identification of ligands 142730975 and 21568811 as strong interactors with the receptors may provide valuable leads for the development of new cannabinoid-based therapies.

DEDICATION

"To my cherished family, whose unwavering support have been the bedrock of my perseverance, and to my husband, who has stood by me with an unfailing commitment and encouragement that have fuelled my ambition and soothed my moments of doubt.

All your sacrifices have not gone unnoticed, and your belief in me has been a guiding star on this arduous journey of discovery.

To my cultural heritage and divine intervention, which have imbued me with the resilience and passion to pursue knowledge in the vast and intricate field of Cannabis research, I dedicate this work.

May the outcomes of these endeavours not only push the boundaries of scientific research but also serve as a bridge between the wisdom of tradition and the promise of innovation, paying homage to our roots as we navigate towards future discoveries."

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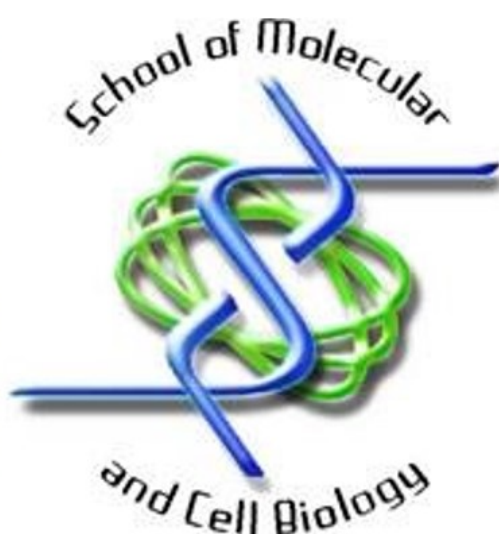
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RESEARCH OUTPUTS

1 ORIGINAL PUBLICATIONS

1.1 Journal Computational Biology and Chemistry

- Title: Exploring the Evolutionary Trajectory and Functional Landscape of Cannabinoid Receptors: A Comprehensive Bioinformatic Analysis
- Published

1.2 Chemistry Africa

- Title: Computationally Characterising the Conformational Dynamics of Pseudo-Endocannabinoid Receptors CB₁ and CB₂ with Cannabidiol
- To be submitted to journal

1.3 Journal of Molecular Graphics and Modelling

- Title: Comparative Analysis Between Natural and Acetylated Phytocannabinoids Interacting with Pseudo-Endocannabinoid Receptors CB₁/CB₂: Insights from Computational Modelling Studies
- Accepted by journal and currently under review

1.4 Journal of Computational Biology and Chemistry

- Title: High-Throughput Virtual Screening of Cannabidiol Diacetate Analogues using Aqueous/Membrane-Partitioned Pseudo-Endocannabinoid CB_{1/2} Receptor Models
- To be submitted to journal

2 CONFERENCES

2.1 6th Annual BioAfrica Convention

- Science Communication Competition by Dr Chris Smith “The Naked Scientist”.
- Winner - The Naked Scientists Fellowship at the University of Cambridge with Dr Smith

2.2 Molecular Biosciences Research Thrust Symposium

- Poster presentation

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LIST OF ABBREVIATIONS AND ACRONYMS

Δ^9 -THC: Δ^9 -Tetrahydrocannabinol

Δ^9 -THCA: Δ^9 -Tetrahydrocannabinolic acid

Δ^9 -THCOA: Δ^9 -Tetrahydrocannabinol-O Acetate

2-AG: 2-Arachidonoylglycerol

2D: Two-dimensional

3D: Three-dimensional

5-HT1A: Serotonin 1A receptor

ADORA2A: Adenosine A2a Receptor

AEA: Anandamide

AMBER: Assisted Model Building with Energy Refinement

B.C.E: Before Common Era

BBB: Blood-Brain Barrier

BLOSUM62: BLOcks SUBstitution Matrix at the 62 % similarity level

C.E.: Common Era

cAMP: Cyclic AMP

CB₁: Endocannabinoid Receptor One

CB₂: Endocannabinoid Receptor Two

CBD: Cannabidiol

CBDA: Cannabidiolic acid

CBDDA: Cannabidiol Diacetate

CBDP: Cannabidiphorol

CBGA: Cannabigerolic acid

CBR: Endocannabinoid Receptor

CCD: Conserved Core Database

CD-Search: Conserved Domain Search

CHARMM: Chemistry at HARvard Molecular Mechanics

Cl⁻: Chloride

CNS: Central Nervous System

COG: Clusters of Orthologous Groups

Cryo-EM: Cryo-Electron Microscopy

C_α: Carbon Backbone

DNA: Deoxyribonucleic Acid

DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

DRD2: Dopamine Receptor D2

ECS: Endocannabinoid System

FAAH: Fatty Acid Amide Hydrolase

FDA: Food and Drug Administration

FDR: False Discovery Rate

GABA: γ -aminobutyric acid

GlgA: Glycogen synthase

Glide: Grid-based Ligand Docking with Energetics

GNAI1: Guanine Nucleotide Binding Protein, Alpha Inhibiting Activity Polypeptide 1

GNB1: Guanine Nucleotide Binding Protein, Beta Polypeptide 1

GNG2: Guanine Nucleotide Binding Protein, Gamma 2

GO: Gene Ontologies

GOT: Geranylpyrophosphate:Olivetolate Geranyltransferase

GPCR: G-Protein Coupled Receptor

GPP: Geranyl-Pyrophosphate

GPR55: G Protein-Coupled Receptor 55

GRK: GPCR kinases

GRK: GPCR Kinases

GROMOS: GRONingen MOlecular Simulation

H: Hydrogen

HIV/AIDS: Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome

HTVS: High-Throughput Virtual Screening

ICL: Intracellular

IFD: Induced-Fit Docking

IPR: InterPro

IQ: Intelligence quotient

JWH: John W. Huffman

Lig-RMSD: Ligand Root-Mean-Square Deviations

MD: Molecular Dynamics

MGLL: Monoacylglycerol Lipase

MM-GBSA: Molecular Mechanics-Generalised Born and Surface Area

MM/PBSA: Molecular Mechanics/Poisson–Boltzmann Surface Area

MM2: Molecular Mechanics 2

Na⁺: Sodium

NADA: *N*-arachidonoyl dopamine

NCBI: National Centre for Biotechnology Information

NK: Natural Killer

NMR: Nuclear Magnetic Resonance

NPS: New Psychoactive Substances

NPT: Isothermal-Isobaric

nr: non-redundant

NVE: Microcanonical Ensemble

NVT: Canonical Ensemble

O: Oxygen

OA: Olivetolic Acid

OH: Hydroxyl

OPLS: Optimised Potentials for Liquid Simulations

p-CBR: Pseudo-endocannabinoid Receptor

PC: Principal Component

PCA: Principal Component Analysis

Pfam: Protein families database

PKR: Protein Kinase Resource

PNS: Peripheral

POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

POPE: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

POPG: 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol

PPI: Protein-Protein Interaction

PTM: Post-Translational Modifications

PTSD: Post-Traumatic Stress Disorder

QM/MM: Quantum Mechanics/Molecular Mechanics

Rap1: Ras-proximate-1

RCSB PDB: Research Collaboratory for Structural Bioinformatics Protein Data Bank

RefSeq: Reference Sequence Database

RMSD: Root-Mean-Square Deviation

RMSF: Root-Mean-Square Fluctuation

RoG: Radius of Gyration

RPS-BLAST: Reverse Position-Specific Basic Local Alignment Search Tool

S1P1: Sphingosine 1-Phosphate Receptor-1

SASA: Solvent Accessible Surface Area

SC: Synthetic Cannabinoids

SDF: Structure-Data File

SP: Standard Precision

THCP: Tetrahydrocannabiphorol

TIGRFAM: The Institute for Genomic Research

TIP3P: Transferable Intermolecular Potential with Three-Points

TM: Transmembrane

U.S.: United States of America

U.V: Ultraviolet

UTR: Untranslated Region

vdW: van der Waals

VSW: Virtual Screening Workflow

XP: Extra Precision

ZC3H12D: Zinc Finger CCCH-Type Containing 12D

AMINO ACIDS TABLE

Amino Acid	1 Letter Symbol	3 Letter Abbreviation
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamic acid	Q	Glu
Glutamine	E	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

KEY TERMS

Cannabinoids: Chemical compounds that interact with the endocannabinoid receptors throughout the body

Endocannabinoids: Cannabinoids naturally produced by the body

Phytocannabinoids: Cannabinoids found naturally in plants

Synthetic Cannabinoids: Cannabinoids that have been synthesised or modified to enhance the effect of natural cannabinoids

INTRODUCTION

1 BACKGROUND

Endocannabinoid receptors (CBRs) are part of the endocannabinoid system (ECS), a complex network of receptors—distributed throughout the human body—that play a pivotal role in regulating various physiological processes. There are two primary CBRs, with CB₁ being predominantly found in the central nervous system (CNS) and CB₂ mainly located in peripheral tissues, specifically those linked to the immune system (Howlett, 2002). The therapeutic potential of targeting these receptors is vast, with implications for pain management, inflammation reduction, and management of neurological disorders. However, the activation of CB₁ by cannabinoids such as *Cannabis* is associated with the psychoactive effects (Mackie, 2006). Delta⁹-tetrahydrocannabinol (Δ^9 -THC), the primary psychoactive compound in *Cannabis*, binds to CB₁ receptors, initiating a signal cascade that can affect cognition, memory, pleasure, and time perception contributing to its potential for abuse. Conversely, CBD, another significant phytocannabinoid found in *Cannabis*, interacts differently with CBRs and is currently studied for its potential therapeutic benefits while avoiding psychoactive effects (Marzo & Petrocellis, 2006; Satterlund et al., 2015).

The binding of cannabinoids to CBRs triggers a series of intracellular (ICL) signals that lead to the diverse effects observed, from therapeutic benefits to psychoactive experiences. Given the broad effects that CBR activation can have, there is a need to study these interactions in detail. Understanding the precise mechanisms by which cannabinoids influence signal cascades and produce varied effects is crucial for the development of new medications that harness the benefits of CBR activation while minimising adverse outcomes (Maurya & Velmurugan, 2018). This knowledge is particularly relevant as *Cannabis* phytocannabinoids and its derivatives emerge in the market and are becoming increasingly accepted and available, demanding a nuanced approach to regulation, medical application, and public health.

2 PROBLEM STATEMENT

Recognising the potential of CBD, there has been a notable surge in its market demand. From oils and edibles to topical creams and lotions infused with CBD, the market in Gauteng and parts of South Africa has seen an increase of these products. Major retail chains like Dischem and Clicks, alongside specialised CBD outlets, have stocked their shelves with these sought-

after products. Catering to individuals with ailments such as chronic pain, insomnia, anxiety, and stress, the impact of the *Cannabis* industry is significant, as evidenced by its estimated value of ~R28 billion industry is in South Africa (Smit, 2021). Parallel to these developments, the *Cannabis* market landscape has been rapidly evolving worldwide.

The emergence of unregulated synthetic and modified cannabinoids in the market presents a growing public health concern, especially when there is a lack of pharmacological data available. With an increasing number of these compounds being synthesised or modified by individuals seeking to exploit gaps in drug regulation, the risks to consumers are escalating (Spaderna et al., 2013). Many of these substances, lacking regulatory oversight, are distributed in the market without adequate pharmacological profiling, leaving users vulnerable to unexpected and potentially dangerous side effects. Two modified compounds, cannabidiol diacetate (CBDDA) and Δ^9 -THC-O acetate (Δ^9 -THCOA) have been detected in the *Cannabis* market in edibles and vapes consumed by individuals who then experienced severe adverse reactions, including seizures, hospitalisation and hallucinations (Holt et al., 2022; Valentine, 1996). Individuals purchasing these products likely have little awareness of the modified cannabinoids they contain and the lack of information on their pharmacological and toxicological profiles only compounds the danger (Bonn-Miller et al., 2017). Therefore, highlighting the need to study acetylated phytocannabinoids as there is currently no pharmacological data available (Holt et al., 2022).

There is further anecdotal evidence to suggest that Δ^9 -THCOA exhibits heightened effects and adverse effects, raising concerns about the broader implications of phytocannabinoid modification. (Assi et al., 2020; Benowitz et al., 2023). The presence of both Δ^9 -THCOA and CBDDA in edibles without any available data on how it interacts with CBRs is particularly alarming (Holt et al., 2022). It indicates a need for comprehensive research on modified cannabinoids such as CBDDA which is currently circulating in unregulated markets. The challenge lies in responding rapidly to the proliferation of these substances with thorough scientific investigation and policy development. It is imperative to elucidate the effects of modified phytocannabinoids on the CBRs and to establish regulatory frameworks that can keep pace with the rapid innovation and distribution of these compounds.

3 RATIONALE

With the rapid spread of acetylated phytocannabinoids in consumer products, particularly those like CBDDA and Δ^9 -THCOA, it becomes critically important to understand their pharmacological effects and interactions with receptors. Natural CBD has attracted substantial attention for its therapeutic benefits without the psychoactive impacts of Δ^9 -THC. Nonetheless, modifying CBD into acetylated forms such as CBDDA may change how it affects CBRs, possibly introducing psychoactive effects and raising safety concerns. CBD is generally regarded as non-psychoactive and safer than Δ^9 -THC as it does not have adverse health effects such as psychosis, paranoia, and a decrease in intelligence quotient (IQ) (Maurya & Velmurugan, 2018). Therefore, the legal status of CBD, which differs internationally, allows for the use and commercialisation of CBD in certain countries such as South Africa and some states in the United States of America (U.S.) (Child, 2018). However, due to the lack of data, mislabelling of products and shortage of *Cannabis* education within this industry, Consumers might unwittingly use modified phytocannabinoids without understanding the potential changes in physiological effects.

The lack of regulation and awareness regarding *Cannabis* compounds exacerbates the issue. Therefore, pharmacological research is imperative to inform and assist lawmakers in promptly regulating modified phytocannabinoids by rapidly acquiring data on the interaction of these novel compounds with CBRs. Therefore, conducting computational studies to investigate molecules like CBDDA compared to its natural counterpart—CBD—provides a swift and economically feasible means to ascertain their binding affinities and difference in activity at CBRs (Willems et al., 2020). Such studies can offer preliminary insights into receptor-ligand indications and conformational dynamics of CBRs when bound to these compounds, which is paramount given the lag in legislative response to their unregulated distribution in the *Cannabis* market. The rationale for this study is rooted in the need to pre-emptively determine the effects of acetylated phytocannabinoids on CBRs to address the potential health risks of modified phytocannabinoids and to inform policy makers, healthcare providers, and consumers about their effects, ensuring public safety amidst the evolving landscape of cannabinoid use.

4 AIMS AND OBJECTIVES

4.1 Aim One

To evaluate the evolutionary trajectory and functional landscape of CBRs.

4.1.1 Objective One

Identify conserved domains and residues within the receptor family and their variation across species.

4.1.2 Objective Two

Characterise the structure and function of CBRs and pseudo-CBRs (p-CBRs).

4.1.3 Objective Three

Identify and collate biological and molecular pathways associated with CBRs.

4.2 Aim Two

To computationally characterise the conformational dynamics of p-CBRs with CBD.

4.2.1 Objective One

Perform induced-fit ligand docking (IFD) with known ligands and CBD.

4.2.2 Objective Two

Conduct MD simulations and subsequent post-MD analysis using the top scoring conformations from IFD to determine the structural dynamics of p-CBRs upon ligand binding for 250 ns.

4.3 Aim Three

To elucidate the change in conformational dynamics of CBRs when bound to natural versus acetylated phytocannabinoids using a lipid-bilayer surrounded by an aqueous solution system.

4.3.1 Objective One

Determine the variations in binding affinity using IFD to compare natural phytocannabinoids and their acetylated derivatives.

4.3.2 Objective Two

Perform MD simulation analysis to determine the dynamics of the receptor-ligand complex upon ligand binding over a 500 ns simulation.

4.4 Aim Four

To identify compounds that are structurally similar to CBDDA and to determine changes in CBR dynamics in comparison to CBDDA.

4.4.1 Objective One

Perform high-throughput virtual screening HTVS with standard precision (SP) and extra precision (XP) steps to identify the energetically optimal conformation and compound using Molecular Mechanics energies combined with Generalised Born and Surface Area solvation (MM-GBSA) scores.

4.4.2 Objective Two

Run MD simulations and subsequent analyses on the highest-scoring compounds for both CBRs in comparison to CBDDA.

5 NOVELTY OF THE STUDY

The innovation of this study lies in its comparative analysis of CBR interaction differences between natural cannabinoids and its acetylated derivative, focusing specifically on CBD versus CBDDA. This research incorporates multiple innovative elements, using bioinformatics tools, IFD, MD simulations, and HTVS. These methodologies were essential in providing a dynamic and detailed view of the molecular interactions at play when ligands bind to CBRs. HTVS expands the potential to screen a vast number of compounds efficiently by examining CBDDA structures as it has biopharmaceutical potential by targeting CBRs. Together, these methodologies and simulations are critical for predicting how emerging cannabinoid compounds might interact with CBRs, thereby allowing informed regulations to be made and opening new avenues for *Cannabis* research and its therapeutic potential.

The study began with a thorough bioinformatics approach, extracting extensive data and analysing evolutionary information related to specific functional domains across species. It also compared these findings between natural CBRs and p-CBRs. This analysis provided insights

into the protein-protein interactions (PPIs), biological pathways, and functional domains related to CBRs. The collected evolutionary data enhanced understanding of how these receptors have changed over time and how they physiologically function, creating a comprehensive foundation for future research into CBR-cannabinoid-ligand interactions.

Next the p-CBRs were validated by examining the binding interactions of the p-CBR models with known agonists, inverse agonists, CBD, and *N*-arachidonoyl dopamine (NADA—an endogenous cannabinoid). This establishes a foundation for using p-CBRs to examine cannabinoids and investigate their conformational dynamics. This advancement in computational modelling significantly deepens our grasp of CBR interactions at a molecular scale. Building on this, the study deepened its investigation into how acetylated phytocannabinoids affect the structural dynamics of CBRs compared to their natural counterparts. It compared the interactions between CBRs with Δ^9 -THC and its acetylated version, Δ^9 -THCOA, as well as CBD with its acetylated form, CBDDA. The study monitored changes in the root-mean-square deviation (RMSD) of the carbon backbone (C_α), C_α root-mean-square-fluctuation (C_α RMSF), radius of gyration (RoG), and ligand RMSD (lig-RMSD). These metrics indicate conformational changes in the protein upon ligand binding that could signal that the chemical alterations of natural phytocannabinoids might lead to different biological effects, as some anecdotal evidence suggests. These methods and simulations play an essential role in predicting the interaction between new cannabinoid compounds and CBRs, thus facilitating the establishment of informed regulations and policies. They also pave the way for advancing *Cannabis* research and exploring its therapeutic possibilities.

Lastly, the study used HTVS to identify compounds that target the ECS but are not derived from *Cannabis*, addressing the complex legal issues surrounding CBD and its analogues. HTVS identified structures similar to CBDDA with potential for medical use while complying with legal restrictions related to *Cannabis*-based substances. This approach harmonises scientific research with regulatory guidelines, allowing for the exploration of new therapeutic options without legal barriers.

6 OVERVIEW OF THE THESIS

This PhD thesis offers a comprehensive *in silico* analysis of the conformational dynamics of CBRs when bound to natural versus acetylated phytocannabinoids within a lipid bilayer and solvation model. The study addresses significant gaps in our understanding of CBRs, particularly in the context of emerging acetylated phytocannabinoid derivatives, mainly CBDDA. Chapter one outlines the project, defining the central issue and the objectives of the study, and provides a roadmap for the thesis. Chapter two presents an extensive literature review, tracing the evolution of the *Cannabis* industry with an emphasis on regulations, phytocannabinoids and synthetic cannabinoids. Additionally, the chapter discusses the principles behind the molecular mechanics of the computational methods employed in the study and justifying their selection.

Next, chapter three delves into the bioinformatics of CBRs, exploring their conserved functional domains and contrasting natural receptors with p-CBRs. It also elaborates on the role of the ECS in physiological biological functions. This chapter has been published by the “Journal Computational Biology and Chemistry” (Appendix A, Fig. A1). In Chapter four, computational methods rationalise the conformational dynamics of p-CBRs in relation to CBD, known agonists, inverse agonists, and NADA, a paper will be submitted to “Chemistry Africa” (Appendix A, Fig. A2). Chapter five addresses the core research question, analysing the change in conformational dynamics of CBRs when interacting with natural versus acetylated phytocannabinoids. This work has been accepted for review by the “Journal of Molecular Graphics and Modelling” (Fig. A3). Chapter six introduces the HTVS component, identifying compounds structurally similar to CBDDA and examining dynamic changes relative to CBDDA. It utilises HTVS with standard and extra precision steps, applying MM-GBSA scores to find the energetically optimal conformations and compounds, an article will be submitted to the “Journal of Computational Biology and Chemistry”.

Finally, chapter seven synthesises the findings, drawing conclusions from the outcomes and proposing directions for future research and empirical studies. This thesis concludes with a comprehensive list of references and an appendix providing supplementary information.

LITERATURE REVIEW

1 HISTORY OF *CANNABIS*

1.1 Origins and Initial Domestication

The *Cannabis* plant, classified under the family Cannabaceae, has two primary species named *Cannabis sativa* and *Cannabis indica*, with a third, lesser known, *Cannabis ruderalis* (McPartland, 2018). This genus, originating from the fertile regions of Central Asia (Fig. 2.1), is speculated to have first been domesticated in regions now known as Mongolia and southern Siberia around 12,000 Before Common Era (BCE) (Li, 1974; Pierro Neto et al., 2023). Early humans valued *Cannabis* not for its psychoactive effects, but for its practical benefits—the strong fibres of its stalks were used for making textiles, and its seeds were consumed for nutrition (Kriese et al., 2004). These versatile plants were then transported along ancient trade routes, reaching, and integrating into the diverse cultural tapestry of South Asia. The reputed Chinese emperor Shen Nong is believed to have documented the medical uses of *Cannabis* around 2737 BCE in the “Pen Ts'ao Ching”—a Chinese materia medica book (Touw, 1981). Similarly, in the Indian subcontinent, Hindu scriptures called the Atharvaveda referred to *Cannabis* as one of the “five sacred plants,” thereby integrating it into religious and spiritual rites (Courtwright, 2001). During Classical Antiquity, from the 5th – 3rd centuries BCE, *Cannabis* was known among the learned Greeks and Romans (Sumler, 2018). A notable Greek physician, Dioscorides, mentioned *Cannabis* in his medical texts as a remedy for various health issues (Dioscorides, 1 CE). The sprawling Roman Empire further aided the spread of *Cannabis* throughout Europe, leading to its adoption in different regional customs and practices (Sumler, 2018).

During the Middle Ages and Renaissance, Islamic laws strictly forbade any form of intoxication (Robins, 2016). Nevertheless, the medicinal value of *Cannabis* was recognised and persisted through these times. Avicenna (Ibn Sina), a philosopher and physician of the 11th century, praised the healing properties of *Cannabis* in his medical text, the “Canon of Medicine” (Mahdizadeh et al., 2015). At the same time in Europe, there was a surge in scholarly attention to plants for medicinal purposes. Scholars meticulously examined *Cannabis*, documenting its characteristics in comprehensive writings. As European countries expanded their territories from the 15th to the early 20th centuries, the strong fibres of hemp became highly valued by

continents, it typically merged with local customs without formal bans. However, within Islamic empires, religious rules were implemented against *Cannabis* use (Robins, 2016). This resulted in a complex legal stance where some regions practised tolerance, and others implemented strict prohibitions at various times. During the Age of Discovery, European colonists introduced hemp to the New World, valuing it for its sturdy fibres (Fike, 2016). Laws were enacted not to restrict, but to encourage its cultivation; in Jamestown Colony, Virginia, in 1619, a mandate required settlers to grow Indian hemp seed (Browne-Marshall, 2020). These legislative efforts were aimed at supporting an industry rather than controlling substance abuse. In the 19th century, as medical research into *Cannabis* began to flourish, there were still no laws against its therapeutic use as figures like William Brooke O'Shaughnessy advocated for its medical application, thus reinforcing its legitimacy (MacGillivray, 2017).

The early 20th century witnessed a significant shift in the legal landscape of *Cannabis* in the U.S. with the Marijuana Tax Act of 1937, which imposed strict regulations on the possession and sale of *Cannabis* and effectively initiated the war on drugs (Anslinger & Cooper, 1937; Bonnie & Whitebread, 1970). This act served to criminalise *Cannabis*, thereby setting an international benchmark for drug legislation. The controversial perceptions surrounding *Cannabis* and its derivative products have deep historical and socio-political roots (Carrier & Klantschnig, 2012). Multiple factors played a role in this. For one, the Mexican Revolution of 1910 led to an influx of Mexican immigrants into the U.S., many of whom used *Cannabis* for recreational purposes (Campos, 2012). The unfamiliar custom of smoking *Cannabis* was subsequently linked to migrants, creating xenophobic overtones and associations with deviant behaviour (Bonnie & Whitebread, 1970). Simultaneously, media portrayal played a crucial role in escalating these fears. With the propagation of propaganda films like "Reefer Madness" (1936), *Cannabis* became synonymous with criminality, insanity, and societal decay (Steiner et al., 2019). Additionally, Harry J. Anslinger, played a pivotal role in the inception of this legislation and championed the vilification of *Cannabis*, often using racial undertones in his arguments (Anslinger & Cooper, 1937; McWilliams, 1990). The international implications of this stance became evident in 1961 when the Single Convention on Narcotic Drugs was established. Spearheaded by the U.S., this treaty classified *Cannabis* alongside potent narcotics, urging member countries to adopt stringent regulations (Boister, 2021). Many nations acceded to these recommendations, aligning their national policies to this international directive. Additionally, In the mid-20th century, the popularity of *Cannabis* as a recreational drug burgeoned amidst the 1960s counterculture movements by groups known as "hippies", leading

to stricter laws (Wesson, 2011). The U.S. Controlled Substances Act of 1970 Classified *Cannabis* as a Schedule I drug, attributing to it a high potential for abuse and no recognised medicinal value, reinforcing the prohibitive stance (Gabay, 2013).

The close of the 20th century and dawn of the 21st brought about a renewed understanding and appreciation for the medicinal value of *Cannabis*, due to researchers such as Organic Chemist Dr Raphael Mechoulam, the “Father of *Cannabis* Research”. He aided in the discovery of the structure of CBD and pioneered the work in the 1960s that led to the detection of delta⁹-tetrahydrocannabinol (Δ^9 -THC), the primary psychoactive compound in *Cannabis*, and later the endocannabinoid system (ECS), which plays a crucial role in human physiology (Devane et al., 1988; Gaoni & Mechoulam, 1964; Mechoulam & Shvo, 1963). This advancement in *Cannabis* research began to shed light on the potential therapeutic applications of *Cannabis* and its components, challenging the prevailing views that *Cannabis* was solely a substance of abuse. This growing body of evidence laid the foundation for a re-evaluation of *Cannabis* laws. As the medical benefits became more widely understood and accepted, many countries started to reform their *Cannabis* policies, easing legal restrictions and, in some cases, allowing for its use and research, particularly for medical reasons (Sciences et al., 2017). This shift was further supported by changing public opinion and advocacy for patients' rights to access *Cannabis* for medical conditions.

1.3 South Africa's Stride Toward Liberation

In South Africa, *Cannabis*, locally known as "dagga," has been intertwined with the culture for centuries but faced prohibition under the 1922 Dangerous Drugs Act (Chattopadhyaya, 2019; Nkosi et al., 2020). This prohibition continued under the 1971 Abuse of Dependence-producing Substances and Rehabilitation Centres Act (Waetjen, 2022). The landscape shifted dramatically when, in 2018, Constitutional Court of South Africa ruled that the private use and cultivation of *Cannabis* by adults was no longer a criminal offence, thereby decriminalising it for private use (Child, 2018). The law now reflects a society moving towards embracing the potential of the plant, with regulations being developed for its commercial use and trade.

The legislative trajectory on *Cannabis* in South Africa reflects both indigenous customs and external influences. Historically, the use of *Cannabis* was entrenched in various South African cultures, with both recreational and medicinal applications (Du Toit, 1975). However, during the 20th century, as global sentiment shifted towards criminalisation, South Africa also

followed (Chattopadhyaya, 2019; Waetjen, 2022). The Abuse of Dependence-producing Substances and Rehabilitation Centres Act of 1971 criminalised the possession and use of dagga. This was exacerbated during the apartheid era, where drug laws, including those on dagga, were often employed as tools of social control, disproportionately targeting Black and Coloured communities (Waetjen, 2022).

However, the dawn of the 21st century brought about a notable shift. In 2018, the Constitutional Court of South Africa decriminalised the private use and cultivation of *Cannabis*, acknowledging the cultural significance and the human rights aspects intertwined with its use (Child, 2018; Mia, 2020). The judgement symbolised not just a legal recalibration but a nuanced understanding of *Cannabis* within the socio-cultural framework of South Africa. The recent relaxation of laws around *Cannabis*, influenced by global recognition of its potential therapeutic benefits, has opened avenues for scientific study and research. This legislative change facilitates investigations into the therapeutic efficacy of *Cannabis*. It permits the rigorous exploration of its medical applications, advancing our understanding of how *Cannabis* can be used to treat various health conditions. This research is essential not only for developing *Cannabis*-based medications but also for informing policy, regulations, guiding clinical practice, and ensuring that the use of *Cannabis* in treatment is both safe and effective.

2 THE CANNABIS PLANT

2.1 Chemical Compounds in *Cannabis*

The *Cannabis* genus is a biochemical powerhouse, synthesising a spectrum of compounds that contribute to its adaptability and utility. These compounds—characterised as cannabinoids, terpenoids, terpenes, flavonoids, nitriles, and other plant compounds—have over 545 distinct compounds identified (ElSohly & Gul, 2014; Lowe et al., 2021). Cannabinoids are of particular interest, with approximately 104 varieties discovered thus far (ElSohly & Gul, 2014). Among these, terpenes such as myrcene, limonene, and pinene each contribute unique aromas to the plant—myrcene with its earthy undertones, limonene with a burst of citrus, and pinene with a refreshing pine scent. These terpenes do more than engage the senses as they can enhance the therapeutic effects of the plant—a phenomenon known as the entourage effect—in full-spectrum *Cannabis* oils (Russo, 2011). Moreover, the plant is abundant in flavonoids like cannflavins, which provide vibrant colours and defend against environmental stressors while attracting pollinators (Shomali et al., 2022). Beyond their aesthetic contribution, these

flavonoids are recognised for their antioxidant and anti-inflammatory properties, which are of increasing interest in pharmacological research (Shomali et al., 2022).

Popular phytocannabinoids, Δ^9 -THC and CBD stand out for their unique and contrasting effects on the human body. Δ^9 -THC, the primary psychoactive ingredient, is the compound responsible for altering perceptions and mood (Marzo & Petrocellis, 2006; Satterlund et al., 2015). Its interaction with CBRs in the brain disrupts normal neural communication, affecting cognitive functions and emotional states. This psychoactivity was first elucidated by Dr. Raphael Mechoulam and Dr. Yechiel Gaoni in the 1960s, who isolated and synthesised Δ^9 -THC from Lebanese hashish (Gaoni & Mechoulam, 1964). Initially, Adams and colleagues embarked on an effort to elucidate the structure of Δ^9 -THC, but their attempts did not yield success (Adams, Pease, et al., 1940). Subsequently, another team led by Wollner also attempted to determine the structure but faced similar challenges (Wollner et al., 1942). Ultimately, it was Mechoulam and his team who succeeded in identifying and clarifying the structure of Δ^9 -THC. Δ^9 -THC was found to feature a closed cyclic ring with a pentyl chain (Fig. 2.2) (Gaoni & Mechoulam, 1964). On the other hand, CBD offers a contrasting profile. Non-intoxicating in nature, CBD was first isolated in 1940 by Roger Adams and his team through a rigorous extraction process (Adams, Hunt, & Clark, 1940; Adams, Pease, et al., 1940). Yet, its structure remained elusive until the subsequent work of Mechoulam's team, which utilised nuclear magnetic resonance (NMR) imaging to reveal the stereochemistry of CBD which identified the open benzene ring (Fig. 2.2), thereby unlocking the secret to its non-psychoactive yet therapeutically promising attributes (Mechoulam & Shvo, 1963). Unlike Δ^9 -THC, CBD does not bind to CBRs in the same way, explaining its lack of intoxicating effects and its potential in treating a range of conditions, from inflammation and pain to anxiety and seizures. Additionally, CBD influences other receptors, such as serotonin and vanilloid receptors, contributing to its vast and varied therapeutic potential (White, 2019).

The discovery of the exact structures of Δ^9 -THC and CBD, through advanced imaging techniques such as NMR spectroscopy and crystallography, have had profound implications. They paved the way for the synthesis of phytocannabinoids, allowing for controlled scientific studies, and further research into the human ECS (Devane et al., 1988; Herkenham et al., 1990). The distinction between the psychoactivity Δ^9 -THC and the therapeutic potential of CBD continues to shape the legal landscape of *Cannabis*. For instance, in regions where *Cannabis* remains illegal, CBD products might be permitted if they contain minimal levels of Δ^9 -THC,

particularly in contexts where medicinal use of *Cannabis* has gained legal acceptance (Mead, 2017). Furthermore, South Africa's amendment of the Medicines and Related Substances Act in 2017 to permit the prescription of medicinal *Cannabis* reflects the global shift towards embracing the therapeutic aspects of cannabinoids, influenced by the growing body of scientific evidence (Child, 2018). This evolving legal framework, recognising the delineation between the effects of Δ^9 -THC and CBD, highlights a societal move towards a nuanced understanding of *Cannabis*, balancing regulatory considerations with the need for therapeutic access.

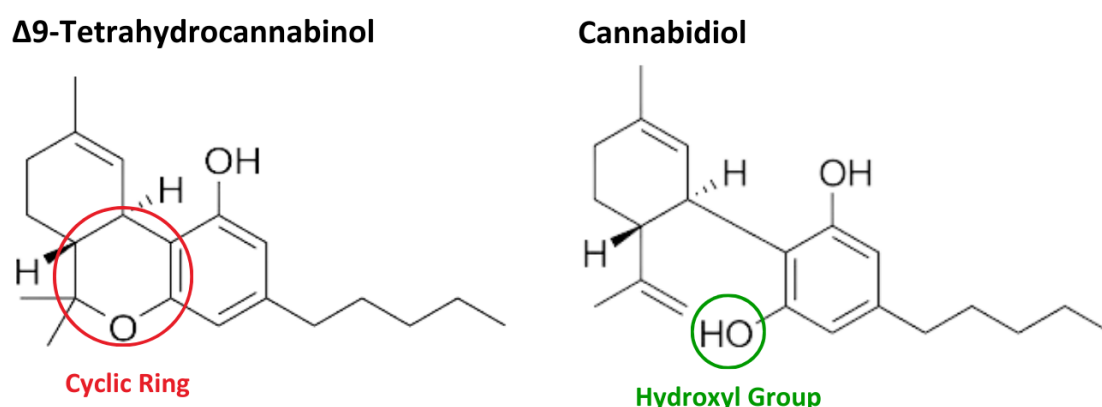
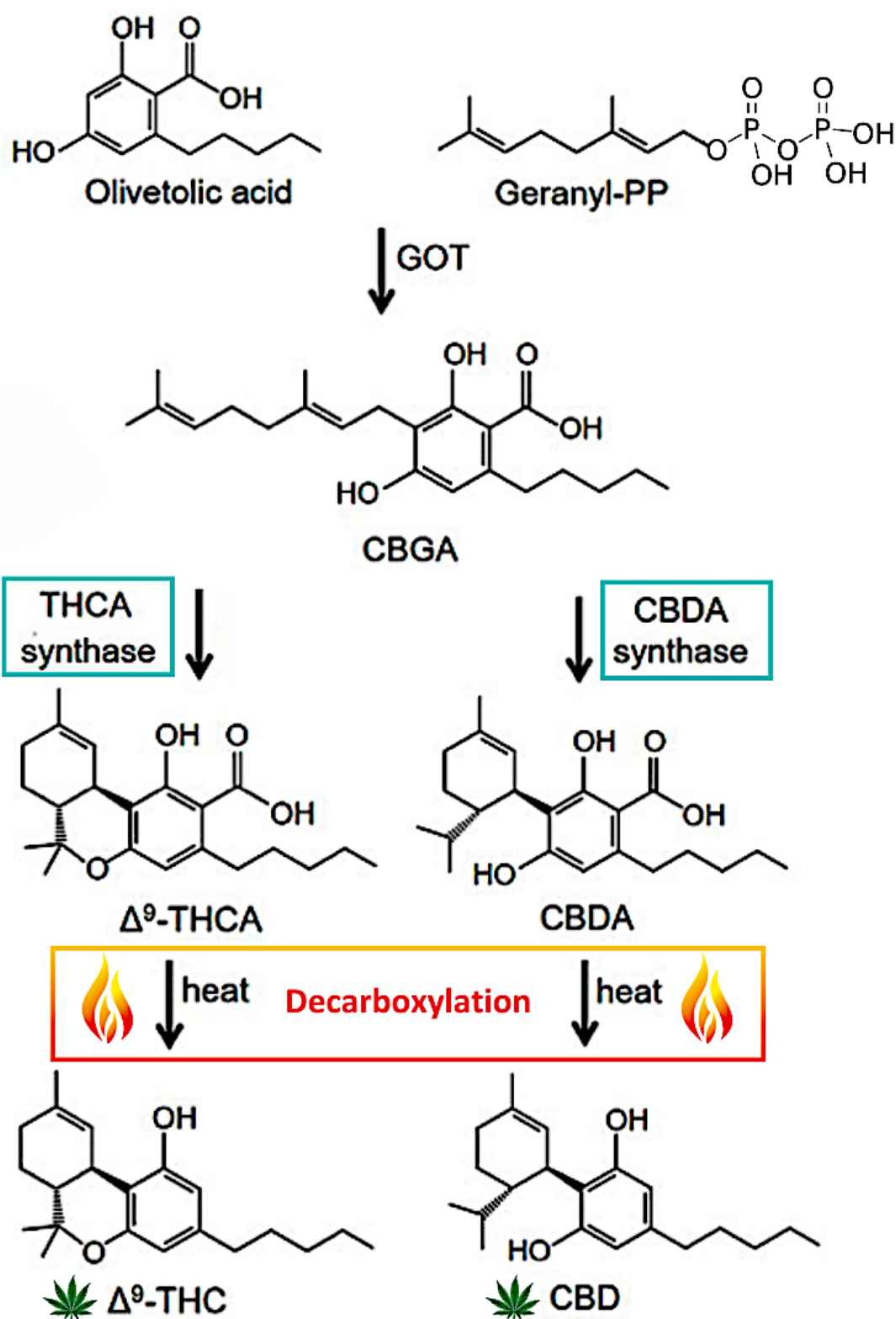


Figure 2.2: Chemical structures of Δ^9 -tetrahydrocannabinol and cannabidiol differences. highlighting the cyclic ring in Δ^9 -THC (red) and hydroxyl group in CBD (green) that opens the cyclic ring that is present in Δ^9 -THC. These molecular differences contribute to their distinct pharmacological properties. Created using ChemDrawJS and Canva.

2.2 Biosynthesis of Phytocannabinoids

The biosynthesis of phytocannabinoids within the *Cannabis* plant exemplifies a complex interplay of organic chemistry and plant biology. This process, initiating from basic molecular building blocks, leads to the synthesis of an array of pharmacologically active compounds (Gülck & Møller, 2020). At the core of this biosynthetic pathway (Scheme. 2.1) lies the precursor molecule geranyl pyrophosphate (GPP), a fundamental building block for a vast array of plant terpenoids and essential oils. GPP engages in a chemical partnership with olivetolic acid (OA), catalysed by the enzyme geranyl pyrophosphate: olivetolate geranyl transferase (GOT) (Walsh & McKinney, 2021). This enzymatic action is highly precise, ensuring the accurate assembly of these molecules to form cannabigerolic acid (CBGA), the so-called "Mother of all Phytocannabinoids," serving as the scaffold from which all other phytocannabinoids are derived (Hanuš et al., 2016).

From this pivotal point, CBGA is met with distinct enzymes (Scheme. 2.1) that direct it towards specific cannabinoid acids. Δ^9 -Tetrahydrocannabinolic acid (THCA) synthase, for example, converts CBGA into Δ^9 -THCA, the non-psychoactive precursor of Δ^9 -THC. Parallel to this, cannabidiolic acid (CBDA) synthase transforms CBGA into CBDA, leading to the synthesis of CBD upon decarboxylation. The specificity of THCA and CBDA synthases is significant, with each enzyme influencing the final phytocannabinoid composition found within a given *Cannabis* strain (De Meijer et al., 2003). This enzymatic specificity is a primary factor in determining the chemotype of the plant, which can be selectively bred for specific therapeutic or recreational properties. The decarboxylation process, essential for activating these phytocannabinoids, involves the removal of a carboxyl group from their acid forms, a reaction typically induced by exposure to heat or ultraviolet (UV) light (Scheme. 2.1) (Walsh & McKinney, 2021). It is during this chemical reaction that THCA is converted into the psychoactive Δ^9 -THC, and CBDA into the therapeutically valued CBD. These processes are fundamental to the life cycle of the plant and to the utility of the compounds they produce.



Scheme 2.1: Biosynthetic pathway of CBD and Δ^9 -THC. The alkylation of olivetolic acid with geranyl-pyrophosphate (GPP) produces cannabigerolic acid (CBGA) using geranyl transferase:olivetolate geranyltransferase (GOT). Subsequently, CBGA is converted to both cannabidiolic acid (CBDA) and Δ^9 -tetrahydrocannabinolic acid (THCA) by CBDA synthase and THCA synthase, respectively. Heat-induced

decarboxylation then transforms CBDA and Δ^9 -THCA into CBD and Δ^9 -THC, respectively. Adapted from (Walsh & McKinney, 2021) using Canva.

The pathway to Δ^9 -THC and CBD biosynthesis is highly sensitive to both intrinsic and extrinsic factors. Environmentally, factors such as light exposure, particularly UV-B radiation, are known to increase Δ^9 -THC concentrations (Gülck & Møller, 2020). CBD levels can be influenced by the developmental stages of the plant and by external stressors such as drought or physical damage to the plant, which can prompt a shift in the production of phytocannabinoids. Genetically, the deoxyribonucleic acid (DNA) of the plant contains the information to produce the synthase enzymes that guide the production of specific phytocannabinoids. Different cultivars of *Cannabis* have different proportions of these enzymes, leading to the characteristic phytocannabinoid profile of each cultivar. This can be seen throughout the different species of *Cannabis* as each of these species has been utilised for different purposes based on their unique characteristics (McPartland, 2018).

Cannabis sativa, with its characteristic long, narrow leaves, thrives in equatorial climates and has been historically valued for its fibres and seeds (Fig. 2.3). The fibres are strong and durable, making them ideal for textiles and ropes, while the seeds are rich in oil and protein, beneficial for nutrition (Pierro Neto et al., 2023). *Sativa* strains typically have higher levels of Δ^9 -THC compared to CBD, contributing to their energising effects that may enhance creativity and focus along with its psychoactive effects (ElSohly & Gul, 2014). This makes them favourable for daytime use, both medicinally and recreationally. *Cannabis indica*, in contrast, has broad leaves and a compact stature, originating from the Hindu Kush mountain region (Fig. 2.3) (De Meijer et al., 2003). It is renowned for its higher CBD content relative to Δ^9 -THC, which is associated with its more sedative effects, offering relaxation and aiding sleep. This makes the *indica* species particularly sought after for evening medicinal use, providing relief for conditions like pain, anxiety, and insomnia. *Cannabis ruderalis* is smaller and hardier, suited to the more challenging climates of Central and Eastern Europe and Russia (Fig. 2.3). *Cannabis ruderalis* plants are generally lower in Δ^9 -THC but are prized for their auto-flowering capability, which is a valuable trait in breeding programs to produce new hybrid strains. The varying ratios of Δ^9 -THC and CBD in these *Cannabis* species influence their distinct effects (McPartland, 2018) as Δ^9 -THC causes the “high” associated with *Cannabis* use, while CBD is non-psychoactive and is being studied for a variety of therapeutic effects, including anti-inflammatory and anxiolytic properties. These phytocannabinoids interact with the ECS in humans, which regulates a variety of functions, from pain sensation to mood and appetite (Rezende et al., 2023).

The elucidation of these biosynthetic routes offer invaluable insights into how the *Cannabis* plant produces such a diverse and potent group of chemical compounds. It allows for targeted manipulation of phytocannabinoid profiles, enhancing specific traits desired for medical efficacy or user experience in full spectrum oils (De Meijer et al., 2003). Moreover, the understanding of phytocannabinoid biosynthesis is not only of botanical or biotechnological interest but also crucial for advancements in medicinal chemistry. As these pathways are explored further, novel approaches can be developed to harness the therapeutic properties of these phytocannabinoids. This includes selective breeding techniques, genetic modification, or even synthetic biology to optimise the production of specific phytocannabinoids like Δ^9 -THC and CBD, which have shown potential in treating an array of medical conditions (Mackie, 2006).

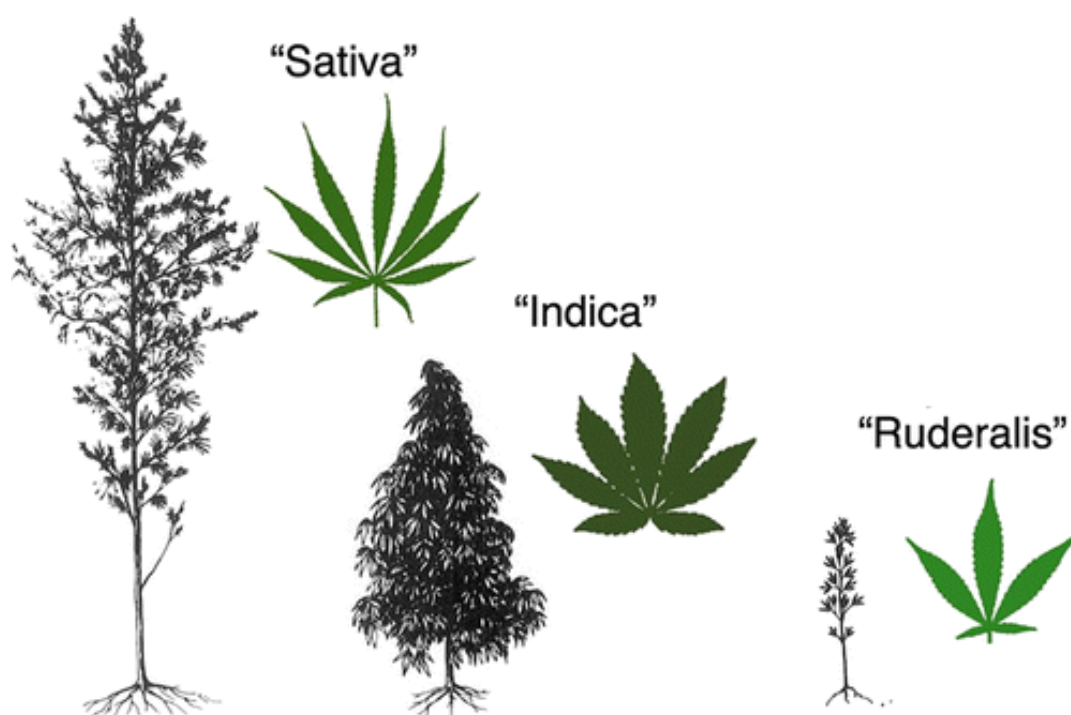


Figure 2.3: *Cannabis* species plant varieties. "Sativa," characterised by a tall, slender profile; "Indica," noted for its shorter and bushier appearance; and "Ruderalis," distinguished by its small stature and thin leaves. Each variety is represented alongside its typical leaf morphology. Sourced from (McPartland, 2018)

3 THERAPEUTIC EFFECTS OF PHYTOCANNABINOIDS

3.1 Δ^9 -Tetrahydrocannabinol Therapeutics

The compound Δ^9 -tetrahydrocannabinol is recognised as the main psychoactive component of the *Cannabis* plant with significant therapeutic interest. It operates primarily by interacting with

the ECS in the body, which includes a network of CBRs that regulate various physiological processes (Sciences et al., 2017). The binding of Δ^9 -THC to these receptors is fundamental to its ability to influence these bodily functions, thereby contributing to its therapeutic benefits. By engaging with this intricate system of receptors thereby engaging signalling molecules, Δ^9 -THC can alter physiological states, offering potential benefits in the management of conditions characterised by pain, mood disorders, appetite loss, and cognitive impairment (Leinen et al., 2023; Mackie, 2006).

One of the most validated medical attributes of Δ^9 -THC are its analgesic—or pain-relieving—properties. Through various clinical trials, Δ^9 -THC has demonstrated a significant reduction in different types of pain, notably neuropathic pain, which frequently proves resistant to standard pain medications (Slomski, 2018). The mechanism behind pain-relieving capability of Δ^9 -THC involves the activation of CB₁ receptors in the CNS, which results in the suppression of neurotransmitter release and, consequently, the attenuation of pain signals sent to the brain (Slomski, 2018). The role of Δ^9 -THC in pain management is multifaceted. It modulates pain perception not just by binding to receptors in the brain, but also by engaging with receptors in the peripheral nervous system (PNS) (Manzanares et al., 2006). This dual action is particularly beneficial for those suffering from chronic and neuropathic pain, which are notoriously challenging to treat with conventional pain relief methods. By diminishing pain intensity, Δ^9 -THC has the potential to enhance the overall quality of life for those affected by persistent pain conditions.

The antiemetic properties of Δ^9 -THC, which are highly valuable in mitigating nausea and vomiting, particularly when these symptoms are a consequence of chemotherapy treatments (Leinen et al., 2023; Sciences et al., 2017). By targeting specific brain regions responsible for nausea regulation, Δ^9 -THC offers significant relief for cancer patients undergoing chemotherapy. Concurrently, the role of Δ^9 -THC as an appetite stimulant presents therapeutic advantages, especially for individuals contending with cachexia, a wasting syndrome often seen in serious illnesses such as human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) and cancer (Hammond et al., 2021). The interaction of Δ^9 -THC with CB₁ receptors is understood to be a key factor in its capacity to enhance appetite and increase food intake, offering a two-fold benefit in managing both the direct and collateral effects of these conditions (Mackie, 2006).

Emerging research indicates that Δ^9 -THC may offer neuroprotective benefits, presenting a potential therapeutic avenue for conditions such as multiple sclerosis, Parkinson's disease, and traumatic brain injury (Maurya & Velmurugan, 2018; Sarne et al., 2011). The neuroprotective properties of Δ^9 -THC could be attributed to its anti-inflammatory actions and its ability to support neurogenesis—the growth and development of neuronal tissue (Valeri & Mazzon, 2021). This dual capability not only suggests a role for Δ^9 -THC in safeguarding neural structures but also in fostering the recovery and regeneration of the brain, opening new possibilities for the treatment of neurodegenerative and neuro-traumatic conditions (Valeri & Mazzon, 2021).

The psychotropic effects of Δ^9 -THC are typically labelled as side effects, yet they do possess potential therapeutic benefits. For patients grappling with post-traumatic stress disorder (PTSD) or those in the throes of palliative care, the induced euphoria or altered states of consciousness from Δ^9 -THC may provide substantial psychological solace (Rehman et al., 2021). Instead of solely focusing on the negative aspects of psychotropic impact of Δ^9 -THC, it is important to recognise the value it may have for individuals dealing with severe mental health conditions or the profound stress of terminal illness. Despite its potential, the therapeutic use of Δ^9 -THC is complicated by its psychoactive effects, which can include impaired memory, altered judgement, and in some cases, anxiety, or paranoia (Maurya & Velmurugan, 2018; Volkow et al., 2014). The psychotropic effects of Δ^9 -THC can vary widely among individuals, influenced by factors such as dose, the method of administration, and the specific ECS of the user. The therapeutic window for Δ^9 -THC—that is, the dosage range in which it is effective without causing unacceptable side effects—is narrow and can be difficult to determine (Campos et al., 2022). This presents a challenge for clinicians who prescribe Δ^9 -THC-containing medications, as they must carefully balance the desired therapeutic effects with the potential for psychoactive side effects (Parmar et al., 2016). In addition, users of Δ^9 -THC-based drugs may develop an addiction as a euphoric feeling is experienced due to the increase of serotonin (Baltz & Le, 2020). This can lead some long-term users to develop depression as Δ^9 -THC also inhibits the reuptake of serotonin. Long-term use of Δ^9 -THC may cause detrimental health issues such as cognitive impairment which can decrease IQ especially during constant adolescence use (Maurya & Velmurugan, 2018; Volkow et al., 2014). There is also an increased risk of activating chronic psychosis disorders in individuals that are genetically predisposed to these disorders such as schizophrenia (Volkow et al., 2014).

The medicinal value of Δ^9 -THC is rooted in its molecular interaction with the ECS, a fundamental aspect of human physiology. However, realising the full therapeutic promise of Δ^9 -THC demands careful navigation of its psychoactive effects, diligent scientific inquiry, and a responsive legal framework. As research progresses, the aim is to optimise the use of Δ^9 -THC in medicine, enhancing its efficacy, and minimising risks, thus improving the quality of life for patients across a spectrum of health conditions. However, despite its medical potential, the status of Δ^9 -THC as a controlled substance in many parts of the world restricts research opportunities and patient access. However, changing legislation in various countries reflects a growing acknowledgment of the medicinal value of Δ^9 -THC and is gradually expanding the boundaries of its clinical application.

3.2 Cannabidiol Therapeutics

Cannabidiol stands out as a significant compound within the *Cannabis* plant due to its extensive range of potential therapeutic properties (Thomas et al., 2007). Unlike Δ^9 -THC, which is known for its mind-altering effects, CBD does not induce a state of intoxication. This characteristic of CBD is particularly compelling for those seeking the medicinal benefits of *Cannabis* without the altered mental state that Δ^9 -THC produces (Maurya & Velmurugan, 2018). The non-intoxicating nature of CBD has broadened its appeal and acceptance, allowing for wider usage among various demographics, including those who may be sensitive to the effects of Δ^9 -THC or those who wish to maintain clarity of mind while treating their symptoms. This has led to a growing interest in CBD as a viable option for a multitude of conditions, potentially providing relief for individuals suffering from chronic pain, anxiety, inflammation, seizures, and other ailments without cognitive disruption (Scuderi et al., 2009; White, 2019). The specific interactions of CBD with the ECS and its modulatory effects on various physiological pathways highlight its therapeutic promise and have made it a focal point of cannabinoid-based medical research.

The role of CBD in mediating inflammatory responses in the body has been a subject of scientific interest. For instance, CBD has demonstrated that transdermal CBD application had therapeutic potential for relief of arthritis pain-related behaviours and inflammation without evident side-effects (Hammell et al., 2016). It is also found that CBD treatment significantly reduced chronic inflammation and neuropathic pain in rodents (Philpott et al., 2017). The analgesic properties of CBD have also been substantiated through research evidence suggesting that CBD reduces pain by enhancing the activity of the natural endocannabinoids in the body

and by interacting with pain receptors in the nervous system (Frane et al., 2022; Manzanares et al., 2006). These findings collectively indicate that the impact of CBD on inflammation and pain may offer a basis for developing new anti-inflammatory and analgesic drugs.

The neuroprotective effects of CBD have shown promise in the treatment of various neurological conditions. Specifically, its efficacy in epilepsy has led to the FDA approval of Epidiolex, a CBD-based medication for the treatment of two rare forms of childhood epilepsy, Dravet syndrome and Lennox-Gastaut syndrome (Stockings et al., 2018). The clinical trials that supported the approval of Epidiolex showed the therapeutic potential of CBD. One pivotal study revealed that CBD significantly reduced the frequency of seizures in patients with Dravet syndrome compared to a placebo group (Devinsky et al., 2017). This double-blind, placebo-controlled trial was instrumental in demonstrating the effectiveness of CBD in a rigorous scientific approach (Koubeissi, 2017). Additionally, research into the role of CBD multiple sclerosis has indicated that it may help manage symptoms like muscle spasticity and chronic pain, common in this condition (Haddad et al., 2022). The potential of CBD to aid in Alzheimer's disease is also being explored, with preclinical studies suggesting that it could help reduce inflammation and promote cell repair, which are considered beneficial in neurodegenerative diseases (Watt & Karl, 2017). These studies, along with ongoing clinical trials, continue to inform the scientific community about the extent of the potential of CBD as a neuroprotective agent.

The anxiolytic and antipsychotic properties of CBD, offering potential therapeutic benefits for individuals with anxiety and psychotic disorders (White, 2019). For anxiety, a study found that participants who were given CBD experienced significantly reduced anxiety levels during a public speaking test compared to the placebo group (Bergamaschi et al., 2011). In the realm of psychosis, CBD has been studied as a potential treatment for schizophrenia. When investigating the effects of CBD on schizophrenia and psychosis, three studies displayed a decrease in the symptoms of schizophrenia while consuming CBD along with their normal anti-psychosis medication (Boggs et al., 2018; Leweke et al., 2012; McGuire et al., 2018). In addition, CBD has also shown antipsychotic properties (Zuardi et al., 1995). A study compared CBD to amisulpride—a potent antipsychotic—in a group of patients with schizophrenia (Leweke et al., 2012). The study reported that CBD was as effective as amisulpride in treating psychotic symptoms and had a more favourable side-effect profile, including lower incidences of extrapyramidal symptoms, weight gain, and increases in prolactin levels. Moreover, the

possible moderating of CBD effects on Δ^9 -THC-induced psychosis have been an area of research interest where CBD is found to protect against the adverse effects of Δ^9 -THC (Niesink & van Laar, 2013). These studies, along with ongoing research, suggest that CBD could play a valuable role in managing conditions related to anxiety and psychosis without the significant side effects associated with traditional pharmaceutical agents. However, the exact mechanisms by which CBD exerts these effects are not yet fully understood, and further research is needed to determine optimal dosing and administration methods.

The potential of CBD as a treatment for addiction has garnered increasing research interest, with studies suggesting it could play a role in reducing cravings and withdrawal symptoms associated with substance abuse disorders. For example, a study found that CBD significantly reduced the cue-induced cravings and anxiety of heroin users (Hurd et al., 2019). Moreover, these effects appeared to be sustained, with the impact on cravings lasting a week after the last administration of CBD. Another study focused on the potential of CBD in addressing tobacco addiction (Morgan et al., 2013). The study observed a significant reduction in the number of cigarettes smoked by tobacco users who were administered CBD compared to the control group. Furthermore, research is also exploring how CBD may impact the behavioural and physiological aspects of addiction. A review published in *Substance Abuse: Research and Treatment* in 2015, collated preclinical and clinical data and discussed CBD as an intervention for addictive behaviours, proposing mechanisms such as the modulation of neuronal circuits involved in addiction (Prud'homme et al., 2015). These studies provide promising insights into the role of CBD in addiction therapy. However, this research is in the developmental stages, and further studies are needed to fully understand the effectiveness and mechanism of CBD in the context of addiction treatment.

The challenges surrounding CBD therapy are multifaceted and are often centred on the pharmacodynamics and pharmacokinetics of the compound, as well as regulatory issues. A pivotal study that delves into the mechanisms of CBD shedding light on the complexity of the action of CBD, noting its interaction with various receptor systems beyond the well-known CBRs, such as G protein-coupled receptor 55 (GPR55) and serotonin 1A receptor (5-HT1A) (Bih et al., 2015). Additionally, regulatory issues and the subsequent impact on product quality are also major concerns in CBD therapy. A study revealed labelling inaccuracies in CBD products, which raises substantial concerns about the reliability of CBD product content on the market (Bonn-Miller et al., 2017). These challenges emphasise the need for ongoing research

to clarify the therapeutic mechanisms of CBD, personalised medicine approaches to account for individual variability, and stricter regulatory standards to ensure the quality and consistency of CBD products. Only with rigorous scientific research and appropriate regulatory measures can the therapeutic potential of CBD be fully realised and safely integrated into medical practice. Accessing the latest research and regulatory information will require consultation with current databases and regulatory agency publications.

Moreover, the therapeutic potential of CBD is expansive, with research continually uncovering new applications and benefits. Its non-intoxicating nature makes it particularly attractive for therapeutic use. As research progresses, it is likely that CBD will become increasingly integrated into medical treatments, with the potential to offer relief for a myriad of conditions. The future of CBD in therapeutics is promising, though it will require rigorous scientific research, standardised regulations, and clinical trials to fully understand and harness its capabilities.

4 SYNTHETIC AND MODIFIED PHYTOCANNABINOIDS

The remarkable therapeutic benefits of CBD and Δ^9 -THC have catalysed the search for agents that can replicate or enhance the effects of natural phytocannabinoids (Russo & Guy, 2006). This pursuit started in Europe in the 2000s and has led to the emergence of synthetic and modified phytocannabinoids—laboratory-synthesised compounds aimed at interacting with the ECS akin to the naturally occurring compounds in *Cannabis* (Brown et al., 2021). The motivation for creating these synthetic cannabinoids (SCs) stems from legal restrictions surrounding *Cannabis* as well as variability in natural *Cannabis* products, whose phytocannabinoid content can fluctuate due to genetic differences, cultivation practices, and post-harvest processing. These inconsistencies can pose challenges when using full spectrum oils for medicinal use, where precise dosing and predictable outcomes are imperative. Additionally, the complexity and expense of purifying natural phytocannabinoids to the high purity levels demanded by medical protocols often make synthetic alternatives more viable (Romano & Hazekamp, 2013). Synthetic methods can achieve greater control over the resulting compounds, offering potential cost advantages and consistency in the final product. Furthermore, synthetic and modified cannabinoids can provide a pathway for legal research and therapeutic application in regions where *Cannabis*-related legalities impose strict controls. SCs, which are part of the new psychoactive substances (NPS) group, aim to bind to the same receptors in the brain as Δ^9 -THC. Although SCs were originally developed for valid research

purposes, their unregulated status and similarity to controlled substances have made them a part of the illegal drug market (Castaneto et al., 2014). The complexity of their effects, combined with the lack of oversight, means that these substances can pose significant risks to users. As such, understanding and regulating these compounds remains a critical issue for public health and safety.

Synthetic cannabinoids, such as JWH-018 and Spice K2, represent a category of chemically produced compounds designed to mimic the pharmacological effects of Δ^9 -THC. Chemist John W. Huffman (JWH) developed the synthetic compound called JWH-018 in the early 1990s (Huffman et al., 1994). It was one of the first of its kind to be discovered and was synthesised with the intent of advancing the scientific study of the ECS, particularly for its therapeutic potential in regulating immune responses and pain. However, due to its potency and easy accessibility, JWH-018 has been misused as a recreational drug with effects similar to Δ^9 -THC but with a higher risk of adverse reactions due to its stronger affinity for CBRs (Castaneto et al., 2014). Spice refers to a wide variety of herbal mixtures that produce experiences similar to *Cannabis* and that are marketed as "safe," legal alternatives to that drug. Sold under many names, including K2, fake weed, Yucatan Fire, Skunk, Moon Rocks, and others—and labelled "not for human consumption"—these products contain dried, shredded plant material and chemical additives that are responsible for their psychoactive effects (Vandrey et al., 2012). Despite being labelled not for human consumption, Spice products are smoked by users (Spaderna et al., 2013). These chemicals are analogues of Δ^9 -THC and were initially created for therapeutic research. However, their recreational use has led to numerous cases of severe side effects, including acute psychosis, kidney injury, and even seizures, due to their unpredictable nature and lack of quality control in production (Spaderna et al., 2013).

These SCs are marketed as legal highs and have been sold under the guise of “herbal incense” or “potpourri” (Assi et al., 2020). The labelling often disguises their true intention and side steps regulatory requirements. They exploit legal loopholes that allow them to be sold legally until legislation catches up, at which point the molecular structure of the cannabinoid is often slightly altered to create a new compound that again temporarily bypasses legal restrictions. The use of these substances represents a significant public health concern due to their potency, the potential for addiction, and their side effects (Bilici, 2014; Brown et al., 2021). Their existence has prompted new legislation and has posed significant challenges to law enforcement, public health officials, and drug testing organisations.

Furthermore, modified phytocannabinoids are appearing in the market with increasing frequency, often as a result of individuals attempting to enhance the effects of natural phytocannabinoids (Huffman et al., 1994). This includes increasing their efficacy, reducing psychoactive effects, enhancing bioavailability, and reducing side effects. This practice can lead to the availability of compounds with heightened potency compared to their unmodified counterparts. However, the lack of comprehensive pharmacological data, particularly from authoritative bodies such as the U.S. Food and Drug Administration (FDA), poses significant challenges.

Tetrahydrocannabiphorol (THCP) is a phytocannabinoid recently discovered in *Cannabis*, typically present in minute quantities. Research shows that THCP binds to the CB₁ receptor with an affinity 30 % higher than that of Δ^9 -THC, indicating a potential potency significantly greater than Δ^9 -THC (Citti et al., 2019). This is attributed to the molecular structure of THCP, which features an extended alkyl side chain, believed to enhance its binding affinity and, therefore, its potency. Following its discovery, there has been a move to synthesise THCP from high-CBD hemp, circumventing the legal limitations linked to Δ^9 -THC and allowing exploration of its therapeutic potential, especially for conditions that may benefit from a stronger cannabinoid effect. The discovery of THCP calls for more research to understand its safety and therapeutic applications fully. Similarly, cannabidiphorol (CBDP), another phytocannabinoid isolated alongside THCP, has caught scientific interest. Like THCP, the unique structure of CBDP, with a longer side chain compared to CBD, may increase its binding affinity, hinting at more pronounced effects (Citti et al., 2019). Yet, the impact and possible benefits of CBDP remain largely unexplored, signalling the need for extensive research to uncover its pharmacological properties and potential uses in medicine.

The stronger effects of these modified cannabinoids are due to their increased affinity for cannabinoid receptors in the brain compared to their unmodified counterparts. These compounds raise significant safety, regulatory, and therapeutic questions. While they may offer the potential for more robust therapeutic effects, the increased potency also comes with an elevated risk of adverse effects, highlighting the need for careful study and regulation. Additionally, THCP and CBDP have undergone some level of study, acetylated phytocannabinoids, specifically CBDDA have not been thoroughly researched, raising concerns about their safety and effects (Citti et al., 2019; Holt et al., 2022).

4.1 Acetylated Phytocannabinoids

Modified cannabinoids, such as acetylated phytocannabinoids, have emerged as new entities in the spectrum of cannabinoid substances. These are phytocannabinoids that have been chemically altered, usually to enhance their potency, alter their effects, or to skirt legal restrictions. Among the modified cannabinoids, acetylated versions such as THCOA and CBDDA have gained attention (Sabaghi, 2022). THCOA is synthesised from THC and is believed to be more potent than its parent compound due to its acetylated form which affects its bioavailability and strength of interaction with CBRs (Holt et al., 2022; Pardridge, 2012). The synthesis involves the reaction of THC with acetic anhydride, yielding THCOA, which has been reported to produce intense psychoactive effects (Holt et al., 2022; Pardridge, 2012). Additionally, various types of THC are being acetylated such as Δ^8 -THC, Δ^9 -THC, and Δ^{10} -THC as each have their characteristic double bond placement on different carbons (Fig. 2.4) with the most common being Δ^9 -THC converted to Δ^9 -THCOA.

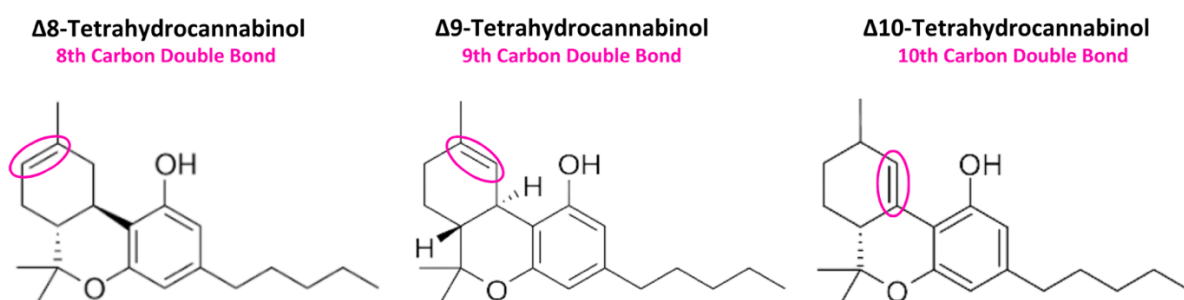


Figure 2.4: Structures of THC cannabinoid isomers: Δ^8 -THC, Δ^9 -THC, and Δ^{10} -THC. Each structure is highlighted to show the location of the double bond: at the 8th carbon for Δ^8 -THC, 9th carbon for Δ^9 -THC, and 10th carbon for Δ^{10} -THC. The position of this double bond is critical for the pharmacological and psychoactive properties of these compounds.

Similarly, CBDDA is an acetylated form of CBD, created through a comparable synthetic process (Holt et al., 2022). The acetylation potentially affects its pharmacokinetic properties, including its potency and bioavailability (Pardridge, 2012). However, comprehensive research and clinical data on these acetylated phytocannabinoids are limited, and there is a need for detailed study to understand their pharmacology, efficacy, and safety profiles fully. The development and presence of Δ^9 -THCOA and CBDDA in the market pose challenges due to their unregulated status and the potential for unpredictable and potent effects (Holt et al., 2022). This raises concerns about consumer safety, especially when these substances are sold without proper labelling or quality assurance, often misleading users about their potential effects and risks.

As acetylated phytocannabinoids (Sugawara et al., 2023) are gradually making their way into the market, primarily through unregulated channels. They are attracting attention due to their modified effects compared to their natural counterparts, with some anecdotal reports suggesting Δ^9 -THCOA may produce more intense psychoactive effects. These compounds are not just a point of interest for consumers looking for novel experiences; they also offer potential as research tools for better understanding the ECS and the effects of cannabinoids. However, their emergence in the market is outpacing the regulatory frameworks necessary to ensure safety and efficacy. With little to no clinical studies, particularly on CBDDA, the full scope of their pharmacological effects remains unknown. This gap presents significant challenges in determining their therapeutic potential and the risks they may pose to users. From an ethical and legal standpoint, the synthesis and modification of cannabinoids raise critical questions. The lack of a comprehensive regulatory framework allows these substances to be distributed and consumed without sufficient understanding of their impacts, which could lead to adverse health outcomes. This situation necessitates a balance between encouraging scientific innovation and ensuring public safety.

Looking to the future, the advancement of the *Cannabis* industry will play a pivotal role in both the development and regulation of synthesised and modified cannabinoids. There is a pressing need for robust scientific research and clinical trials to understand these compounds thoroughly. With proper regulation and in-depth study, synthesised and modified cannabinoids could have a significant impact on the pharmaceutical industry and medical practice, potentially offering new therapeutic options for patients. However, this future is contingent upon a proactive approach to the challenges they present, ensuring they are safe, effective, and beneficial for those who choose to use them.

5 THE ENDOCANNABINOID SYSTEM

The ECS is a complex cell-signalling system identified in the early 1990s by researchers exploring Δ^9 -THC (Fig. 2.5) (Devane et al., 1988; Herkenham et al., 1990). The ECS is crucial for maintaining homeostasis within the body, which means it plays a vital role in keeping the internal environment stable, regardless of external changes (Pacher et al., 2006). It is involved in regulating a range of functions and processes, including sleep, mood, appetite, memory, reproduction, and fertility (Howlett, 2002). The ECS consists of three core components: endocannabinoids, receptors, and enzymes. Endocannabinoids are molecules produced by the body that bind to CBRs but are produced endogenously. The key endocannabinoids are

anandamide (AEA), 2-arachidonoylglycerol (2-AG) and *N*-arachidonoyl dopamine (NADA). These endocannabinoids bind to receptors found throughout the body to perform various actions (Rezende et al., 2023). The two primary receptors are CB₁ receptors, which are predominantly found in the CNS, and CB₂ receptors, which are mainly found in the PNS, especially immune cells. CB₁ receptors are responsible for the psychoactive effects of cannabinoids, while CB₂ receptors are associated with anti-inflammatory and immune-enhancing effects (Howlett, 2002; Mackie, 2006).

The Δ^9 -THC compound binds to both CBRs but has a higher affinity for CB₁ receptors, which leads to the psychoactive effects of feeling "high." On the other hand, CBD has a lower affinity for both CB₁ and CB₂ receptors (Howlett, 2002; Mackie, 2006; Maurya & Velmurugan, 2018). The modified cannabinoids like Δ^9 -THCOA and CBDDA, due to their acetyl groups, may affect the ECS differently. The addition of acetyl groups may potentially enhance their ability to cross the blood-brain barrier (BBB), which could result in different efficacy and potency compared to their non-acetylated counterparts (Calapai et al., 2020; Pardridge, 2012). While Δ^9 -THCOA could potentially exert stronger psychoactive effects due to its enhanced interaction with CB₁ receptors, the effects of CBDDA are still being explored (Holt et al., 2022). The ECS is a critical target for therapeutic agents due to its widespread influence on human physiology (Leinen et al., 2023). The importance of studying CBRs within the ECS cannot be overstated. These receptors are the points of contact where cannabinoids exert their effects, influencing a vast array of physiological processes. Deepening our understanding of CBRs can help in comprehending not only the effects of *Cannabis* consumption but also the broader applications of the ECS in human health.

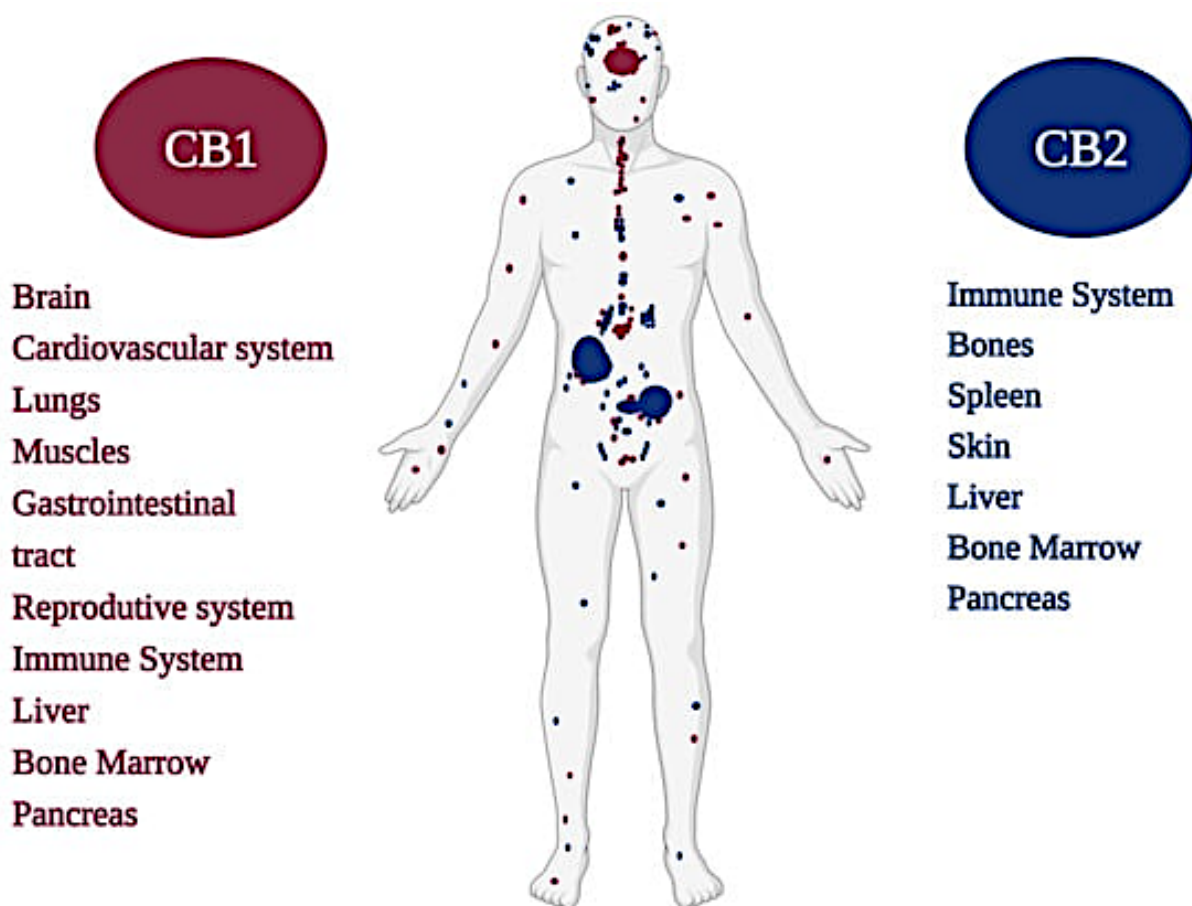


Figure 2.5: Distribution of CBRs in the human body. CB₁ receptors are predominantly found in the brain, cardiovascular system, lungs, muscles, gastrointestinal tract, reproductive system, immune system, liver, and bone marrow. CB₂ receptors are primarily associated with the immune system and are located in the spleen, bones, skin, liver, bone marrow, and pancreas. This illustration indicates the regions and organ systems where each receptor type is prevalent. Sourced from (Rezende et al., 2023)

Studying the ECS and its receptors is vital for unlocking the full potential of *Cannabis*-derived compounds in medicine. It holds the promise of new treatments for chronic and debilitating conditions, with the caveat that rigorous scientific research and thoughtful regulation must keep pace with pharmacological innovation. The exploration of the ECS, a pivotal biological system, reveals its profound influence on human health (Pacher et al., 2006). Furthermore, with the pace of emerging cannabinoids the existing experimental methods, while invaluable, have their limitations. They may not provide the granular, molecular-level insights required to unlock the full therapeutic potential of interactions between cannabinoids and the ECS and these methods are often time-consuming (Willems et al., 2020). This is where advanced computational methods come into play. Computational biology and molecular mechanics can simulate and model the interactions between cannabinoids and ECS receptors with great precision (Aviz-Amador et al., 2021). These methods can predict how modifications to cannabinoid molecules

might change their shape, behaviour, and affinity for receptors, which is crucial for the design of new therapeutic agents.

5.1 Discovery of Endocannabinoid Receptors

The discovery and structural elucidation of the human CBRs, are pivotal achievements in molecular biology and pharmacology. The path to uncovering the structures of these receptors has been marked by significant technological advancements and collaborative research efforts across various disciplines. The identification of the CB₁ receptor in the late 1980s marked the beginning of a new era in cannabinoid research (Devane et al., 1988). After which, the first CB₁ receptor was cloned by utilising radiolabelled THC to identify cannabinoid-binding sites in the rat brain, researchers provided the first evidence of a molecular target for cannabinoids within the brain (Matsuda et al., 1990). This discovery not only unveiled a new receptor but also hinted at the complex interactions between cannabinoids and the brain. Shortly thereafter, the CB₂ receptor was discovered, initially noted for its primary expression in the immune system. This finding suggested a broader role for cannabinoids, extending beyond neuromodulation to include immune regulation (Munro et al., 1993). The identification of these receptors was crucial for understanding the comprehensive role of the ECS in the body.

Historically, the structural elucidation of GPCRs like CB₁ and CB₂ has been fraught with challenges. Their membrane-bound nature and conformational flexibility made it difficult to determine their structures using traditional crystallography methods (Carpenter et al., 2008). Early efforts relied on homology modelling and indirect biophysical techniques to infer structural features. The field of structural biology transformed in the 2000s and 2010s with the advent of advanced crystallography, cryo-electron microscopy (cryo-EM), and computational modelling (Berman et al., 2018). These technological breakthroughs enabled the direct visualisation of the structures of receptors. A landmark achievement was the publication of the first crystal structure of the human CB₁ receptor in 2016, revealing detailed insights into its ligand-binding pocket and conformational changes upon ligand binding, setting the stage for targeted drug design (Hua et al., 2016). Further advancements led to the elucidation of the CB₂ receptor structure and additional complex structures of CB₁ with various ligands, enhancing our understanding of ligand-receptor interactions, receptor activation, and the selectivity and efficacy of cannabinoid ligands (Li et al., 2019). The detailed structural characterisation of CBRs has significantly impacted drug discovery and development. By facilitating the design of

novel cannabinoids with improved specificity, efficacy, and safety profiles, these discoveries hold promise for therapeutic applications in a range of conditions.

6 COMPUTATIONAL BIOLOGY

The history of computational structural biology represents a significant development in scientific research, transitioning from initial theoretical models to advanced simulations and analyses over several decades. This progression has fundamentally altered the approach to understanding biological processes at the molecular level (Berman et al., 2018; Trewavas, 2006). The history of molecular mechanics traces its roots back to the mid-20th century, reflecting a journey through the development of computational methods to model and predict the behaviour of molecules. This field has evolved significantly over the years, marked by several key milestones and innovations.

Starting in the 1950s, computational biology began to take shape with the development of computational and mathematical models aimed at unravelling biological processes. This era saw the first uses of computational methods in biology, notably in the simulation of biochemical pathways, signifying the integration of the field into biological research (Trewavas, 2006). The subsequent decade witnessed the need for computational methods such as bioinformatics to analyse the increasing sequences of proteins and nucleic acids. The first computerised protein sequence database introduced the scientific community to the concept of molecular evolution, marking a pivotal advancement in the field (Masic, 2016). The foundations of molecular mechanics were laid in the 1940s and 1950s, as chemists sought to understand the forces governing molecular structures and reactions (Trewavas, 2006). The 1960s and 1970s witnessed significant advancements in the field with the development of comprehensive force fields, which are sets of equations and parameters used to calculate the potential energy of a molecular system. These force fields made it possible to model molecular geometry, vibrations, and other properties more accurately. Notably, the development of the Molecular Mechanics 2 (MM2) force field marked a significant milestone in molecular mechanics, providing a robust framework for calculating molecular energies and properties (Allinger, 1977). The 1970s further advanced the discipline with significant developments in sequence alignment, particularly through dynamic programming algorithms such as the Needleman-Wunsch and Smith-Waterman algorithms (Needleman & Wunsch, 1970).

Subsequently, the field saw substantial growth in the 1980s with the advent of 3D structure prediction and modelling of proteins. Homology modelling, in particular, allowed for the prediction of protein structures based on their similarity to known structures, facilitated by the expanding database of protein structures in the Protein Data Bank (PDB) (Berman et al., 2000; Greer, 1980; Venselaar et al., 2010). This period also marked the emergence of bioinformatics as a distinct discipline, integrating computational biology with a rapidly growing database of genomic and protein sequences. The focus expanded to include the development of algorithms for gene prediction, multiple sequence alignment, and in-depth genomic sequence analysis. During the 1980s and 1990s, molecular mechanics expanded rapidly, thanks to the increasing power of computers and the development of more sophisticated force fields. This period saw the emergence of new force fields, such as AMBER and CHARMM, designed for specific types of molecules, such as biomolecules (González, 2011). These advancements allowed structural biologists to simulate large biological molecules, including proteins and nucleic acids, with greater accuracy (Poltev, 2015; Trewavas, 2006).

Entering the genomics era in the early 2000s, the completion of the Human Genome Project and other genomic projects provided a wealth of data, leading to the development of sophisticated tools for analysing large-scale genomic data (Collins & Fink, 1995). In the late 20th and early 21st centuries, molecular mechanics began to be integrated with other computational methods, such as quantum mechanics, in hybrid approaches. These methods, known as quantum mechanics/molecular mechanics (QM/MM) simulations, allowed for the accurate modelling of systems where a detailed quantum mechanical treatment of a portion of the molecule is necessary, while the rest of the system can be efficiently described by molecular mechanics (Field et al., 1990).

The impact of computational biology on protein studies has been insightful, enabling groundbreaking discoveries that shed light on the molecular basis of diseases, advance drug discovery, and facilitate the design of novel proteins with specific functions. As the field continues to evolve, driven by advancements in computational power, algorithms, and interdisciplinary collaboration, its future appears incredibly promising. The integration of computational biology with experimental techniques is leading to novel insights into biological systems, emphasising its critical role in advancing the life sciences. Today, molecular mechanics is a fundamental tool in computational chemistry, materials science, and molecular biology. It is used extensively in the pharmaceutical industry for drug design and in materials science for designing new

materials with specific properties (De Vivo et al., 2016). The field continues to evolve, with ongoing research focused on developing more accurate force fields, improving computational algorithms, and applying molecular mechanics to increasingly complex systems (Vanommeslaeghe & Guvench, 2014). The history of molecular mechanics is a testament to the collaborative effort of chemists, physicists, and computer scientists. As computational power continues to grow and algorithms become more sophisticated, molecular mechanics will undoubtedly play an even more significant role in advancing our understanding of the molecular world, offering new insights into complex chemical and biological systems.

6.1 Classical and Quantum Computing

Classical computing and quantum computing represent two different paradigms in the field of computation, each with its unique principles, capabilities, and intended applications (Kitaev et al., 2002). While classical computing has been the backbone of digital technology for decades, quantum computing emerges as a revolutionary approach with the potential to solve certain types of problems much more efficiently than classical systems (Jaeger, 2007). Understanding the distinctions between these two computing types is crucial for grasping the future trajectory of computational technology.

Classical computing is based on the binary digit or bit, the fundamental unit of information, which can be either 0 or 1. Classical computers perform operations using bits to represent and manipulate data. This paradigm encompasses everything from the earliest computers to modern supercomputers, which rely on integrated circuits and transistors to process complex calculations. The efficiency of classical computing has seen exponential growth, often described by Moore's Law, which observes that the number of transistors on a microchip doubles approximately every two years, though this pace has slowed in recent years (Schaller, 1997). Classical computing excels at executing a vast range of tasks, from basic word processing to complex simulations in weather forecasting, financial modelling, and more. However, it faces limitations when dealing with problems that require exploring a vast number of potential states simultaneously, such as factoring large prime numbers, simulating quantum systems, or optimising complex systems in operations research (Kitaev et al., 2002).

Classical computing and quantum computing differ fundamentally in their underlying principles and capabilities. Classical computers operate based on the principles of classical physics and use bits whereas quantum computers are grounded in quantum mechanics, utilising

qubits that can exist in a state of superposition, meaning they can represent both 0 and 1 simultaneously, and can be entangled with one another (Brassard et al., 1998). When it comes to processing information, classical computers perform operations sequentially, whereas quantum computers, through the phenomena of superposition and entanglement, can process various probabilities and execute multiple operations at once. This allows for potentially enormous increases in computing power, especially for certain complex tasks (Kitaev et al., 2002). In terms of error correction and stability, classical bits are relatively robust and can be easily copied, which facilitates data storage and simple error correction. Quantum states, however, are more delicate and can be disrupted by the slightest environmental changes, a problem known as decoherence (Brandt, 1999). Measuring quantum states without altering them is another significant challenge, making quantum error correction a crucial and complex field of research necessary for the development of dependable quantum computers. The practical uses of these two computing paradigms differ. Classical computers are incredibly versatile and capable of handling a wide array of tasks. Quantum computers, while not universally applicable, hold the promise of outperforming classical computers in certain domains, such as quantum simulations, specific optimisation problems, and tasks that are exponentially complex for classical computers to solve (Jaeger, 2007; Kitaev et al., 2002).

Since classical and quantum computing offer different strengths, it is unlikely that quantum computers will replace classical computers for everyday tasks. Instead, quantum computing is expected to complement classical computing, tackling specific problems that are currently beyond reach. As quantum technology continues to develop, it holds the promise of opening new frontiers in science, medicine, cryptography, and beyond, reshaping our computational capabilities and understanding of the natural world.

7 MOLECULAR MECHANICS

Classical computing provides a foundation for molecular mechanics, a field that applies classical physics principles to model and predict the behaviour of atoms and molecules (Levitt et al., 1995). This contrasts with quantum mechanics, which examines particles at the atomic level. Molecular mechanics simplifies these particles to mass points linked by springs, representing bonds, and incorporates the influence of non-bonded interactions (Fig. 2.6) (Vanommeslaeghe & Guvench, 2014).

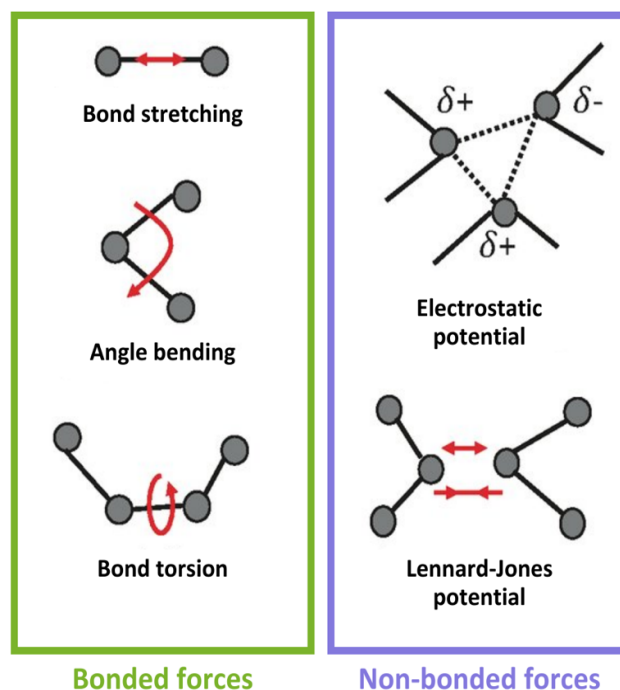


Figure 2.6: Illustration of molecular interactions in bonded and non-bonded forces. The left (green) displays bonded forces, including bond stretching with arrows indicating the direction of force applied to stretch a bond, angle bending showing the force applied at the vertex atom to alter the bond angle, and bond torsion depicted by circular arrows indicating the rotation around the bond axis. The right (purple) represents non-bonded forces, with electrostatic potential characterised by delta symbols ($\delta+$ and $\delta-$) denoting partial charges attracting or repelling each other, and Lennard-Jones potential illustrated with arrows representing the balance of attractive and repulsive forces between neutral atoms or molecules. (Waidyasooriya et al., 2017)

7.1 Molecular Mechanics Principles

The core of molecular mechanics is rooted in Newtonian mechanics, which describes the motion of particles based on forces acting upon them (Levitt et al., 1995). This framework allows the prediction of molecular structures and dynamics by treating atoms as particles moving according to the forces derived from potential energy functions (Kitaev et al., 2002). Newton's second law of motion (Eq. 1) is fundamental to this, where the force (F) acting on an atom is equal to the mass (m) of the atom multiplied by its acceleration (a).

$$F = m \cdot a \quad (1)$$

In the context of molecular mechanics, this translates to calculating the acceleration of atoms within a molecule from the forces acting on them, which arise from potential energy gradients in the system. Central to MD simulations is Eq. 2, which expresses the force exerted on each atom within a molecular system (Tuckerman & Martyna, 2000). This force is derived from the

potential energy surface (U), a scalar field representing all interatomic interactions in a system as a function of atomic positions (Allen & Tildesley, 2017). The gradient operator ∇ provides of this potential energy surface, and thus the negative of this gradient indicates the direction in which the forces act on each atom, attempting to bring the system to a state of minimal energy (Leach, 2001). Additionally, $r_1, r_2, \dots, r_N$ denotes the positions of the N atoms within the molecular system. The force on each atom F_i then determines its acceleration, and by extension, its motion, according to Newton's second law (Eq. 1), allowing for the computation of trajectories via numerical integration methods such as the Verlet algorithm (Eq. 3) (Verlet, 1967).

$$F_i = \nabla U(r_1, r_2, \dots, r_N) \quad (2)$$

$$r(t + \Delta t) = 2r(t) - r(t - \Delta t) + a(t)\Delta t^2 \quad (3)$$

The Verlet algorithm is a computational technique integral to MD simulations, serving to predict the trajectories of particles by integrating their equations of motion over time. In essence, the algorithm provides a means to calculate the future position of a particle, denoted as $r(t + \Delta t)$, by using its current position $r(t)$, its position at the previous timestep $r(t - \Delta t)$, and its current acceleration $a(t)$. This acceleration is derived from the forces currently acting on the particle divided by its mass, expressed by the formula: $a(t) = \frac{F(t)}{m}$. The timestep Δt is a carefully chosen small interval that allows for the simulation to progress in discrete but sufficiently fine-grained increments. One of the key strengths of the Verlet algorithm lies in its time-reversible and symplectic geometry, which ensures the conservation of energy within the system across extensive simulation periods (Feng & Qin, 2010). This attribute is especially critical for accurately modelling and understanding the molecular dynamics that dictate physical phenomena on a microscopic scale, ranging from chemical reactions to the intricate processes of protein folding (Poltev, 2015; Verlet, 1967).

The forces between atoms are considered conservative, meaning that the work done in moving an atom along a path is independent of the path taken, depending solely on the initial and final positions. This allows the definition of potential energy functions for various interactions (bond stretching, angle bending, torsional angles, non-bonded interactions), whereby the total energy of a system can be calculated (Patodia et al., 2014). Additionally, the total energy of a system is defined by the Hamiltonian. One of the critical components of this potential energy is bond

stretching, which is modelled after Hooke's Law. This law posits that the force required to stretch or compress a spring by a certain distance is proportional to that distance (Giuliodori et al., 2009). In molecular mechanics, chemical bonds are analogised to springs, where force constants (k_b) mirror bond stiffness, and deviations from bond equilibrium length (r_0) are seen as displacements of the spring. The bond stretching potential energy can be expressed (Eq. 4) where k_b is the bond force constant, r is the actual bond length during the simulation, and r_0 is the equilibrium bond length, the state where the bond is at its energetic minimum. This model is pivotal in molecular mechanics as it provides the basis for understanding how bonds behave under various forces, an essential factor in the dynamics and stability of molecular structures.

$$U_{bonds} = \frac{1}{2} k_b (r - r_0)^2 \quad (4)$$

The angle in the context of molecular mechanics is applied to the angles formed between bonds. The energy associated with bending a bond angle away from its preferred (equilibrium) angle is proportional to the square of the displacement, similar to how the potential energy of a string depends on the displacement from its rest length (Patodia et al., 2014). In this equation (Eq. 5), k_θ is the angle force constant where θ is the actual bond angle, and θ_0 is the equilibrium bond angle. This term contributes to the potential energy when a bond angle deviates from its equilibrium value.

$$U_{angles} = \frac{1}{2} k_\theta (\theta - \theta_0)^2 \quad (5)$$

The torsional angle (Eq. 6) accounts for the energy associated with the rotation around bonds, typically represented by a Fourier series-like expression. The potential energy for torsion considers the barriers to rotation around a bond, which are periodic in nature. In this equation, V_n represents the barrier height to torsional rotation, n is the periodicity of the torsional barrier, ϕ is the dihedral angle, and δ is the phase shift. These barriers correspond to different conformations that the molecule can adopt.

$$U_{dihedrals} = \sum_n \frac{V_n}{2} [1 + \cos(n\phi - \delta)] \quad (6)$$

In molecular mechanics, non-bonded interactions are crucial for modelling the behaviour of molecules, and among these, the van der Waals forces are particularly significant. They encompass both the attraction and repulsion between atoms and molecules that are not bonded

covalently. The Van der Waals interactions are represented by the Lennard-Jones potential (Fig. 6), which is a mathematical expression that captures the balance between attractive and repulsive forces as a function of the distance between two atoms or molecules (Jones, 1924). The potential is described by Eq. 7 where ε is a parameter that represents the depth of the potential well, corresponding to the strength of the attraction when two atoms are at an optimal intermediate distance. The σ parameter corresponds to the distance at which the potential between the two particles is zero, typically representing the point at which the repulsive forces begin to dominate as the particles approach each other. The r term represents the actual distance between the two particles.

$$U_{vdW} = 4\varepsilon \left[\left(\frac{\sigma}{r} \right)^{12} - \left(\frac{\sigma}{r} \right)^6 \right] \quad (7)$$

The Lennard-Jones potential consists of two main parts (Fig. 2.7): the repulsive part, which is proportional to $\frac{1}{r^{12}}$ and the attractive part, proportional to $\frac{1}{r^6}$. The repulsive part dominates at short ranges when atoms are very close to one another, while the attractive part governs the interaction at longer ranges. These Van der Waals forces are simplified into classical potentials for molecular mechanics from their more complex descriptions in quantum mechanics and electromagnetism (Vanommeslaeghe & Guvench, 2014). They play a critical role in determining the physical properties of molecules, such as boiling points, solubility, and the structure of molecular crystals, as well as the behaviour of biological systems, including protein folding, ligand binding, and the formation of cell membranes.

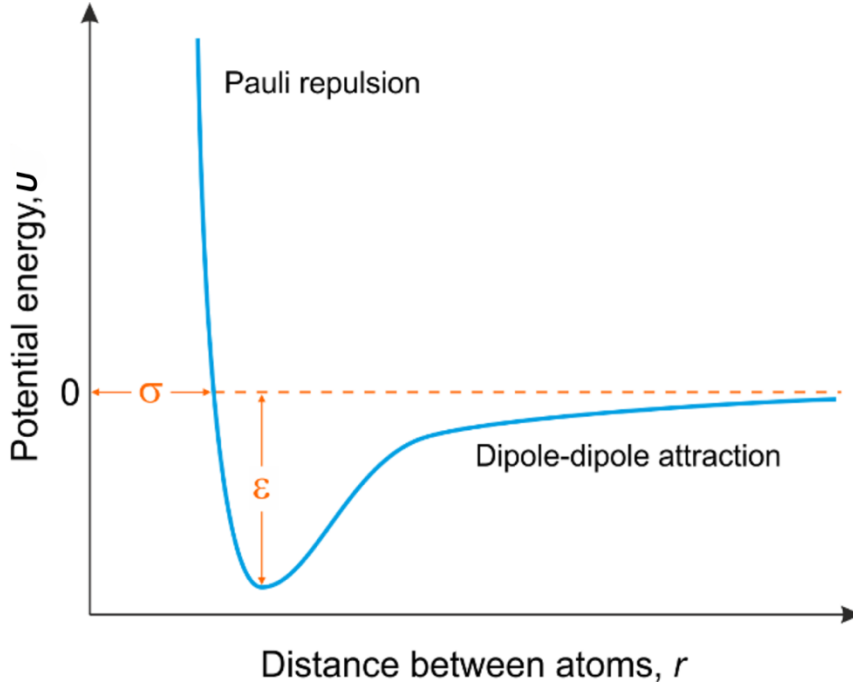


Figure 2.7: the Lennard-Jones potential describing the relationship between the potential energy (U) and the interatomic distance (r). It showcases two key forces: the Pauli repulsion at short ranges due to electron cloud overlap, which causes the potential to rise sharply as atoms approach each other, and the dipole-dipole attraction at longer ranges, responsible for the attractive part of the interaction. The potential energy minimum at ϵ indicates the most energetically favourable distance between two atoms. The distance at which the potential energy crosses zero is marked by σ , representing the point beyond which the interaction becomes repulsive. Adapted from (Generalic, 2022).

Coulomb's Law is fundamental in molecular mechanics for modelling electrostatic interactions, which are pivotal for the structure and stability of molecular systems (George et al., 2022). The law posits that the force F between two charged particles is directly proportional to the product of their charges q_i and q_j , and inversely proportional to the square of the distance r between them, encapsulated in the formula (Eq. 8) where ϵ represents the vacuum permittivity, which is a measure of the resistance encountered when forming an electric field in the vacuum (Patodia et al., 2014).

$$F = \frac{1}{4\pi\epsilon_0} \cdot \frac{q_i q_j}{r^2} \quad (8)$$

In the context of molecular mechanics, electrostatic potential energy $V_{\text{electrostatic}}$ (Eq. 9) rather than force is typically considered as it includes ϵr which is the relative permittivity or dielectric constant of the medium, which adjusts the interaction for effects due to the environment surrounding the charges (George et al., 2022). Molecular mechanics utilises this equation to calculate the electrostatic component of the potential energy for a system of charged particles.

This energy contributes to the overall potential energy landscape that dictates how molecules interact, fold, and react.

$$U_{electrostatic} = \frac{1}{4\pi\epsilon_0} \cdot \frac{q_i q_j}{\epsilon_r r} \quad (9)$$

The total potential energy (Eq. 10) U_{total} in a MD simulation is the sum of various energy contributions. U_{bonds} accounts for energy due to bond stretching, U_{angles} for bond angle deformations, $U_{dihedrals}$ for torsions or rotations around bonds, U_{vdW} for van der Waals forces which are weak attractions or repulsions between non-bonded atoms, and $U_{electrostatic}$ for electrostatic or Coulombic interactions between charged species. Each term sums the potential energy contributions from individual interactions of its type across the whole molecule or system. This collective sum provides a comprehensive view of the forces acting within a molecular system, which is essential for predicting molecular behaviour through simulation (Karplus & Petsko, 1990).

$$U_{total} = \sum U_{bonds} + \sum U_{angles} + \sum U_{dihedrals} + \sum U_{vdW} + \sum U_{electrostatic} \quad (10)$$

Molecular mechanics uses these physical principles to create a framework that allows for the efficient computational modelling of molecular structures and dynamics. By applying classical approximations to molecular systems, it provides valuable insights into the physical properties and behaviours of molecules, which are essential for fields ranging from chemistry and biology to materials science and pharmacology. Molecular mechanics, while powerful, has its limitations and cannot fully describe processes that are inherently quantum mechanical in nature, such as electronic transitions or bond formation/breaking (Kitaev et al., 2002). For these phenomena, a hybrid approach that incorporates quantum mechanics (QM/MM) is often used. This enables an accurate description of electron behaviour for parts of the system where such detail is necessary, while maintaining the computational efficiency of classical mechanics for the rest of the system.

7.2 Force Fields

Force fields are a critical component in molecular mechanics. They are essentially collections of equations and parameters that describe the potential energy of a system of atoms, providing a means to calculate the forces acting on the atoms and thus predict the molecular geometry,

physical properties, and reactions (González, 2011). Each force field has its own set of parameters tailored for specific kinds of molecules or interactions.

Several force fields are commonly used in molecular simulations to model the behaviour of atoms and molecules. The MM2 and MM3 force fields are some of the earliest force fields used predominantly for organic molecules (Allinger, 1977). They include parameters for bond stretching, angle bending, torsional angles, and non-bonded interactions, with values derived from both empirical measurements and quantum mechanical calculations. Next, AMBER, which stands for Assisted Model Building with Energy Refinement, is a force field frequently used to simulate biomolecules like proteins and nucleic acids. It is known for accurately representing hydrogen bonds and includes specialised parameters for various biomolecular components (Case et al., 2005). The CHARMM force field, which stands for Chemistry at HARvard Molecular Mechanics, is extensively used for biomolecules, providing detailed parameters for proteins, nucleic acids, lipids, and carbohydrates (Vanommeslaeghe et al., 2010). It is commonly used in drug discovery and biophysical simulations. GROMOS, the force field from the GRONingen MOlecular Simulation package, is tailored for simulating biomolecular systems in solution (Scott et al., 1999). It has a simpler functional form compared to CHARMM or AMBER, which allows for faster computational processing, making it ideal for large systems or long simulation durations. Finally, the Optimised Potentials for Liquid Simulations (OPLS) force field, is known for its broad applicability, including liquids, gases, and some biomolecules (Jorgensen et al., 1996). OPLS combines empirical data and theoretical findings to effectively model van der Waals forces and electrostatic interactions.

The OPLS-2005 force field represents a significant update, providing improvements in the accuracy of intermolecular interactions, particularly for liquid simulations (Jorgensen et al., 1996). It was optimised using a broad range of thermodynamic data, quantum chemistry, and experimental measurements. The enhancements in OPLS-2005 include more accurate modelling of hydrophobic interactions and the treatment of specific H-bonding and ionic interactions (Shivakumar et al., 2012). These advancements make OPLS-2005 suitable for studying complex organic systems, including those relevant to pharmaceutical research and material science. The development and refinement of force fields are ongoing processes, with each new version aiming to address limitations and expand applicability to different molecular systems. In the case of OPLS-2005, its robust parameter set, and broad validation make it a versatile tool for a wide array of molecular mechanics simulations.

7.3 Protein Conformational Dynamics

Molecular dynamics (MD) simulations are computational methods that model the movements of atoms and molecules based on classical mechanics. At the core of MD simulations is Newton's second law of motion which is used to predict how atoms and molecules will move over time (Eq. 1). In practice, MD simulations apply force fields such as AMBER, CHARMM, or OPLS to determine the forces on each atom, based on their positions and the parameters of the force field (González, 2011). The simulation unfolds in small increments, updating the positions and velocities of atoms at each step according to the calculated forces.

Before conducting the main MD simulations, the system undergoes equilibration, which includes minimising energy and gradually heating to the desired temperature (Ormeño & General, 2024) (Tuckerman & Martyna, 2000). Proteins are often constrained initially to allow lipids and water to settle into a natural configuration around them. Once equilibrated, the production phase begins, where the movement of the protein, lipids, and water is simulated over time, providing valuable data for analysis (Karplus & Petsko, 1990). Such analyses encompass a range of metrics, including RMSD, RMSF, RoG, and lipid order parameters, to examine the behaviour of the system. The trajectories from MD simulations are invaluable in providing a dynamic 3D picture of how membrane proteins function and interact with other molecules like drugs (De Vivo et al., 2016). This level of detail is often difficult to attain with experimental techniques alone, thus computational data from MD simulations is essential for interpreting experimental results and designing further experiments. MD remains an essential tool for the detailed study of biological systems.

In the context of MD simulations, RMSD, is a crucial measure that helps structural biologists understand how a molecule changes over time (Eq. 11). It calculates the average distance that selected atoms, often the backbone atoms of the protein (C_α), have moved from a set reference position (Carugo & Pongor, 2001). To perform this calculation, first a group of atoms is selected and then its position is recorded at a given time t , denoted as $r_i(t)$, and is compared to its position in the reference structure, r_i^0 . This comparison is made for all chosen atoms, which are typically numbered N in total.

$$RMSD(t) = \sqrt{\frac{1}{N} \sum_{i=1}^N (r_i(t) - r_i^0)^2} \quad (11)$$

The RMSD value is a single number that captures the overall deviation of the current conformation of the molecule from the reference conformation. It provides insight into the structural stability or flexibility of the molecule during the simulation. A small RMSD value indicates that the molecule has undergone little change, suggesting structural stability, while a larger RMSD value can indicate significant structural alterations, pointing to flexibility or conformational changes in response to environmental conditions or molecular interactions (Arnittali et al., 2019).

Root-Mean-Square Fluctuation, or RMSF (Eq. 12), is a statistical measure used in MD simulations to understand the flexibility of atoms within a protein or other molecular structures with reference to amino acid residues (Karplus & Kuriyan, 2005). By calculating the RMSF, regions of a molecule that are rigid and or more dynamic can be elucidated. RMSF measures how much the position of each individual atom varies around its average position over time. To calculate RMSF, the position of each atom i at every time point t throughout the simulation is tracked (Ghahremanian et al., 2022). These positions are then compared to the average position of the atom, denoted as $\bar{r}_i$ over the entire simulation. The fluctuation is calculated for all the time points T in the simulation. The higher the RMSF value for a particular atom or residue, the more it deviates from its average position, indicating greater flexibility or disorder in that region of the molecule. This information is particularly valuable in understanding protein dynamics, as regions with high RMSF values may be important for protein function, interaction with other molecules, or allosteric regulation.

$$RMSF(i) = \sqrt{\frac{1}{T} \sum_{t=1}^T (r_i(t) - \bar{r}_i)^2} \quad (12)$$

The radius of gyration (RoG) (Eq. 11) is a term used in MD to describe how a mass of a molecule is distributed around its centre of mass. It is a measurement of the compactness of the protein. Essentially, RoG is the mass-weighted root mean square distance of a group of atoms from the centre of mass of the molecule (Lobanov et al., 2008). To compute the RoG, each position and mass of each atom are considered. The position of atom i , denoted by r_i , and its mass, denoted by m_i , are used in the calculation, along with the position of the molecule's centre of mass, r_{cm} . By squaring the distance of each atom from the centre of mass, weighting it by the atom's mass, and then averaging it over the total number of atoms N , the radius of gyration provides a single value that gives us a sense of how stretched out or compact the molecule is. A larger radius of gyration implies a more extended molecule, while a smaller one indicates a

molecule that is more compact or folded (Lobanov et al., 2008). This measurement is particularly useful in understanding the folding patterns of proteins and the structural integrity of complex molecular assemblies.

$$R_g = \sqrt{\frac{1}{N} \sum_{i=1}^N |r_i - r_{cm}|^2} \quad (13)$$

To mimic real-world conditions, MD simulations control temperature and pressure through algorithms called thermostats and barostats, respectively (Karplus & Kuriyan, 2005). These tools ensure the simulated environment aligns with the desired physical conditions. Used in theoretical and computational chemistry, biophysics, and materials science, MD simulations offer insights into the dynamic properties and behaviours of molecular systems, providing a detailed view of the interactions and changes that occur within them over time (Nadendla, 2004; Zhao & Caflisch, 2015). For these simulations, the term "ensemble" refers to a specific set of macroscopic conditions that statistically define the state of a simulated system (Fig. 2.8). The concept of ensembles is borrowed from statistical mechanics, where an ensemble represents a large set of virtual copies of a system, considered all at once, each of which represents a possible state that the real system might be in (Tuckerman & Martyna, 2000). Using ensembles allows scientists to simulate how a system behaves under different controlled conditions. For example, in the canonical ensemble (NVT), temperature is a controlled variable, which is useful for simulating biological systems that naturally exist at a stable temperature. The microcanonical (NVE) ensemble, on the other hand, is used for simulating isolated systems, where no energy is exchanged with the environment. This can be useful for understanding fundamental properties of systems without the influence of external factors. For membrane proteins, which are embedded in the lipid bilayer of the cell, the isothermal-isobaric (NPT) ensemble is often the preferred choice (Merz Jr, 1997). This ensemble keeps the number of particles, pressure, and temperature constant, allowing the volume to fluctuate. This is advantageous for simulating membrane proteins as it enables the lipid bilayer to adapt its area and volume naturally, mimicking the dynamic physiological environment where the pressure and temperature are consistent, but the shape and size of the membrane can change. This flexible simulation context provided by the NPT ensemble is essential for understanding the function and structure of membrane proteins in realistic conditions. Moreover, each ensemble type provides a framework within which the MD simulation is conducted, and this framework determines how the properties like energy, pressure, volume, and temperature are treated during the simulation.

They are essential for ensuring that the simulation accurately reflects the real-world conditions under which the system being studied would normally exist.

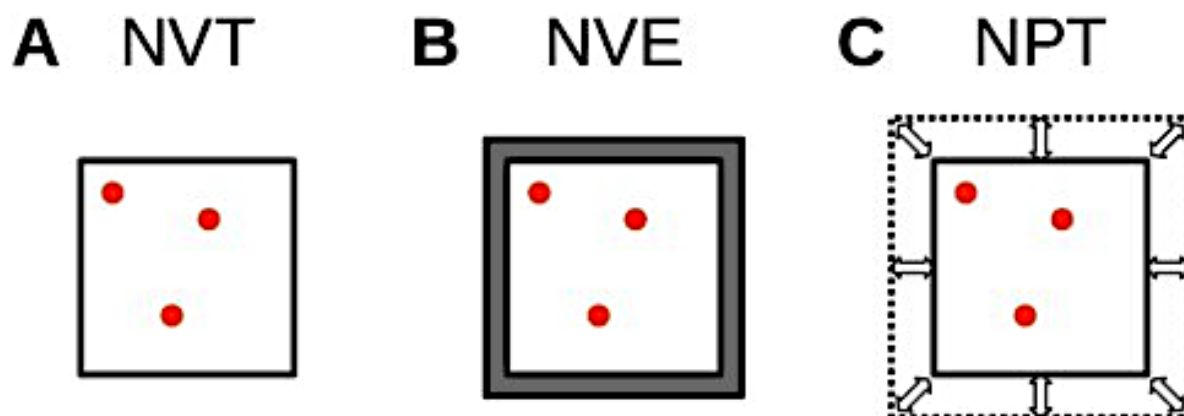


Figure 2.8: Illustration of thermodynamic ensembles used in molecular dynamics simulations. A. NVT ensemble (canonical), where the number of particles (N), volume (V), and temperature (T) are held constant. B. NVE ensemble (microcanonical), in which the number of particles (N), volume (V), and energy (E) are conserved. C. NPT ensemble (isothermal-isobaric), where the number of particles (N), pressure (P), and temperature (T) are maintained constant, allowing the volume to fluctuate to maintain pressure. The dashed lines in C. represent the ability of the system to change volume. Sourced from (van Gils et al., 2023)

When conducting MD simulations for membrane proteins, such as ion channels or GPCRs, the proteins are typically embedded within a lipid bilayer (Fig. 2.9) that replicates their natural cellular environment which is vital in terms of structural dynamics and protein stability (Merz Jr, 1997; Shahane et al., 2019). POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) is a phospholipid commonly selected for these bilayers due to its unsaturated fatty acid chain, which offers a balance of fluidity that is akin to natural biological membranes, making it suitable for general studies of membrane protein dynamics (Spooner & Gale, 2015). Other phospholipids like DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and POPG (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol) have different properties. For instance, DPPC has saturated fatty acid chains, resulting in a more rigid bilayer, which is representative of the pulmonary surfactant in lung tissue, hence its frequent use in respiratory system studies. POPG, carrying a net negative charge, and POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), which forms a more conical shape, are not typically used alone for GPCR simulations because they can significantly alter the membrane properties like charge and curvature, which may not accurately reflect the native lipid environment of GPCRs. Selecting the appropriate lipid composition is crucial for accurately simulating the behaviour of membrane proteins within their lipid environment (Shahane et al., 2019).

To investigate membrane proteins, such as ion channels or GPCRs, within MD simulations, it is crucial to integrate them into a lipid bilayer, replicating their natural cellular environment. POPC is frequently chosen for this purpose because it resembles the bilayer found in cells (van Meer & de Kroon, 2011). Surrounding the POPC bilayer and embedded protein with a water box, typically using the TIP3P model, ensures necessary hydration (Jorgensen et al., 1983). This model represents water molecules with three sites, effectively simulating the oxygen (O) and hydrogen (H) atoms of water.

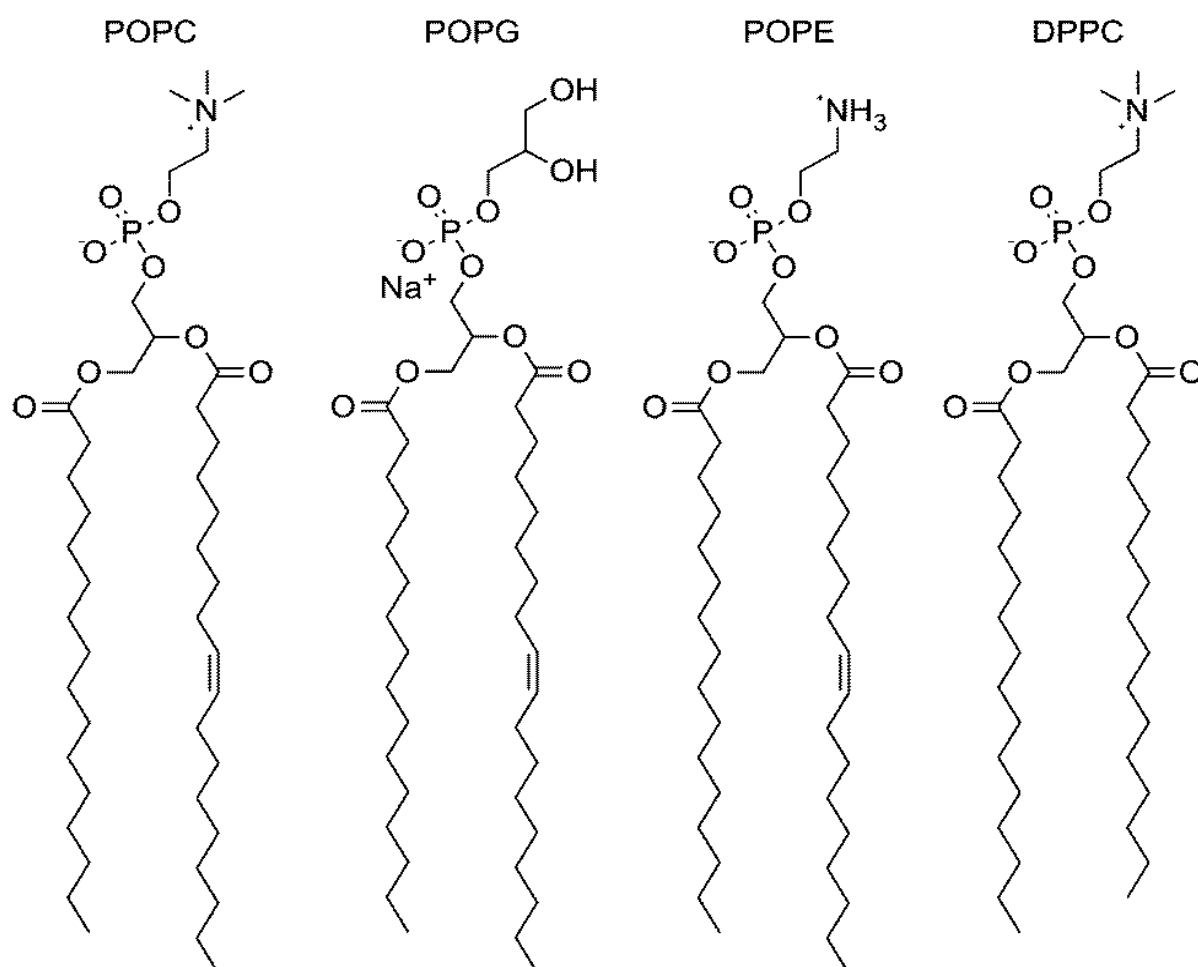


Figure 2.9: Chemical structures of common phospholipids found in biological membranes: POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine), POPG (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine). Each structure is depicted with its head group, glycerol backbone, and fatty acid tails, highlighting the variations in head group composition that contribute to the diversity of membrane lipid properties. Sourced from (Spooner & Gale, 2015).

8 BINDING FREE ENERGY ESTIMATION

Thermodynamics is fundamentally linked to computational biology, particularly in processes like docking, HTVS, MM/GBSA calculations, and MD simulations. Each of these computational techniques relies on thermodynamic principles to predict and understand molecular interactions and stability. The fundamental thermodynamic metric computed during docking is the free energy of binding (ΔG) (Eq. 14). It is a measure of the spontaneity of the ligand binding to the target and is estimated through docking scores which reflect the enthalpic and entropic contributions ($T\Delta S$) to binding (Dragan et al., 2017).

Molecular docking utilises scoring functions—mathematical algorithms—to predict the binding affinity between a ligand, and a receptor. These functions aim to determine the fit of the ligand within a binding site of the protein, which correlates with its potential to bind effectively (Wani et al., 2022). The calculations mainly involve enthalpy (ΔH), which includes energy changes from intermolecular forces such as hydrogen bonding and van der Waals forces. Enthalpic factors are more straightforward to compute and traditionally form the focus of many scoring functions. Additionally, entropy (ΔS) is considered, representing the change in disorder when a ligand binds to a protein. This binding event generally reduces entropy, as it constrains the movement of the ligand and possibly the protein. However, factors like solvation and conformational changes introduce complexities to entropy calculations, making them more challenging to accurately quantify (Verteramo et al., 2019). However, the Molecular Mechanics/Generalised Born Surface Area (MM/GBSA) and Molecular Mechanics/Poisson–Boltzmann Surface Area (MM/PBSA) approaches provide a deeper, more nuanced computation of binding free energy (Ghahremanian et al., 2022). These methods account for both enthalpic and entropic contributions, factoring in the crucial influence of solvation effects to render a detailed thermodynamic profile of molecular interactions (Zhang, 2022).

The enthalpy (Eq. 15) is determined by the internal energy of the ligand is represented by $E_{internal}$, which encompasses the energy within the molecule itself. The electrostatic and van der Waals energies are denoted by $E_{electrostatic}$ and E_{vdW} , respectively, indicating the energetic contributions from ionic interactions and induced dipole attractions between the ligand and its environment. The solvation free energy (Eq. 16), $G_{solvant}$, accounts for the ligand's interaction with the solvent, and is divided into polar and nonpolar parts. The polar component, G_{polar} , is calculated using either the Generalised Born (GB) or Poisson–

Boltzmann (PB) equations based on the selected method. The non-polar contribution, $G_{nonpolar}$ is typically estimated from the solvent accessible surface area (SASA) of the ligand, reflecting the energetics related to the molecular surface exposed to the solvent.

$$\Delta G = \Delta H - T\Delta S \quad (14)$$

$$\Delta H = E_{internal} + E_{electrostatic} + E_{vdW} + G_{solvent} \quad (15)$$

$$G_{solvent} = G_{polar} + G_{non-polar} \quad (16)$$

8.1 Induced-Fit Docking

Induced fit docking (IFD) addresses a key challenge in the field of molecular docking by elucidating the interactions of proteins with ligands. Traditional docking methods, often referred to as rigid docking, operate under the assumption that both the ligand and the receptor are rigid bodies (Laddach et al., 2018). This "lock and key" model (Fig. 2.10) assumes that the ligand fits into the receptor exactly as a key fits into a lock, without either changing shape (Sherman, Beard, & Farid, 2006). However, this approach does not accommodate the inherent flexibility of proteins and often fails to predict binding accurately when large conformational changes are involved (Koshland Jr, 1995). IFD is a more nuanced computational technique that allows both the ligand and the receptor to adapt their conformations during the docking process (Fig. 10). Additionally, ligand flexibility is captured by exploring different conformational states within the rotational space using docking software, while the IFD algorithm specifically focuses on optimising the residues of the receptor to better adapt to the binding orientation of the ligand.

This process can be understood by comparing it to rigid docking which sees both the ligand and the receptor as unchanging shapes, making it quick and suitable for screening many compounds (Desta et al., 2020). However, it may not find the right binding modes if the real binding process involves changes in shape. The potential advantage of IFD is that it can mimic the natural adaptability seen in biological interactions. This makes it more realistic, as ligand binding often makes the receptor change its shape to fit the ligand (Herschlag, 1988). By considering these changes, IFD can more accurately predict how and how well the ligand and receptor fit together and which residues of the protein interact with the ligand, which is important for developing new drugs. Moreover, IFD models are often more congruent with experimental data from

techniques like X-ray crystallography or NMR spectroscopy, especially when significant conformational changes occur during ligand binding. This congruence provides researchers with a clearer understanding of how drugs will function within the body.

Rigid Docking



Induced-Fit Docking

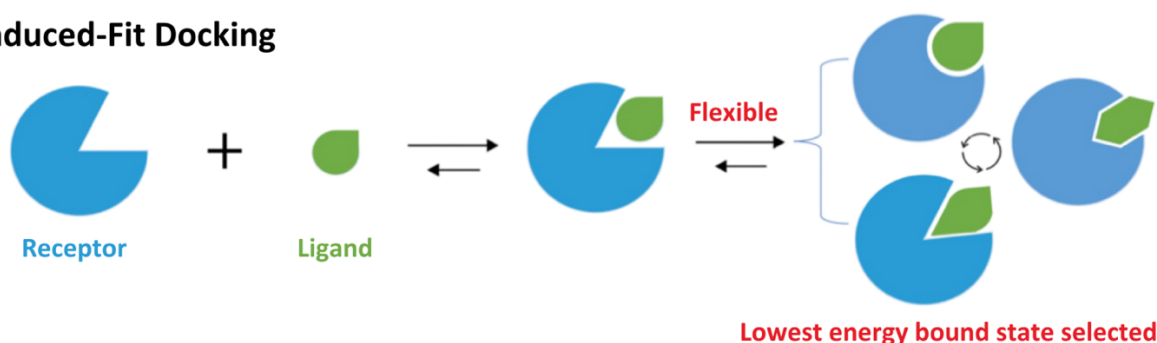


Figure 2.10: Comparison of rigid docking and induced-fit docking models. Rigid docking illustrates the interaction between a receptor and a ligand without structural changes in either molecule, symbolising a lock-and-key model. Induced-fit docking demonstrates a more dynamic interaction where the receptor and ligand undergo conformational adjustments to achieve an optimal binding state, with the lowest energy bound state being selected. This represents a more accurate model of molecular recognition in biological systems. Adapted from (Yang et al., 2022) using Canva.

The fundamental principle behind IFD is that the binding site of the receptor is not a static entity but can adapt its shape to accommodate the ligand in a more energetically favourable manner. Grid-based Ligand Docking with Energetics (Glide) is a particular docking software developed by Schrödinger that performs ligand-receptor docking (Friesner et al., 2004). It uses a grid to represent the active site of the receptor and evaluates how a ligand fits into this site based on various energetic parameters (Friesner et al., 2004). Glide can be used for rigid docking, where the protein is held fixed, or it can be incorporated into an IFD protocol, allowing for receptor flexibility to better predict how a ligand will interact with a protein in a more realistic dynamic environment. Therefore, IFD represents a powerful approach in molecular docking, particularly well-suited for systems where the conformational changes of a receptor play a critical role in ligand binding.

8.2 High-Throughput Virtual Screening

High-Throughput Virtual Screening (HTVS), Standard Precision (SP), and Extra Precision (XP) are various tiers of accuracy and computational intensity used in docking simulations to screen large libraries of compounds against a particular target, such as a receptor protein. These methods vary in their thoroughness and computational demand, with HTVS being the fastest and least computationally expensive, and XP being the most detailed and costly in terms of computational resources (Vilar et al., 2011; Willems et al., 2020). These different levels are often used in a funnel-like approach, starting with HTVS to narrow down the number of potential candidates and moving towards more precise methods like SP and XP for the most promising compounds. HTVS is designed to rapidly evaluate many compounds. It uses a simplified scoring function and limited conformational sampling for each ligand (Vilar et al., 2011). The goal is to identify potential hits from large libraries by quickly eliminating compounds with poor docking scores. The compounds that pass this initial screening are then subject to more rigorous docking protocols. After HTVS, compounds that show promise are often re-evaluated using SP docking. This method uses a more accurate scoring function and allows more extensive sampling of ligand conformations. SP docking can provide a better approximation of the binding affinities of the ligands for the receptor and offers a more reliable ranking of the compound library. XP docking is used for a detailed analysis of the most promising candidates from SP docking. It involves an even more accurate scoring function and extensive sampling of both ligand and receptor flexibility. XP docking can account for subtle changes in ligand and receptor conformations upon binding, water displacement effects, and entropic contributions. Due to its computational intensity, XP is typically applied to a much smaller set of compounds (Vilar et al., 2011).

In all three processes, docking scores are used to rank the ligands based on their predicted affinity for the target receptor. These scores are derived from the force field calculations and consider various interaction energies such as hydrogen bonding, hydrophobic interactions, electrostatic interactions, and desolvation effects. The MM/GBSA method is a post-docking approach used to estimate the free energy of binding of ligand-receptor complexes with a higher level of accuracy. After docking, selected complexes can be further refined using MM/GBSA, which is a popular choice for re-scoring docked complexes due to its balance between computational efficiency and accuracy (Zhang, 2022). MM/GBSA scores provide a more nuanced picture of the binding interaction than standard docking scores because they offer a

more comprehensive calculation of the free energy changes associated with ligand binding. Therefore, HTVS, SP, and XP provides a tiered approach to virtual screening, enabling the efficient filtration of compound libraries to identify potential leads. Docking scores from these methods help prioritise compounds for further study, while MM/GBSA scores can offer more detailed insights into the energetics of binding, informing the subsequent stages of drug development.

9 COMPUTATIONAL BIOLOGY IN *CANNABIS* RESEARCH

Computational methods are used to understand the dynamics of CBRs. For instance, they can model how receptors change shape when they bind to different cannabinoids, or how these changes might trigger downstream effects within the cell (Jiang, 2008). By simulating different systems, hypotheses can be generated about how cannabinoids exert their effects, which can then be tested experimentally. Moreover, molecular mechanics can assist in the design of cannabinoids that have improved properties, such as greater selectivity for a particular receptor subtype or reduced ability to cross the BBB, to minimise psychoactive effects. By leveraging computational biology and molecular mechanics could dramatically accelerate the pace of cannabinoid research, allowing for the rational design of novel cannabinoids with desired therapeutic profiles, thereby overcoming some of the current challenges faced by researchers in the field (Willems et al., 2020).

The computational exploration of CBRs has markedly advanced the frontier of neuropharmacology, unravelling the intricate molecular interplay these receptors mediate within the CNS and peripheral tissues. Such investigations have utilised a suite of computational techniques, including docking, MD simulations, homology modelling, and virtual screening, to illuminate the structural dynamics, ligand interactions, and therapeutic potentials of these receptors. Many studies have contributed to our current understanding of CBRs, highlighting their findings and implications for cannabinoid research and therapeutic development both computationally and experimentally. One example is the study of CBD with CB₁ receptor (Laprairie et al., 2015), which employed MD simulations to examine the interaction mechanisms between the CB₁ receptor and the cannabinoids CBD and Δ^9 -THC and endocannabinoids. This study revealed distinct binding modes and interactions for CBD and Δ^9 -THC, shedding light on the differential effects these compounds exert on CB₁ receptor dynamics (Laprairie et al., 2015). Such insights are crucial for understanding the psychoactive

properties of Δ^9 -THC in contrast to the non-psychoactive, therapeutic profile of CBD, offering a molecular basis for their pharmacological distinctions.

In parallel, the structural and functional elucidation of the CB₂ receptor has been significantly advanced through computational studies. A homology model of the CB₂ receptor to identify key interaction sites for non-classical cannabinoid agonists (Dhopeshwarkar & Mackie, 2016). Through computational docking and mutagenesis, they identified critical amino acids essential for ligand binding and receptor activation, enhancing our understanding of the role of the CB₂ receptor immunological responses and its therapeutic potential. Additionally, novel CBR receptor ligands were determined using virtual screening which represents a significant stride in cannabinoid therapeutics (Vilar et al., 2011). This research utilised virtual screening and molecular docking to sift through a vast compound library, unearthing several promising candidates for CB₁ receptor modulation (Aviz-Amador et al., 2021). These ligands hold potential for treating conditions signifying the power of computational methods in discovering new therapeutic agents.

Collectively, these studies exemplify the diverse computational strategies employed in cannabinoid receptor research, from dynamic simulations that capture the fluid nature of protein-ligand interactions to virtual screening that expands the horizon of potential therapeutic compounds. The insights garnered from *Cannabis* studies not only deepen our comprehension of CB₁ and CB₂ receptor function but also pave the way for the development of novel cannabinoid-based therapies. As these techniques continue to evolve and converge with experimental approaches, the full therapeutic potential of cannabinoid receptors becomes an achievable goal, signalling a new era of precision medicine with cannabinoid-based treatments. With the intersection of computational research and policy, the insights obtained are vital for shaping regulations surrounding the development and therapeutic use of cannabinoids. By providing robust scientific evidence, computational biology can inform safe and ethical cannabinoid development, ensuring responsible implementation within therapeutic contexts. Hence, the advancement of computational methodologies stands at the forefront of cannabis research, essential for unlocking the therapeutic promise of cannabinoids and guiding their integration into clinical practice and regulatory frameworks.

Exploring the Evolutionary Trajectory and Functional Landscape of Endocannabinoid Receptors: A Comprehensive Bioinformatic Analysis

	1 Endocannabinoid Receptors	2 Sequence Extraction	3 Structural Data Mining	4 Bioinformatic Analysis
Endocannabinoid Receptor 1	UniProtKB: P21554 52,858 Da GPCR: CNR1 472 Amino Acids 52,858 Da	Amino Acid Sequence UniProt PDB PROTEIN DATA BANK Primary structure Secondary structure Tertiary structure Quaternary structure α-helices β-sheets		Gene Ontology Evolutionary Tree
Endocannabinoid Receptor 2	UniProtKB: P34972 39,681 Da GPCR: CNR2 360 Amino Acids 39,681 Da	PDB: 5UO9 PDB: 5ZTY	Biological and Molecular Function Analysis Sequence Alignment	

The bioinformatics analysis of CBRs, CB₁ and CB₂ reveals a detailed picture of their structure, evolution, and physiological significance within the endocannabinoid system (ECS). The study highlights the evolutionary conservation of these receptors evidenced by sequence alignments across diverse species including humans, amphibians, and fish. Both endocannabinoid receptors (CBRs) share a structural hallmark of seven transmembrane (TM) helices, characteristic of class A G-protein-coupled receptors (GPCRs), which are critical for their signalling functions. The study reports a similarity of 44.58 % between both CBR sequences, which suggests that while their evolutionary paths and physiological roles may differ, there is considerable conservation in their structures. Pathway databases like KEGG, Reactome, and WikiPathways were employed to determine the involvement of the receptors in various signalling pathways. The pathway analyses integrated within this study offer a detailed view of the CBRs interactions within a complex network of cannabinoid-related signalling pathways. High-resolution crystal

structures (PDB ID: 5U09 for CB₁ and 5ZTY for CB₂) provided accurate structural information, showing the volume and surface area of the receptors binding pocket, essential for ligand interaction. The comparison between these receptors' natural sequences and their engineered pseudo-CBRs (p-CBRs) showed a high degree of sequence identity, confirming the validity of using p-CBRs in receptor-ligand interaction studies. This comprehensive analysis enhances the understanding of the structural and functional dynamics of cannabinoid receptors, highlighting their physiological roles and their potential as therapeutic targets within the ECS.

1 INTRODUCTION

G-protein-coupled receptors (GPCRs) constitute the most expansive family of membrane receptors, playing a pivotal role in human physiology and pathophysiology. These receptors can be activated by an array of ligands, from photons and ions to small molecules and proteins, precipitating a domino effect of cellular responses. Characterised by their seven-transmembrane (TM) domain structure, GPCRs have the remarkable ability to activate heterotrimeric G proteins, which then modulate a variety of intracellular signalling pathways (Hanson et al., 2012). Given their implication in a multitude of diseases and disorders, GPCRs have become a focal point for medical research and drug discovery. Their pharmacological significance is further highlighted by the fact that a vast proportion of marketed drugs target GPCRs, offering therapeutic solutions for conditions as varied as cardiovascular and metabolic diseases to mental health disorders (Hauser et al., 2017; Ibsen et al., 2017).

To understand the significant role of GPCRs, it is essential to look at the basics of cellular signalling – the complex system that manages all essential activities of life. Cellular signalling is an intricate and vital mechanism for the survival and proper functioning of living organisms (Cascieri et al., 1996; Ibsen et al., 2017). It involves a complex network of signals and responses that allow cells to communicate and adapt to their ever-changing environment. Signalling pathways are the communication conduits through which cells regulate gene expression, modulate enzymatic activity, and orchestrate developmental processes. Initiated by ligand-receptor interactions, these pathways trigger a sequence of biochemical reactions leading to various cellular responses, such as growth, division, or apoptosis. Dysregulation of these pathways can result in diseases, highlighting the importance of precise control in cellular signalling systems (Gómez et al., 2008; Tuteja, 2009).

This complex communication network is exemplified in the ECS, a key player in the regulation of cellular signalling and homeostasis. The ECS epitomises the critical role of cellular signalling in maintaining homeostasis (Garcia-Arencibia et al., 2019). It modulates numerous physiological processes through a complex network of receptors, endogenous ligands, and enzymes. ECS signal transduction is initiated by the binding of endocannabinoids to CBRs, which are part of the GPCR subset, eliciting a spectrum of cellular responses from neurotransmitter release to inflammation and metabolic regulation (Garcia-Arencibia et al., 2019; Gómez et al., 2008). This delicately balanced signalling system highlights the ECS role in maintaining equilibrium across various bodily systems, illustrating the influence of cellular communication on overall health.

The ECS operates through its specialised receptors, each playing a distinct role in modulating these vital cellular communications. Within the ECS, the CB₁ and CB₂ receptors are vital for cellular signalling and homeostasis (Graham et al., 2009). CB₁ receptors, predominantly located in the brain and CNS, are critical in modulating neurotransmitter release and neuronal activity. They influence functions such as cognition, memory, and pain perception. CB₂ receptors, although also present in the brain, are more abundant in immune cells and are involved in regulating immune responses and inflammation (Graham et al., 2009; Howlett, 2002; Mackie, 2008). Together, CB₁ and CB₂ receptors are key mediators in the ECS, orchestrating a vast array of physiological processes through their specific signalling pathways.

The significance of CB₁ and CB₂ receptors extends beyond their physiological roles, presenting a frontier for bioinformatics research that holds promise for innovative therapeutic applications. The study of CB₁ and CB₂ receptor bioinformatics is essential for advancing research in areas such as molecular docking and MD simulations (Bayat, 2002). Their prominent role in the physiological effects of cannabinoids, which are active compounds in *Cannabis*, makes them particularly relevant in today's context of a growing *Cannabis* market with the discovery of novel natural and SC (Holt et al., 2022). A deep understanding of how these receptors interact with various cannabinoids is crucial for determining their therapeutic potential and facilitating the development of new pharmacological interventions. Given the significant involvement of these receptors in key biological processes, modulating their activity with novel cannabinoids could offer targeted approaches for conditions such as pain, inflammation, anxiety, and seizure disorders, potentially revolutionising the medical landscape (Hauser et al., 2017; Mackie, 2008; Reggio, 2010).

The purpose of this study was to systematically characterise and perform analyses of CB₁ and CB₂ using bioinformatic tools, to serve as a repository for information that assists when performing computational studies using crystal structures of CBRs. Through data mining, sequence analysis, and the mapping of biological pathways and gene ontologies (GO), the research was designed to unravel the structural details, evolutionary history, and functional attributes of these receptors. This study aimed to identify conserved domains and patterns within receptor sequences, thereby establishing a comprehensive framework that elucidated their potential physiological impacts when targeted by ligands. By providing a detailed bioinformatics compendium on CBRs, this research can facilitate future studies into receptor-ligand dynamics, aiding in the development of computational models and therapeutic strategies that harness the physiological implications of cannabinoid interactions.

2 MATERIALS AND METHODS

2.1 Evolutionary History and Phylogenetic Exploration

To comprehend the evolutionary history of CBRs, an evolutionary tree was constructed. This analysis was predicated on the conserved domains identified within the CBR sequences using the NCBI Conserved Domain Search (CD-Search) service (Lu et al., 2020; Marchler-Bauer et al., 2017; Sayers et al., 2022). The amino acid sequences retrieved from UniProtKB (CB₁ ID: P21554) (CB₂ ID: P34972) were submitted to CD-Search, which utilised default settings and databases such as Protein families database (Pfam), Simple Modular Architecture Research Tool (SMART), Clusters of Orthologous Groups (COG), Protein Kinase Resource (PKR), and The Institute for Genomic Research (TIGRFAM) (Altschul et al., 1997; Altschul et al., 2005; UniProtKB, 2023; Wang et al., 2023). An Expect Value threshold of 0.01 was set for detecting domains with significant homology. The Reverse Position-Specific Basic Local Alignment Search Tool (RPS-BLAST) algorithm compared the sequences against conserved domain models, outputting a list of domains with E-values and graphical domain architectures. The evolutionary tree was generated using the Fast Minimum Evolution method and the Grishin distance metric, specific for protein sequences. This phylogenetic representation clarified the relationships between mammalian species and highlighted the extensive research on the CB₁ receptor, particularly concerning human evolution. The cladogram depicted various mammalian groups, from rodents to primates, highlighting the evolutionary significance of the CB₁ receptor. Together, these methodologies provided a foundation for bioinformatics research, allowing for the detailed study of protein function, especially regarding receptor-ligand

interactions. They contributed to elucidating the structural basis of receptor function and informed the development of targeted therapies to modulate receptor activity.

2.2 Sequence Extraction and Analysis

The CBRs were examined by extracting their amino acid sequences from the UniProtKB database. These sequences were then subjected to homology searches using the National Centre for Biotechnology Information (NCBI) BLASTp algorithm, version BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005). The non-redundant protein sequences (nr) database was selected for its extensive collection of sequences from various sources, including GenBank, SwissProt and Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), enhancing the possibility of identifying homologous sequences and providing an evolutionary framework for the receptors (Berman et al., 2000). Sequences of CB₁ and CB₂ were entered into the NCBI BLASTp search input field, applying filters to remove models and sequences from uncultured or environmental samples to refine the search results. The BLASTp algorithm, set with a high threshold for interaction confidence, was used to identify significant matches, indicating potential homology and functional relationships (Bayat, 2002; Sayers et al., 2022; Zhang & Wang, 2023).

Upon initiating the BLASTp search, the resulting alignments were analysed based on sequence similarity to identify conserved domains and regions, which was essential for establishing evolutionary relationships between CBRs and predicting their functional domains (Bayat, 2002). Particular attention was paid to the conservation of specific amino acid residues that may signify regions of functional importance across different species and between the two receptors. The BLASTp results were sorted by score and E-value, with lower E-values denoting more statistically significant alignments. Alignments were visualised using tools from NCBI, highlighting conserved amino acids to infer structural and functional relationships. The substitution scoring system utilised matrices such as BLOcks SUBstitution Matrix at the 62 % similarity level (BLOSUM62) and standard gap penalties. Positive scores from these matrices indicate amino acid substitutions that occur more frequently in aligned protein sequences with similar functions than by chance, suggesting functional acceptability (Altschul et al., 1997; Altschul et al., 2005).

2.3 Protein Feature Examination

To elucidate the primary features and post-translational modifications (PTMs) of CBRs, comprehensive data mining was conducted using repositories such as UniProtKB, RCSB PDB, and InterPro (Berman et al., 2000; Paysan-Lafosse et al., 2023; UniProtKB, 2023). This process involved querying the databases with the receptors names. The UniProtKB database provided extensive information on protein sequences, structure, function, and PTMs, while PDB contributed data on the three-dimensional (3D) structures of proteins. InterPro offered insights into the protein families, domains, and functional sites, enriching the understanding of protein characteristics. Extracting this data was crucial for revealing the molecular makeup and diversity of the receptors. The significance of this step lies in establishing a foundational understanding of protein structure and function, which is vital for investigating how they interact with various ligands.

2.4 Protein-Protein Interaction and Gene Ontology Evaluation

The study of CBRs protein networks entailed a detailed protein-protein interaction (PPI) analysis utilising the STRING database, which is known for its extensive compilation of known and predicted protein interactions (Mering et al., 2003; Szklarczyk et al., 2021). By using the full STRING network, it allowed the study to capture a comprehensive overview of both physical and functional interactions. Analytical parameters were stringently set, with interaction scores fixed at a high threshold of 0.900 and the visualisation restricted to a maximum of 10 direct interactors in the first shell. This strategic limitation was imposed to maintain focus on the primary interactions and to eschew the potential noise from peripheral associations. The inclusion of diverse sources of interaction evidence, such as text mining, experimental results, and gene co-occurrence, ensured the construction of a robust PPI network (Szklarczyk et al., 2021).

Exploring the GO terms associated with the proteins in the PPI network provided a comprehensive overview of the biological processes, molecular functions, and cellular components linked to the CB₁ and CB₂ receptors (Aleksander et al., 2023; Ashburner et al., 2000). The analysis was based on the count in the network—contrasting the number of proteins annotated with specific GO terms against the background dataset. The strength of enrichment, expressed as $\text{Log}_{10}(\text{observed}/\text{expected})$, quantified the extent of overrepresentation of GO terms within the network. In this expression, a positive log ratio suggests a meaningful

enrichment, which is considered "good," indicating a significant biological process or function is at play. Conversely, a negative log ratio would indicate underrepresentation, which may be less immediately biologically interesting. Meanwhile, the False Discovery Rate (FDR), corrected for multiple testing through the Benjamini-Hochberg procedure, validated the statistical significance of these observations. An FDR value below a typical threshold of 0.05 or 0.01 would be "good," signifying a high level of confidence in the results and a low probability of the findings being false positives due to random chance in the multiple tests performed. This integrative approach to PPI and GO analysis is essential for unravelling the intricate web of interactions that govern biological functions, with a low FDR reinforcing the validity of the observed enrichment in the network (Aleksander et al., 2023; Ashburner et al., 2000; Szklarczyk et al., 2021). It is important in characterising the roles of CB₁ and CB₂ receptors, examining the gene ontologies they influence, and interpreting the pathways they engage with. Such an examination is critical not only for understanding how these receptors might influence various pathophysiological conditions but also for determining potential therapeutic targets. The findings derived from this analysis are pivotal for deepening our comprehension of the ECS functionality and propelling forward the frontier of drug discovery.

3 RESULTS

3.1 Evolutionary Relationships

In evolutionary biology, cladograms serve as insightful visual representations that map out the evolutionary relationships among various species (Kitching, 1998). The intricate details of the cladogram focusing on CB₁ (Fig. 3.1A), provides an indication into the widespread distribution and evolutionary conservation of CB₁ within the mammalian kingdom. The presence of the CB₁ receptor in diverse taxa, such as bats, rodents, carnivores, and even-toed ungulates, was clearly depicted, with varying numbers of leaves indicating the extent of studies or sequence variations within each group. For instance, the inclusion of six leaves under bats and 18 under whales, dolphins, and ungulates signals a considerable degree of research interest and genetic diversity in these taxa. This diversity highlighted the evolutionary adaptability of the receptor and suggested its pivotal role in the survival and development in these mammals.

Human CB₁ receptors were represented with particular emphasis, denoting a multitude of studies that reflect extensive research interest as there were multiple entries such as Chain R, CNR1 protein, and CNR1 synthetic construct. The close clustering of these receptors signifies

a high degree of sequence similarity, indicative of functional conservation. This diversity was not limited to humans alone; other primates such as the common chimpanzee, the western gorilla, and predicted receptors in the *Angolan colobus* and the sooty mangabey are also included. The evolutionary closeness of these species CB₁ receptors to those of humans suggested a shared heritage and functional conservation among primates. This trajectory suggested that the evolutionary journey of the receptors has been integral to the adaptation and survival of mammals, dating back to a common ancestor. Additionally, this cladogram did more than just trace the evolutionary lineage of the CB₁ receptor; it highlighted the essential role of the receptor in the evolutionary history of mammals and highlights the vast array of research dedicated to understanding its function and diversity, particularly within humans.

The second cladogram (Fig. 3.1B) traced the evolutionary history of CB₂ across various species, showcasing its divergence and distribution within the animal kingdom (Kitching, 1998). The evolutionary path of the human CB₂ receptor can be inferred from the positioning of the nodes and the associated leaves which represent the number of sequences within each taxonomic group. The proximity of human CB₂ sequences to those of other primates suggests a recent common ancestor, indicative of a relatively recent evolutionary divergence within this group. The presence of multiple sequences within the primate cluster highlights the conservation and potential variation of the CB₂ receptor within this group, suggesting that while the receptor is conserved, there are differences that have arisen through primate evolution. The human CB₂ receptors, represented by the highlighted nodes, are closely related to those of other mammals, as seen in the clustering of sequences from placental mammals. This suggested that the basic structure and function of the CB₂ receptor have been maintained throughout mammalian evolution, reflecting its essential role in physiological processes.

The tree branching patterns also suggest the evolutionary timeline and adaptation processes that the CB₂ receptor has undergone. Notably, the tree indicates that the CB₂ receptor has a broad distribution across different mammalian taxa, including rodents, carnivores, and ungulates, with each branch representing an evolutionary lineage that has adapted the CB₂ receptor to its specific ecological niche and physiological needs. Overall, the cladogram illustrated that the CB₂ receptor in humans shares a common evolutionary origin with those in other species, highlighting the interconnectedness of life and the shared molecular mechanisms that maintain the ECS across different organisms. The CB₂ receptor has been subject to evolutionary forces

that have conserved its critical functions while allowing for the diversity necessary for adaptation to various biological roles.

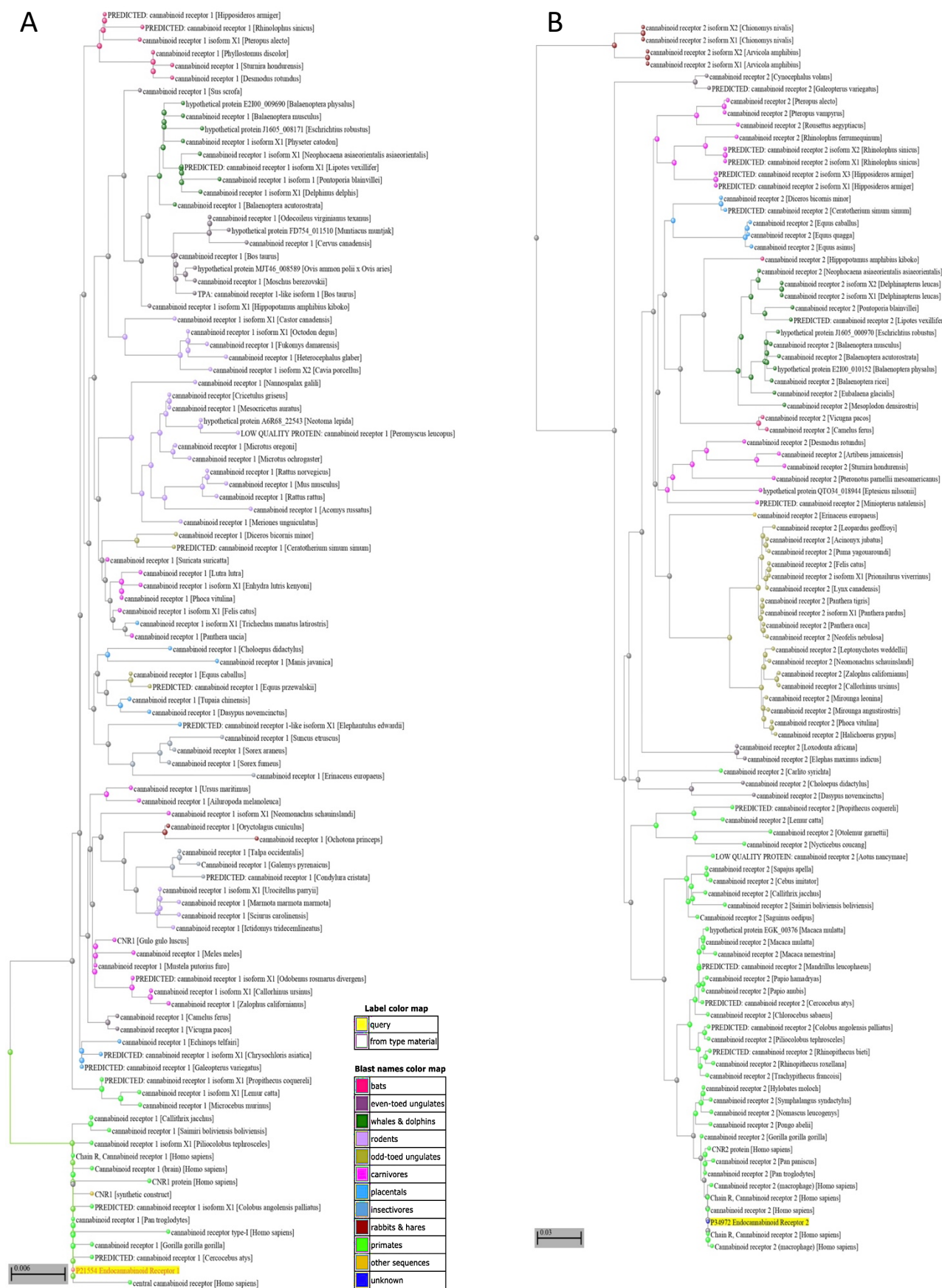


Figure 3.1: The cladogram A presented here provides a comprehensive view of the evolutionary relationships of CB₁ within various mammalian taxa. Each colour in the colour key corresponds to a specific taxonomic group, allowing for an intuitive understanding of the phylogenetic distribution. The use of the Fast Minimum Evolution method, paired with the Grishin distance metric for protein sequences, ensures an accurate representation of evolutionary distances with a maximum sequence difference of 0.85. The colours range from magenta, representing bats with six distinct CB₁ sequence studies, to light blue for the diverse 18 sequence studies of whales, dolphins, and even-toed ungulates, reflecting the breadth of research across species. Rodents are represented in green with a total of 17 leaves, denoting a significant focus on CB₁ receptor variations within this group. Carnivores are split across dark blue and red hues, indicating separate sub-groups within this category. Primates, including humans, are in green and the highlighted yellow is the sequence of interest UniProtKB ID: P21554 for CB₁, a prominent human-specific reference sequence. The colour-coding not only distinguishes different groups but also highlights the depth of research and diversity within each clade. The cladogram B. presented visualises the evolutionary divergence of CB₂ across a variety of species, employing the Fast Minimum Evolution method coupled with the Grishin distance metric for protein sequences. The evolutionary distances are calibrated with a maximum sequence difference set at 0.85, and the scale at the bottom, marked as 0.03, denotes the genetic distance between the nodes. The colour of each node corresponds to a specific taxonomic group, as indicated by the label colour map, aiding in the identification of evolutionary trends across different biological classifications. The red nodes pinpoint the initial query sequences, while the green nodes represent sequences from type materials, which serve as reference points or standards in taxonomic studies. The number of leaves at each node indicates the number of sequences within that taxonomic group, providing a view of the distribution and prevalence of CB₂ sequences among the groups. Notably, branches with the label "LOW QUALITY PROTEIN" indicate sequences that may not adhere to certain quality standards, alerting researchers to exercise caution when interpreting these particular data points. This serves as a map to trace the evolutionary path and the spread of CB₂ receptors, offering insights into the molecular evolution of this key component of the ECS. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005).

3.2 Conserved Domains

Figure 3.2 illustrates the comparative sequence alignment of the amino acid sequences for CB₁ and CB₂ across various species. The CB₁ alignment (Fig. 3.2.1) includes sequences from humans, African clawed frog, zebra finch, and torafugu (a type of pufferfish), focusing on conserved regions crucial for the receptors structure and function. These conserved domains are part of the 7tmA_CB₁ family (GenBank Accession Number: cd15340), a subset of class A GPCRs known for their seven transmembrane (TM) helices, which are essential for the structural and signalling functions of the CB₁ receptors (Eric W. Sayers, 2020; Hanson et al., 2012). Similarly, the CB₂ alignment (Fig. 3.2.2) encompasses sequences from humans, Norway rats, green sea turtles, and western clawed frogs, emphasising the conserved domains that are fundamental to the overall architecture and functionality of the receptor. These domains, identified as part of the 7tmA_CB₂ family (cd15341), are also characteristic features of class

A GPCRs (Eric W. Sayers, 2020). The accession number cd15341 in NCBI CDD specifically identifies the conserved domain unique to the CB₂ receptors (Lu et al., 2020; Marchler-Bauer et al., 2017). The alignments for both CB₁ and CB₂ highlight the evolutionary conservation of these receptors within the GPCR superfamily, reflecting their importance in maintaining receptor activity and integrity across diverse species.

For both CBRs the putative ligand-binding sites (Fig. 3.1), labelled as Feature 1 and highlighted in yellow, were inferred from their similarity to the antagonist binding site of the S1P1 receptor. The conservation of these sites suggests their critical role in the recognition and binding of ligands, which is required for the physiological operations of the receptor. Moreover, the seven TM helices, labelled TM helix 1 – TM helix 7 and depicted in shades of purple for CB₁ (Fig. 3.2.1) and pink for CB₂ (Fig. 3.2.2) respectively, define the structure of GPCRs. They traverse the cellular membrane and are essential for the role of the receptor in signal transduction from external to internal cellular environments. The evolutionary conservation seen in the alignment across various species highlights the TM regions significance in the biological functions of CBRs throughout vertebrates. The amino acids in these TM regions and ligand-binding sites are presumably critical for receptor interactions with specific ligands and signal transduction processes. Alterations in these conserved areas could affect receptor function, impacting its physiological significance and potential as a drug target.

Lastly, the alignment indicates functional domains within the CBRs receptor, possibly involving ligand binding, G-protein coupling, receptor dimerisation, and regulatory modifications such as phosphorylation (Cascieri et al., 1996). These domains are crucial for the receptors signalling mechanisms and interactions within the cell. Therefore, analysing this alignment provides insights into the structural and functional dynamics of CBRs, which is essential for further experimental validation and has significant implications for receptor signalling and the development of pharmacological interventions.

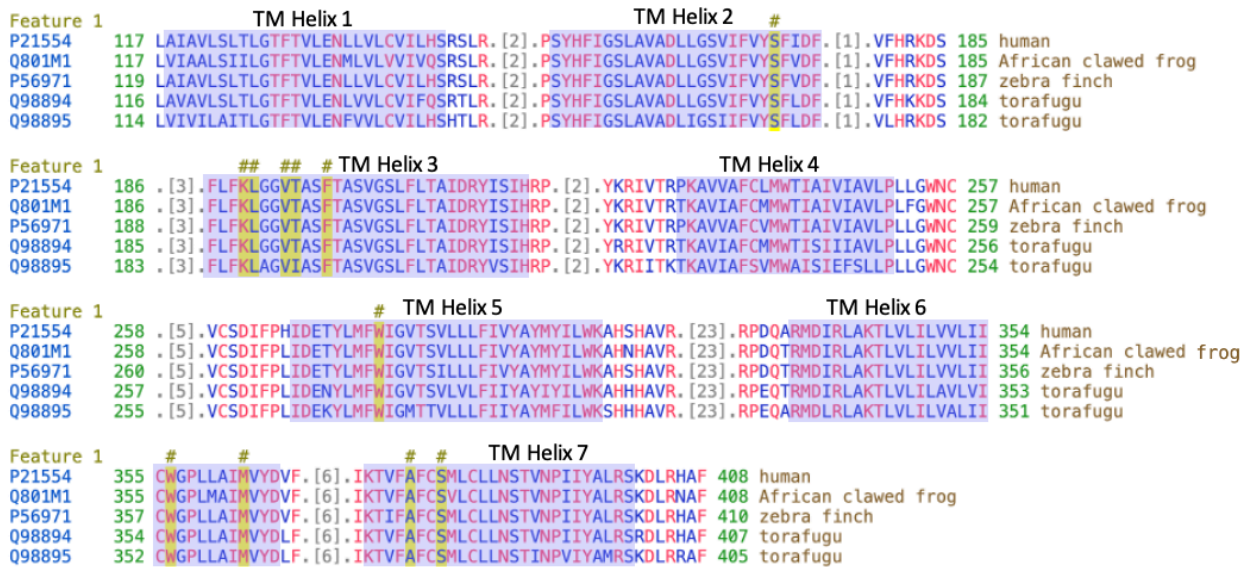


Figure 3.2.1: This figure displays the conserved domains of the CB₁ receptor as identified by a BLASTp search within the Conserved Protein Domain Family 7tmA_CB₁ (Accession number: cd15340). Highlighted in yellow are the regions representing Feature 1, the putative ligand binding sites, which are predicted based on sequence similarity to the S1P1 receptor's antagonist binding site. The seven TM helices, which are characteristic structural motifs of this receptor family, are indicated in purple. Each helix is sequentially numbered from TM helix 1 to TM helix 7, depicting the integral parts of the receptors seven-transmembrane helix domain. This alignment suggests evolutionary conservation of the structure of the receptor, particularly in the transmembrane regions crucial for its function. The sequence comparison spans across different species, including humans, African clawed frog, zebra finch, and torafugu, illustrating the preservation of the putative ligand binding site and seven TM helices domains across diverse taxa. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005).

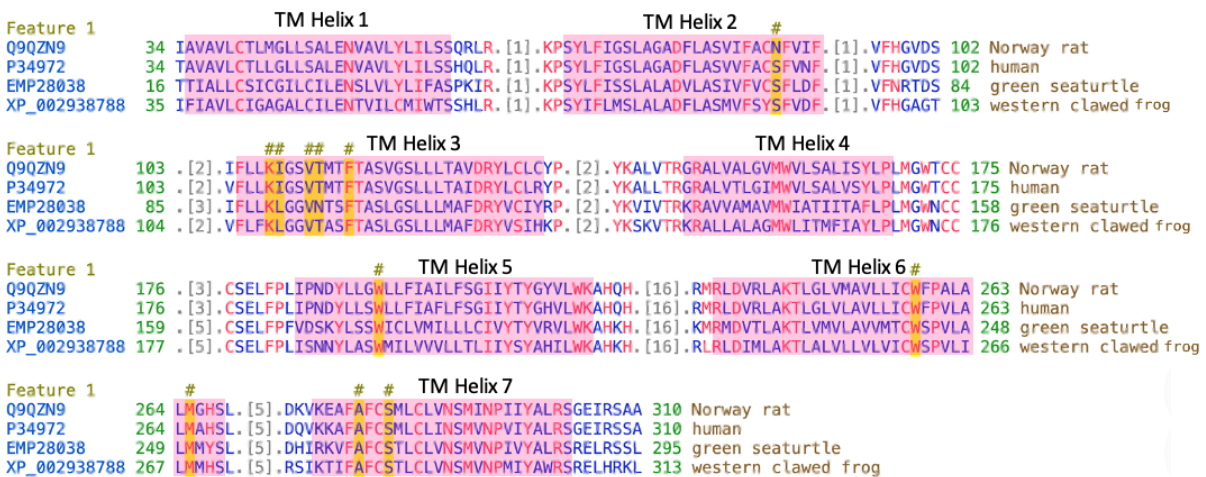


Figure 3.2.2: This figure illustrates the sequence alignment of the CB₂ receptor within the Conserved Protein Domain Family 7tmA_CB₂, (Accession number: cd15341). Highlighted in yellow are the regions corresponding to feature 1, the putative ligand binding sites, inferred from sequence similarity to the S1P1 receptor when bound to the selective antagonist ML56. Also depicted are the seven TM helices, labelled as TM helix 1 through TM helix 7 and coloured in various shades of pink. These helices represent a key structural motif found across the receptor family, playing an essential role in receptor function by spanning the cell membrane. The conservation of

these motifs across different species, as shown in the alignment, emphasises the evolutionary importance of the putative ligand binding site and seven TM domains in maintaining receptor activity and integrity. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005).

The alignment (Fig. 3.3.1) comparing FASTA sequences of both CBRs, as obtained from the UniProtKB database illustrated the similarities and differences between the amino acid sequences of the two receptors, which share a similarity of 44.58 %. An alignment score of 278 bits and an E-value of $1e-94$ highlight a significant level of similarity, pointing to a potential common evolutionary origin and possibly similar functions or structural characteristics. The alignment shows 148/332 identical amino acids, indicating conserved regions that are possibly crucial for the structural integrity and function of the receptors. Additionally, 207/332 amino acids are positively scoring substitutions. These are non-identical residues that are conservatively substituted and may maintain similar properties, such as charge, hydrophobicity, or size, which could be important for maintaining receptor function despite sequence variation. The presence of gaps in 26 out of 332 positions (7 % gaps) suggests that there have been insertions or deletions in the evolutionary history of these proteins. Such events can lead to structural variations that might affect the receptors ligand-binding properties or their interaction with other proteins.

The comparison of CB₁ and CB₂ receptor sequences suggests that while they have evolved to potentially have different physiological roles, there is a considerable degree of conservation. This conservation might reflect shared mechanisms of action, such as binding similar endogenous or exogenous ligands, or coupling to similar intracellular signalling pathways. Therefore, understanding these similarities and differences is crucial for designing selective drugs that target one receptor over the other, which is particularly important in the development of treatments for conditions where the ECS plays a significant role.

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1   MKSILDGLADTTFRITITDLLYVGSNDIQYEDIKGDMSKLGYPQKFPLTSFRGSPFQEKMTAGDNPQLVPADQVNITE  80
1   -----MEECWVTE  8

81  FYNKSLSSFKENEENIQCGENFMDIECFMVLNPSQQLAIAVLSLTGFTVLENLLVLCVILHSRSLRCRPSYHFIGSLA  160
9   IANGSKDGLDSNP-----MKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRKPSYLFFIGSLA  77

161 VADLLGSVIFVYSFIDFHVHFRKDSRNVFLFKLGGVTASFTASVGSFLTAIDRYISIHRLAYKRIVTRPKAVVAFCLM  240
78  GADFLASVVFACSFVNFHVHGVDSKAVFLLKIGSVMTFTASVGSLLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIM  157

241 WTIAIVIAVLPLLGNCEKLQSVCSDFPHIDETYLFWIGVTSVLLLFIVYAYMYILWKAHSHAVRMIQRGTQKSIIH  320
158 WVLSALVSYLPLMGWTC--CPRPCSELFPLIPNDYLLSWLLFIAFLFSGIITYTYGHVLWKAHQHVASL--SGHQDR----  229

321 TSEDGKVQVTRPDQARMDIRLAKTLVLILVLLIICWGPLLAIMVYDVFGKMNKLIKTVFAFCSMCLCLNSTVNPIIYALR  400
230 -----QVPGMARMRLDVR LAKTLGLVLAVLLICWFPVLALMAHSLATTLSDQVKKAFACSMCLCLINSMVNPVIYALR  302

401 SKDLRHAFRSMFPSCGTAQPLDNSMGDSCLLHKHANNAASVHRAAESCIKSTVKIAKVT-MSVSTDTSAEAL  472
303 SGEIRSSAHHCLAHWKCKVRGLGSEAKEE-----APRSSVTETeadgKITPWPDSRDLDLSDC  360

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Figure 3.3.1: A sequence alignment between CB₁ and CB₂, highlighting the conserved and variable regions across the two sequences. Amino acids (red) indicate identical matches between the sequences while residues (blue) represent different regions. Grey colouring denotes gaps or regions where alignment is not possible due to insertions or deletions. The overall similarity score of 44.58 % suggests a moderate level of conservation, indicative of shared evolutionary origins and possibly common functional characteristics between these two CBRs. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005).

Figures 3.3.2 and 3.3.3 provide sequence alignments for the CB₁ and CB₂ receptors, comparing their FASTA sequences from UniProtKB with their corresponding engineered pseudo-endocannabinoid receptors (p-CBRs) from PDB entries 5U09 and 5ZTY, respectively (Li et al., 2019; Shao et al., 2016). These alignments reveal high sequence identities of 99.53 % for CB₁ and 98.51 % for CB₂, signifying that the primary amino acid structures of the p-CBRs closely mirror those of the natural receptors. Conserved amino acids are highlighted in red, emphasising the structural integrity maintained in the p-CBRs which is critical for their functionality.

The term p-CBRs encapsulates the concept of these engineered receptors which, while highly representative of the natural receptors, include modifications to enhance stability and facilitate high-resolution structural studies. These changes are paramount when crystallising the receptors for in-depth studies, with the understanding that such modifications can influence the pharmacological profile in binding and functional assays. Therefore, these were rigorously tested post-crystallisation to validate the structures (Li et al., 2019; Shao et al., 2016). The alignments affirm the potential of p-CB₁ and p-CB₂ receptors as robust models for studying receptor-ligand interactions and as viable targets for the development of novel therapeutic agents aimed at regulating the ECS.

```

1   MKSILDGLADTTTFRITITDLLYVGSNDIQYEDIKGDMSKLGYPQKFPLTSFRGSPFQEKMTAGDNPQLVPADQVNITE 80
   -----G-----

81  FYNKSLSSFKEENEIQCGENFMDIECFMVLNPSQQLAIAVLSLTGTTVLENLLVLCVILHSRSLRCRPSYHFIGSLA 160
2   -----KENEENIQCGENFMDIECFMVLNPSQQLAIAVLSLTGTTVLENLLVLCVILHSRSLRCRPSYHFIGSLA 72

161 VADLLGSVIFVYSFIDFHVHRKDSRNVFLFKLGGVTASFTASVGSFLTAIDRYISIHRPLAYKRIVTRPKAVVAFCLM 240
73  VADLLGSVIFVYSFIDFHVHRKDSRNVFLFKLGGVTASFTASVGSFLTAIDRYISIHRPLAYKRIVTRPKAVVAFCLM 152

241 WTIAIVIAVLPLLGNCEKLQSVCSDFPHIDETYLMFWIGVTSVLLLFIYAYMYILWKA----- 301
153 WTIAIVIAVLPLLGNCEKLQSVCSDFPHIDETYLMFWIGVTSVLLLFIYAYMYILWKA----- 232

302 -----HSHA-VRMIQRG 312
233 KKSLLSKFGMDEGVTFMFIGRFDRGQKGVLDVLLKAIEILSSKKEFQEMRFIIIGKGDPELEGWARSLEKHGNNVKVITEM 312

313 TQKSII-----IHTSEDG----- 325
313 LSREFVRELYGSVDFVIIPSYFEPFGLVALEAMCLGAIPASAVGGLRDIITNETGILVKAGDPGELANAILKALELSRS 392

326 -----KVQVTRPDQARMDIRLAKTLVLILVVLIIICWGPELLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPI 395
393 DLSKFRENCKRAMSFSDQARMDIRLAKTLVLILVVLIIICWGPELLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPI 472

396 IYALRSKDLRHAFRSMFPSCEGTAQPLDNSMGDSDDLKHANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEAL 472
473 IYALRSKDLRHAFRSMFPSCEGTAQPHHHH-----HHHHH----- 508

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Figure 3.3.2: Sequence alignment of CB₁ and p-CB₁ receptors. This alignment compares the primary amino acid sequences of the UniProtKB (P21554) CB₁ receptor (top) with the PDB (5U09) p-CB₁ receptor (bottom). Identical residues are highlighted in red, indicating a high degree of similarity with 99.53 % identity between the two sequences and blue showing different regions. The dashes represent sequence gaps introduced to optimise alignment. The figure illustrates the remarkable conservation of the CB₁ receptor sequence in the p-CB₁ receptor, which is engineered to retain structural integrity for bioinformatics analysis and functional studies. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005).

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1  MEECWVTEIANGSKDGL---DSNPMKDYMILSGPQKTAVAVLCTLLGLLSALENAVLYLILSSHQLRRKPSYLFIGSLA 77
1  MK----TIIALSYIFCLVFAGAPPMKDYMILSGPQKTAVAVLCTLLGLLSALENAVLYLILSSHQLRRKPSYLFIGSLA 76

78  GADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTASVGSLLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIM 157
77  LADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTASVGSLLLTAIDRYLCLRYPPSYKALLTRGRALVLLGIM 156

158  WVLSALVSYLPLMGWTCPRPCSELPFLIPNDYLLSWLLFIAFLFSGIITYGHVLWKAHQHVASL----- 223
157  WVLSALVSYLPLMGWTCPRPCSELPFLIPNDYLLSWLLFIAFLFSGIITYGHVLWKAHQHVASNIFEMLRIDEGLRLK 236

224  -----SGHQDRQVPGM----- 234
237  IYKDTEGYTIGIGHLLTKSPSLNAAKSELDKAIGRNTNGVITKDEAEKLFNQDVDAAVRGILRNAKLKPVYDSLDAVRR 316

235  -----ARMRLDVRLAKTLGL 249
317  AALINMVFQMGETGVAGFTNSLRMLQQKRWDEAAVNLAWSRWYNQTPNRAKRVITTFRTGTWDAYARMRLDVELAKTLGL 396

250  VLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCMLCLINSMVNPVIYALRSGEIRSSAHHCLAHWKKCVRGLS--E 327
397  VLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCMLCLINSMVNPVIYALRSEIRSSAHHCLAHWKKCVRGLEFLE 476

328  AKEEAPRSSVTETeadGKITPWPDSRDLDLSDC 360
477  VLFQGPHHHHHHHHH-----DYKDDDDK-- 500

```

Figure 3.3.3: Sequence alignment of CB₂ (UniProtKB ID: P34972) and p-CB₂ receptor (PDB ID: 5ZTY). This figure presents an alignment between the FASTA amino acid sequence of the CB₂ receptor, as sourced from UniProt (P34972), and the p-CB₂ receptor derived from the PDB entry 5ZTY. Residues that are conserved between the two sequences are highlighted in red, signifying a sequence identity of 98.51 %. Grey gaps in the alignment, indicated by dashes, are introduced for maximum sequence alignment.

3.3 Characteristic Protein Features

The domain architecture of proteins is essential in defining their role in cellular processes. InterPro (IPR) classifications for CBRs and associated proteins shed light on their specific structural and functional characteristics within the cell (Paysan-Lafosse et al., 2023). Considering the structural specifics, the GPCR rhodopsin-like domain (IPR000276), which is shared among five proteins: Dopamine Receptor D2 (DRD2), CB₁, CB₂, Adenosine A2a Receptor (ADORA2A), and G protein receptor 55 (GPR55). These proteins belong to the GPCR superfamily, more precisely, the class A rhodopsin-like receptors. The presence of this domain, with a strength of 1.12 and an FDR of 0.0456, indicates a significant and reliable association. It points to common structural and functional traits among these proteins, such as the seven-TM domain architecture (Fig. 3.4.1), enabling them to respond to a variety of ligands, including light. This structural motif is further specified in the GPCR, rhodopsin-like, 7TM domain (IPR017452). This domain reiterates the seven-TM structure characteristic of these GPCRs. With the same proteins in focus and identical statistical measures, it shows their role in cellular signalling, facilitated by ligand binding and subsequent G-protein activation.

Upon further analyses of CBRs, it becomes evident that their distinct domain architecture, especially the cannabinoid receptor family domain (IPR002230), is what equips them to interact with specific ligands, revealing the intricacies of the ECS. This domain, found specifically in CBRs, highlights the unique structural features that these proteins possess, enabling the binding of cannabinoid molecules. The strength of 3.25 and FDR of 0.0122, against an observed gene count of 2 in a background of 2, suggests a highly significant association specific to the ECS.

Integral to receptor functionality, both CB₁ and CB₂ utilise a toggle switch (Table. 3.1) mechanism critical for their transition between active and inactive states, essential for downstream signalling. In their inactive states, these receptors are constrained in a conformation that prevents active signalling. The binding of an agonist induces conformational changes, leading to the "toggle" of these critical residues and facilitating the transition to the active state (Lucchesi et al., 2014). For CB₁, activation involves the synergistic movement of twin toggle switch residues Phe3.36 and Trp6.48, whereas CB₂ activation is governed by the single toggle switch residue, Trp6.48 (Ji et al., 2021). This difference shows the unique activation dynamics and signalling pathways of these related receptors. The tryptophan residue W6.48, located in the sixth TM domain and denoted by the Ballesteros-Weinstein numbering, acts as a pivot in the toggle switch, guiding the receptors shift from inactivity to its active form, which is capable of interacting with G-proteins or other effectors (Ji et al., 2021; Lucchesi et al., 2014). The precise operation of this switch is fundamental to the physiological functions mediated by CB₁ and CB₂, from neuromodulation to immune responses, reflecting the complexity and specificity of ligand-receptor interactions.

These IPR domain analyses point to the specialised roles that CBRs play, mediated by their distinctive domain structures that facilitate physiological responses to cannabinoids (Paysan-Lafosse et al., 2023). The association strengths and low FDRs across these domains attest to their significant physiological roles and potential as therapeutic targets. Understanding these contributions of the domains to the function of cannabinoid receptors is fundamental to grasping their mechanism of action and developing targeted interventions within the endocannabinoid signalling pathways.

The crystal structures (PDB ID: 5U09 for CB₁ and 5ZTY for CB₂) were selected based on their superior resolution available, ensuring the most accurate structural information for analysis. These structures, despite being p-CBRs due to the presence of stabilising modifications, their quality remains intact. The modifications were incorporated to enhance the stability of the

receptors during the crystallisation process, a common technique in structural biology (Li et al., 2019; Shao et al., 2016). The CB₁ receptor (Fig. 3.4.1), displayed a 3D conformation with seven (TM helices, each colour-coded for clarity. The N-terminal region was marked in red and the C-terminal in purple, providing a visual demarcation of the terminals of the receptor. Similarly, the CB₂ structure of the receptor (Fig. 3.4.2) demonstrated the same level of detail, with an emphasis on the TM helices and the key intracellular region. The modifications to these receptors, although potentially altering their natural state, were carefully chosen to not impact the key regions involved in ligand binding and G protein-coupling, preserving the functional aspects necessary for computational studies.

Table 3.1: Attributes of CB₁ and CB₂

Attributes	Endocannabinoid Receptor One (CB ₁)	Endocannabinoid Receptor Two (CB ₂)
UniProtKB ID	P21554	P34972
Species	<i>Homo sapiens</i>	<i>Homo sapiens</i>
Protein Type	GPCR	GPCR
Gene Name	CNR1	CNR2
Protein Name	Endocannabinoid receptor one (CB ₁)	Endocannabinoid receptor two (CB ₂)
Mass (Da)	52 858	39 681
Length	472 Amino acids	360 Amino acids
Toggle Switch	Phe3.36 and Trp6.48	Trp6.48

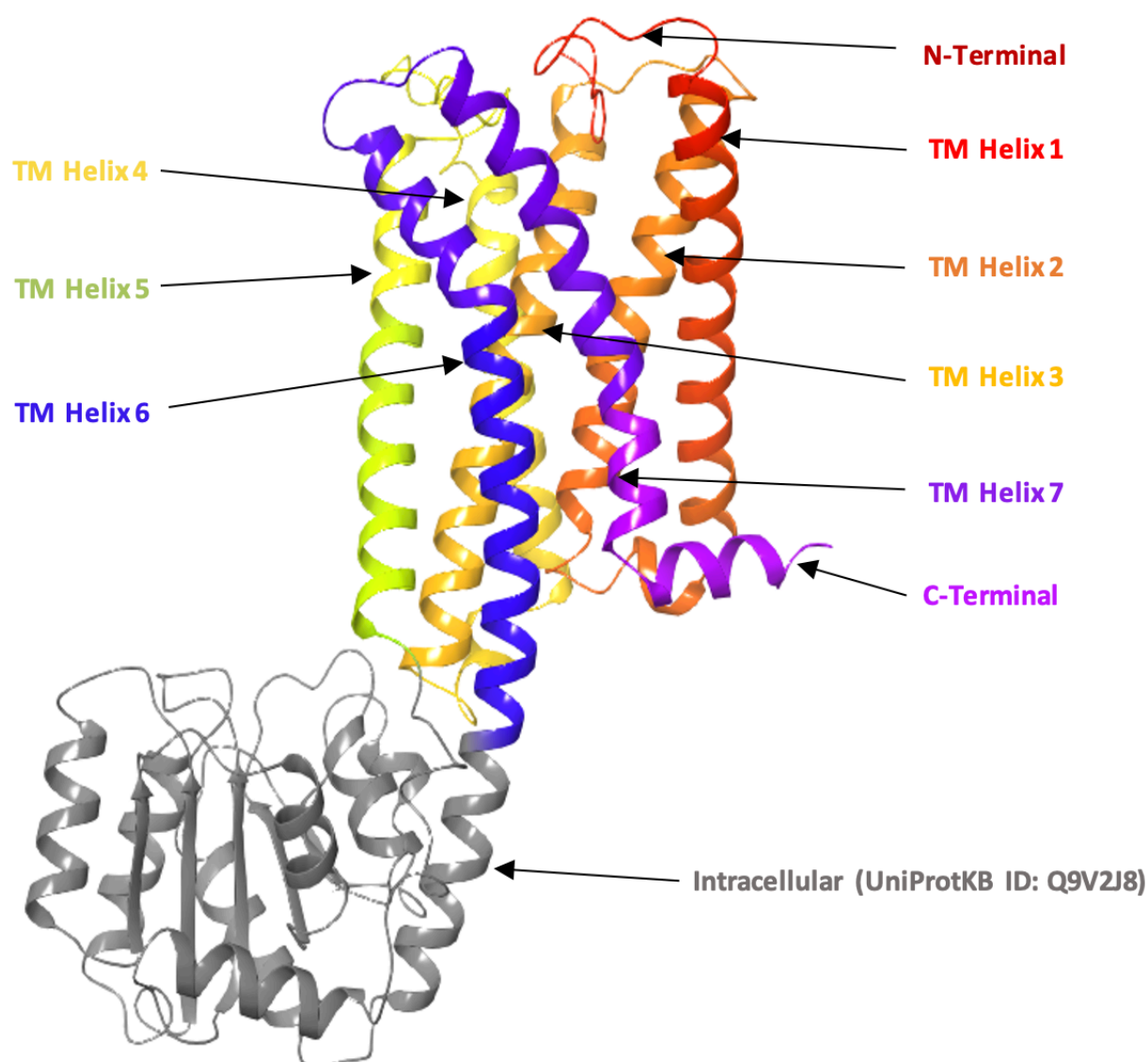


Figure 3.4.1: 3D Structural representation of the CB₁ receptor (PDB ID: 5U09). The receptor is shown with its seven TM helices, colour-coded to differentiate each helix. The N-terminal and C-terminal regions are indicated, with the N-terminal shown in red and the C-terminal in purple. An intracellular stabilising region (grey) contributes to the stability of this receptor structure.

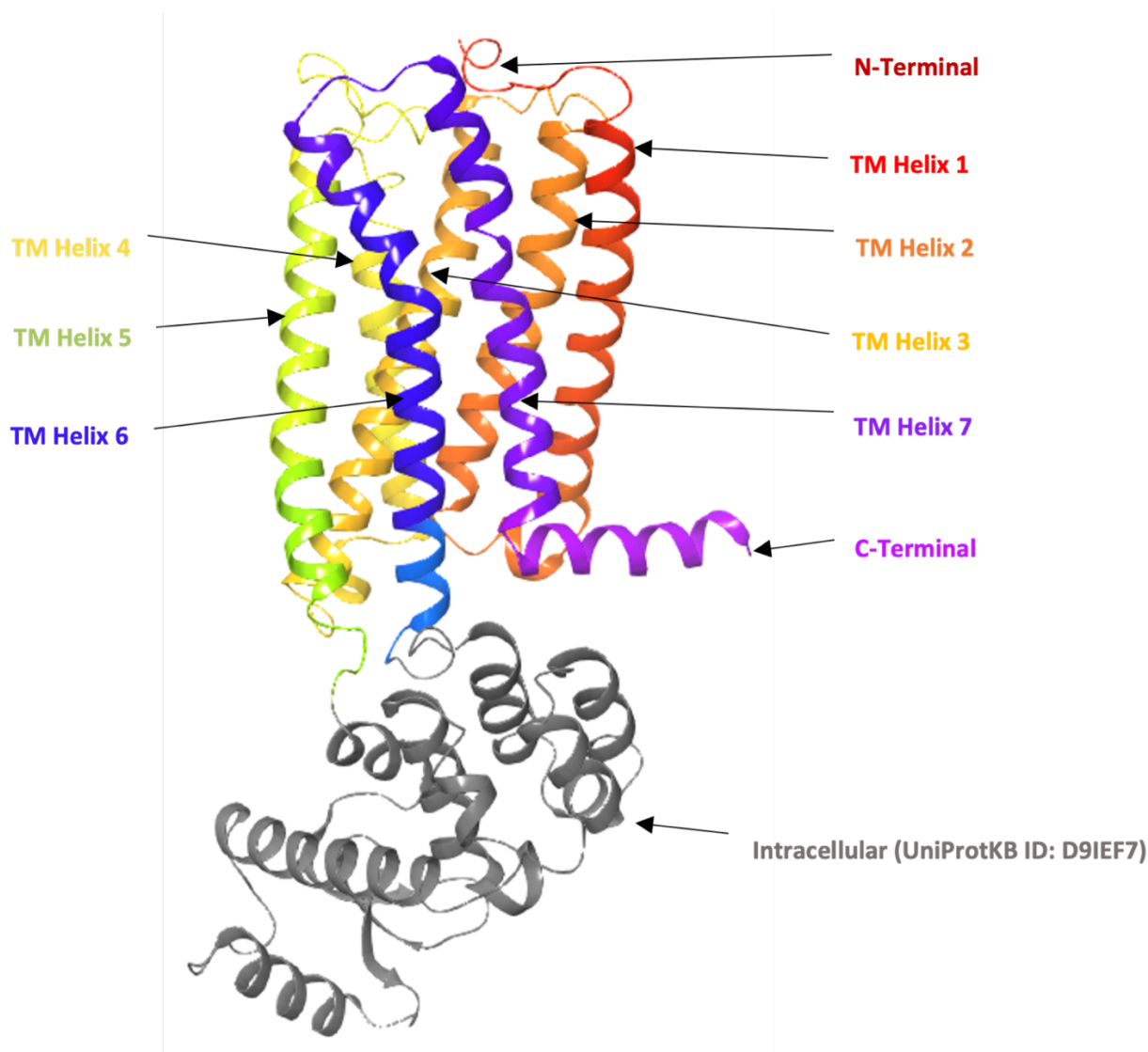


Figure 3.4.2: 3D Configuration of the CB₂ (PDB ID: 5ZTY). This representation features the seven TM helices that are fundamental to the receptor's structure, each helix presented in a distinct colour for visual clarity. The N-terminal region is shown at the top, transitioning through the helices to the C-terminal region at the bottom. The intracellular domain (grey), which contains a modification for stabilisation during the crystallisation process. In studies utilising these modified receptors, they can be referred to as p-CBRs, acknowledging the artificial stabilisation region.

The high-resolution crystal structure of the human CB₁ (Fig. 3.5) shows the surface area and volume of the binding pocket, which are crucial parameters for understanding how the receptor interacts with its ligands, including endocannabinoids, agonists, inverse agonists, and antagonists. The surface area of the binding pocket, at 624.481 Å², and a volume of 321.277 Å³, as identified by CASTp 3.0 with Pocket ID 1, suggest a spacious active site that can accommodate the binding of sizeable ligand molecules (Tian et al., 2018). The shape and charge distribution within this pocket are critical for the specificity and affinity of ligand binding. Since

CB₂ is not available on CASTp 3.0, analyses were inferred by analysing the CB₁ structure given their homology and shared functional roles within the ECS. Although the specific amino acid composition and the exact conformation of the binding pocket may differ between CB₁ and CB₂, the overall architecture of the GPCR family they both belong to often shares common features, such as the presence of TM helices surrounding a central ligand-binding pocket towards the N-terminal (Li et al., 2019; Shao et al., 2016).

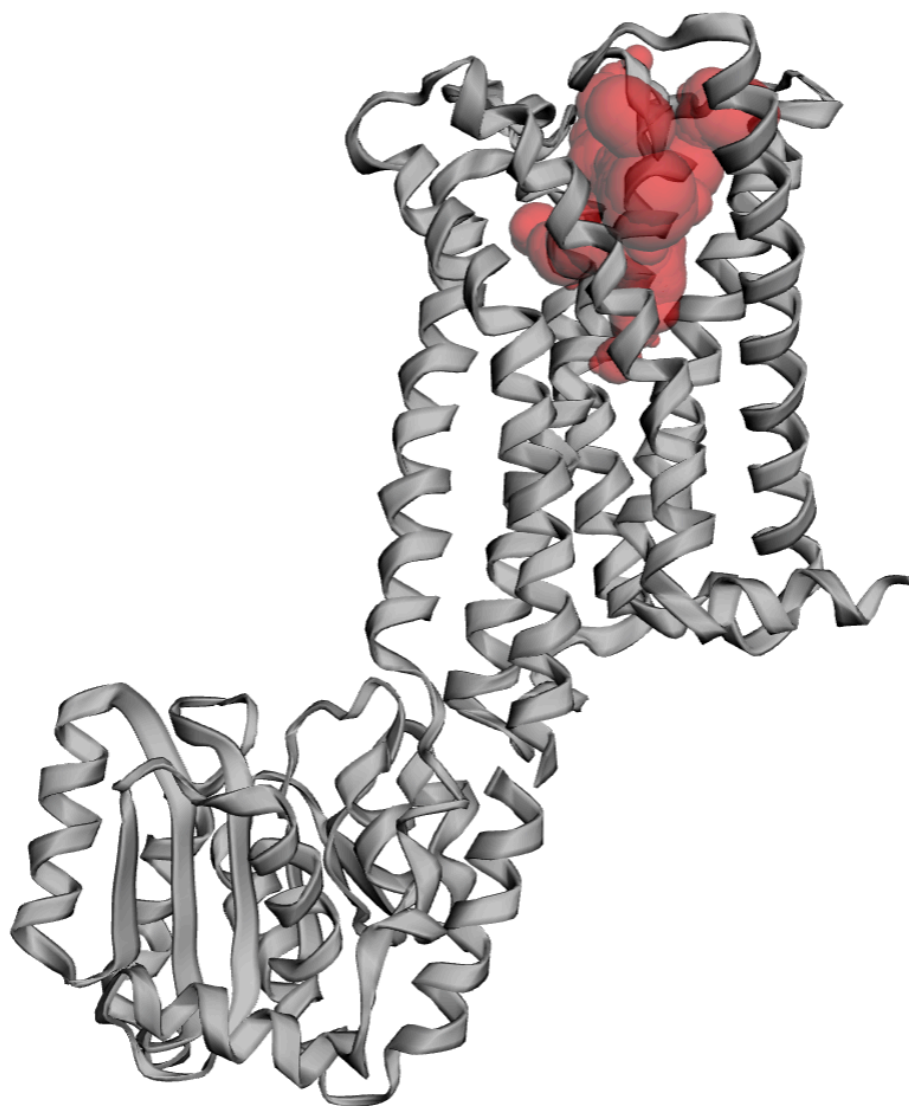


Figure 3.5: A high-resolution crystal structure of the human CB₁ receptor, highlighting its binding pocket in red. The binding pocket, as identified by CASTp 3.0 with Pocket ID 1, showcases a surface area of 624.481 Å² and a volume of 321.277 Å³, providing insights into the potential interactions within the active site of the receptor. Sourced from CASTp 3.0 (Tian et al., 2018).

3.4 Post-Translational Modifications

For the CB₁ receptor, the process of *N*-linked glycosylation occurs at asparagine residues 77 and 83. This form of glycosylation, which attaches a glycan chain to the nitrogen (N) atom on the side chain of asparagine, is essential for the proper folding, stability, and surface expression of the protein. The addition of *N*-linked glycans plays a pivotal role in the transport of receptors to the cell membrane, its ability to bind ligands, and its interactions with other cellular proteins. These glycosylation sites on the CB₁ receptor have been predicted through sequence analysis, which is a manual assertion indicating that the modifications are inferred based on the known amino acid sequence of the receptor (UniProtKB, 2023).

Next, phosphorylation at serine residue 316, as identified by PRIDE (PXD012174), plays a regulatory role in CB₁ receptor function (Perez-Riverol et al., 2022; UniProtKB, 2023). This post-translational modification (PTM) suggests potential impacts on receptor interaction with G proteins, its internalisation, or downstream signalling pathways. Since phosphorylation is a reversible process, it is crucial for the dynamic regulation of cellular signalling, often resulting in the activation or deactivation of protein functions. Additionally, phosphorylation at serine residues 425 and 429 is inferred by similarity (UniProtKB: P47746), meaning that analogous phosphorylation events have been observed in related proteins or in homologous proteins across different species such as *Mus musculus* (mouse). Such phosphorylation is presumed to be involved in receptor activation, desensitisation, or internalisation, thereby influencing the receptors signalling pathways. The 'by similarity' annotation indicates that these PTMs are predicted based on conserved phosphorylation sites seen in other proteins within the same family or functional group.

Lipidation at cysteine residue 415, more specifically S-palmitoylation, is a post-translational modification that involves the covalent attachment of a palmitate, which is a fatty acid, to the sulphur atom of the cysteine side chain (Oddi et al., 2012). This modification is known to increase the hydrophobicity of the receptor, which significantly affects its localisation within the cell membrane and can alter its interactions with signalling molecules. This lipidation process has been confirmed through experimental evidence and as indicated by manual assertions in scientific studies. The S-palmitoylation at cysteine 415 plays a crucial role in the functionality of the CB₁ receptor, particularly in its ability to localise within the membrane and its capacity for signal transduction (Oddi et al., 2012).

When analysing CB₂, the PTM of glycosylation at asparagine residue 11 is likely part of the receptor's extracellular domain, which could significantly affect the receptor's ability to recognise and bind ligands. This glycosylation site was identified through sequence analysis, suggesting that it is a manual assertion based on the known amino acid sequence of the protein, highlighting the targeted and specific nature of this modification in receptor functionality (UniProtKB, 2023). Phosphorylation of serine residues 335 and 336 in the CB₂ receptor has been documented through experimental evidence and supported by large-scale data, including UniProtKB (ID: P47936) and PRIDE databases (PXD012174), suggesting a conserved modification seen in related proteins, particularly *M. musculus* (Perez-Riverol et al., 2022). Similarly, threonine at position 338 is also phosphorylated, a modification that could regulate the receptor's structure and its interactions with G proteins and other signalling entities, thereby influencing signalling efficiency and specificity. Moreover, serine at position 352 undergoes phosphorylation, a PTM with direct links to the biological responses of the CB₂ receptor. This phosphorylation is involved in the regulatory mechanisms of the receptor, especially its responses to inverse agonists, and can significantly affect the receptors internalisation, desensitisation, and downstream signalling pathways (Bouaboula et al., 1999). These phosphorylation events collectively highlight the complex regulation of CB₂ receptor function and the complex network of intracellular signalling cascades in which it participates.

3.5 Protein-Protein Interactions

Figure 3.6.1 reveals insights into how CB₁ and its associated proteins contribute to cellular function and how they might be targeted for therapeutic purposes centring around CB₁. This network has 11 nodes, each representing a protein, and 25 edges that show the interactions between them. The filled nodes suggest these proteins have a known or predicted 3D structure, which can be important for understanding their function and interactions (Kovács et al., 2019; Szklarczyk et al., 2021). Empty nodes, on the other hand, represent proteins without a known 3D structure, potentially highlighting areas where further structural biology research might be needed. The colour coding in this network is used to differentiate between primary proteins and their direct interactors. The average node of the network degree is 4.55, which means that each protein, on average, interacts with 4.55 others. This is a relatively high degree of connectivity, suggesting that these proteins are likely to be part of a complex and highly interconnected system. The local clustering coefficient of 0.87 again indicates a compact cluster of interactions, which are typical for biological networks where proteins often function in groups or complexes.

Most notably, the PPI enrichment p-value of 0.000129 is highly significant. This means there is a very low probability that such a dense network would be seen by chance, which implies that the interactions are biologically meaningful. Therefore, this network represents a group of proteins that are functionally related and may work together in related pathways, such as those involved in signalling, metabolism, or cellular response related to the endocannabinoid system (Szkłarczyk et al., 2021).

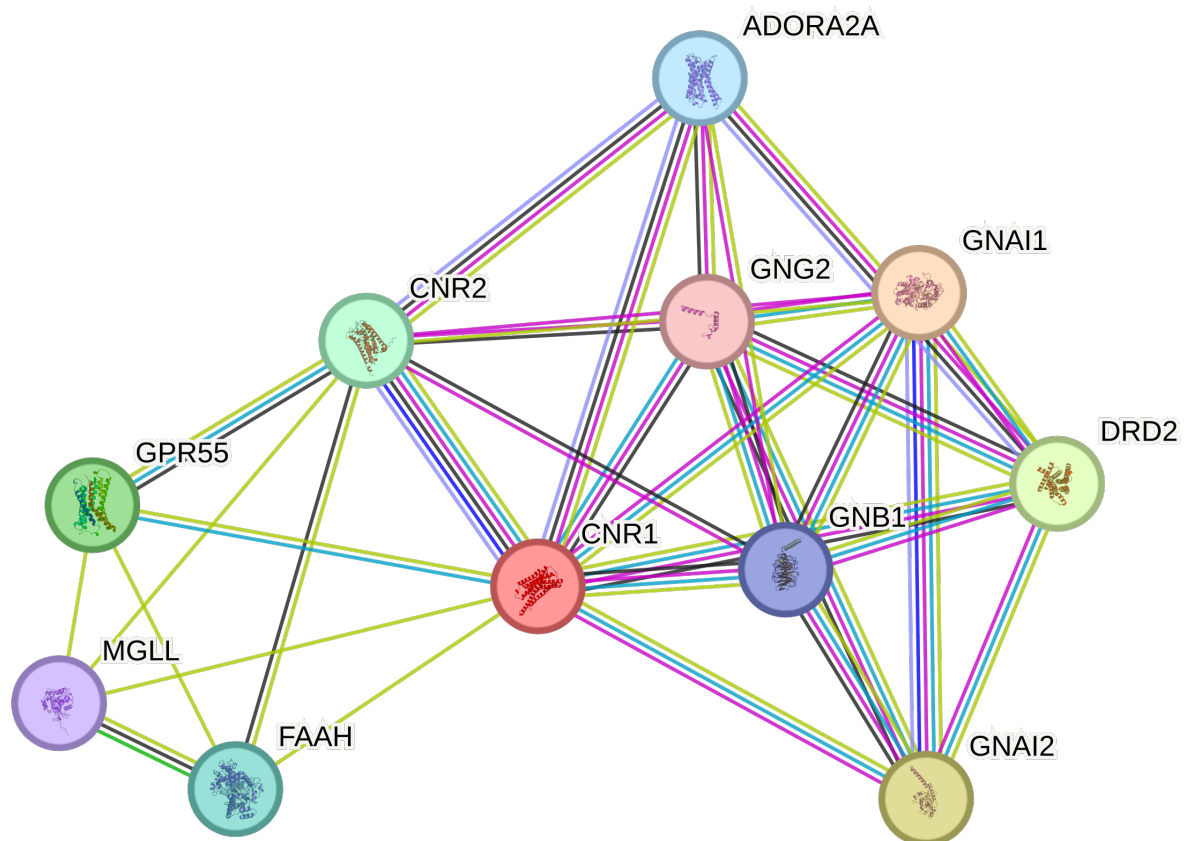


Figure 3.6.1: A protein-protein interaction network with CB₁ represented by its gene name CNR1 at its core. It features 11 nodes, each representing a protein. Proteins with a known or predicted 3D structure are depicted as filled nodes, while those without are empty. Coloured nodes represent the primary protein and its direct interactors. The network contains 25 edges that denote the interactions between proteins, which are more numerous than expected by chance, as indicated by a PPI enrichment p-value of 0.000129. This suggests a biologically significant clustering of interactions, implying these proteins may work together in related pathways. The average node degree is 4.55, with a high local clustering coefficient of 0.87, reflecting the network's dense connectivity.

Figure 3.6.2 depicts a PPI network. The nodes Guanine Nucleotide Binding Protein, Beta Polypeptide 1 (GNB1), CB₁, CB₂, Guanine Nucleotide Binding Protein, Alpha Inhibiting Activity Polypeptide 1 (GNAI1), Zinc Finger CCCH-Type Containing 12D (ZC3H12D) represent different proteins, and the edges (lines connecting the nodes) represent the

interactions between these proteins. The average node degree of 2.8 means that, on average, each protein interacts with ~ 2.8 other proteins in this network. This can be a measure of the complexity or connectivity of the network. The average local clustering coefficient of 0.9 is quite high, which typically indicates that proteins within the network are highly interconnected, forming compact clusters. This can be a sign of a functionally or physically interacting group of proteins, like a protein complex or pathway. An expected number of edges count of four suggests that if this network were random, only four interactions would be expected. However, this network has more than that, indicating a more complex interaction pattern than what would be expected by chance. However, the PPI enrichment p-value of 0.12 suggests that the observed number of interactions does not reach statistical significance by conventional standards, such as $p < 0.05$. Consequently, the detected interconnectivity may not be substantially greater than what could be expected from random association, which raises questions about the biological relevance of these links. Nevertheless, this lack of statistical significance does not necessarily invalidate the biological importance of the protein selection. It is possible that these proteins are simply not well-represented in current research, and as such, their interactions may not be adequately reported in the STRING database.

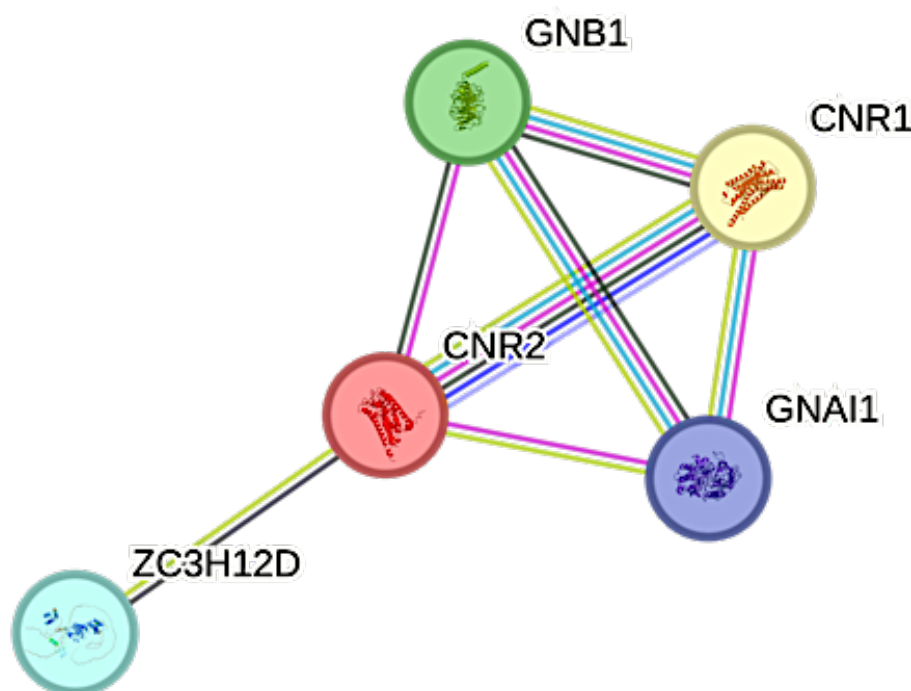


Figure 3.6.2: A protein-protein interaction network with five nodes and seven edges with CB_2 in the centre represented by its gene name CNR2. Each node represents a protein, and the edges indicate known or predicted interactions. The average node degree of 2.8 reflects the average number of connections per protein, and the high average local clustering coefficient of 0.9 suggests a close-knit group of interactions. An expected number of edges

count of four indicates the anticipated number of interactions in a random network of this size, but with a PPI enrichment p-value of 0.12. Source STRING Database

3.6 Tissue Specificity and Isoform Expression

The CB₁ receptor, known for its involvement in the CNS, is characterised by a wide expression pattern, with the highest levels found in both foetal and adult brain tissues (Reggio, 2010). This correlates with the extensive role that CB₁ plays in neural development and regulation of neuronal function. The high expression levels in the brain indicate that CB₁ receptors are crucial for a variety of brain functions, including cognition, memory, pain sensation, and motor control. Interestingly, the expression levels of isoforms two and three of the CB₁ receptor are significantly lower than those of isoform one (Reggio, 2010). Isoform one is thus suggested to be the predominant form active in the brain, which might indicate a more significant role in the physiological processes governed by this receptor (Ryberg et al., 2005). The presence of multiple isoforms could reflect a mechanism allowing precise receptor function or a means to diversify the physiological roles of CB₁ across different tissues and developmental stages. The tissue-specific expression of CB₁, particularly its dominance in the brain, highlights the potential of the receptor as a therapeutic target in neurological conditions, where modulation of CB₁ activity could lead to beneficial outcomes (Mackie, 2008). Understanding the differential expression of its isoforms also provides deeper insight into the nuanced regulation of CB₁ receptor function and its implications in health and disease.

The tissue specificity of the CB₂ indicates a preferential expression in cells related to the immune system, with notably higher levels found in B-cells and natural killer (NK) cells. This expression profile is aligned with the known involvement of the receptor in modulating immune responses. The presence of CB₂ in skin, particularly in the suprabasal layers and hair follicles, as well as in various lymphoid tissues like the tonsil, spleen, and peripheral blood mononuclear cells, emphasising its role in peripheral systems (Casanova et al., 2003; Liu et al., 2009). The reported absence of CB₂ expression in normal brain tissue suggests that, unlike CB₁, which is prevalent in the nervous system, CB₂ might play a more significant role outside the CNS (Benito et al., 2003). However, its expression in perivascular microglial cells and dorsal root ganglion sensory neurons indicates that CB₂ may still contribute to immune surveillance within the brain and sensory signalling, respectively (Anand et al., 2008; Matias et al., 2002; Núñez et al., 2004).

Furthermore, the existence of two CB₂ isoforms, resulting from alternative promoter usage and differing only in the 5' untranslated region (UTR), also points to the functional versatility of the receptor. Isoform CB2As predominant expression in the testis and some regions of the brain, compared to isoform CB2Bs distribution in the spleen and leukocytes, suggests differential regulation and potential functional specialisation of these variants in various tissues (Galiègue et al., 1995). This intricate expression pattern of CB₂, particularly its prominent role in immune cells, its peripheral tissue distribution, and its isoform variation, offers valuable insights for therapeutic targeting, especially for conditions that involve the immune system and peripheral tissues.

3.7 Cellular Components and Subcellular Location

The GO data for cellular components (Fig. 3.7) enrich our understanding of localisation of CB₁ and CB₂ receptors within cells and their pivotal roles in physiological processes (Aleksander et al., 2023; Binns et al., 2009). For the CB₁ receptor, its identification as an integral component of the presynaptic membrane (GOCC:0099056/GO:0042734), alongside DRD2 and ADORA2A, and its considerable strength of 1.86 and low FDR of 0.0025 suggest a significant role in modulating neurotransmitter release, indicating presynaptic inhibition as a crucial aspect of synaptic transmission regulation (Hua et al., 2020; Oddi et al., 2012; Shao et al., 2016). This is echoed in its presence in neuron projections (GO:0043005), which underlines its involvement in neuron communication and plasticity, essential for neurological functions (Ferreira et al., 2012). Furthermore, the CB₁ receptors localisation to the plasma membrane (GO:0005886), membrane (GO:0016020) and synapses (GO:0045202) accentuates its accessibility to extracellular ligands and its central role in neurotransmission and synaptic plasticity (Mendizabal-Zubiaga et al., 2016; Oddi et al., 2012; Sarnataro, 2005).

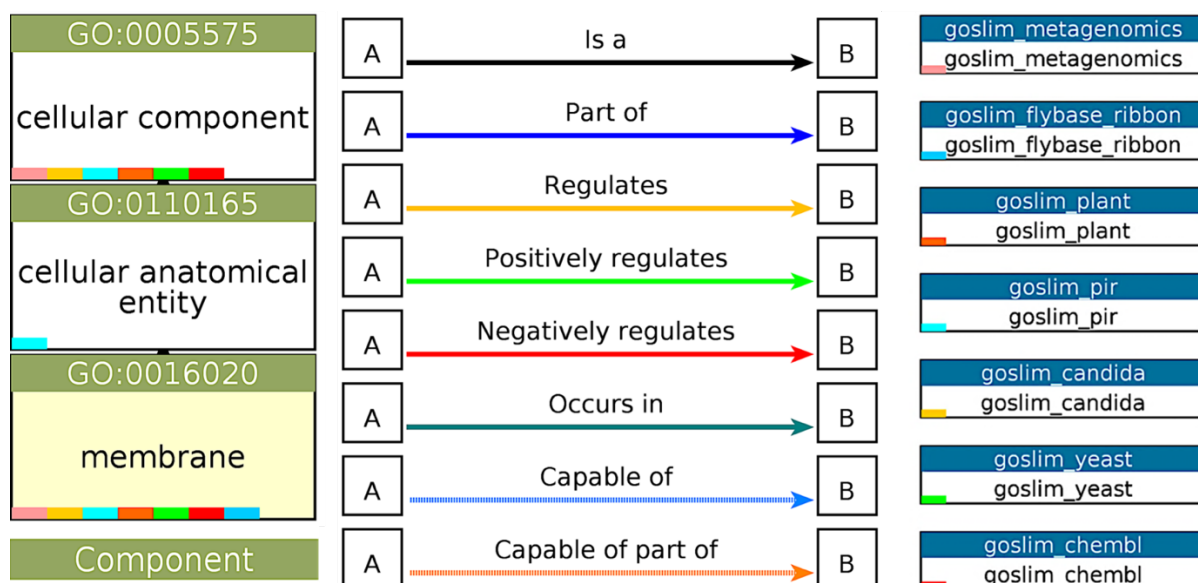


Figure 3.7: Hierarchical representation of Gene Ontology (GO) terms associated with the cellular component category for the membrane (GO:0016020) in relation to CB₁ and CB₂ as classified by InterPro. The diagram illustrates membrane classification as a cellular component (green), which is a subset of a cellular anatomical entity within the broader category of cellular components. The membrane serves as the principal location for CB₁ and CB₂ receptors, reflecting their role in signal transduction across the cell boundary. The specific GO term for the membrane is highlighted in yellow, indicating its central role in the structure and function of these GPCR. The displayed relationships (e.g., 'is a', 'part of', 'regulates', 'positively regulates', 'negatively regulates', 'occurs in', 'capable of', 'capable of part of') describe the interaction between the different GO terms. The colour-coded bars at the bottom represent various GO subsets used for annotation. Sourced from EBI QuickGO.

Conversely, the CB₂ receptor is significantly associated with the extrinsic component of the cytoplasmic side of the plasma membrane (GO:0031234), which highlights its interaction with intracellular G proteins and its integral part in signal transduction from external cannabinoid ligands to intracellular responses (Bie et al., 2018). This localisation is particularly critical in immune cells, where CB₂ predominantly resides, emphasising its functionality in immune responses.

Combining the subcellular location data with GO analysis, the strategic cellular positioning of both CB₁ and CB₂ receptors becomes apparent, highlighting their critical roles in cellular communication. The subcellular location data for the CB₁ receptor and related proteins, which include Fatty Acid Amide Hydrolase (FAAH), Monoacylglycerol Lipase (MGLL), GNAI2, Guanine Nucleotide Binding Protein, Gamma 2 (GNG2), DRD2, CB₁, CB₂, GNB1, ADORA2A, GNAI1, and GPR55, underline their association with membrane structures, particularly within the 'Membrane' (GO:0016020) and 'Plasma membrane' (GOCC:0005886) categories (Hua et al., 2020; Oddi et al., 2012). These proteins have been identified with

compelling statistical support, as evidenced by the strength (0.54) of their associations and remarkably low FDRs (0.00036), reinforcing their essential membrane localisation. Such localisation is not only pivotal for their function as sites of cellular interactions and signalling pathways but also for their roles in the modulation of neurotransmission and immune responses (Nezhady et al., 2020; Radoux-Mergault et al., 2023).

This individual functionality, reflected in the capacity of the receptor to regulate neurotransmission and participate in immune system modulation, highlights the versatility of CBRs. Their importance spans a variety of physiological processes, evident in their involvement in both the central nervous system and peripheral immune functions. The low FDR values add a layer of confidence to these findings, suggesting that the membrane and plasma membrane compartments have a significant biological impact on the functionality of these proteins. This is particularly true within the context of cannabinoid signalling and the activities of GPCRs, where precise localisation is crucial for effective signal transduction from external stimuli to intracellular actions.

3.8 Biological and Molecular Function

The molecular function of CBRs, as defined by the GO term "Cannabinoid Receptor Activity" (GO:0004949), is crucial for the understanding of how both CB₁ and CB₂ receptors interact within the ECS and affect a range of physiological processes. This GO term encompasses the specificity of interaction between the receptors and their ligands, which include endogenous, plant-derived, and synthetic cannabinoids.

For the CB₁ receptor, the observed gene count of 3 against a background of 4, with a strength of 3.13 and an incredibly low false discovery rate (FDR) of 2.23e-05, reflects a strong, non-random association with cannabinoid receptor activity. This data highlights the significant role of CB₁ in core components of cannabinoid signalling, such as pain sensation, mood regulation, appetite, and memory, and suggests a focused involvement in mediating the effects of endocannabinoids (Zou & Kumar, 2018). The inclusion of the GPR55 receptor alongside the traditional CB₁ and CB₂ receptors implies a broader, more complex landscape of cannabinoid signalling than previously understood (Lauckner et al., 2008). The high strength and low FDR of the CB₁ receptor in this context underline its physiological importance and hint at its potential pharmacological relevance, particularly in developing targeted therapies aimed at modulating the ECS to treat various diseases.

In parallel, the GO term (GO:0004949) for the CB₂ receptor demonstrates an explicit role in cannabinoid receptor activity with an observed gene count of 2 against the same background gene count of 4. The strength of 3.29 and an FDR of 0.0038 for the CB₂ receptor further emphasises the specificity and biological relevance of this receptor in the ECS. The strong association of the CB₂ receptor with cannabinoid receptor activity reflects its crucial role in immune system regulation, pain sensation, and inflammation (Zou & Kumar, 2018). It also suggests possible effects on mood and behaviour, although these are more commonly associated with the CB₁ receptor. The significant FDR coupled with the specificity regarding the role of the CB₂ receptor in this molecular function supports its targeted therapeutic potential, especially given its predominant expression in immune cells and its involvement in immunoregulatory functions. As such, CB₂ emerges as a promising target for pharmacological intervention in inflammatory and autoimmune diseases, offering therapeutic benefits without the psychoactive side effects often linked to CB₁ activation. Moreover, combining the analyses for CB₁ and CB₂, it is seen that their roles are distinct yet interconnected, with the CB₁ receptor primarily influencing central nervous system processes and the CB₂ receptor more involved in peripheral and immune functions.

The biological processes GO data (Fig. 3.8) for the CB₁ receptor presents a comprehensive view of the wide-ranging influence on biological processes, laying the groundwork for understanding neurophysiological complexities and identifying pharmacological targets. Starting with the Adenylate cyclase-activating G protein-coupled receptor signalling pathway (GO:0007189), the data reveals a strong association between CB₁ and the modulation of cyclic AMP (cAMP) levels (Childers & Deadwyler, 1996; Eldeeb et al., 2016). The strength of 1.94 for the pathway, against a gene count of 7 in comparison to a background of 145, along with a remarkably low false discovery rate (FDR) of 3.56e-09, firmly establishes the CB₁ receptor as a significant player in this signalling pathway. This robust association is far from coincidental and highlights the role of the receptor in cellular responses to various stimuli, facilitated by cAMP, a pivotal second messenger in cellular signalling.

Additionally, the CB₁ receptor also significantly influences the regulation of synaptic transmission, particularly γ -aminobutyric acid GABAergic transmission (GO:0032228), as evidenced by a strength value of 2.3 and an FDR of 1.86e-05. This indicates a substantial role in modulating GABA activity, which affects neuronal excitability and the balance within neural circuits. The involvement of the receptor is crucial in preventing excitotoxicity and maintaining

neurological function by keeping a check on the balance of excitatory and inhibitory signals in the nervous system (Hoffman & Lupica, 2000). In the context of positive regulation of neurotransmitter transport (GO:0051590), the CB₁ receptor is implicated in the movement of neurotransmitters across cellular membranes, impacting synaptic signalling (Viveros et al., 2007). With a strength of 2.35 and a low FDR of 0.00042, this suggests that the association of the CB₁ receptor in this biological function is statistically significant and not due to random chance. Therefore, this shows the potential impact of the receptor on synaptic communication and its pivotal role in synaptic plasticity and neurophysiological processes.

The participation of the CB₁ receptors in the regulation of catecholamine secretion (GO:0050433) is also noteworthy, with a strength of 1.98 and an FDR of 0.0026, indicating a significant influence on the release of stress-related neurotransmitters like dopamine. This could affect various physiological functions, including stress responses, alertness, and neuroendocrine regulation, pointing to the biological relevance of CB₁ in catecholamine-mediated pathways (Cobellis et al., 2016; Niederhoffer et al., 2001). Furthermore, the regulation of synaptic vesicle exocytosis (GO:2000300) with a strength of 1.92 and an FDR of 0.0032, signifies the substantial influence of CB₁ on neurotransmitter release. This process is essential for signal transmission across synapses, affecting learning, memory, and overall brain function (Ramirez-Franco et al., 2014). The statistical significance of this observation emphasises the reliability of the association and the importance of understanding how the CB₁ receptor regulates synaptic vesicle dynamics (Patzke et al., 2021). The negative regulation of dopamine secretion (GO:0033602) points to a significant role of the CB₁ receptor in modulating brain dopamine levels, with a strength of 2.86 and an FDR of 0.0032. This suggests an essential function in potentially dampening dopamine release, a critical mechanism for disorders characterised by dysregulated dopamine signalling, such as schizophrenia, addiction, and Parkinson's disease (Dunnett & Björklund, 2010; Parsons & Hurd, 2015). The statistical robustness of this association highlights the importance of CB₁ receptors in neural circuit balance and emotional regulation. Lastly, the regulation of synaptic transmission via glutamatergic pathways (GO:0051966) with a strength of 1.85 and an FDR of 0.0046, highlighting the significant role of the receptor in excitatory neurotransmission. This shows the key role of CB₁ in synaptic plasticity, fundamental for learning, memory, and cognitive function (Zhang et al., 2022). The statistical significance of this relationship further illustrates the critical role of CB₁ in the network of neurotransmitter systems, providing valuable insights for therapeutic targeting in cognitive disorders.

The GO data pertaining to CB₂ (Fig. 3.8) provides substantial evidence for the receptor's involvement in a myriad of biological functions. Though it shares similarities with the CB₁ receptor in terms of cellular signalling pathways, it distinguishes itself through unique physiological responses and implications. In the context of the adenylyl cyclase-modulating G protein-coupled receptor signalling pathway (GO:0007188), the observed gene for CB₂ with a count of 4 against a background of 232, a strength of 1.83, and an FDR of 0.0016 illustrate its role in modulating adenylyl cyclase activity. This modulation is pivotal for cyclic AMP level alterations, which in turn affect diverse signalling cascades downstream (Schatz et al., 1997). Particularly critical in immune responses, this pathway highlights the CB₂ integral function of CB₂ in the modulation of immune regulation (Dhopeshwarkar & Mackie, 2016). Furthermore, the adenylyl cyclase-activating protein-coupled receptor signalling pathway (GO:0007189), with a strength of 1.91 and an FDR of 0.0145, points to CB₂ direct activation role in enhancing adenylyl cyclase activity (Schatz et al., 1997; Turcotte et al., 2016). This contrasts with its modulatory role, indicating a dual and complex interplay with the intracellular signalling mechanisms (Turcotte et al., 2016). The data suggests a sophisticated regulatory capacity of the CB₂ receptor, capable of both upregulating and downregulating crucial signalling pathways.

CB₂ plays several roles in negative regulation such as the negative regulation of action potential (GO:0045759), CB₂ involvement signifies a strength of 3.05 and an FDR of 0.0145, denoting its part in modulating neuronal excitability. Although primarily linked with immune function, this receptor may also affect nervous system activities, particularly by modulating neurotransmission and playing a potential role in neuroinflammation (Ma et al., 2019; Turcotte et al., 2016). The negative regulation of mast cell activation (GO:0033004) indicates CB₂ engagement in mediating anti-inflammatory responses with a strength of 2.75 and an FDR of 0.0145. This is particularly relevant in the context of allergic reactions where mast cells are crucial participants, suggesting CB₂ therapeutic potential in treating allergic and inflammatory diseases (Facci et al., 1995; Osorio-Perez et al., 2023). The negative regulation of nitric-oxide synthase activity (GO:0051001) is another critical pathway with a high strength of 2.99 and an FDR of 0.0145. This process points to the role of CB₂ in controlling nitric oxide production, a mediator integral to inflammation and vasodilation, thus reflecting the potential impact of the receptor on cardiovascular health and inflammatory responses (McCollum et al., 2007; Shmist et al., 2006). Lastly, the response to organonitrogen compounds (GO:0010243) with a strength of 1.21 and an FDR of 0.0435 indicates the broader role of the receptor in responding to nitrogen-containing biomolecules (Yang et al., 2013). This could encompass a variety of

neurotransmitters or pharmacologically active drugs, emphasising the relevance of CB₂ in pharmacological interventions.

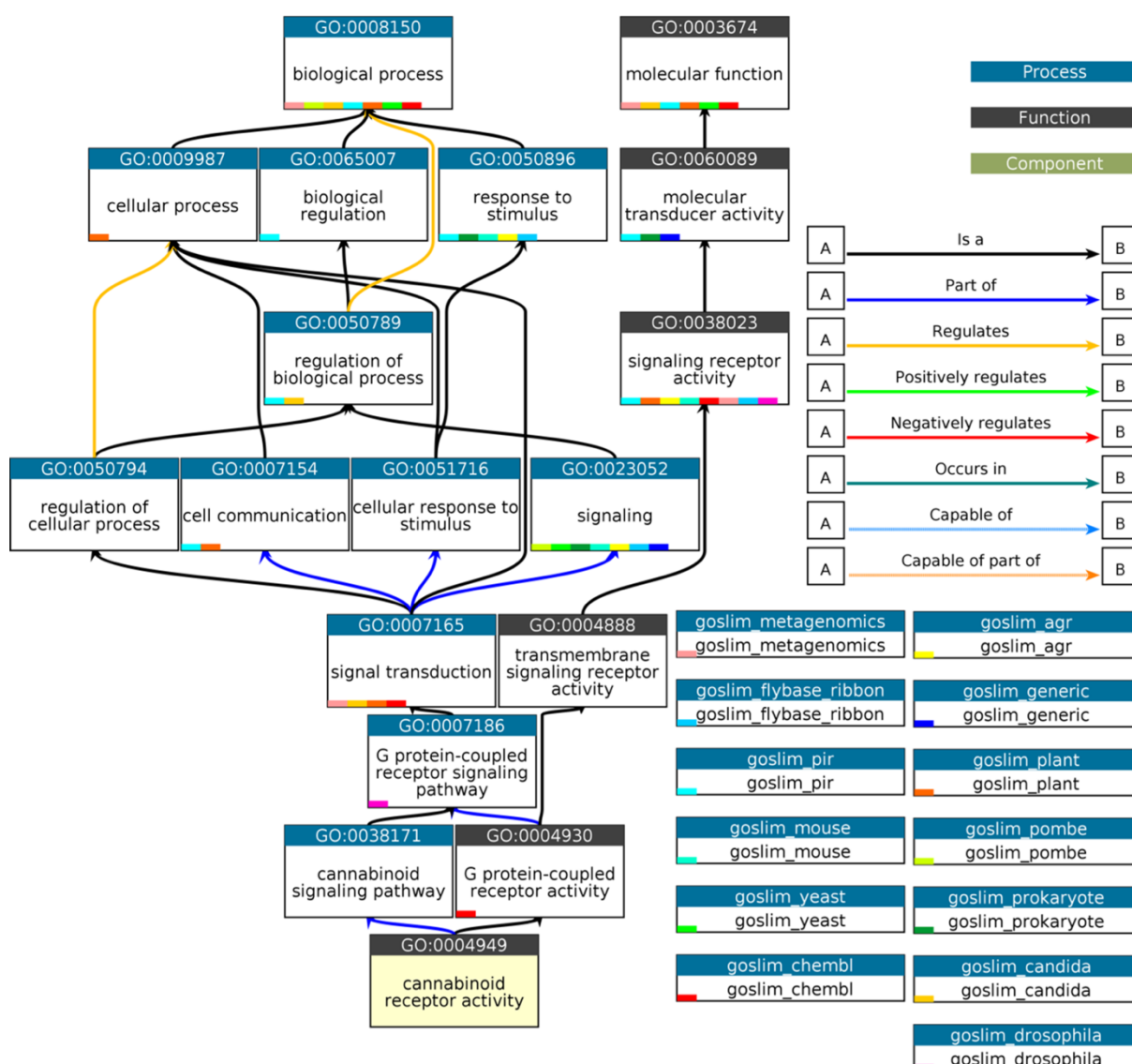


Figure 3.8: Hierarchical representation of Gene Ontology (GO) terms associated with the cellular component category for the membrane (GO:0016020) in relation to CB₁ and CB₂ as classified by InterPro. The diagram illustrates the membrane's classification as a cellular component (green), which is a subset of a cellular anatomical entity (grey) within the broader category of cellular components (blue). The membrane serves as the principal location for CB₁ and CB₂ receptors, reflecting their role in signal transduction across the cell's boundary. The specific GO term for the membrane is highlighted in yellow, indicating its central role in the structure and function of these G-protein-coupled receptors. The displayed relationships (e.g., 'is a', 'part of', 'regulates', 'positively regulates', 'negatively regulates', 'occurs in', 'capable of', 'capable of part of') describe the interaction between the different GO terms. The colour-coded bars at the bottom represent various GO subsets used for annotation. Sourced from EBI QuickGO.

3.9 Protein Function and Signalling Pathways

The intricate involvement of CB₁ in the endocannabinoid signalling pathway is a focal point of study in various bioinformatics analyses, including KEGG, Reactome, and WikiPathways (Szkarczyk et al., 2021). The KEGG pathway analysis shines a light on the integral role of CB₁ in the endocannabinoid signalling pathway, especially within the context of retrograde endocannabinoid signalling (HSA-04723) (Leo & Abood, 2021). The exceptionally low false discovery rate (p-value: 1.32e-10) emphasises the significance of CB₁ in modulating neurotransmission. This modulation is crucial for the regulation of synaptic strength and plasticity, pivotal to learning, memory, and other neural functions. Additionally, the CB₁ receptors involvement in the Ras-proximate-1 (Rap1) signalling pathway (HSA-04015), with its potential influence on cell adhesion, growth, and proliferation, suggests a role in mediating cellular responses essential for neuroplasticity and neuroprotection (He et al., 2005; Jordan et al., 2005). The presence of CB₁ in the neuroactive ligand-receptor interaction pathway (HSA-04080), with a p-value of 0.00038, underlines its importance in the pharmacodynamics of various neuroactive substances. This pathway encompasses a wide range of physiological responses, from neuronal communication to muscle contraction and glandular secretion, highlighting the widespread impact of CB₁ on cellular processes.

Complementing these findings, the Reactome pathway analysis provides a detailed view of the role of the CB₁ receptor in various signalling pathways, demonstrating its extensive involvement in GPCR functions as represented by the 'signalling by GPCR pathway' (HSA-372790). This involvement carries significant weight, as suggested by the compelling p-value of 8.06e-11, indicating that the role of CB₁ is not due to chance but is a robust association with a broad spectrum of GPCR-mediated responses. The receptors engagement continues with 'GPCR downstream signalling' (HSA-388396), reflecting its participation in the complex signalling cascade that occurs inside the cell after initial GPCR activation (Childers & Deadwyler, 1996; Eldeeb et al., 2016). The pathway 'G alpha (*i*) signalling events' (HSA-418594) highlights the receptor interaction with inhibitory G-proteins, which is essential for modulating the levels of the secondary messenger cAMP within cells. The 'GPCR ligand binding' pathway (HSA-500792), with a p-value of 5.48e-07, emphasises the receptors selectivity for certain ligands, crucial for controlling a myriad of physiological processes. Lastly, the receptors inclusion in 'Class A/1 (Rhodopsin-like receptors)' (HSA-373076) aligns it with a group of receptors known for their structural role in phototransduction and various

other signalling functions (Pasquaré et al., 2023). These findings from the Reactome pathways, taken together, reinforce the pivotal role of the CB₁ receptor in cellular communication and suggest a multitude of potential therapeutic targets within these pathways for conditions associated with the ECS.

Building on this, WikiPathways data provides a more nuanced picture of the CB₁ receptors signalling network, underscoring its pivotal role in cannabinoid signalling, as seen in pathway WP3869 (Ehrhart et al., 2021). The strong association indicated by a low false discovery rate (FDR) reveals a tightly knit cluster of proteins, including FAAH, CB₁, CB₂, and ADORA2A, which are integral to endocannabinoid processes and the physiological effects of cannabinoids. Further highlighting the receptors influence, pathways WP455 and WP117 delve into the roles of class A rhodopsin-like GPCRs and other diverse GPCRs, confirming the CB₁ receptors extensive contribution to central nervous system signalling (Cascieri et al., 1996). The data also presents an intriguing perspective on the cell-type-dependent selectivity of receptor signalling (WP3679), involving CNR1 and GNAI1, and showing the complexity of targeting specific tissue types in drug development. Moreover, pathway WP247s focus on small ligand GPCRs, with notable inclusion of CB₁ and CB₂, reinforces the pharmacological importance of CBRs within the vast signalling landscape of small molecules through GPCRs. These WikiPathways data points collectively illustrate a highly interconnected network of cannabinoid-related signalling pathways, with significant biological and pharmacological implications. The robustness of these associations, as confirmed by the low FDRs and high strengths, underscores the potential of these pathways to impact our understanding of cannabinoid physiology and the development of targeted therapies.

Taken together, these pathway analyses present a comprehensive picture of the multifaceted role of CB₁ in cellular communication, its interaction with a wide array of proteins, and its significant contribution to physiological and pathological processes. The convergence of these pathways, with their low p-values and FDRs, not only substantiates the biological significance of the CB₁ receptor's interactions but also reinforces its potential as a therapeutic target within the endocannabinoid system, with far-reaching implications for health and disease.

The integration of Reactome and WikiPathways analyses offers a comprehensive understanding of the CB₂ receptors function in GPCR signalling. Reactome pathways analysis sheds light on two noteworthy routes involving the CB₂ receptor, G alpha (*i*) signalling events (HSA-418594) and GPCR ligand binding (HSA-500792). The first pathway demonstrates the role of CB₂ in

inhibitory G protein (G_i) signalling, an essential modulator of cellular responses including the downregulation of cAMP via adenylyl cyclase inhibition (Ye et al., 2019). This pathway has been noted for its observed gene count of 4 in a background of 309, boasting a strength of 1.71 and an impressively low false discovery rate of 0.00070. Such figures highlight the pathways substantial relevance and the dependability of these interactions within the functionality of CB₂. The second pathway, GPCR ligand binding, spotlights the nascent activation step of the CB₂ receptor, where ligand binding initiates a domino effect of intracellular signalling events. With an observed gene count of 3 against a background of 459 and a strength of 1.41 accompanied by an FDR of 0.0217, this pathway underscores the CB₂ receptors broader implication in diverse GPCR-mediated signalling pathways beyond classic G_i protein interactions (Tuteja, 2009). These pathways collectively emphasise the crucial involvement of CB₂ in cellular signalling, reinforced by quantifiable metrics that emphasise its potential for therapeutic intervention in various conditions, including inflammatory and neurodegenerative diseases, where GPCR signalling modulation can be advantageous.

Complementing the insights from Reactome, the WikiPathways analysis provides a granular perspective on the CB₂ receptor within the GPCR signalling context, notably through small ligand GPCRs (WP247) and Cannabinoid receptor signalling (WP3869) pathways. The Small ligand GPCRs pathway, with its observed gene count of 2 against a background of 19, reveals a strength of 2.62 and an FDR of 0.0042, accentuating the significant role of cannabinoid receptors in the specificity of small molecule interactions. Cannabinoid receptor signalling further delineates the pathways importance with a strength of 2.45 and an FDR of 0.0044, focusing on the interactions of cannabinoids with CBRs. This specificity of the pathway highlights the unique influence of cannabinoid receptors on cellular responses, such as neurotransmitter release, immune function, and pain perception. The statistical data from WikiPathways, including the observed gene counts, strength, and FDR, highlight the vital and specialised roles of CBRs, suggesting a robust involvement in both broad and GPCR signalling pathways. This analysis reinforces the significance of CBRs in physiological and pathophysiological processes and their potential as therapeutic targets in diseases related to pain, inflammation, and neurological disorders, thereby enriching the comprehensive understanding of the CB₂ receptor's role in GPCR signalling.

4 DISCUSSION

This investigation highlights the significance of researching CBRs by emphasising their critical functions in cellular signalling and their prospective utility as therapeutic agents within the rapidly evolving *Cannabis* industry. The objective was to investigate the mechanisms and physiological responses when cannabinoids interact with these receptors, prompted by the swift emergence of new cannabinoids and the increased accessibility of *Cannabis*-derived products. Such an inquiry is vital for elucidating the complex interplay between cannabinoids and receptors, thereby laying the groundwork for future therapeutic innovations and drug development initiatives in alignment with the expanding cannabis marketplace.

Proceeding to evolutionary characteristics, a comprehensive bioinformatics analysis of the evolutionary history and conserved domains of CBRs was conducted. Through the use of phylogenetic trees, constructed via the Fast Minimum Evolution method and Grishin distance metric, it traces the development and divergence of these receptors across species. This analysis reveals the broad distribution and adaptation of CBRs across mammalian taxa, indicating their critical role in conserved physiological processes. Additionally, the study highlights the identification of conserved domains within CB₁ and CB₂ receptors through comparative sequence alignment across a variety of species. Moreover, structural conservation often suggests a similar mode of action among the receptors across different species, which is fundamental for their function in signal transduction. The seven-TM helices, in particular, are a characteristic feature of GPCRs and are known for their involvement in various signalling pathways, further suggesting that these receptors have maintained their structural integrity to effectively mediate their roles in physiological processes such as pain sensation, mood regulation, and immune responses (Cascieri et al., 1996). Studies show that CBRs emerged from an ancestor shared by extant chordates, and in the marine organism *Ciona intestinalis* (a sea vase), a receptor similar to CBRs is found in the axons. This suggests that CBRs have long been involved in controlling the communication between nerve cells. (Elphick, 2012). By connecting the evolutionary and molecular profiles of these receptors with their biological functions, the analysis significantly contributes to the deeper comprehension of their role in physiology and medicine, supporting ongoing investigations into cannabinoid-based therapies.

In exploring the receptor structure, a detailed analysis of the structural characteristics of CB₁ and CB₂ receptors and their alignment were assessed within the broader context of the GPCR superfamily. The comparison between CB₁ and CB₂ receptors reveals a significant level of

similarity, indicating a common evolutionary origin and potentially similar structural characteristics of 44.58 % (Munro et al., 1993). The presence of conserved regions, notably the seven TM helices, is shown to be essential for the structural integrity and function of these receptors (Howlett, 2002). These conserved domains facilitate the ability of the receptor to respond to a variety of ligands, including endocannabinoids, by activating G-proteins and initiating intracellular signalling cascades (Ibsen et al., 2017). These structural features in maintaining receptor activity and integrity across diverse species, emphasising the evolutionary conservation of these motifs. The identification of specific conserved domains, such as the GPCR rhodopsin-like domain shared among CBRs, and other GPCRs, points to common functional traits that enable signal transduction through ligand binding and G-protein activation (Ehrhart et al., 2021; Tuteja, 2009).

The cellular positioning of these receptors plays a critical role in the pathways they affect, thereby influencing physiological and pathological states. For instance, the CB₁ receptors involvement in the CNS and its impact on cognition, memory, and pain perception, contrast with CB₂ receptor's predominant role in the immune system, influencing immune responses and inflammation (Anand et al., 2008; Felder et al., 1995). This distinction between the receptor locations and their specific biological impacts underlines the complexity of the ECS in maintaining homeostasis and its influence on a wide array of physiological processes (Nezhady et al., 2020). The localisation of CBRs is therefore not only imperative for their function as sites of cellular interactions and signalling pathways but also for their roles in modulating neurotransmission, immune responses, and a variety of physiological processes (Nezhady et al., 2020). This emphasises the need for a detailed understanding of the cellular and subcellular locations of these receptors to fully grasp their roles in health and disease, offering insights into potential therapeutic targets within the ECS.

The necessity for continuous research into CBRs is increased by the expanding *Cannabis* market and the potential therapeutic benefits of cannabinoids (Holt et al., 2022). This stresses the importance of ongoing studies to unravel the intricate mechanisms of action and the physiological processes influenced by these receptors. This is particularly important when considering an expanding array of cannabinoids entering the market and consumed by *Cannabis* users, necessitating a deeper understanding of their interactions with the ECS. In the case of computationally studying CBRs, the crystal structures available in the PDB with the current best resolution contain stabilising artefacts called p-CBRs (Li et al., 2019; Shao et al., 2016).

This approach emerges as a promising avenue to advance our understanding of cannabinoid effects on CBRs and their potential physiological effects. Such studies could shed light on the complex signalling pathways and receptor-ligand dynamics, paving the way for innovative therapeutic approaches targeting conditions modulated by the ECS.

The research into CBRs contributes not only to our molecular understanding but also to the potential discovery of novel drug candidates. By leveraging insights from these studies, it is possible to develop targeted interventions for a range of diseases, including those related to pain, inflammation, and neurodegeneration, thereby harnessing the therapeutic potential of cannabinoids in a precise and effective manner (Howlett, 2002).

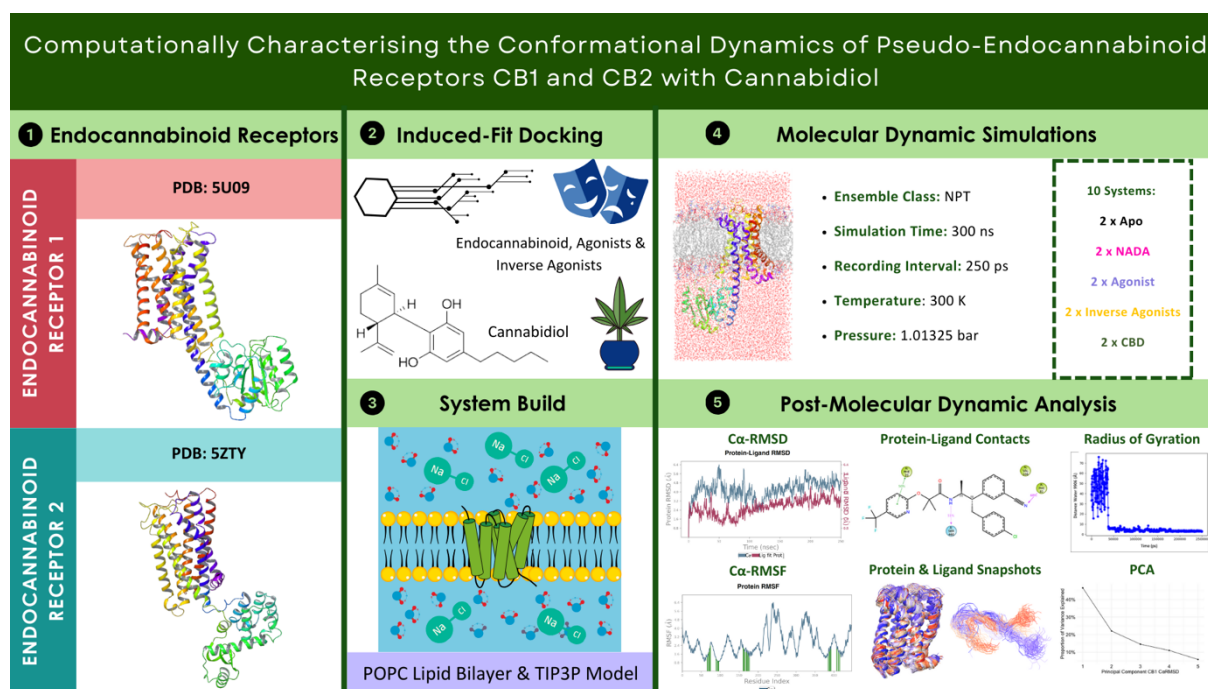
5 CONCLUSION

This study showcases an enhanced understanding of CBRs through the application of bioinformatic methods. The statistical robustness of the findings, paired with the integration of detailed pathway analyses, not only strengthens the biological significance of these receptors but also accentuates their viability as pharmacological targets. The integration of bioinformatics precision with biological insight constitutes a considerable advancement in the interdisciplinary field of computational biology and pharmaceutical research. The depth of this study provides a detailed perspective on the role of cannabinoid receptors in complex biological pathways, laying out a strategic framework for drug discovery and the development of new therapies. It highlights the capacity of bioinformatics to dissect the complexities of receptor signalling and its broader implications for health and pathology, thereby confirming the essential role of these receptors in the realm of medical science. Such a comprehensive understanding has the potential to revolutionise personalised medicine, offering a robust, data-driven basis for crafting innovative treatments that target the ECS.

CHAPTER 4

COMPUTATIONALLY CHARACTERISING THE CONFORMATIONAL DYNAMICS OF PSEUDO-ENDOCANNABINOID RECEPTORS CB₁ AND CB₂ WITH CANNABIDIOL

GRAPHICAL ABSTRACT



ABSTRACT

This study presents an extensive analysis of the conformational dynamics of pseudo-endocannabinoids (p-CBRs) CB₁ and CB₂ in response to ligand binding with *N*-arachidonoyl dopamine (NADA), cannabidiol (CBD) and known agonists (FUB-MDMB and E3R) and inverse agonists (Taranabant & AM630) to validate p-CBRs models for further cannabinoid analysis. Utilising homology models based on crystal structures (PDB ID: 5U09 for CB₁ and 5ZTY for CB₂), molecular docking, and molecular dynamics (MD) simulations, the research validates the structural integrity and dynamic behaviours of these p-CBRs in response to certain ligands. Induced fit docking results revealed NADA as the ligand with the highest binding affinity for both receptors, showing significant docking scores of −13.92 kcal/mol for CB₁ and −13.32 kcal/mol for CB₂, facilitated by key hydrogen (H) bond interactions. The MD simulations, extending over 250 ns, demonstrated successful equilibration at ~298 K, with energy properties indicating the most stable interaction for NADA, further validating the

models. CB₁ and CB₂ receptors exhibited distinct conformational responses to ligands, as elucidated by alpha carbon root-mean-square deviation (C_α RMSD) and alpha carbon root-mean-square fluctuation (C_α RMSF) analyses, suggesting a more rigid binding pocket for CB₁ and a flexible binding pocket for CB₂ that could accommodate a wider range of ligands, potentially impacting immune modulation. The study highlights the significance of receptor behaviour and dynamics in the development of cannabinoid-based therapeutics. It supports the potential of CBD as a modulator of CB₁ and with NADA as a modulator to CB₂ due to its unique binding profile and interaction with water molecules, providing a comprehensive computational foundation for the design of novel therapeutic agents targeting the endocannabinoid system (ECS) and paving the way for more cannabinoids to be computationally researched.

1 INTRODUCTION

G protein-coupled receptors (GPCRs) are membrane proteins involved in cellular signalling and various physiological processes. They interact with transducers such as G-proteins, GPCR kinases (GRKs), and arrestins, leading to specific intracellular signalling pathways and functional outcomes. GPCRs are extensively studied for their therapeutic potential as they mediate the effects of many human hormones and drugs ("UniProt: the universal protein knowledgebase in 2023,"). CB₁ and CB₂ hold particular significance within this family. CB₁ receptors are ubiquitous in the body but are predominantly expressed in the CNS while CB₂ receptors are mainly located in immune cells and peripheral tissues (Herkenham et al., 1991; Mackie, 2008). In addition, the distinct locations of these receptors explain why cannabinoids have such diverse effects on the body. The discovery of CBRs has shed light on the ECS, a complex signalling network involved in various physiological functions, such as pain modulation, immune response, appetite regulation, and mood control (Kano et al., 2009; Marsicano & Lutz, 2006; Turcotte et al., 2016). Furthermore, the activation of CBRs has been associated in the therapeutic management of various medical conditions, including cancer, anxiety disorders, chronic pain, neurodegenerative diseases, and other health complications (Scuderi et al., 2009; Slomski, 2018; White, 2019).

The dynamic behaviour of GPCRs is essential for their biological function in cell signalling. These receptors located in the cell membrane, act as intricate molecular switches that toggle between active and inactive conformations in response to ligand binding which is a fundamental aspect of their role in signal transduction (Haubrich, 2021). These changes are crucial for

activating ICL G-proteins, which cause a signal cascade inside the cell, leading to varied physiological responses. For example, when CB₁ binds to its endogenous ligand AEA, it undergoes a structural change that enables it to activate an associated G-protein. The activated G-protein trimer then dissociates into subunits, which can inhibit adenylate cyclase activity, leading to decreased levels of the second messenger cAMP (Eldeeb et al., 2016; Vaidehi, 2010). This cascade modulates neurotransmitter release, affecting physiological processes such as pain sensation, appetite, and memory. Similarly, the CB₂ receptor, when engaged by a phytocannabinoid such as CBD, can influence immune cell behaviour, potentially reducing inflammation through its impact on G-protein signalling pathway (Hilger, 2021). Ligands that act as agonists stabilise the receptor in its active conformation, enhancing signalling. Conversely, inverse agonists stabilise the receptor in its inactive state, dampening signalling (Haschek-Hock et al., 2021; Haubrich, 2021). CBRs are naturally activated by endogenous cannabinoids called AEA and 2-AG (Mechoulam et al., 1998). These lipid-based neurotransmitters are naturally produced in the body and initiate a cascade of ICL signalling events involved in maintaining homeostasis and regulating various physiological processes. Due to the extensive range of physiological effects CBRs modulate, an in-depth understanding of the structure, function, and dynamics of CBRs is essential for developing cannabinoid-based therapeutics, as these receptors serve as key targets for exogenous phytocannabinoids derived from the *Cannabis sativa* L. plant, including Δ^9 -THC and CBD (Adams, Hunt, & Clark, 1940; Adams, Pease, et al., 1940). The interaction of CBRs with phytocannabinoids can lead to various therapeutic effects, such as pain relief, anti-inflammation, and modulation of mood and appetites. Additionally, synthetic cannabinoids (SCs) are also being developed to enhance their potency and effects on CBRs (Holt et al., 2022).

Presently, considerable progress has been made in the fields of computational biology and molecular modelling, leading to a deeper understanding of the sequence and 3D structures of CBRs. The availability of crystal structures and computational models has paved the way for structure-based drug discovery, rational drug design, and virtual screening approaches, thereby aiding in the identification of novel ligands with enhanced specificity and efficacy (Gromiha, 2010; Zhao & Caflisch, 2015). Currently, several crystal structures of CBRs have been identified, providing valuable insights into their 3D conformations (Manandhar et al., 2022). The first crystal structure of a CB₁ receptor was determined in 2016 using X-ray crystallography, while the first crystal structure of a CB₂ receptor was resolved in 2018 (Hua et al., 2016; Li et al., 2019). To date, multiple crystal structures, with co-crystal ligands, of both

CBRs have been reported, contributing to our understanding of their ligand-binding sites and functional mechanisms.

To computationally study the effects of natural and modified phytocannabinoids on CBRs, it is essential to develop a comprehensive computational system. This involves accurately representing the receptor structure in various states, optimising ligand parameters, and validating simulation protocols (Aminpour et al., 2019). Once established and validated, these systems provide a foundation for investigating the effects of various protein interactions, thereby allowing researchers to simulate and understand how different cannabinoids and their synthetic variants interact with the receptors at a molecular level. Molecular modelling methods, such as molecular docking and MD simulations, are essential for understanding receptor-ligand interactions and predicting functional and dynamic behaviour of receptor-ligand complexes based on the structural dynamics of the protein over time. Molecular docking aids in identifying key interactions and potential binding sites, contributing to the understanding of binding modes and affinities of ligands such as phytocannabinoids from *Cannabis* to CBRs (Nadendla, 2004). Additionally, MD simulations allow studying the dynamic behaviour of the receptor-ligand complex (Karplus & Kuriyan, 2005). By simulating the motion and interactions of atoms within the system, MD simulations can provide insights into the stability, flexibility, and conformational changes of the complex (Berman et al., 2000) (McCammon et al., 1977). This understanding can lead to the design of new compounds with tailored properties, enhancing therapeutic benefits and reducing side effects. Additionally, these computational models expedite the development of new medications that can target specific receptor states, thus opening possibilities for personalised medicine and more effective treatment strategies for a variety of conditions.

For this study, analyses were conducted on CB₁ and CB₂ receptors obtained from the Research Collaboratory for Structural Bioinformatics: Protein Data Bank (RCSB: PDB) where the crystal structures were deposited by the X-ray crystallographers (Berman et al., 2000; Hua et al., 2020; Shao et al., 2016). Currently, the crystal structure of the CB₁ receptor (PDB ID: 5U09) stands as the model with the best resolution of 2.6 Å available on the UniProtKB database (Shao et al., 2016). This structure was obtained by X-ray diffraction and can be used for cannabinoid research due to strategic modifications that preserve its functional integrity while facilitating the lipidic cubic phase crystallisation process. These alterations do not affect the receptor as they ensure that the active site of the receptor and binding domains remain unchanged,

providing a reliable framework for examining cannabinoid interactions and their potential therapeutic applications. First, the N-terminal was truncated, and the third ICL loop (ICL3) was replaced with a thermostable PGS domain (1.8 Å), a glycogen synthase (GlgA) from *Pyrococcus abyssi*. These alterations, along with the T210A mutation, were crucial for crystallisation despite lower affinity for certain agonists. The binding properties remain sufficiently intact, as shown by binding assays, and the structure maintains key aspects of the receptor that are key for interaction with ligands like CBD (Shao et al., 2016). This makes the modified structure a reliable model for studying ligand-receptor interactions and designing new modulators for therapeutic use. The CB₂ receptor (PDB ID: 5ZTY) underwent a similar process while being crystallised by X-ray diffraction by (Li et al., 2019). For computational studies, this structure is valuable as it retains functional integrity despite modifications for crystallisation. The modifications include truncating a part of the N-terminus and incorporating T4 lysozyme into the ICL3. These changes do not impact the functionality or ligand binding of the receptor, as confirmed by radioligand binding assays and receptor stabilisation showing negligible effect on affinity. Therefore, this CB₂, which holds the current best resolution (2.8 Å) on UniProtKB, is suitable for computational studies of phytocannabinoids as the empirical data support its utility in drug design and understanding receptor-ligand interactions. Due to these changes, in this study CBRs were referred to as pseudo-CBRs (p-CBRs).

This study specifically focused on exploring each p-CBRs in complex with various ligands (NADA, CBD, FUB-MDMB/E3R, Taranabant/AM630) to enable further investigations of phytocannabinoids such as modified phytocannabinoids emerging in this fast-paced *Cannabis* market. This comprehensive approach allowed for a deeper understanding of receptor-ligand binding behaviour and its associated dynamics. By unravelling the intricate mechanisms underlying the binding and activation of p-CBRs, novel therapeutic strategies can be developed to target these receptors and modulate their functions, potentially leading to the development of more effective and specific natural, modified, or synthetic cannabinoid-based treatments for various diseases and conditions.

2 MATERIALS AND METHODS

All computational work using Maestro Software 13.0 Schrödinger suite Inc was performed using the hardware and software specifications described in appendix C.

2.1 Protein Crystal Structures Preparation

Crystal structures for CB₁ and CB₂ were first selected from the UniProtKB database (UniProtKB ID: P21554) and (UniProtKB ID: P34972) with sequences obtained from PDB (PDB ID: 5U09) and (PDB ID: 5ZTY), respectively (Li et al., 2019; Shao et al., 2016) (Fig.4.1). Homology models (Fig. 4.2) were generated using SWISS-MODEL to help predict structure of any missing regions (Waterhouse et al., 2018), after which the p-CBRs were prepared using the Protein Preparation Wizard tool implemented in Maestro Software 13.0 Schrödinger suite Inc. (Madhavi Sastry et al., 2013). This step involved several optimisation procedures to improve the protein structures, including the addition of H atoms, filling in missing side chains using Prime, the assignment of bond orders from the conserved core domain (CCD) database, and the optimisation of side chain orientations (Jacobson et al., 2002; Jacobson et al., 2004). This is conducted to enhance the accuracy of protein models for computational studies by correcting structural details not resolved in the original data. Both apo proteins were prepared for IFD and MD simulations. The aim of this preparation was to ensure that the protein structures were in a suitable conformation for accurate docking and simulation studies.

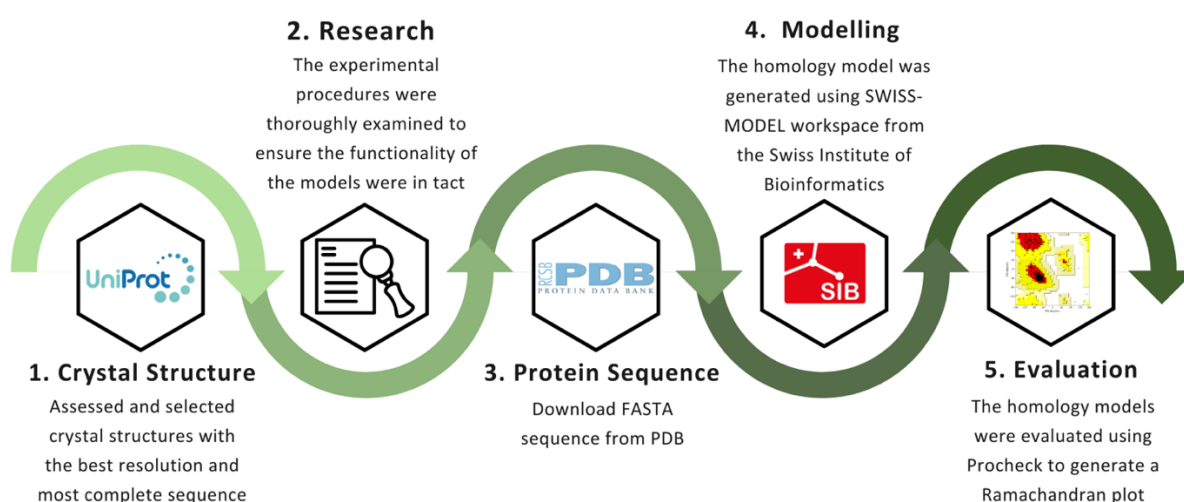


Figure 4.1: The process for selecting and modelling the crystal structure of p-CBRs. Step 1 involves the assessment of crystal structures from UniProtKB for optimal resolution and completeness. Step 2 ensures thorough verification of experimental procedures to maintain model functionality. The amino acid sequence of the protein was acquired from PDB in Step 3, followed by homology modelling using the SWISS-MODEL workspace in Step 4, associated with the Swiss Institute of Bioinformatics (SIB). The process concludes with Step 5, where the homology models are evaluated through PROCHECK to generate a Ramachandran plot, ensuring the structural accuracy of the models.

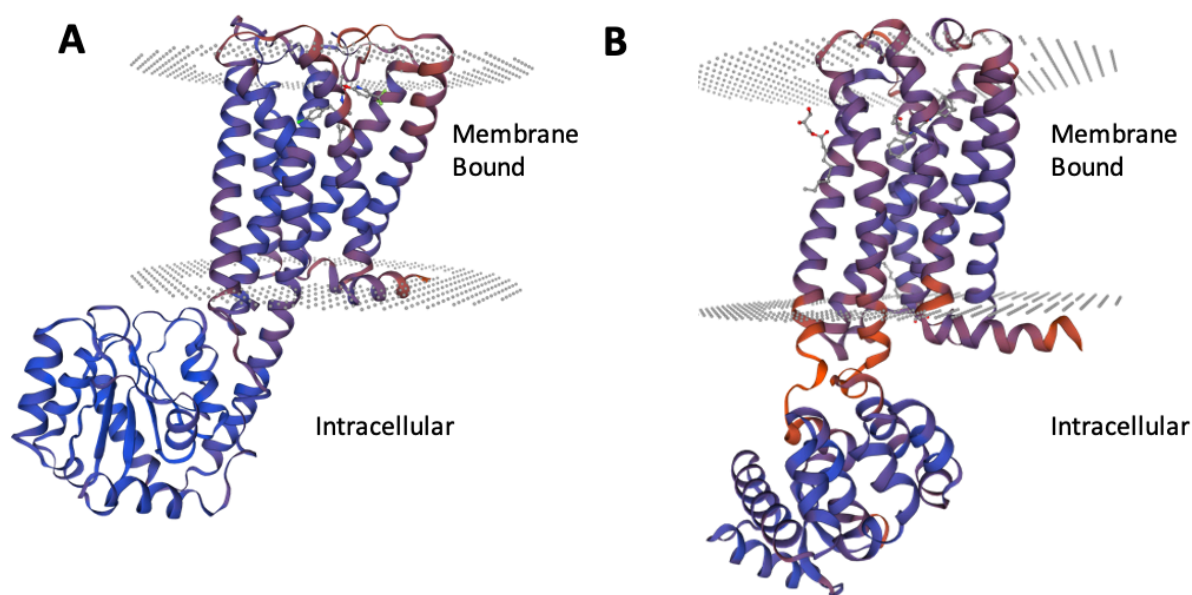


Figure 4.2: Homology models of p-CBRs CB₁ and CB₂. Each model displays the respective receptor membrane-bound and intracellular regions with the highest confidence levels depicted as blue and the lowest as red. A. CB₁, based on the crystallographic template of PDB entry 5U09. B. CB₂, receptor derived from the PDB entry 5ZTY. Generated using the SWISS-MODEL workspace on the ExPASy server (Waterhouse et al., 2018).

2.2 Preparation of Ligands

All ligands (Fig. 4.3) underwent preparation using the LigPrep tool implemented in Maestro using the OPLS2005 force field (Jorgensen et al., 1996). LigPrep generates tautomers and stereoisomers and low-energy conformations of the ligand while optimising ligand protonation states at physiological pH conditions of 7.0 ± 2.0 using Epik (Bochevarov et al., 2016; Johnston et al., 2023; Shelley et al., 2007). This critical step was aimed at ensuring the ligands had suitable conformations for precise docking analyses.

2.3 Induced-Fit Docking

The prepared p-CBR structures underwent IFD procedures using Schrödinger's Glide software integrated into the Maestro platform with various ligands (Fig. 4.3) (Farid et al., 2006; Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004; Sherman, Beard, & Farid, 2006; Sherman, Day, et al., 2006; Yang et al., 2021). IFD is a docking technique allowing the binding site of the protein to adapt flexibly to the lowest energy conformation of the ligand throughout the docking process. To ensure precision, the IFD box centre for each protein was strategically defined based on the residues intricately interacting with their corresponding co-crystal ligands.

The complex selected for further analysis was the top conformer with the most favourable docking score, which was subsequently utilised for subsequent MD simulations.

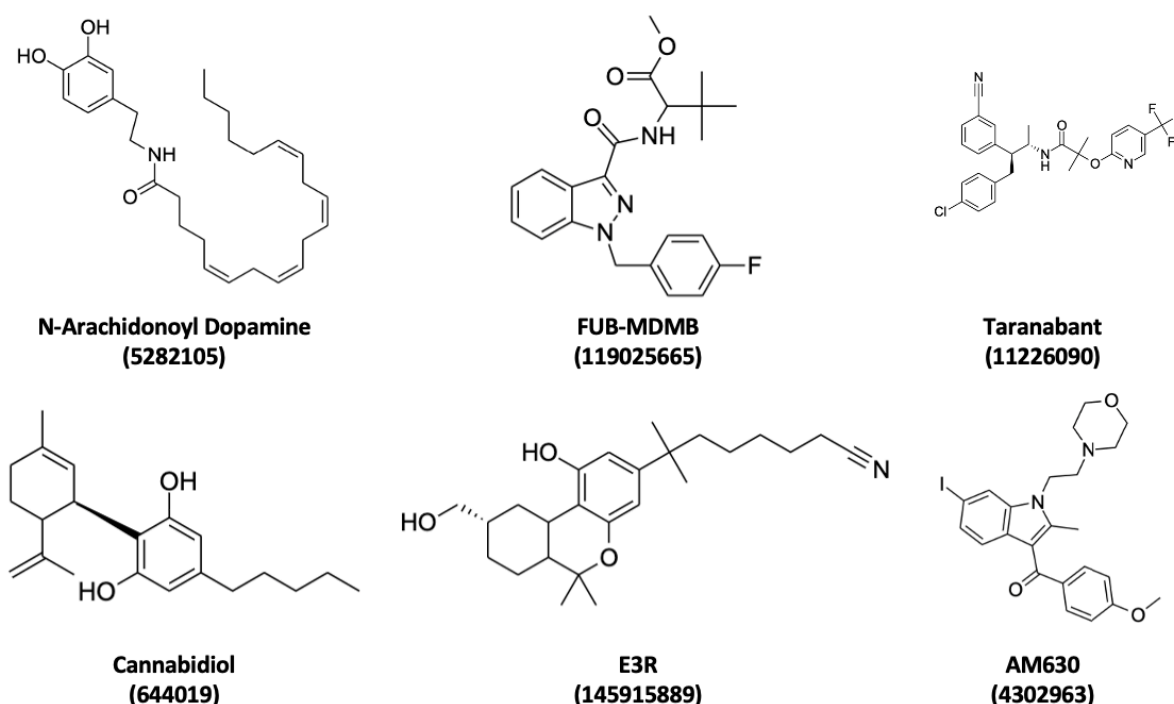


Figure 4.3: Two dimensional (2D) structures of ligands. NADA, a CB₁ and CB₂ endocannabinoid. FUB-MDMB a CB₁ Agonist. Taranabant, a CB₁ inverse agonist. CBD, a CB₁ and CB₂ phytocannabinoid. E3R, a CB₂ Agonist. AM360, a CB₂ inverse agonist. Drawn with ChemDraw JS. Compound CID acquired from Pubchem.

2.4 System Builder

The Desmond Molecular Simulation Software version System Builder tool was used to prepare a total of 10 systems for MD simulations of the p-CBRs (Bowers et al., 2006). The protein-ligand complexes for these systems are the most favourable conformations identified through IFD. Each system was constructed while being embedded in a POPC lipid bilayer model, and explicit TIP3P water molecules were introduced around the system with a minimum 10 Å buffer distance (Fig. C1). To replicate physiological conditions, sodium (Na⁺) and chloride (Cl⁻) ions were added at a concentration of 0.15 M to neutralise the net charge of the system. This comprehensive approach aimed to establish a realistic and biologically relevant environment, facilitating the exploration of dynamic behaviours within the protein-ligand complex during MD simulations.

2.5 Molecular Dynamics Simulations

Desmond in Maestro was used to run all MD simulations which were 250 ns long to explore system dynamics (Bowers et al., 2006). Following the initial solvation and ionisation in an explicit solvent setup, the system proceeded through a multi-stage MD production phase. The process entailed simulation specifics across eight stages, with stages one to seven focusing on equilibration via brief simulation intervals and stage eight being the 250 ns simulation stage. The type and parameters of the solvated system were established in the first stage, followed by a 100 ps simulation at 10 K, applying restraints to solute-heavy atoms in the second stage under NVT conditions using Brownian dynamics. The third stage continued under similar NVT conditions for 12 ps. The series of simulations for stages four (12 ps), six (12 ps), and seven (24 ps), while omitting the pocket solvation at stage five, involved short steps under NPT conditions, with temperature and atom restraints integrated in stages four and six. The seventh stage heavy atoms had no restraints, paving the way for the final eighth stage, which adopted an NPT ensemble at a consistent 300 K temperature.

2.6 Post-Molecular Dynamic Analysis

While performing post-MD simulation analysis, several key parameters were examined to gain insights into the conformational dynamics and interactions within the protein-ligand complexes. The C_{α} RMSD was calculated to assess the overall structural stability of the protein-ligand complexes over the course of the simulation. Additionally, the C_{α} RMSF was computed to quantify the local flexibility of residues and to identify regions with significant conformational changes. RoG analysis was conducted to monitor the compactness of the protein-ligand complexes during the simulation, providing information on structural changes and potential unfolding events. Principal component analysis (PCA) was performed utilising RStudio by Posit Software, PBC (version 2023.09.1+494), incorporating the ggfortify package to assess all C_{α} values in relation to their corresponding apo structure (RStudio, 2020; Tang et al., 2016). The creation of scree plots based on the PCA outputs was also executed within the R environment, facilitating a visual interpretation of the variance distribution among the principal components (Aboytes-Ojeda et al., 2016; Tang et al., 2016; Team, 2010). Ligand-specific analyses were also performed. The lig-RMSD was calculated to evaluate the stability of the ligands within the binding pocket and to identify any deviations from their initial positions. These trajectory analyses collectively provided a comprehensive view of the dynamic behaviour and intermolecular interactions within the protein-ligand complexes post-MD

simulations, shedding light on the stability and potential binding mechanisms of the ligands to CBRs.

3 RESULTS

3.1 Crystal Selection and Homology Modelling

After the appropriate structures were selected (Fig. 4.1) homology models were generated (Fig. 4.2). Figure 4.3 illustrates the structural alignment of p-CBRs, highlighting their similarities and differences. The superimposition of CB₁ (purple) and CB₂ (pink) models allows for a visual comparison of their 3D structures. This comparison can elucidate variations in their conformations that may relate to their distinct physiological roles, ligand specificities, and signalling mechanisms. Differences in structure between CB₁ and CB₂ are key to understanding their unique interactions with various ligands, which ultimately influences their differing functions in the body. For the p-CB₁ receptor (Appendix C, Fig. C1), the ICL region spans residues 214-409, while the segment embedded within the membrane encompasses residues 12-213 and 410-498. Similarly, the ICL region of p-CB₂ receptor extends from residues 222-381, with its membrane-bound segment comprising residues 20-221 and 382-472 (Fig. C2). The Ramachandran statistics in Table 4.1 indicate that for CB₁ 94.2 % of amino acid residues in the favoured regions, thereby suggesting a reliable model consistent with known protein structural conformations (Kleywegt & Jones, 1996). Similarly, Table 4.1 shows the CB₂ receptor model, with 94.4 % of residues occupying energetically favourable regions, signifying a well-structured protein model. Additionally, a small percentage of residues are in the additional allowed regions, 5.5 % for CB₁ and 5.4 % for CB₂, while an even smaller fraction, 0.2 % for both receptors, are in generously allowed regions. Notably, there are no residues in disallowed regions for either receptor, which further underscores the structural integrity of the models. The distribution of residues within these plots (Fig. C3) provides a qualitative measure of the overall quality of the protein homology models. It is a critical step in validating structural predictions for functional analyses.

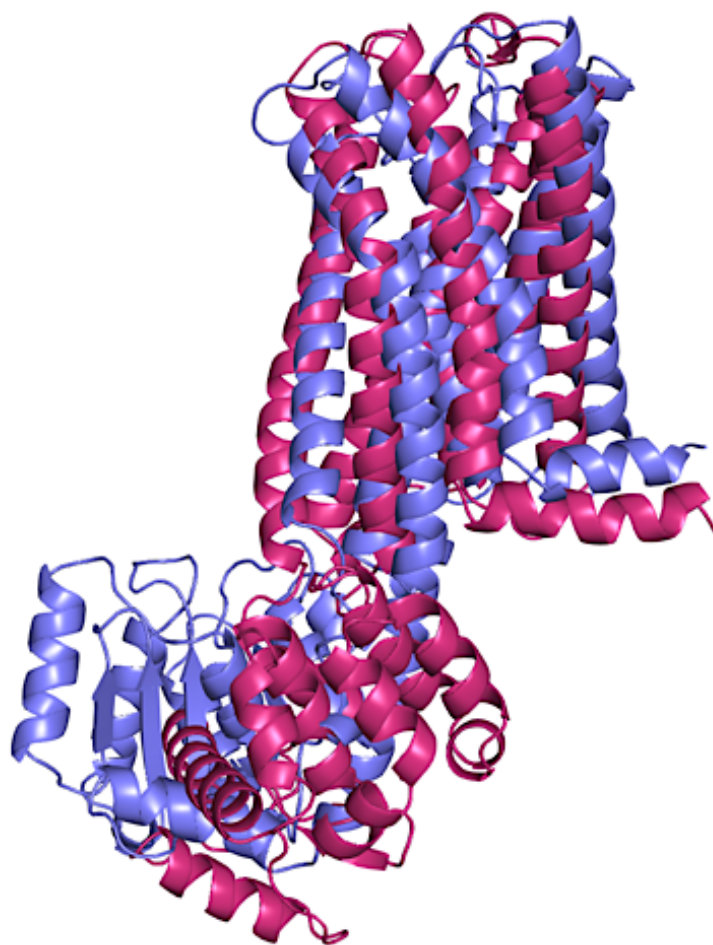


Figure 4.4: Alignment of endocannabinoid receptors CB₁ and CB₂. Superimposed models of CB₁ (purple) and CB₂ (pink). Superimposed with PyMOL (Schrodinger, 2015).

Table 4.1: Ramachandran plot statistics for homology models of pseudo CB₁ and CB₂ receptors

Plot Statistics	Endocannabinoid Receptor 1		Endocannabinoid Receptor 2	
Residues in most favoured regions	409	94.2 %	385	94.4 %
Residues in additional allowed regions	24	5.5 %	22	5.4 %
Residues in generously allowed regions	1	0.2 %	1	0.2 %
Residues in disallowed regions	0	0.0 %	0	0.0 %
Number of residues present in the structure	478	-	449	-

3.2 Solvation and Membrane System

In computational biology, the structural analysis of proteins using MD simulations provides invaluable insights into their functional mechanisms. The CBRs are of particular interest, as their interaction with various ligands can be influenced by the lipid membrane environment. By

comparing simulations of these proteins with and without a POPC membrane using the TIP3P water model, the role of the membrane in modulating receptor behaviour can be discerned.

For the CB₁ receptor, MD analyses shed light on the stability and dynamics of the protein. C_α RMSD plots over time (Fig. C4A) suggest that the CB₁ receptor within the POPC membrane environment deviates less from its initial conformation, indicating enhanced stability in comparison to the receptor in a purely aqueous TIP3P environment. The C_α RMSF (Fig. C4B) extends this notion, demonstrating that residues of the CB₁ receptor exhibit decreased fluctuations in the presence of the membrane, a sign of increased rigidity and structural restraint. Moreover, the compactness of the receptor, inferred from the RoG (Fig. C4C), appears more consistent and reduced within the membrane, highlighting a more stable, compact structure. The PCA plots and corresponding scree plots (Fig. C5) for the CB₁ receptor supplement these findings by illustrating a less diverse conformational landscape when the receptor is associated with the POPC membrane.

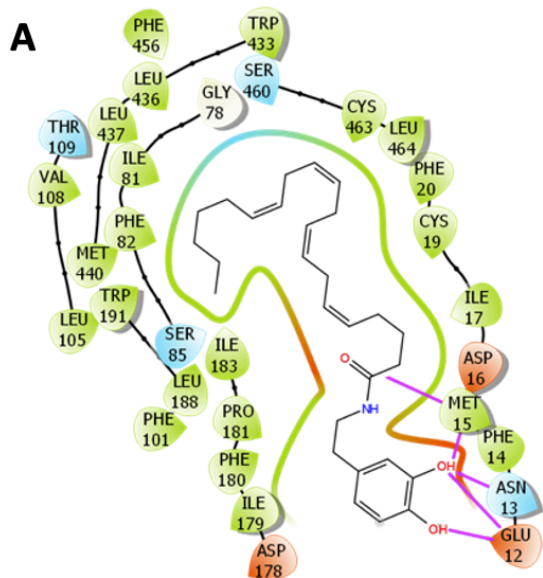
Turning to the CB₂ receptor, similarities in the structural stability over time are observed (Fig. C6A). At various intervals, the membrane-bound receptor exhibits reduced C_α RMSD, pointing to sporadic phases of increased stability. The C_α RMSF data (Fig. C6B) echo the trends observed for CB₁, with certain residues demonstrating diminished fluctuations within the membrane context. The RoG (Fig. C6C) reinforces the pattern of membrane-induced compactness. The PCA and scree plots for CB₂ (Fig. C7) also displays a broad array of sampled conformations, though the POPC membrane introduces constraints on the dynamics of the receptor. While a significant proportion of the movements of the receptor are captured by the first single PC, a complex dynamic profile persists in the absence of the membrane.

For both CBRs the presence of a POPC membrane generally confers structural stability and a more compact form on these CBRs. This is evident from the periods of lower C_α RMSD, restricted C_α RMSF, and reduced RoG, indicating that the membrane plays a stabilising role. The PCA and scree plots highlight these findings, revealing a less varied conformational space suggesting a more constrained range of motions for the membrane system. PCA also shows that the membrane causes a lower and more stable C_α RMSD and RoG. These insights show the practical implications for our understanding of receptor function. The stability and compactness influenced by the membrane are likely to be pivotal for interactions of the receptors with ligands, which naturally occur in a membrane environment. Recognising the influence of the

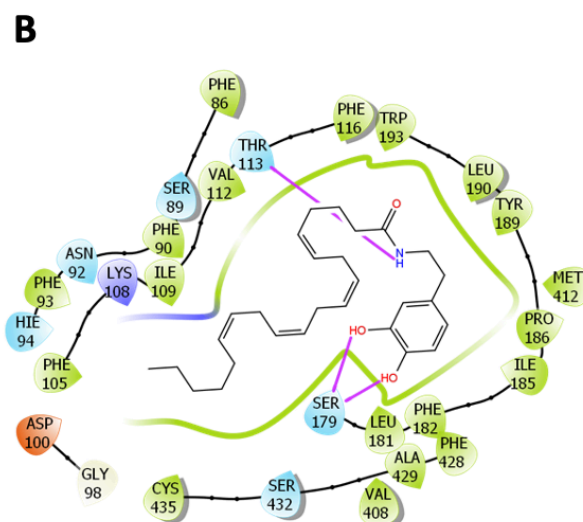
lipid membrane on these interactions is therefore essential for grasping the biological roles of CBRs and can guide the design of therapeutic compounds targeting these receptors.

3.3 Ligand Interaction

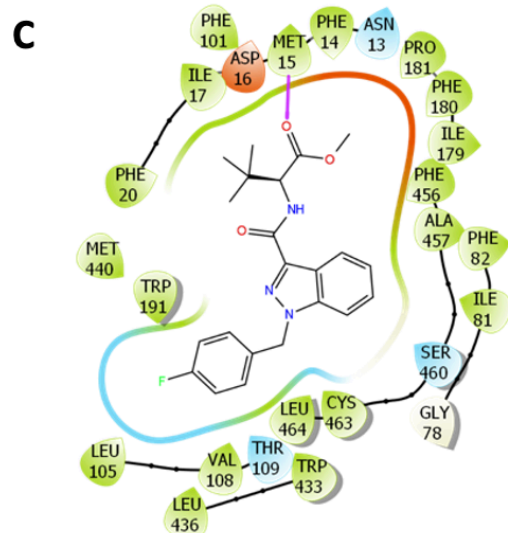
In the analysis of IFD results, various ligands (Fig. 4.3) were evaluated with p-CBRs to determine their interaction profiles (Fig. 4.5). The docking scores provide insights into binding affinities where a negative score indicates a favourable interaction as it represents the free energy change associated with the binding process. The more negative the score, the more likely it is that the ligand will bind strongly to p-CBRs, suggesting a stable and potentially effective interaction in a biological environment. NADA in panel A and B exhibited the highest affinity for both receptors, scoring -13.92 kcal/mol for CB₁ and -13.32 kcal/mol for CB₂. The strong affinity of NADA is supported by H-bond interactions with residues such as Met15, Asn13, and Glu12 for CB₁, and Thr113 and Ser179 for CB₂, with the OH groups of NADA playing a key role necessary for ligand recognition and binding stability. The strong affinity of NADA for both receptors suggest a selective interaction supported by its location in the body, thereby positioning it as a key candidate for *in silico* validation of p-CBRs. FUB-MDMB in panel C shows significant affinity for CB₁, with a docking score of -12.20 kcal/mol through an H-bond interaction with Met15. Taranabant in panel E also displays strong binding to CB₁ with a docking score of -13.28 kcal/mol, due to π - π stacking with Phe20 and Phe82. Similarly, CBD binds to CB₁ through π - π stacking with Phe82 and a H bond with Ser460, resulting in a docking score of -12.26 kcal/mol. For CB₂, alongside NADA, E3R displays a notable docking score of -11.54 kcal/mol with H-bond interactions involving Ser432 and His94, as well as π - π stacking with Phe182 and Phe86. AM630 in panel F, in contrast, exhibits the weakest binding to CB₂ with a score of -8.78 kcal/mol, suggesting a less stable interaction profile and indicating specificity in binding. AM630 formed π -cation bonds with Phe182 and Trp193, a H bond with Thr113, and π - π stacking between Phe116 and Trp405. The moderate affinity of CBD at -9.30 kcal/mol for CB₂ can be attributed to π - π stacking interactions with Trp193.



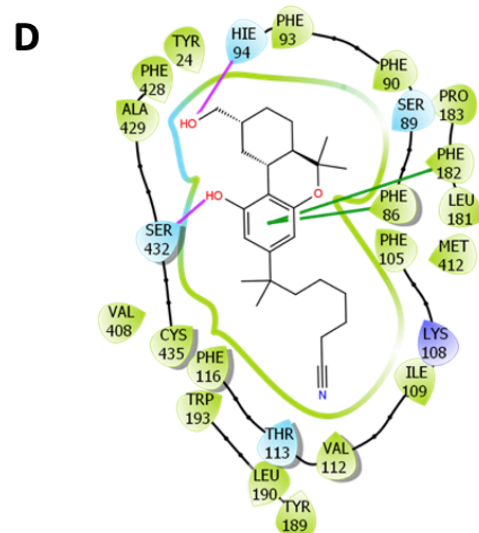
Complex: CB₁ – NADA
 Docking Score: –13.92 kcal/mol



Complex: CB₂ – NADA
 Docking Score: –13.32 kcal/mol



Complex: CB₁ – FUB-MDMB
 Docking Score: –12.20 kcal/mol



Complex: CB₂ – E3R
 Docking Score: –11.54 kcal/mol

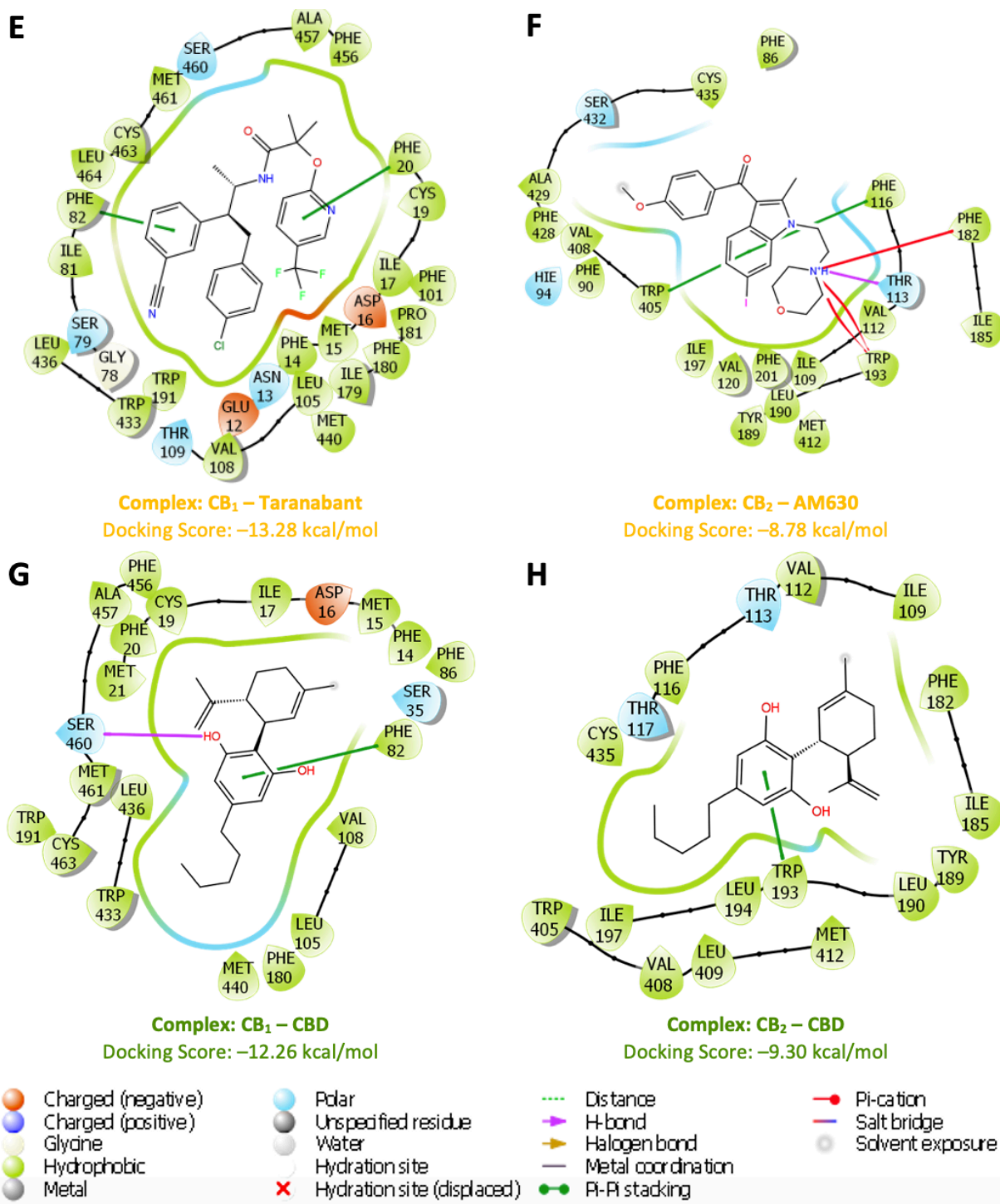


Figure 4.5: Induced-fit docking top conformer 2D ligand interactions. A. and B. illustrate the docking of NADA to CB₁ and CB₂ receptors, respectively. C. Represents the docking of FUB-MDMB to CB₁, and D. shows E3R docked to CB₂, both ligands serving as agonists. E. Depicts Taranabant in complex with CB₁, and F. presents AM630 docking to CB₂, both acting as inverse agonists. G. and H. highlight the docking of CBD to CB₁ and CB₂, with docking scores, expressed in kcal/mol, quantifying the binding affinities, where lower values indicate stronger ligand-receptor interactions. Generated from Maestro Schrödinger Suite.

3.4 Energy Properties

The simulation quality analysis for both CB₁ and CB₂ systems (Table 4.2 and 4.3) over a 250 ns period demonstrates a slope energy of zero for all systems showing successful equilibration, with consistent temperatures at ~298 K. For CB₁, the degrees of freedom peak with Taranabant and NADA, while CB₂ displays higher degrees of freedom when interacting with NADA and E3R suggesting a more flexible simulation environment. This implies that the receptor can adopt a variety of conformations which is essential to the ability of the receptor to bind to a diverse range of ligands or for the activation of different signalling pathways. When examining the energy properties of CB₁-complexes, the average total energy and potential energy displayed a trend where NADA displayed the lowest energies, suggesting the most stable interaction, aligning with the natural affinity of endocannabinoids for CBRs. The CB₁ apo state showed the highest total energy, which is normal as the unbound state of the receptor may not be the most energetically favourable configuration as ligand binding stabilises the conformation of GPCRs. Pressure variations are observed across all simulations which is normal and expected, as the system is dynamic and subject to fluctuations. These variations reflect the constantly changing interactions among molecules in the system and can indicate the level of system compactness or density (Leach, 2001). This is important as it helps ensure that the simulated environment behaves in a physically realistic way, which is crucial for the accuracy of the predictions of the simulation regarding molecular behaviour and interactions. Volumetric analysis shows the largest systems with Taranabant for CB₁ and NADA for CB₂, corresponding to expanded receptor-ligand complexes. Each system is equilibrated, thereby confirming the stability of these dynamic molecular systems. The slope energy stability over time remained at or near zero for all simulations which showed that the receptor maintains a stable conformation across various ligands with minor variations in energy, potentially reflecting different binding affinities and interactions within the binding pocket.

Table 4.2: Simulation energy properties for CB₁ systems

Statistical Parameters	Apo	NADA	FUB-MDMB	Taranabant	CBD
Simulation Period (ns)	250.00	250.00	250.00	250.00	250.00
Degrees of Freedom	141853	161211	153736	164349	146648
Number of Atoms	65690	74517	71224	76178	68022
Average Total Energy (kcal/mol)	-116151.80	-125664.51	-124343.60	-123603.39	-121447.47
Average Potential Energy (kcal/mol)	-158158.88	-173394.12	-169867.49	-172266.69	-164876.64
Temperature (K)	298.04	297.97	298.02	298.00	298.05
Pressure (bar)	0.87	1.47	1.25	1.29	1.30
Volume (Å ³)	638265.35	723028.60	692012.22	739565.77	661813.67
Slope (ps ⁻¹)	-0.001	0.000	0.000	-0.001	0.000

Table 4.3 Simulation energy properties for CB₂ systems

Statistical Parameters	Apo	NADA	E3R	AM630	CBD
Simulation Period (ns)	250.00	250.00	250.00	250.00	250.00
Degrees of Freedom	150195	154383	153622	140182	144228
Number of Atoms	69961	71701	71400	65151	66969
Average Total Energy (kcal/mol)	-128263.10	-126944.28	-127483.56	-118506.84	-119839.14
Average Potential Energy (kcal/mol)	-172746.90	-172660.86	-172976.50	-160023.83	-162550.75
Temperature (K)	298.08	298.03	298.04	298.07	298.05
Pressure (bar)	1.36	0.70	0.75	1.30	0.86
Volume (Å ³)	682471.69	698028.68	695374.07	634755.01	651847.95
Slope (ps ⁻¹)	0.000	0.000	0.000	-0.001	-0.001

3.5 Protein Backbone C_α RMSD

The C_α RMSD data for CB₁, as represented in Graph A (Fig. 4.6.1) along with their corresponding PCA plots A – E (Fig. 4.6.2). The endogenous ligand NADA showed a highly stable C_α RMSD, consistently under between a range of 2 Å, aligning with the tight clustering seen in PCA plot B which is in line with the ECSs natural affinity for NADA. FUB-MDMB and Taranabant also showed stability from 30 ns into the simulation, however Taranabant displayed a decrease in dynamics and FUB-MDMB showed an increase, shown by its broader spread in the respective PCA plot, indicating a change in dynamics when bound yet still is still a stable conformation. In contrast, CBD causes a unique conformational change (Fig. 4.6.1A) where CBD exhibits a different C_α RMSD pattern, indicative of an alternative binding mode

such as a modulator. CB₂ receptor dynamics shown in Graph B (Fig. 4.6.1) and the PCA plot (Fig. 4.6.2) indicate a varied response to ligand interactions. The C_α RMSD for the CB₂ apo form shows natural flexibility, which increases when bound to NADA. These significant C_α RMSD peaks imply that NADA might induce a range of conformations within CB₂, which is supported by the scattered PCA plot. The agonist and inverse agonist exhibit C_α RMSD variability, thereby suggesting diverse conformational shifts. CBD showed moderate C_α RMSD values displaying partially agonistic effect on CB₂ receptor conformation. The larger spread in the PCA plots for CB₂ compared to CB₁, correlates with the higher C_α RMSD values observed for CB₂, suggesting that CB₂ may undergo more pronounced conformational changes upon ligand binding. Additionally, both proteins display more dynamism after ~50 ns.

Finally, the scree plots (Fig. 4.6.3) offer a comparative analysis of the PCs that describe the variance in the C_α RMSD of p-CBRs. For CB₁, PC1 captures a sizable portion of the variance (46.63 %), suggesting a dominant conformational change. The sharp decline to PC2 and gradual descent thereafter indicates that most of the dynamic behaviour is concentrated in the first two PCs. In contrast, the CB₂ protein exhibits a more even distribution of variance across the PCs, with a less pronounced drop between PC1 (39.38 %) and PC2 (26.95 %), which could imply a more complex ensemble of conformational states or a more evenly distributed set of movements. The differences in the variance distribution between CB₁ and CB₂ suggest distinct conformational flexibility and their different mechanisms of action in response to ligand binding.

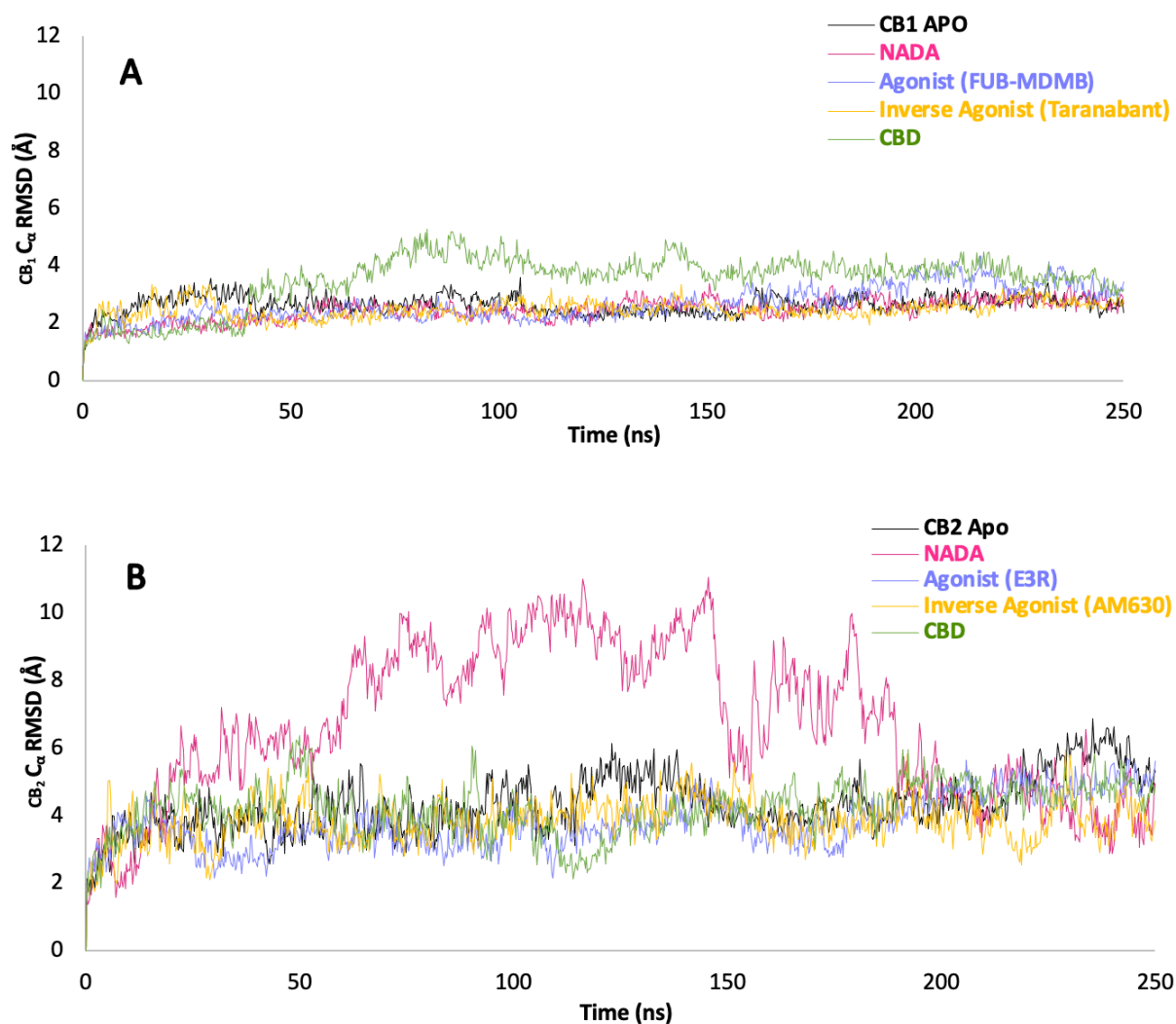


Figure 4.6.1: Stability of ligand-receptor complexes over time C_α RMSD. A. CB₁ and B. CB₂ receptor dynamics represented by C_α RMSD measurements in Å over a 250 ns simulation. Each plot tracks the stability of CBRs in their apo form and in complex with various ligands: NADA, an agonist (FUB-MDMB for CB₁ and E3R for CB₂), an inverse agonist (Taranabant for CB₁ and AM630 for CB₂), and CBD. Higher C_α RMSD values indicate greater deviation from the initial structure, reflecting changes in conformational stability.

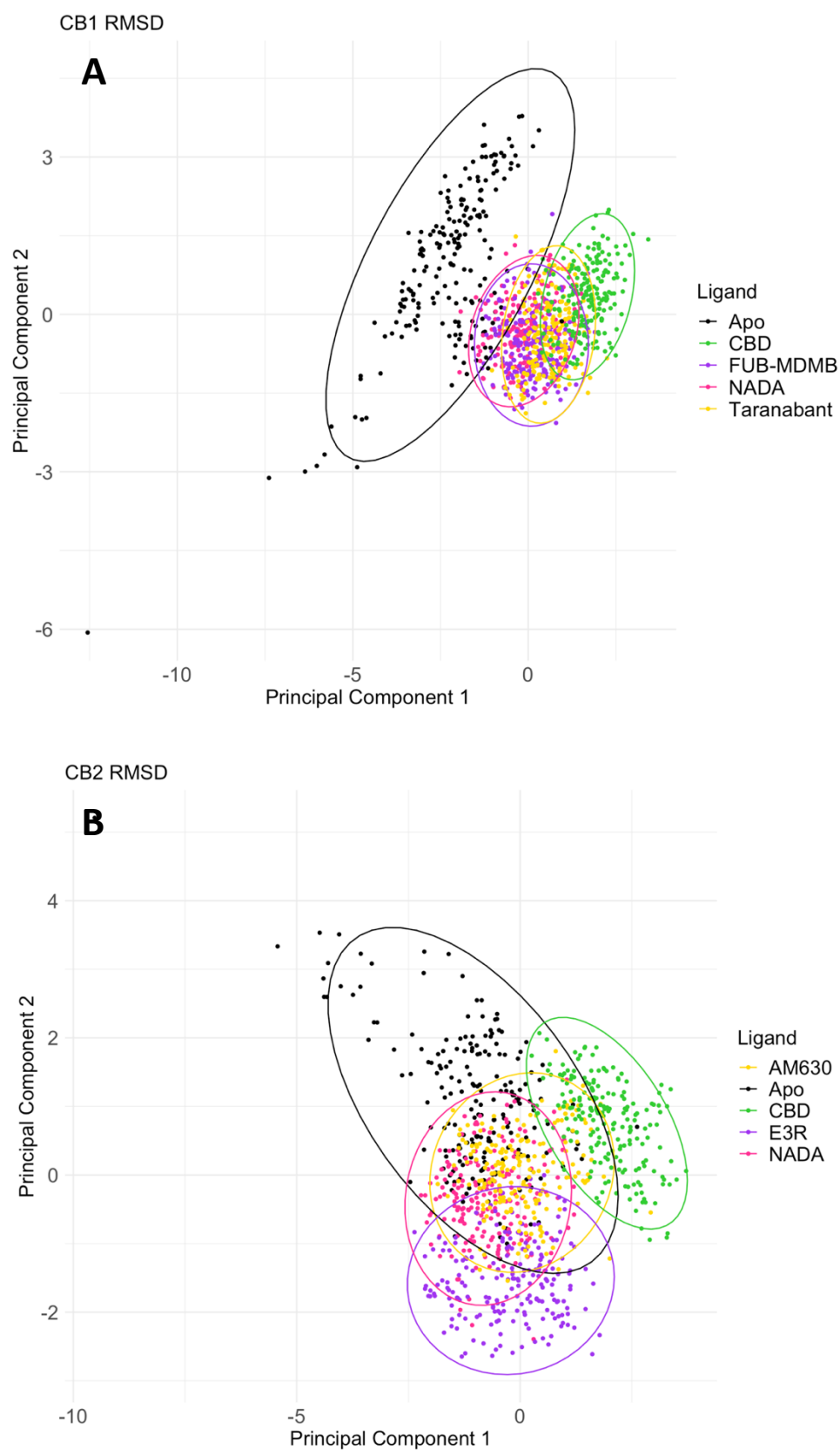


Figure 4.6.2: PCA of CB₁ and CB₂ C_αRMSD receptor dynamics. PCA scatter plots in panel A depict the C_αRMSD of various ligand-bound CB₁ systems in comparison to its apo state, while panel B similarly illustrates the CB₂

receptor dynamics. The spread of data points reflects the conformational diversity sampled during the simulations, with denser clusters indicating more frequently adopted conformations. Generated from R Studios.

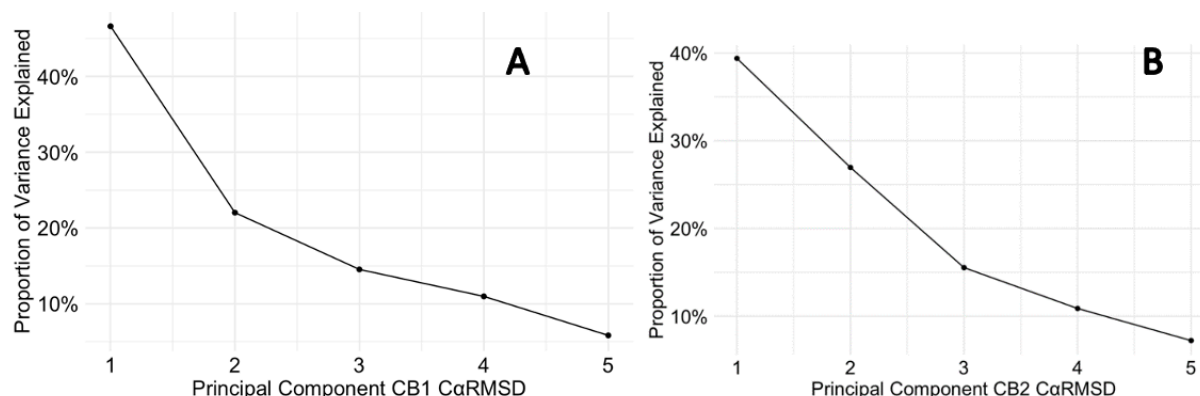


Figure 4.6.3: Scree plots for CB₁ and CB₂ protein C_α RMSD variance. A. Depicts the variance explained by the principal components for the CB₁ protein, while B. shows the same for CB₂. Generated from R Studios.

3.6 Protein Backbone C_α RMSF

The C_α RMSF graph A for CB₁ (Fig. 4.7.1) shows a well-conserved dynamic profile across all residues when in the apo state and when interacting with ligands. NADA has a compact C_α RMSF which indicates a stable receptor-ligand interaction. FUB-MDMB and Taranabant have C_α RMSF peaks in the membrane bound regions that differ when compared to the apo. Therefore, causing subtle conformational changes within the receptor in membrane bound regions. CBD shows a unique C_α RMSF profile to its counterparts especially in the membrane bound region such as between residues, Ile192 – Tyr204 and more residues showing distinct structural changes. Its PCA also suggests a unique binding conformation, highlighting the allosteric or modulatory nature of CBD. The effect of CBD on CB₂ presents an C_α RMSF profile with moderate fluctuations, aligning with its potential partial agonistic effect. However, CBD shows to be more dynamic than a full agonist as substantiated by its PCA (Fig. 4.7.2). CB₂ displays a broader range of conformational adaptability as evidenced by higher C_α RMSF values and the scattered distribution within its PCA plots, especially in the presence of synthetic ligands. This comparative analysis emphasises the differential conformational responses of CB₁ and CB₂ to various ligands and emphasises the importance of considering receptor flexibility in the development of cannabinoid-based therapeutics. These findings suggest that while CB₁ may have a more rigid binding pocket, conferring specificity, CB₂ flexible nature could allow for broader ligand accommodation, which might be significant for its role in immune modulation.

The scree plots provided in figure 4.7.3 offer a detailed view of the dynamical properties of p-CBRs by analysing the eigenvalues across PCs derived from a PCA (Fig. 4.7.2). For CB₁, PC1 accounts for a substantial 82.51 % of the total variance, indicating that a single collective motion characterises the flexibility and dynamic behaviour of the protein within the simulation period. This main fluctuation captured by PC1, may be associated with the protein's functional conformational changes or with its interactions with surrounding molecules. The steep drop to 8.05 % variance explained by PC2 and even lower for subsequent PCs whereby fluctuations in the context of the dynamics of the protein are minimal yet still affect signalling. Similarly, CB₂ protein exhibits a more pronounced dominance in its first PC (91.42 %), suggesting an even more constrained set of motions across residues, indicative of a more rigid or stable structure. Comparatively, CB₂ displays less diversity in its motion patterns than CB₁, as evidenced by the higher proportion of variance explained by its PC1. This could reflect differences in their respective ECS roles, ligand-binding affinities, and stability. The minimal contributions of higher-order PCs (PC2 – PC5) for both proteins suggest that these additional fluctuations play a lesser role in defining the proteins' overall dynamics yet still influences cellular signalling.

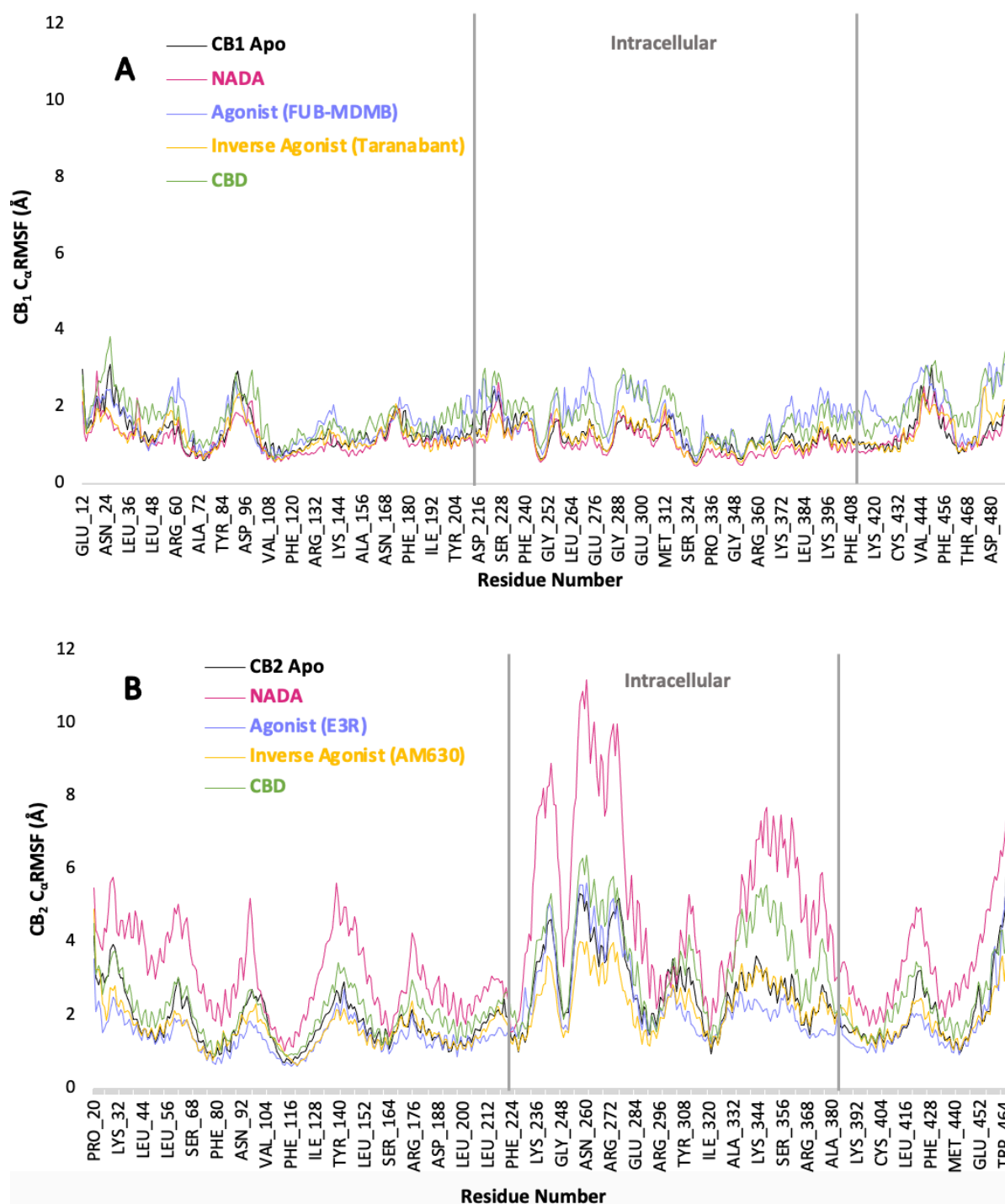


Figure 4.7.1: Residue flexibility of ligand-receptor complexes. A. CB₁ and B. CB₂ C_α RMSF in Å across all residues. These plots illustrate the flexibility of each residue in the apo form and when bound to various ligands: NADA, an agonist (FUB-MDMB for CB₁ and E3R for CB₂), an inverse agonist (Taranabant for CB₁ and AM630 for CB₂), and CBD. The area between the grey lines shows the ICL regions, while the rest represent membrane-bound regions. Higher C_α RMSF values indicate greater flexibility and potential dynamic regions within the protein structure.

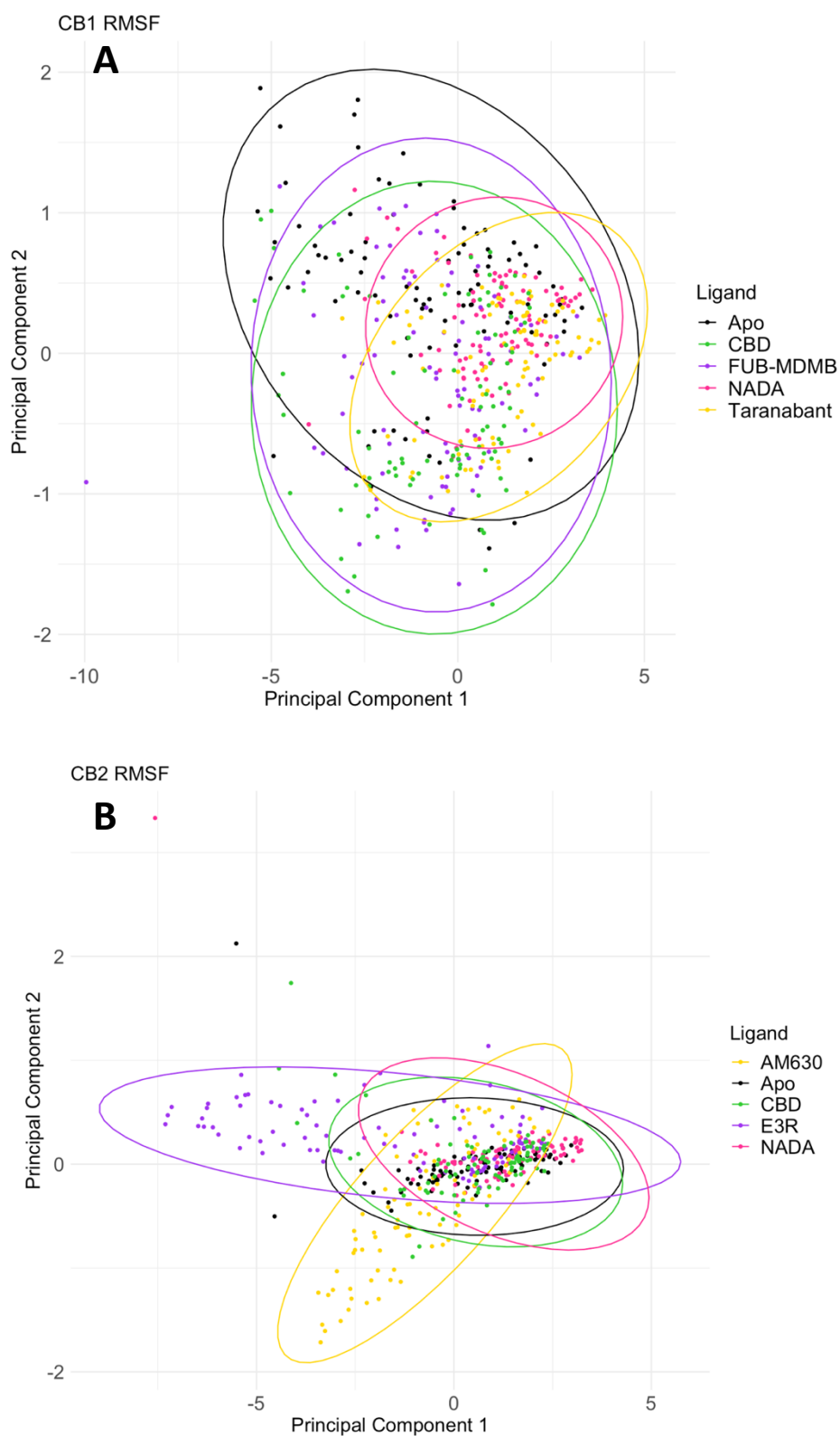


Figure 4.7.2: PCA of CB₁ and CB₂ C_α RMSF receptor dynamics. A. represents the C_α RMSF of the CB₁ receptor in its apo form and complexed with various ligands, while B. mirrors this for CB₂. The scatter of points across the PCA plots reveals the diversity of conformational states examined in the simulations, with areas of higher density indicating a greater frequency of certain conformations by the receptors. These plots provide insights into the flexibility and dynamic behaviour of each receptor-ligand complex. Generated from R Studios.

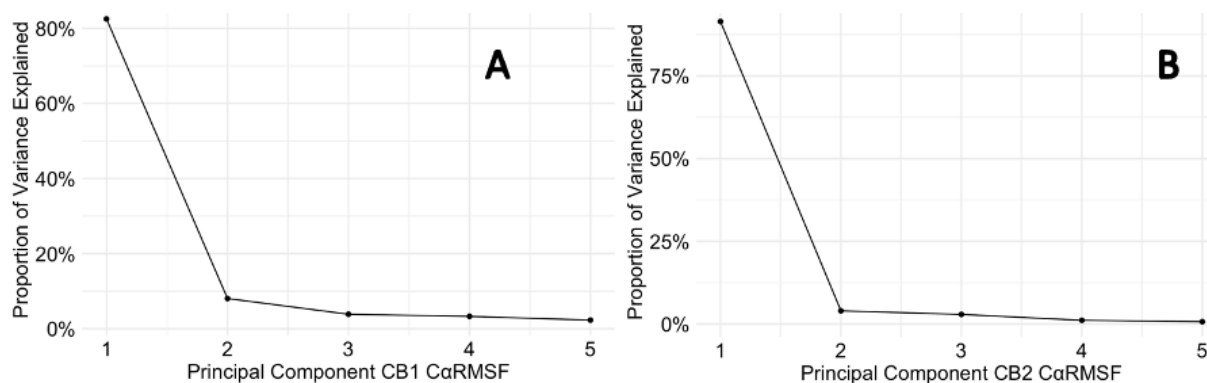


Figure 4.7.3: Scree plots for PCA in CB₁ and CB₂ proteins. A. Presents the scree plot for CB₁, and B. for CB₂. The plots illustrate the proportion of total variance in atomic fluctuations captured by each PC, indicating the relative significance of these components to the overall movement observed in the proteins. Generated from R Studios.

3.7 Protein Backbone C_α Radius of Gyration

The RoG of CB₁ (Fig. 4.8.1 A) remains consistent in the apo form, suggesting a stable and well-folded state. Upon binding with Taranabant, the receptor has a lower level of compactness, as indicated by the RoG values while CBD increases the compactness, implying that these ligands significantly perturb the overall folding and stability of the receptor when compared to the apo. Interestingly, towards 200 ns the interaction of CBD with CB₁ shows a minor increase in RoG variability, suggesting subtle differences in the compactness of the receptor and perhaps slight changes in the folding dynamics, which is reflected in its PCA (Fig. 4.8.2) with a slightly more dispersed cluster. The RoG plot for CB₂ exhibits greater variability, particularly in response to ligand interactions, signifying a more dynamic structural behaviour. The receptor in its apo state already shows a higher degree of fluctuation, indicating intrinsic flexibility. Upon binding with ligands such as NADA and E3R, spikes in RoG values are observed, indicative of transient expansions in the structure of the receptor, which represent regions undergoing conformational changes. The inverse agonist AM630 also causes significant fluctuations in RoG, further emphasising the dynamic nature of CB₂ and its diverse ligand-induced states. These variations in protein compactness and folding dynamics are crucial for understanding the allosteric effects of ligand binding and the resulting functional outcomes.

The PCA of the RoG for p-CBRs (Fig. 4.8.3) provides insightful data on the intrinsic flexibility and the dynamic nature of these proteins. The relatively even distribution of variance explained across the initial five PCs for both CB₁ and CB₂ suggests a complex interplay of gyrations within their structures. Specifically, CB₁ demonstrates a relatively uniform decline in variance explained, with PC1 accounting for 23.9 %, and a gradual decrease to 16.3 % by PC5. This

uniformity in variance distribution could indicate that no single conformational change dominates the dynamic behaviour of CB₁, implying a more distributed and nuanced gyration pattern that may facilitate its interaction with various ligands. Similarly, CB₂ RoG variance follows a close pattern with PC1 explaining 23.7 % of the variance, only marginally less than that of CB₁, and PC5 accounting for 17.1 %. This suggests that CB₂ also does not rely on predominant conformational changes, but rather a combination of motions contributing to its dynamic profile. The slightly higher variance captured by the first PC in both receptors might reflect the overall size and conformational changes, which are critical for understanding how these receptors adapt and respond to different ligands. The subsequent PCs capturing a substantial portion of variance indicate multiple, significant conformational fluctuations rather than a single, dominant motion, which is typical for flexible proteins that interact with diverse molecules.

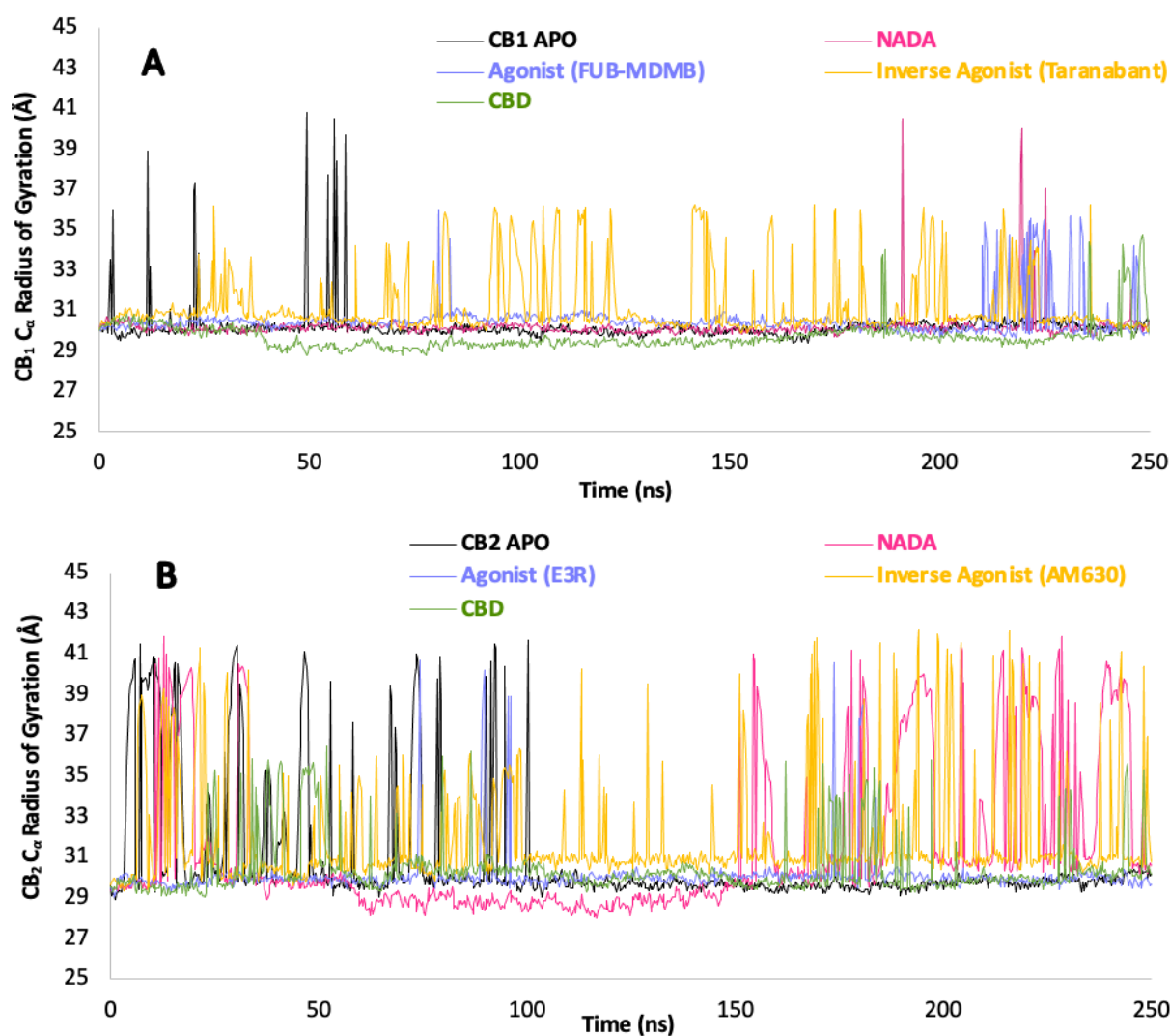


Figure 4.8.1: C_α Protein compactness analysis over time. A. CB₁ and B. CB₂ receptors structural compactness, indicated by the RoG measured in Å over a simulation period of 250 ns. The graphs compare the RoG of the apo CBRs to their complexes with NADA, agonists (FUB-MDMB for CB₁ and E3R for CB₂), inverse agonists (Taranabant for CB₁ and AM630 for CB₂), and CBD.

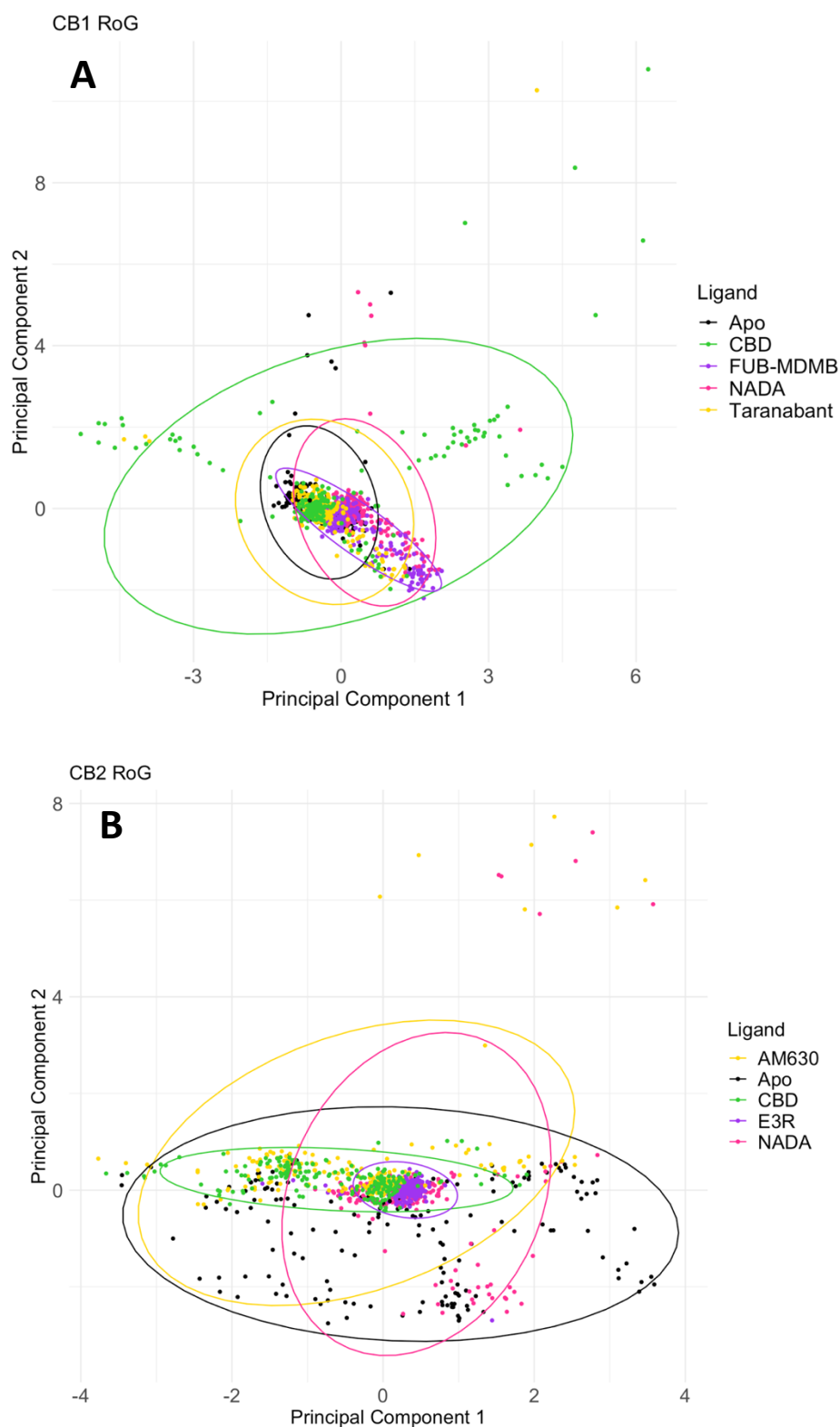


Figure 4.8.2: PCA of CB₁ and CB₂ radius of gyration. The PCA scatter plots A. correspond to the CB₁ receptor in its apo state and are complexed with various ligands: NADA, FUB-MDMB, Taranabant, and CBD. Similarly, B. represent the CB₂ receptor in its apo state and in complex with NADA, E3R, AM630, and CBD. These plots assess the overall compactness of the protein-ligand complexes over the course of simulations, with the RoG serving as an indicator of the protein's structural integrity and folding dynamics. Generated from R Studios.

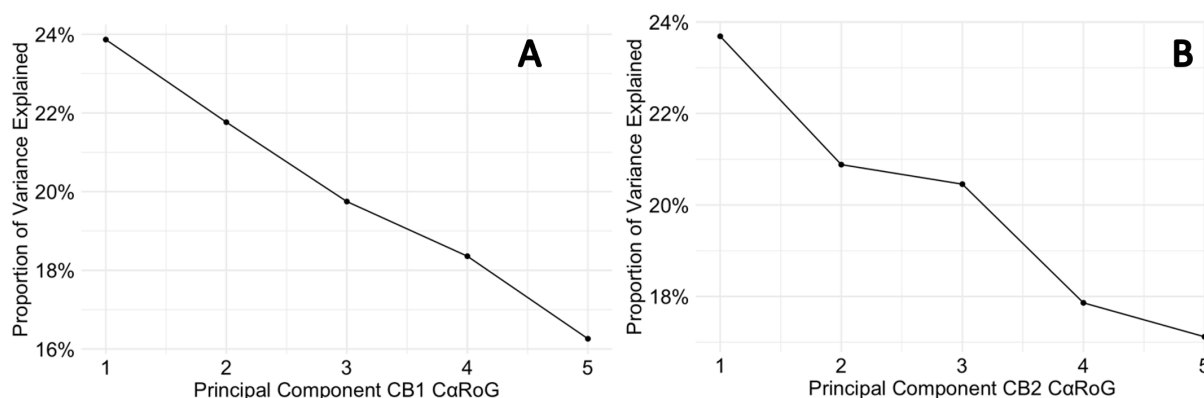
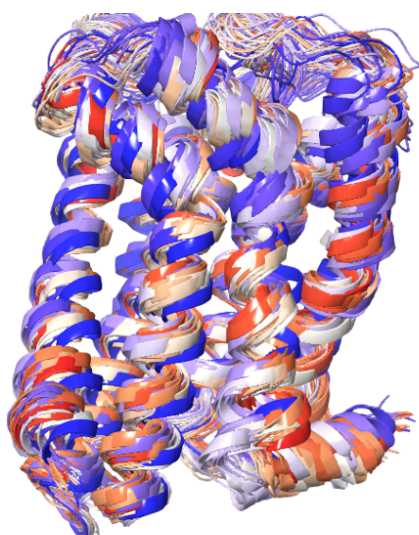
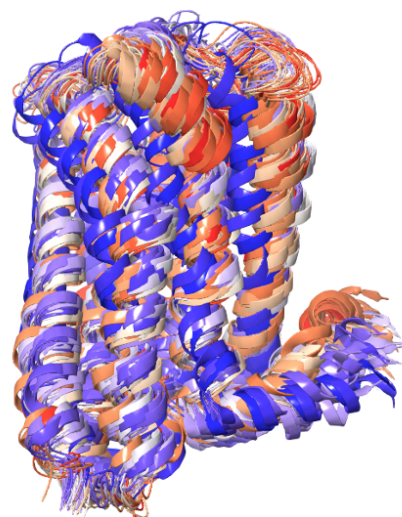
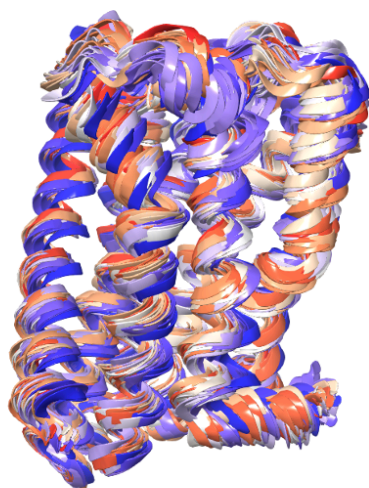
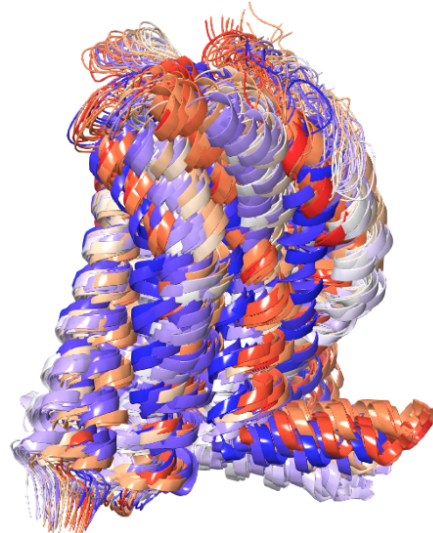
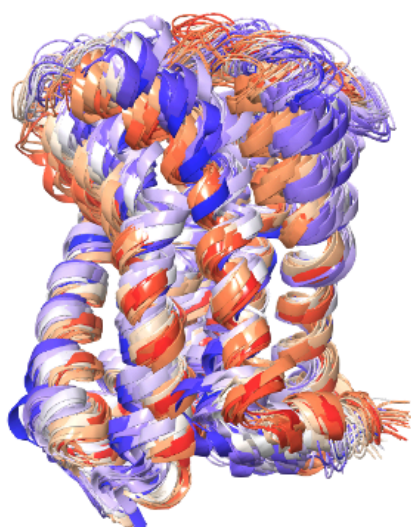
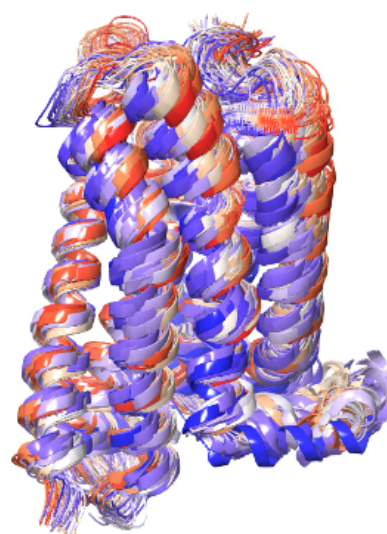


Figure 4.8.3: Comparative PCA of CB₁ and CB₂ radius of gyration. A. The scree plot for the CB₁ receptor's RoG across the first five PCs. B. The scree plot for the RoG of the CB₂ receptor across the first five PCs. Each plot illustrates the percentage of total variance explained by the corresponding PC, highlighting the distribution of conformational flexibility within the protein structure. Generated from R Studios.

3.8 Simulation Trajectory Snapshots

The series of structural snapshots presented in Figure 4.9.1 shows a vibrant depiction of the dynamic nature of CB₁ and CB₂ receptors in the context of their membrane-bound regions over a 250 ns MD simulation. The colour gradient from blue to red effectively illustrates the temporal progression of the simulation, where the blue structures represent the starting conformations, and the red structures signify the conformations at the end of the 250 ns simulation period. For CB₁ in its apo state, the snapshots indicate a high degree of structural conservation with minor deviations from the initial conformation, suggesting a relatively stable receptor in the absence of a ligand. When bound to NADA, CB₁ exhibits subtle conformational adaptations, demonstrating the natural compatibility of the ligand with the active site of the receptor. The structural changes are more pronounced with synthetic ligands such as FUB-MDMB and Taranabant, where the snapshots reveal greater shifts, especially in the transmembrane domains. CBDs interaction with CB₁ has deviations in its structure, thereby showing the structural changes over time influencing the structures signalling properties. In comparison, CB₂ apo snapshots also show a more dynamic conformational baseline, yet the presence of NADA has more noticeable shifts throughout the simulation, indicating a dynamic interplay between the receptor and its endogenous ligand. Synthetic agonists like E3R and the inverse agonist AM630 produce significant conformational changes in CB₂ suggesting a more flexible receptor that can accommodate various ligand-induced states. CBD's interaction with both receptors results in unique changes in CB₁, implying its modulatory role while CBD acts as a partial agonist in CB₂.

A**Complex: CB₁ – Apo****F****Complex: CB₂ – Apo****B****Complex: CB₁ – NADA****G****Complex: CB₂ – NADA****C****Complex: CB₁ – FUB-MDMB****H****Complex: CB₂ – E3R**

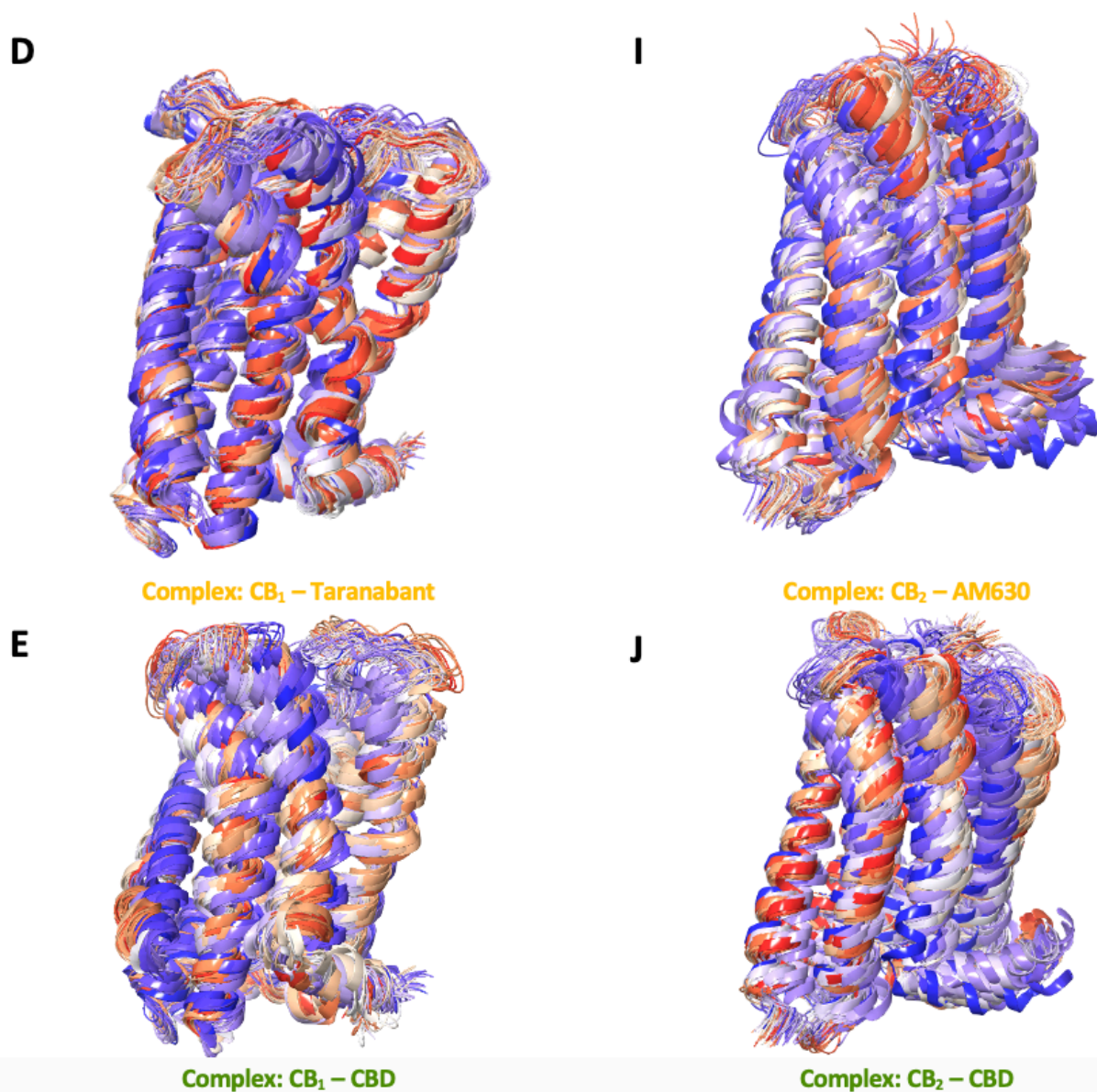
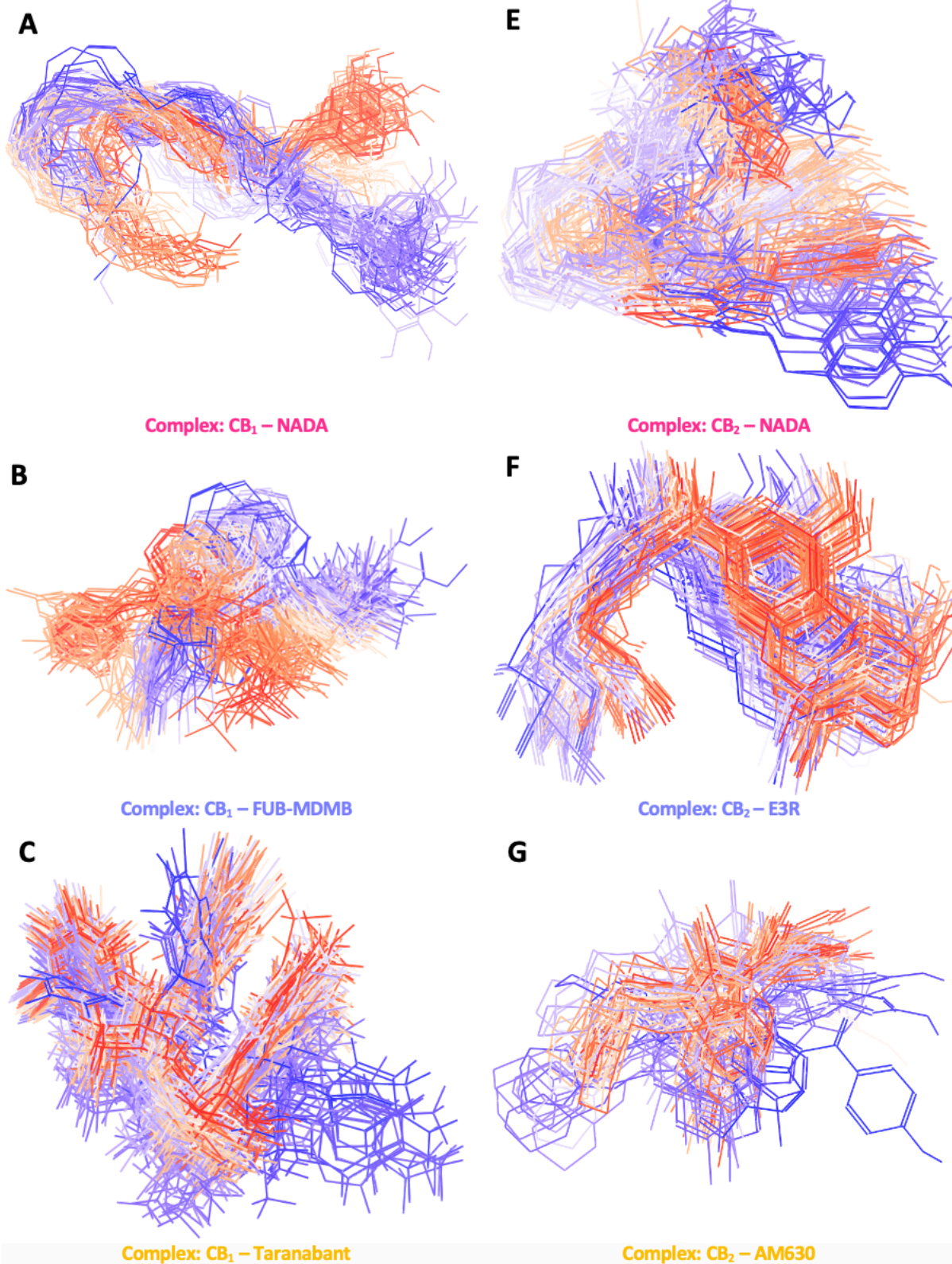


Figure 4.9.1: Membrane-bound conformational snapshots over 250 ns. These figures highlight a series of secondary structural snapshots of CB₁ and CB₂, capturing the conformational changes between the membrane bound region. This illustrates the dynamic behaviour of the receptors in their apo state and when bound to various ligands: A – E NADA, FUB-MDMB, Taranabant, and CBD for CB₁, and F – J NADA, E3R, AM630, and CBD for CB₂. These snapshots highlight the flexibility and the varying degrees of conformational states that each receptor-ligand complex can adopt over 250 ns. Generated from Maestro Schrödinger Suite.

The ligand conformational snapshots over 250 ns in Figure 4.9.2 provide an intricate depiction of how various ligands occupy the binding sites of both p-CBRs, highlighting the dynamic nature of their interactions and the conformational flexibility within the protein environment. In A and C (Fig. 4.9.2), NADA, and the synthetic agonist FUB-MDMB express a range of orientations within the CB₁ binding site, with NADA showing a more focused array of two main conformations. One conformation is predominant at the start of the simulation, whereas

the second stabilises later, indicating a precise fit within its endogenous receptor. FUB-MDMB also follows a similar pattern but shows more alternative binding modes suggesting a more adaptable interaction space. Taranabant, shows an even more diverse conformational spread, potentially reflecting its ability to induce various inactive receptor states, each with distinct structural configurations. NADAs interactions with CB₂ show more flexibility compared to CB₁, with NADA showing more conformational range. This might relate to the role of CB₂ in modulating immune functions, requiring adaptability to a broader spectrum of endogenous ligand shapes. The synthetic agonist E3R shows one main conformation and the inverse agonist AM630 exhibits an extensive range of conformations, indicative of its capacity to explore and stabilise various inactive receptor conformations, which may correspond to different signalling outcomes or binding affinities with other ligands. The visual representation of CBD within the CB₁ binding site reveals a significant conformational range, as denoted by the spread from the initial blue conformations to the final red conformations. The spectrum of conformations suggests that CBD interacts with the CB₁ receptor in a versatile manner, potentially influencing various signalling pathways by inducing multiple receptor states. This aligns with CBDs known pharmacological profile as a compound with multiple mechanisms of action, including its role as a negative allosteric modulator that can alter receptor responsiveness. Similar to its interaction with CB₁, CBD exhibits a variety of conformations within the CB₂ binding site. However, the spread of conformations for CB₂ appears to be slightly more constrained compared to CB₁, which may reflect the distinct roles these receptors play in physiological processes. The dynamic interaction of CBD with CB₂ might contribute to its therapeutic effects related to the immune system and inflammation, as CB₂ is predominantly expressed in immune cells.



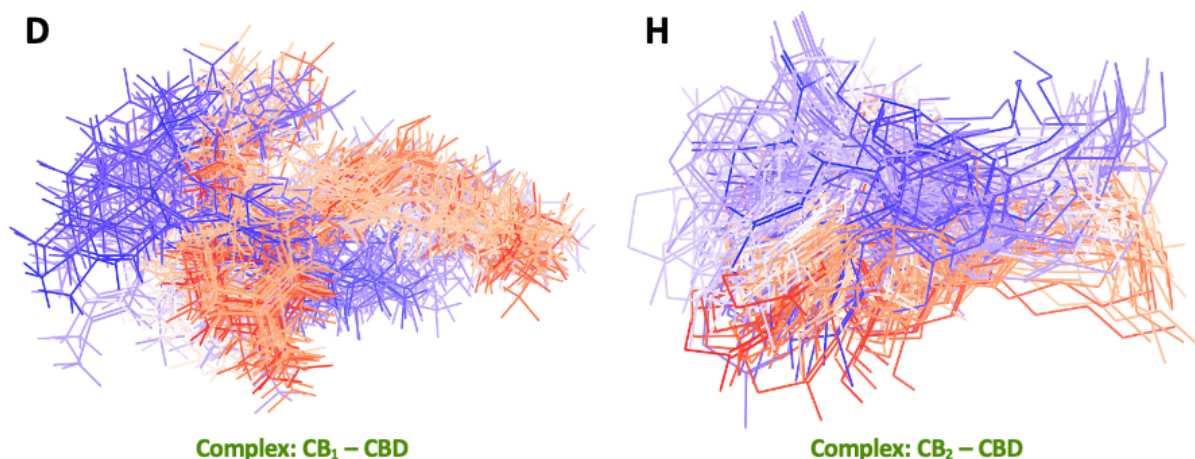


Figure 4.9.2: Ligand conformational snapshots over 250 ns. This figure presents overlaid conformations of ligands within the binding sites of CB₁ and CB₂. The snapshots depict the flexibility and range of orientations for NADA, FUB-MDMB, Taranabant, and CBD when bound to CB₁ A – D and NADA, E3R, AM630, and CBD when bound to CB₂ E – H. These visualisations emphasise the dynamic nature of ligand-receptor interactions and the conformations they exhibit within the protein environment. Generated from Maestro Schrödinger Suite.

3.9 Ligand RMSD and Simulation Protein-Ligand Interactions

Figure 4.10 showcases the lig-RMSD trajectories for both CB₁ and CB₂ receptors over a simulation period of 250 ns, providing a quantitative depiction of the stability and conformational changes of various ligands while bound to CBRs. The lig-RMSD trajectory (Fig. 10) NADA bound to CB₁ demonstrates a stable interaction towards the end of the simulation with all ligands reaching stability at ~185 ns in the simulation. FUB-MDMB, exhibits a slightly higher lig-RMSD, indicating a more flexible interaction compared to NADA but still within a range suggestive of consistent binding. Taranabant, shows increased fluctuations at first then stabilises, reflecting its potential to induce diverse conformations in the receptor, which may correlate with its inverse agonistic activity. CBDs trajectory is characterised by a moderate level of fluctuation, consistent with its role as a modulator, potentially affecting the receptor conformation and thereby its signalling pathways. CB₂ lig-RMSD for NADA with CB₂ shows more pronounced fluctuations compared to CB₁, hinting at a more flexible interaction or multiple binding conformations within this receptor. E3R, another synthetic agonist, presents a lig-RMSD profile with significant variation, suggestive of a dynamic binding that may promote a range of receptor states. AM630, as an inverse agonist, displays a highly variable lig-RMSD, indicative of its ability to stabilise CB₂ in multiple conformations, potentially inactive ones. CBD with CB₂ exhibits a similar pattern to that observed with CB₁, with fluctuations that are indicative of its modulatory influence on the receptor dynamics. The overall lower lig-RMSD values for CB₁ across all ligands imply a

tighter and more consistent binding pocket, which may influence the receptor's signalling specificity. The greater variability in lig-RMSD for CB₂ suggests a more adaptable ligand-binding environment, which could allow for a wider range of physiological responses. The differences in lig-RMSD profiles for synthetic agonists and inverse agonists when compared to NADA underscore the potential for these compounds to elicit varied therapeutic effects.

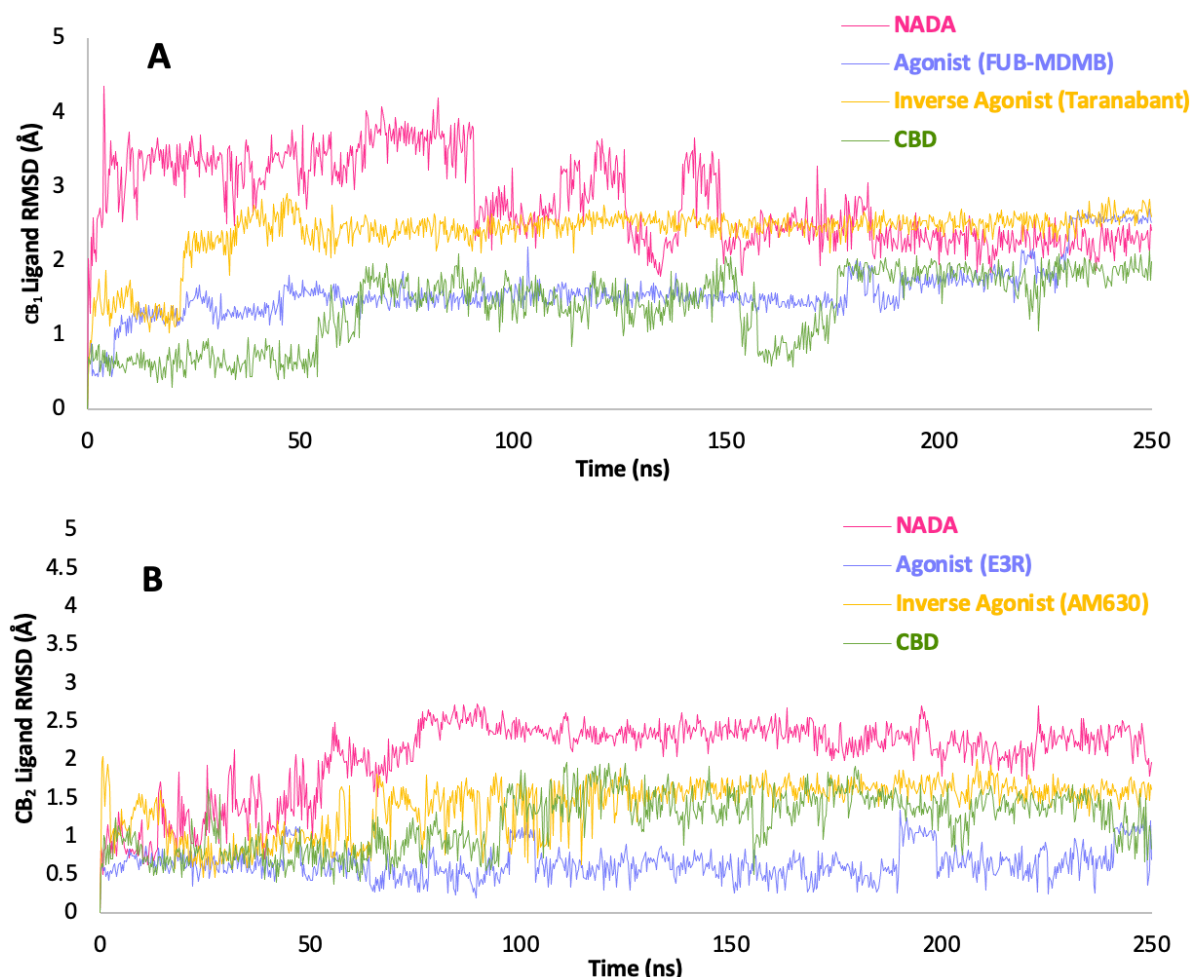
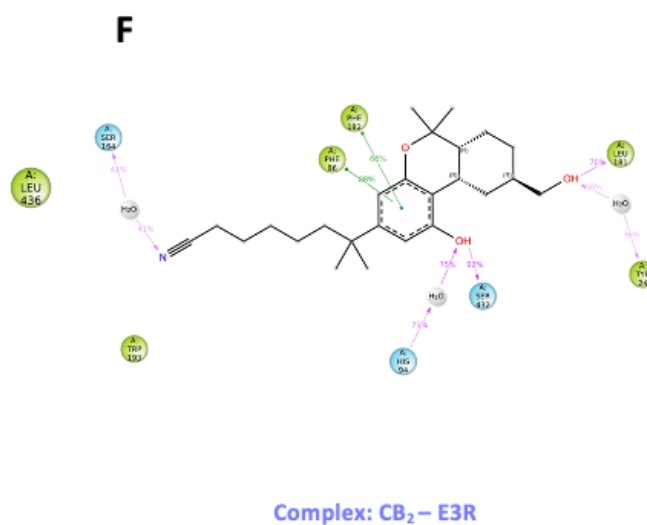
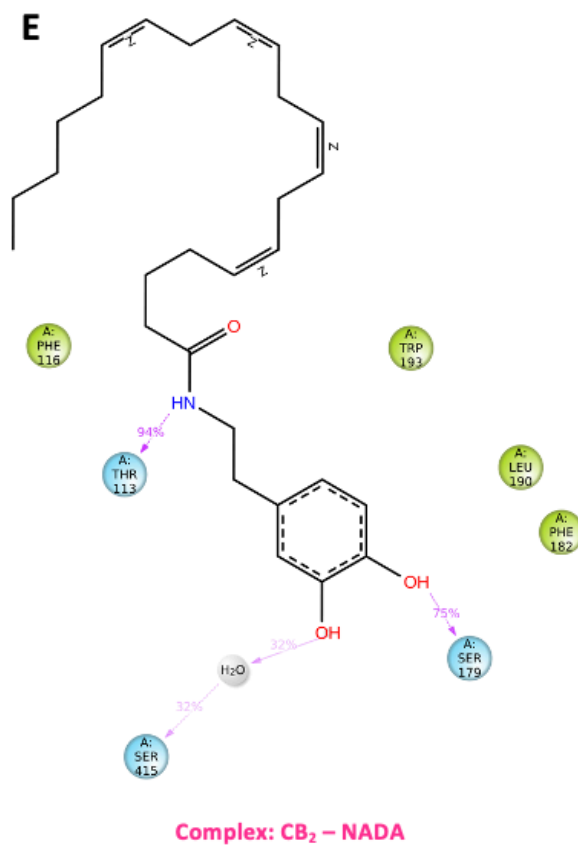
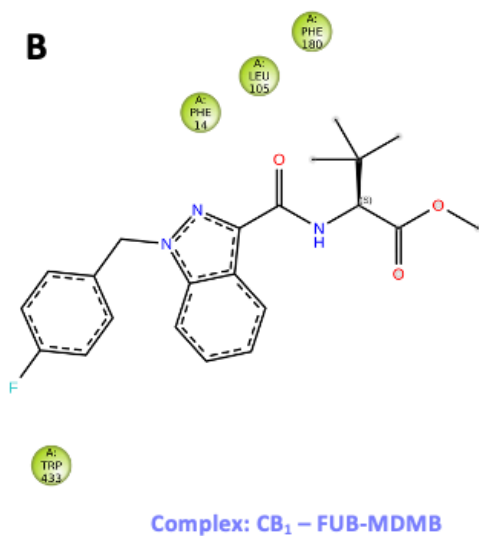
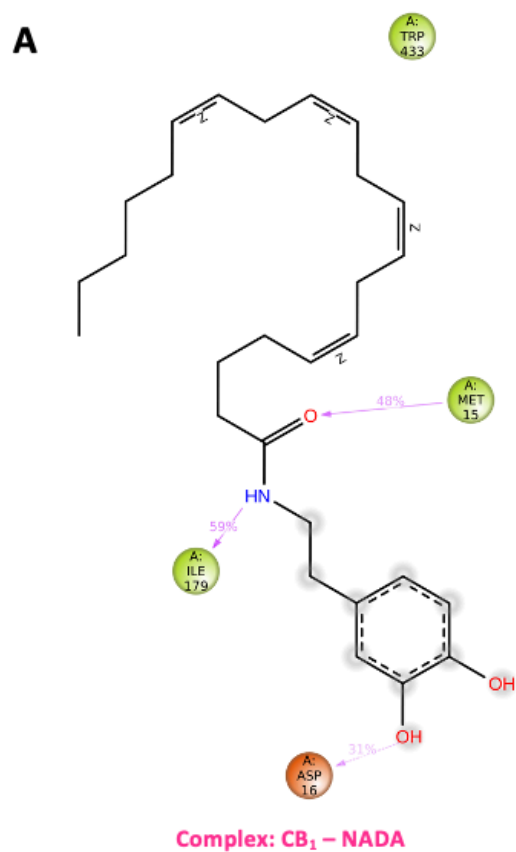


Figure 4.10: Ligand-RMSD trajectories relative to protein. A. illustrates the RMSD of each ligand when bound to the CB₁ receptor, and B. displays the same for the CB₂ receptor, over a simulation time of 250 ns. Each trace represents the RMSD of ligand atomic positions relative to their initial frame, providing a measure of the ligands stability and conformational changes while bound to the receptor. The ligands include NADA, an agonist (FUB-MDMB for CB₁ and E3R for CB₂), an inverse agonist (Taranabant for CB₁ and AM630 for CB₂), and CBD. Stable RMSD values suggest a consistent interaction with the receptor, while fluctuations indicate dynamic binding and conformational adaptability.

The images (Fig. 4.11) depict molecular structures of ligand atom contacts in complex with p-CBRs for more than 30.0 % of the time during molecular dynamic simulations. Each ligand shows a distinct chemical structure with various functional groups that influence their

interaction with the protein residues. NADA in both CB₁ and CB₂ complexes, NADA has a dopamine moiety that can be identified by its catechol structure and a long unsaturated fatty acid tail, which is a characteristic feature of endocannabinoids. NADA complexed with CB₁ has several key interactions such as the longest interaction between Ile179 and the NH group for 59 % of the simulation while the O group attracted Met15 for 48 % of the simulation suggesting a stable hydrogen bond, potentially critical for signal transduction. The NH group of CB₂ complexed with NADA was in contact with Thr113 for 94 % of the simulation while a notable water molecule was in contact with a OH group along with Ser415 (Fig 12). The other OH group was in contact with Ser179 for 75 % of the simulation showing a network of hydrogen bonds that might stabilise specific receptor conformations conducive to signalling.

FUB-MDMB shown in the CB₁ complex, is a synthetic compound with a distinct indazole core with potentially high binding affinity due to its rigid structure. Alternatively, the CB₂ receptor agonist has two distinct π - π stacking bonds with its aromatic ring with residues Phe86 and Phe182 for 88 % and 66 % for the simulation, respectively. This stacking could be pivotal for stabilising the receptor in an active conformation, enhancing signal transduction. For the inverse agonists, Taranabant and CB₁ has a more complex structure with multiple rings, which may contribute to its activity as an inverse agonist, potentially blocking the receptors active site and preventing activation with three main contacts influencing its binding such as π - π stacking with Phe20 while the N group attracted Phe82 and Ser460 attracted the NH group. For CB₂, AM630 has a biphenyl structure linked to a heterocyclic moiety with a key π - π stacking interaction with Phe86 follows a similar pattern as CB₁, with π - π stacking interactions suggesting a potential lock on the receptor's conformation, which may reduce its ability to signal. Present in both CB₁ and CB₂ complexes, CBD is a well-known cannabinoid with a resorcinol core and a pentyl chain. Notably, water molecules in both complexes mediate interactions with the OH groups; in CB₁, they facilitate hydrogen bonding between OH and Met15, as well as OH and Ile179. In the CB₂ complex, water is attached to both the OH group and Ser460. For CB₁, the water-mediated hydrogen bonds suggest a dynamic modulation of receptor conformation. This water bridge could be essential for CBDs ability to modulate receptor activity subtly, rather than inducing a rigid active or inactive state. Such modulation could lead to nuanced signalling outcomes, which aligns with CBDs known diverse physiological effects.



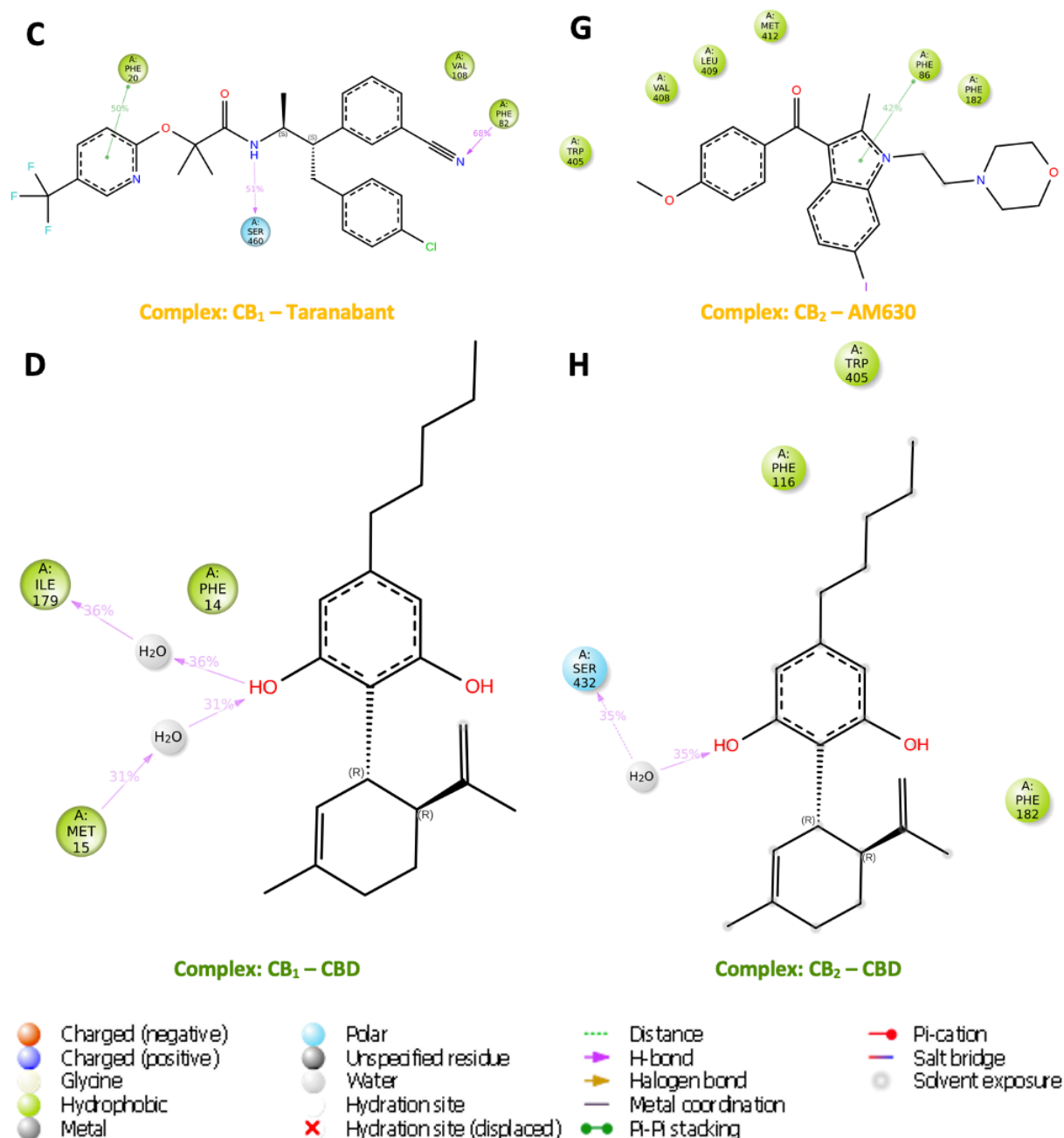


Figure 4.11: Post-MD simulation ligand-contact summary. This figure compiles a visual representation of sustained ligand-protein interactions within the binding sites of CB₁ and CB₂ receptors throughout molecular dynamic simulations. A – D illustrate the contact profiles for the following ligands with CB₁ with E – H depicting the ligand interactions with CB₂: These images capture the intricate contact dynamics and diverse orientation landscapes that ligands adopt within the protein environment, emphasising the variable and complex nature of ligand-receptor interplay. Generated from Maestro Schrödinger Suite.

3.10 Water Analysis

The distance of the water molecule 9906 (Fig. 4.12) demonstrates a notable stabilisation in its role as a bridge between the OH group of NADA and the Ser415 residue of CB₂. Initially, the

distance between the interacting entities fluctuates, as indicated by the variable peaks early in the simulation. However, at $\sim 45,000$ ps into the simulation, we observe a significant change whereby the distance distinctly stabilises, hovering around a much lower value, indicative of a stable interaction being established. This stabilisation suggests that water 9906 has found an energetically favourable position where it can effectively mediate an H bond between the ligand and the receptor. The persistent short distance after 40,000 ps indicates that the water molecule maintains this bridge consistently for the remainder of the simulation. The implications of such a stable water bridge in the context of a lipid bilayer are interesting as the hydrophobic environment of the bilayer typically restricts polar interactions to the periphery of the membrane, yet the persistent interaction of water 9906 deep within the membrane interface suggests a significant structural feature. This water bridge may play a crucial role in maintaining the conformational stability of the ligand-receptor complex, enabling correct signalling function such as a modulator. The provided plot (Fig. 12) captures the intricate fluctuations between water molecule 11077 and residue Ile179 within the CB₁ receptor, complexed with CBD, over 250 nanoseconds. This variability in proximity suggests a non-static interaction, which is significant within the typically hydrophobic environment of the lipid bilayer. The water molecules (11077) recurring proximity to Ile179 suggests its potential influence on the receptor conformation and, consequently, on signal transduction mechanisms. Despite the variability, the intermittent closeness to Ile179 implies episodic bridging interactions that may be integral to the modulation of CBDs influence on the CB₁ receptor. The presence of a water molecule in the hydrophobic environment of a lipid bilayer is noteworthy, as it suggests an energetically favourable interaction that overcomes the hydrophobic exclusion effect (Nickels & Katsaras, 2015). This can be indicative of a polar pocket or channel within the receptor that can accommodate water molecules, potentially impacting the receptor's conformation and function when interacting with CBD to enable it to act as a modulator much like CB₂ in complex with NADA.

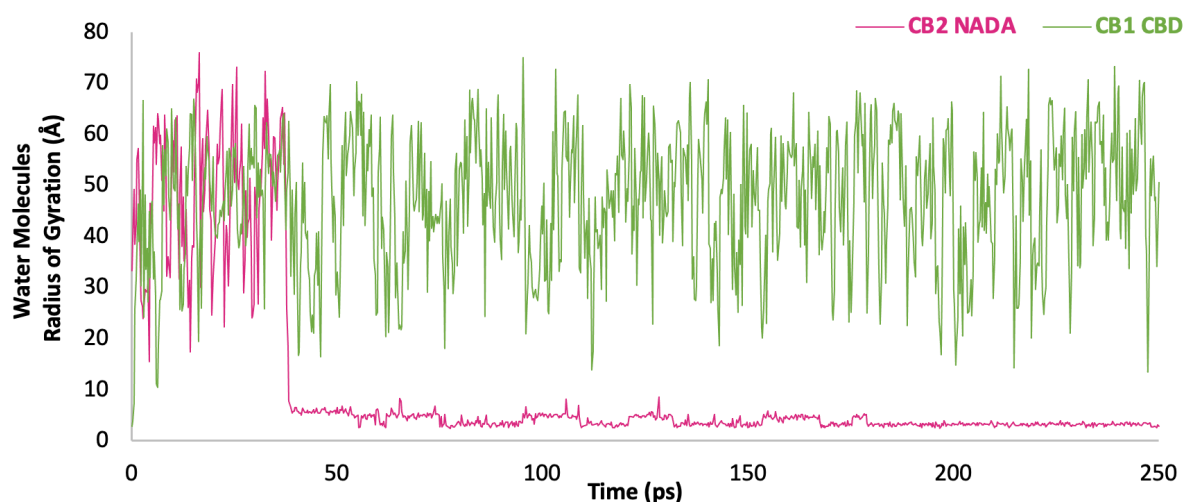


Figure 4.12: Interaction dynamics of water molecules 9906 with CB₂-NADA Complex and Water 11077 with CB₁-CBD Complex. This graph depicts the fluctuating distance (Å) between water molecules over the course of a 250 picosecond molecular dynamics simulation. The water (9906) stabilisation observed after 45 picoseconds highlights the formation of a significant water bridge, integral to the interaction network within the binding pocket. Water 11077 shows the dynamic nature of water molecule interactions within the receptor's binding site, with distance variations suggesting transient stabilising events.

4 DISCUSSION

The purpose of the study was to validate computational models for future research on cannabinoids and related synthetic compounds, establishing a groundwork for discerning the nuances of cannabinoid interactions with these receptors. This foundational work is pivotal for the development of new therapeutics targeting cannabinoid receptors, enhancing our understanding of their pharmacological landscape. The modifications in p-CBR structures, such as truncating the N-terminal and incorporating different stabilising domains in the ICL3, have been essential for retaining functional integrity. This strategic modification is vital for accurate interpretations of cannabinoid receptor behaviour and interaction dynamics (Li et al., 2019; Shao et al., 2016). Taranabant and AM630 were selected for their roles in crystallising CB₁ in its inactive state and as a known inverse agonist for CB₂. FUB-MDMB and E3R, are documented agonists that stabilise the active receptor conformations substantiated by their active-state co-crystal structures (PDB codes 6N4B and 6KPF) (Hua et al., 2020; Kumar et al., 2019). Agonists and inverse agonists were preferred over antagonists to observe conformational changes, as antagonists often block ligands without blocking other ligands or necessarily prompting a significant conformational shift (Haubrich, 2021). The agonists were expected to bind to CBR active site, leading to a change in the receptor's conformation by activating it. This activation results in G-protein coupling and subsequent signal transduction, enhancing receptor

dynamics as it transitions between inactive and active states. In contrast, inverse agonists like AM630 stabilise an inactive receptor conformation, potentially reducing receptor dynamics due to the prevention of the active state formation. As IFD studies show, NADA was selected for its natural and high affinity for CBR while also possessing a benzene ring, a structural similarity to phytocannabinoids yet unique when compared to endocannabinoids (Ibsen et al., 2017; Wojtalla et al., 2012). These interactions, particularly H-bond and π - π stacking interactions with key residues, highlight p-CBR sensitivity to molecular changes, which allows for the development of highly selective cannabinoid-based therapeutics. Furthermore, the agonist dynamics were expected for CB₁ receptor interaction with NADA (Bradshaw et al., 2006). However, while the precise impact of NADA on the CB₂ receptor remains unclear, this study exhibits a distinctive dynamic profile between CB₂ and NADA compared to other ligands, suggesting a potential modulatory effect on the receptor's function. CBDs selection was due to its vast therapeutic potential and growing legal status worldwide. Modulators such as CBD, due to their distinct interaction mechanisms, may not cause the full activation or inactivation of the receptor but could influence its dynamics by altering the receptor's conformational landscape, affecting the efficacy or potency of other ligands. These effects on receptor dynamics are crucial for understanding how different ligands can modulate receptor signalling and subsequent cellular responses.

After MD simulations, the energy properties show that all systems achieved equilibration over a 250 ns simulation period, confirming the stability of the receptor-ligand complexes. Attaining equilibrium is fundamental for the acceptance of these models as valid representations of biological reality (Levitt, 2001). The lowest energy states observed with p-CBR in complex with NADA show a direct correlation between thermodynamic stability and the functional efficacy of the receptor-ligand interactions as NADA is naturally made for CBRs. Therefore, this suggests a direct correlation between thermodynamic stability and therapeutic potency. These stable energy values validate the system for further analysis and application in drug design, providing a credible basis for predicting binding efficiency, ligand interactions and implications. Equilibrium conditions are vital to ensure that the simulated molecular interactions are not transient phenomena but stable states that can provide information for drug efficacy and safety profiles (Jiang et al., 2022). Hence, the equilibrium state of these models supports their function in discovering how subtle molecular modifications can translate into significant therapeutic outcomes, providing a robust framework for the study of cannabinoids. By ensuring that the models have reached equilibrium, it can be trusted that the simulated

behaviours of the molecules reflect a realistic and stable system, thereby enhancing the credibility and applicability of the simulation results in drug discovery processes (Jorgensen, 2004; Levitt, 2001).

The C_{α} RMSD and C_{α} RMSF data provide insight into the dynamic structural behaviour of p-CBR. The stable C_{α} RMSD of NADA and the unique conformational changes induced by CBD on CB₁ yet CB₂ displays an opposite effect which highlights the potential of these ligands in modulating receptor activities differently (Aviz-Amador et al., 2021). The differential responses of CB₁ and CB₂ receptors to these ligands, as indicated by the distinct structural changes observed particularly in membrane-bound regions, emphasise the complexity of receptor-ligand interactions and the necessity to consider this in drug design (Di Marzo & Piscitelli, 2015; Russo & Hohmann, 2012). Since additions were made to the crystal structure in the ICL3, the membrane-bound region of the protein was assessed using both temporal simulation snapshots depicting changes over 250 ns and C_{α} backbone data. The modulation effect by CBD on CB₁, suggested by its unique C_{α} RMSF profile, presents an interesting aspect of cannabinoid pharmacology that could lead to the development of novel therapeutic agents with minimised side effects. Additionally, this data does not only provide insight into the receptor conformational stability and flexibility but also, when combined with protein trajectory snapshots, illustrate the diverse structural dynamics under the influence of different ligands. These snapshots capture the real-time evolution of receptor shapes, highlighting how ligands such as NADA and CBD induce distinct conformational states in the receptors. Moreover, the RoG analysis sheds light on the impact of ligand binding on the overall folding and stability of p-CBRs. For CB₁, the ligand interactions suggest a balance between structural stability and adaptability of its core residues essential for effective receptor function by showing a more compact and folded compared to CB₂ upon ligand binding. The dynamic behaviour of CB₂ suggests a receptor with a high degree of structural flexibility, which might be an adaptive advantage in interacting with a broad range of ligands (Di Marzo & Piscitelli, 2015; Pertwee, 2008). These visualisations are crucial for understanding the complexity of receptor-ligand interactions, offering a perspective for drug design by displaying how subtle variations in ligand structure can lead to significant changes in receptor behaviour which then affects cellular signalling.

The unique conformational responses of CB₁ and CB₂ to various ligands, especially the distinct interactions of CBD, highlight the potential of cannabinoid-based therapies with potential as a

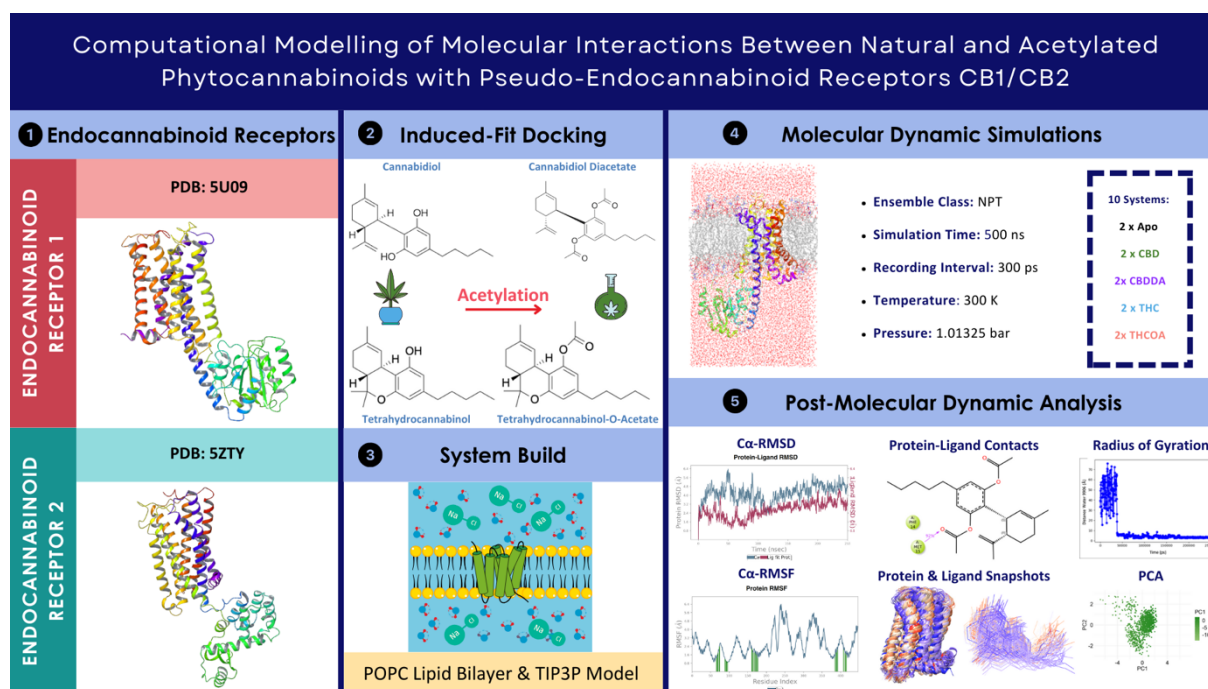
therapeutic agent with fewer side effects compared to traditional agonists or antagonists (Russo & Hohmann, 2012). CB₁ specificity offers targeted therapeutic avenues for neurological disorders, while CB₂ broader ligand profile holds promise for immunological conditions (Russo & Hohmann, 2012). These computational studies bridge the gap to empirical work by rapidly screening and modelling interactions with emerging cannabinoids, thus expediting the drug discovery process. These computational models of p-CBRs can efficiently predict the behaviour of modified cannabinoids, providing a preclinical insight that can guide empirical studies and streamline the path to novel therapeutic agents. This approach is fundamental in a rapidly evolving field where modified cannabinoids are being continuously synthesised and require a swift and reliable method of evaluation for their potential therapeutic or adverse effects (Holt et al., 2022).

5 CONCLUSION

This comprehensive study not only enhances the understanding of the conformational dynamics of CB₁ and CB₂ receptors but also sets the stage for future advancements in cannabinoid science. The detailed structural, energetic, and dynamic analyses of p-CBRs provide a robust foundation for developing effective cannabinoid-based therapeutics, capitalising on the unique properties of these receptors and their ligands. As research continues to unfold, these findings will certainly contribute to a deeper understanding of cell signalling mechanisms and foster the development of innovative therapeutic approaches.

COMPUTATIONAL MODELLING OF MOLECULAR INTERACTIONS BETWEEN NATURAL AND ACETYLATED PHYTOCANNABINOIDs WITH PSEUDO-ENDOCANNABINOID RECEPTORS CB₁/CB₂

GRAPHICAL ABSTRACT



ABSTRACT

The consumer market of phytocannabinoids is rapidly evolving, with synthetic acetylated phytocannabinoids such as cannabidiol diacetate (CBDDA) and delta⁹-tetrahydrocannabinol-O-acetate (Δ⁹-THCOA) gaining attention due to their modified pharmacokinetic profiles, heightened effects, and potential for enhanced therapeutic efficacy. This study presents a comparative analysis of the molecular interactions and pharmacological implications of these modified phytocannabinoids with natural counterparts, cannabidiol (CBD), and delta⁹-tetrahydrocannabinol (Δ⁹-THC), in complex with pseudo-endocannabinoid receptors (p-CBRs), CB₁ and CB₂. Utilising molecular dynamics (MD) simulations, this study examined the C_α RMSD, C_α RMSF, and the RoG to assess the stability, flexibility, and compactness of the ligand-receptor complexes. These findings reveal that acetylation alters the dynamic interaction at both receptors, potentially leading to increased binding affinity and receptor activation. Notably, Δ⁹-THCOA exhibited a tighter binding conformation at CB₁, suggesting a higher

efficacy in receptor modulation, which was paralleled by similar findings at the CB₂ receptor. The study highlights the enhanced potency and therapeutic promise of CBDDA and Δ^9 -THCOA, while also emphasising the critical need for comprehensive safety evaluations to mitigate the risks associated with their higher potency. By bridging the knowledge gap in CBR pharmacology, the research paves the way for future investigations into the clinical applications of acetylated phytocannabinoids, emphasising the need for a cautious approach to the development and regulation of these novel compounds.

1 INTRODUCTION

Cannabis sativa L. has a long history of human usage dating back to 12,000 B.C. (Pierro Neto et al., 2023). It has gained considerable attention due to its active compounds known as phytocannabinoids, which exhibit great pharmacological and industrial potential (National Academies of Sciences & Medicine, 2017). Among these, two prominent compounds are CBD and Δ^9 -THC. CBD, a non-psychoactive phytocannabinoid, has gained significant interest for its potential therapeutic applications. Studies suggest that CBD may have anti-proliferative, anti-inflammatory, analgesic, neuroprotective and anxiolytic properties, making it a candidate for alleviating ailments such as cancer, chronic pain, anxiety disorders, and epilepsy (Maurya & Velmurugan, 2018). In contrast, Δ^9 -THC is known for its psychoactive effects. However, it also has several therapeutic benefits, including pain relief and antiemetic effects. The interaction between phytocannabinoids and the endocannabinoid system (ECS) within the body forms the basis of their therapeutic potential as they affect cellular signalling and various physiological processes (Legare et al., 2022).

Synthetic cannabinoids (SCs) are compounds designed to replicate or enhance the effects of naturally occurring phytocannabinoids. These compounds are typically developed through chemical synthesis and exhibit significant variation in their chemical structures and pharmacological properties. The design of SCs was pioneered by Gaoni & Mechoulam (Gaoni & Mechoulam, 1964), who synthesised a partial constituent of Δ^9 -THC to further investigate the structure and effects of Δ^9 -THC. This pivotal work led to the design of more SCs and eventually to the discovery of CBRs in 1980. Therefore, this initiated an exciting avenue of research by shedding light on the ECS and allowed for elucidating the effects of phytocannabinoids, SCs, and endocannabinoids on signalling pathways and therapeutic outcomes through receptor-ligand interactions (Devane et al., 1988).

Recently, novel SCs such as acetylated phytocannabinoids have emerged in the *Cannabis* consumer market (Holt et al., 2022). Phytocannabinoids such as CBD and Δ^9 -THC are extracted and acetylated to amplify their effects. CBDDA is an acetylated form of CBD, in which two acetyl groups are introduced to the phytocannabinoid structure (Fig. 5.1). Similarly, Δ^9 -THCOA is an acetylated derivative of Δ^9 -THC, the primary psychoactive component of *Cannabis* (Holt et al., 2022; Typek et al., 2023). The modification of natural phytocannabinoids through acetylation or other chemical processes requires thorough investigation to understand their pharmacological properties, potential side effects, and interactions with biological systems (Brown, 1998).

Currently, acetylated phytocannabinoids are gaining attention due to their enhanced potency and potential biopharmaceutical applications. Their ability to cross the BBB more effectively than their natural counterparts suggests a higher efficacy in interacting with the ECS (Calapai et al., 2020; Pardridge, 2012). This could translate to more potent therapeutic effects, offering new avenues for treatment. However, their increased strength also raises concerns about safety and potential adverse effects, which are not well understood due to the novelty of these compounds and the lack of comprehensive pharmacological data. Considering these points, this study focuses on *in silico* investigations of CBD, CBDDA, Δ^9 -THC, and Δ^9 -THCOA interactions with both p-CBRs. This research is imperative as these acetylated phytocannabinoids are newly emerging in the market and are currently being consumed by *Cannabis* users. Reports of visual and auditory effects resulting in hallucinations, paranoia, irrational thinking, and delusions were present in a consumer resulting in an arrest (Holt et al., 2022). This case highlights the potential for severe adverse reactions associated with the consumption of such SCs. The lack of existing pharmacological data on these compounds necessitates a thorough exploration of their effects, interactions, and safety profiles through computational methods before any clinical applications can be considered (Holt et al., 2022). This approach is crucial for ensuring that the therapeutic benefits of SCs are maximised while minimising the risks associated with their use. Additionally, for regulations to be enforced and established, pharmaceutical data on these compounds is required.

To comprehend the intricate interactions between SCs and their respective receptors, advanced computational techniques such as IFD and MD were used to compare the interaction between natural phytocannabinoids and their acetylated analogues to both CB₁ and CB₂ receptors (Schlick & Portillo-Ledesma, 2021; Yadav, 2022). These techniques can provide initial

theoretical results of receptor-ligand complexes in a faster timeframe than laboratory experiments. IFD allows for the study of CBDDA and Δ^9 -THCOA by adapting their conformations to optimally bind to each CBR. This method considers the dynamic nature of both the ligands and the receptors, enabling the identification of specific residues involved in ligand binding and the assessment of potential binding affinities (Sherman, Beard, & Farid, 2006). Following IFD, MD simulations further elucidate the structural and dynamic behaviour of the CBR–SCs complex. These simulations provide an understanding of the ligand-receptor interactions over time at a molecular level, capturing their structural changes and conformational fluctuations. By subjecting the complex to simulated physiological conditions, MD simulations offer insights into the stability, flexibility, and overall dynamics of the receptor-ligand interactions, giving rise to valuable information about the ligands binding mode and its interactions with specific residues within the receptor's binding pocket (Hollingsworth & Dror, 2018).

In this study, CBRs are referred to as p-CBRs as modifications were added in the third intracellular loop (ICL3) during the crystallisation process for stability and functionality purposes. These p-CBR models (PDB:5U09 and 5ZTY) were selected for molecular modelling to compare the structural and interaction dynamics of natural and acetylated phytocannabinoids as they have the current best resolution along with a complete amino acid sequence of CB₁ and CB₂ (2.6 Å and 2.8 Å, respectively) (Li et al., 2019; Shao et al., 2016). These p-CBRs, which have undergone validation binding assays and computational modelling to ensure their reliability, serve as computational proxies to study the interactions of phytocannabinoids with CBRs (Li et al., 2019; Shao et al., 2016). This modelling allows for predictions on how acetyl modifications may alter the binding and efficacy of these compounds at the receptor level, providing valuable insights into their potential pharmacological profiles. This knowledge is crucial for predicting the efficacy, potency, and safety profiles of phytocannabinoids and their derivatives, enabling the design of molecules with desired pharmacological properties (Hollingsworth & Dror, 2018). It also offers insights that could help in the careful modulation of therapeutic effects and the minimisation of adverse side effects in the development of new endocannabinoid-based medications.

2 MATERIALS AND METHODS

Appendix C contains the specifications for the hardware and software used when conducting tasks using Maestro software.

2.1 Protein Preparation

Initial crystallographic data for the p-CBRs CB₁ and CB₂ were retrieved from the UniProtKB repository, corresponding to entries P21554 and P34972 ("UniProt: the universal protein knowledgebase in 2023," 2023). Structural sequences were further sourced from the Research Collaboratory for Structural Bioinformatics: Protein Data Bank (RCSB: PDB), with accession numbers 5U09 for CB₁ and 5ZTY for CB₂ (Li et al., 2019; Shao et al., 2016). Following sequence extraction, SWISS-MODEL was used for homology modelling to infer the structure of segments that were not directly observable in the crystal structures (Waterhouse et al., 2018). Following the homology modelling, a series of refinement techniques were applied using the Protein Preparation Wizard of the Maestro Software 13.0 from the Schrödinger suite (Madhavi Sastry et al., 2013). This refinement process encompassed several enhancements to the protein models, such as hydrogenation, side chain completion via Prime, bond order determinations using the CCD database, and the optimisation of side chain positioning (Jacobson et al., 2002; Jacobson et al., 2004). These enhancements are critical for the precision of computational models, addressing ambiguities inherent in empirical data. The prepared apo forms of the proteins were then set up for subsequent IFD and MD simulations, ensuring the proteins were appropriately configured for precise interaction and dynamics analysis.

2.2 Preparation of Ligands

Each compound (CBD, CBDDA, Δ^9 -THC and Δ^9 -THCOA) depicted in Fig. 5.1 was subjected to a preparation process utilising the LigPrep utility in the Maestro software suite. This process employed the OPLS2005 force field for molecular mechanics calculations. The role of LigPrep was to generate various tautomeric and stereoisomeric forms and low-energy spatial arrangements of the molecules, simultaneously optimising their ionisation states within a pH range of 7.0 ± 2.0 , leveraging the capabilities of Epik (Bochevarov et al., 2016; Johnston et al., 2023; Jorgensen et al., 1996; Shelley et al., 2007). The objective of this procedure was to refine the molecules into configurations that are suitable for accurate and reliable docking studies.

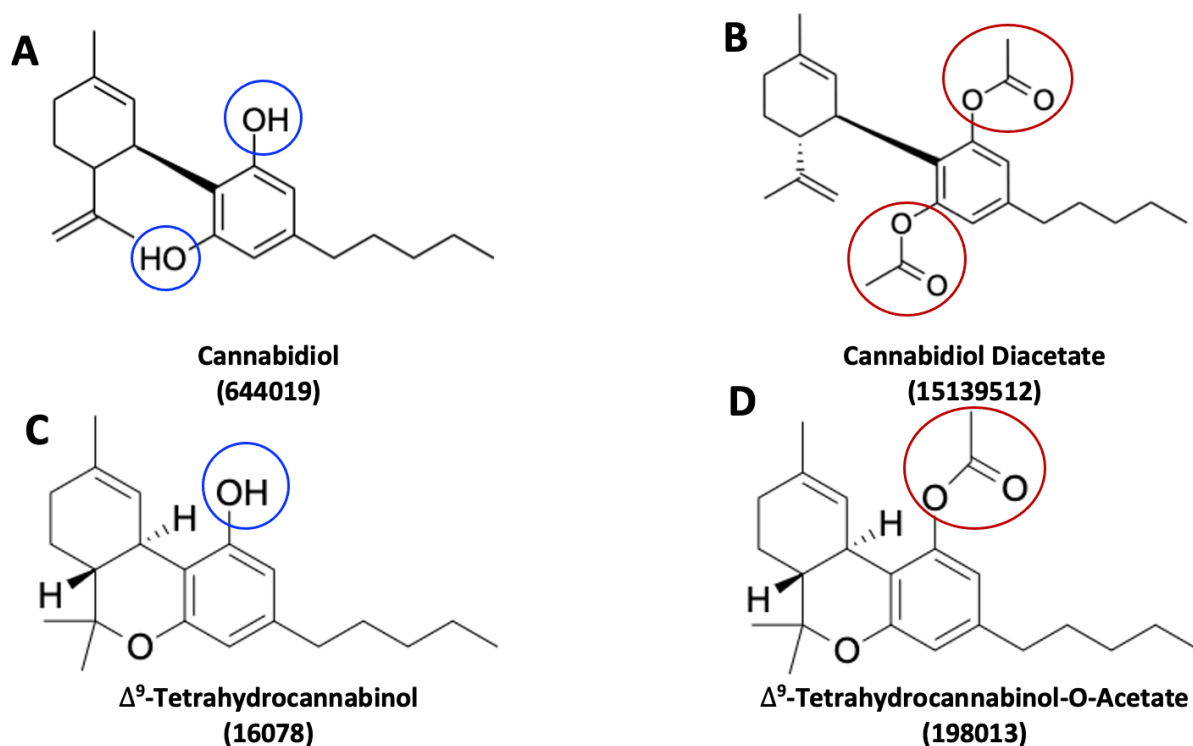


Figure 5.1: Comparative structures of natural and acetylated phytocannabinoid ligands. The top pair shows A. CBD (CID 644019) with hydroxyl groups circled in blue and B. CBDDA (CID 15139512) with acetyl groups circled in red. The lower pair displays C. Δ^9 -THC (CID 16078) and its acetylated version, D. Δ^9 -THCOA (CID 198013). The blue circles identify the original hydroxyl groups in CBD and Δ^9 -THC, while the red circles indicate the added acetyl groups in CBDDA and Δ^9 -THCOA—structures. Created using ChemDraw JS.

2.3 Molecular Docking

Pseudo-endocannabinoid receptors CB₁ and CB₂, were prepared for induced-fit docking (IFD) using the Glide application in the Schrödinger suite via Maestro (Farid et al., 2006; Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004; Sherman, Beard, & Farid, 2006; Yang et al., 2021). This IFD approach docked four specific ligands: CBD, Δ^9 -THC, CBDDA, and Δ^9 -THCOA, enabling the receptor binding sites to adapt to the ligand molecular conformations dynamically. IFD technique facilitated the flexibility of the active site of the protein, enhancing the docking precision by simulating the reciprocal conformational adjustments between the ligand and protein structure. The IFD focus was set on the binding site residues indicated by the co-crystal ligand (Li et al., 2019; Shao et al., 2016). The docking protocol initiated with a Glide docking phase, followed by a refinement stage where the flexibility of ligand and receptor side chains was crucial for optimising the fit. Each resulting ligand-receptor complex was evaluated and ranked based on its docking score, reflecting the binding affinity strength. The

top-ranked conformer for each ligand was identified for subsequent analyses based on their docking score.

2.4 System Builder

The System Builder tool in Maestro was used to set up all 10 simulation environments for CBR-ligand complexes (Bowers et al., 2006). Each complex was situated within a POPC lipid bilayer and surrounded by a solvent of TIP3) water molecules, ensuring a 10 Å separation from the system's edges (Fig. C1). Physiological ion concentrations were mimicked in the simulation by adding sodium (Na^+) and chloride (Cl^-) ions to achieve a 0.15 M ionic strength and to ensure electrical neutrality. This setup was designed to replicate a natural biological environment, providing an accurate framework for studying the dynamic interactions of the protein-ligand complexes throughout the MD simulation process.

2.5 Molecular Dynamic Simulations

Desmond within Maestro software was used to run all MD simulations for 500 ns to investigate the dynamics of each system (Bowers et al., 2006). Beginning with ionisation and solvation in a detailed solvent model, the simulation sequence progressed through a structured multi-stage MD production process. This regimen encompassed eight stages, with the first seven dedicated to system equilibration using incremental simulation durations, leading up to the extensive 500 ns simulation in the eighth stage. The initial stage set the system parameters, succeeded by a 100 ps equilibration at 10 K with restraints on the heavy atoms of solutes, under NVT (constant N = number of moles, V = volume, T = temperature) ensemble conditions utilising Brownian dynamics. Stage three maintained these NVT conditions for another 12 picoseconds. Subsequent stages four, six, and seven, excluding pocket solvation in stage five, involved short NPT (constant N = number of moles, P = pressure, T = temperature) ensemble simulations (12 ps, 12 ps, 24 ps) to gradually increase the temperature, interspersed with strategic applications and removals of atomic restraints. The final preparatory stage removed all restraints from heavy atoms, seamlessly transitioning to the last stage, which then simulated the system under consistent NPT conditions at 300 K for the entire 500 ns duration, providing an in-depth view of the ligand-receptor interaction dynamics.

2.6 Post-Molecular Dynamic Analysis

Conformational stability and compactness were evaluated by examining the protein C α RMSD and RoG, while local residue flexibility and notable conformational shifts were identified via backbone fluctuation assessments (C α RMSF). PCA was conducted in RStudio, provided by Posit Software, PBC (version 2023.09.1+494), using the ggfortify library to investigate the C α metrics (RStudio, 2020; Tang et al., 2016). Visualisation of PCA data through scree plots was implemented within R, offering an interpretative view of the PCs variance contributions (Aboytes-Ojeda et al., 2016; Team, 2010). Additionally, the Lig-RMSD was computed to assess ligand stability within the binding site. Lastly, RoG of water molecules present during the simulation were calculated and assessed to determine their stability of a persistent hydration shell or network (Laage et al., 2017). These analyses provided a comprehensive theoretical overview of the ligand-receptor complex behaviour over the 500 ns simulation.

3 RESULTS

3.1 Induced-Fit Docking

Docking simulations have elucidated the differential binding affinities of phytocannabinoids to p-CBRs, highlighting a clear distinction between the natural compounds and their acetylated analogues (Fig. 5.2). In CB₁, a consistent residue interaction is observed with Met15 (Fig. 5.2 B – D), which forms a H-bond with an O atom present in all ligands signifying its critical role in ligand binding when an O atom is present in a phytocannabinoid. This is due to methionine possessing a side chain that ends in a thioether group, comprising a sulfur atom bonded to two carbon atoms. This configuration allows methionine to engage in van der Waals interactions with O-containing groups due to lone pairs of electrons on the sulfur. These interactions between methionine and O atoms can significantly contribute to the specificity and stability of protein-ligand binding (Lehninger et al., 2005). Such specificity is crucial in drug design and receptor interaction, as it can influence the orientation of the ligand and its binding affinity within the binding site of the receptor, thus impacting the therapeutic efficacy of the drug.

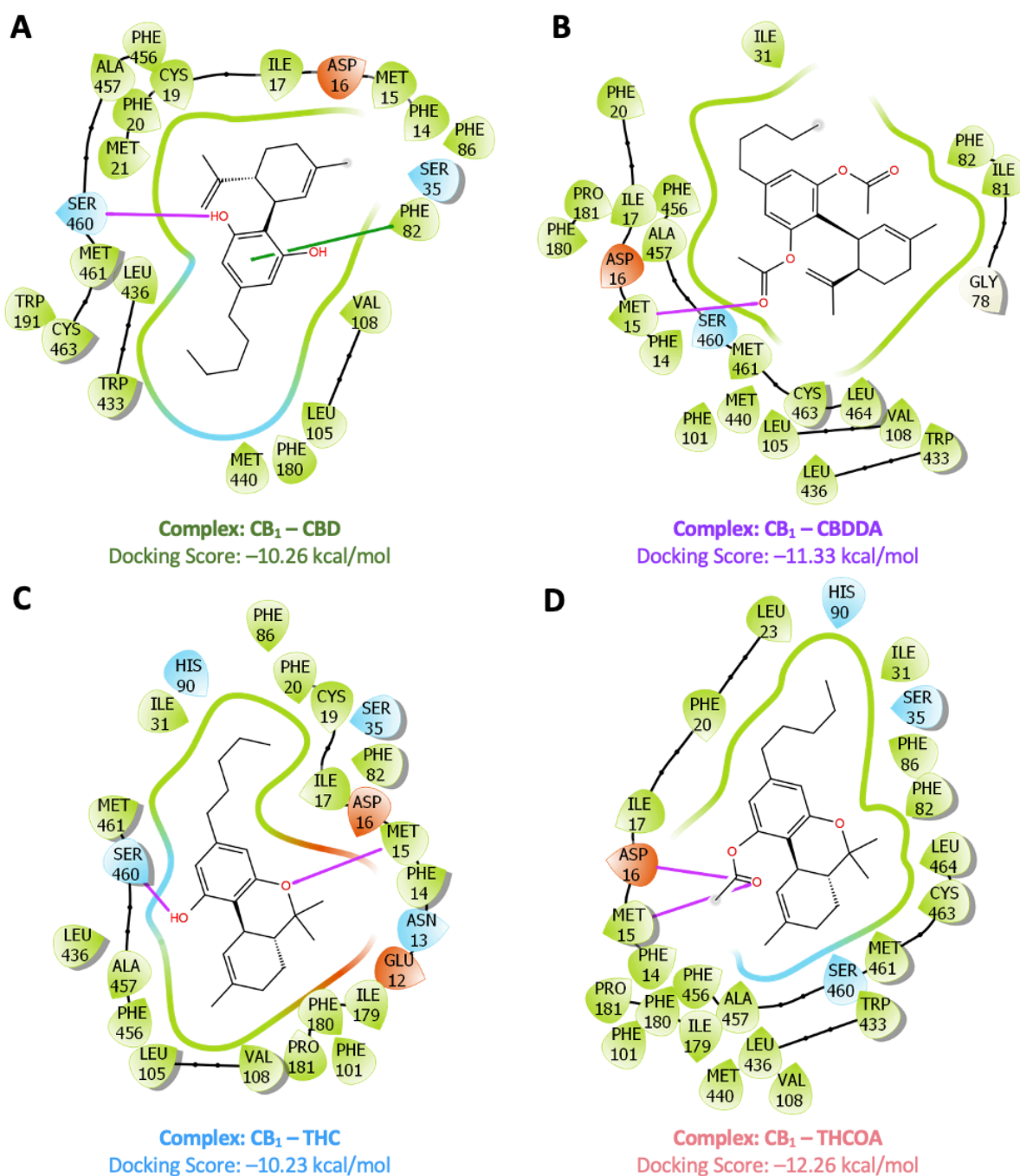
Similarly, Ser460 forms a H-bond when an OH group is present, as seen in the interactions with Δ^9 -THC and CBD (Fig. 5.2 B, D). This is due to the OH group in the serine side chain which can form H-bonds at 2.5 – 3.5 Å due to its polarity, allowing it to interact with other electronegative atoms or functional groups in proteins or other molecules. Alongside the consistent H-bond with Met15, Δ^9 -THCOA interacts via an additional ionic interaction with

Asp16, thereby strengthening the interaction with CB₁ compared to Δ^9 -THC. When comparing the strength of H-bonds formed by aspartic acid and serine between Δ^9 -THC and Δ^9 -THCOA, the H-bond involving the O atom in the aspartic acid side chain, is typically stronger than the H bond involving the OH group in serine (Scheiner et al., 2001). This discrepancy arises due to the higher electronegativity of O compared to H, resulting in a more pronounced polarisation and consequently a stronger H bond. Furthermore, a unique π - π stacking—a non-covalent interaction when two (or more) aromatic rings come within 3.5 – 4 Å of each other that their π orbitals overlap slightly, allowing for delocalised electrons to interact—interaction (Phe82) between CBD and CB₁ (Fig. 5.2A) indicates a specific binding conformation compared to CBDDA, whereas the other ligands only possess H-bond interactions with CB₁ (Deng et al., 2020).

In the case of CB₂, the presence of π - π stacking interactions across all ligands suggests a common mode of aromatic ring engagement within the binding site, with key amino acids playing pivotal roles in binding. Threonine, for instance, possesses a OH group in its side chain, facilitating H-bonding interactions with aromatic rings. This H-bonding, combined with van der Waals forces, facilitates the formation of favourable π - π stacking interactions between threonine and aromatic rings (Lehninger et al., 2005; Liao et al., 2013). Similarly, phenylalanine features an aromatic phenyl ring in its side chain, allowing the π electrons within the phenyl ring to readily engage in favourable π - π stacking interactions with other aromatic rings (Liao et al., 2013). Moreover, both variants of Δ^9 -THC attract residues with π - π stacking interactions (Fig. 5.2G), including Phe182 and Phe86, while forming a polar H-bond with Thr113 and His94 (a histidine with a H on the epsilon nitrogen), respectively. Δ^9 -THCOA has a higher binding affinity due to more electronegative H-bond interaction with His94 and the O atom compared to Δ^9 -THC Thr113 interaction with OH group (Lehninger et al., 2005; Liao et al., 2013). Trp193 is pivotal, engaging in π - π stacking with both CBD and CBDDA (Fig. 5.2E, F), which is essential for CBD binding to CB₂. The additional H-bond interaction at Trp193 in CBDDA compared to CBD implies a stronger affinity, as corroborated by their docking scores.

The docking scores of all ligands reinforce the observations of core residues in the binding site, where acetylated phytocannabinoids display higher binding affinities to p-CBRs than their natural counterparts: CB₁ complexes with CBD and Δ^9 -THC registered docking scores of –10.26 kcal/mol and –10.23 kcal/mol, respectively, whereas CBDDA and Δ^9 -THCOA showed increased affinities with scores of –11.33 kcal/mol and –12.26 kcal/mol, respectively. CB₂

complexes mirrored this trend, with CBD and Δ^9 -THC scoring -9.56 kcal/mol and -9.66 kcal/mol, while their acetylated forms, CBDDA and Δ^9 -THCOA, demonstrated stronger interactions with scores of -10.78 kcal/mol and -10.25 kcal/mol, respectively. Additionally, all ligands show a preference for the CB₁ receptor than the CB₂ receptor. These findings suggest that the acetylation of phytocannabinoids enhances ligand-receptor interactions, potentially due to increased interactions and strength of van der Waals forces of H-bonding, leading to a more energetically favourable binding state.



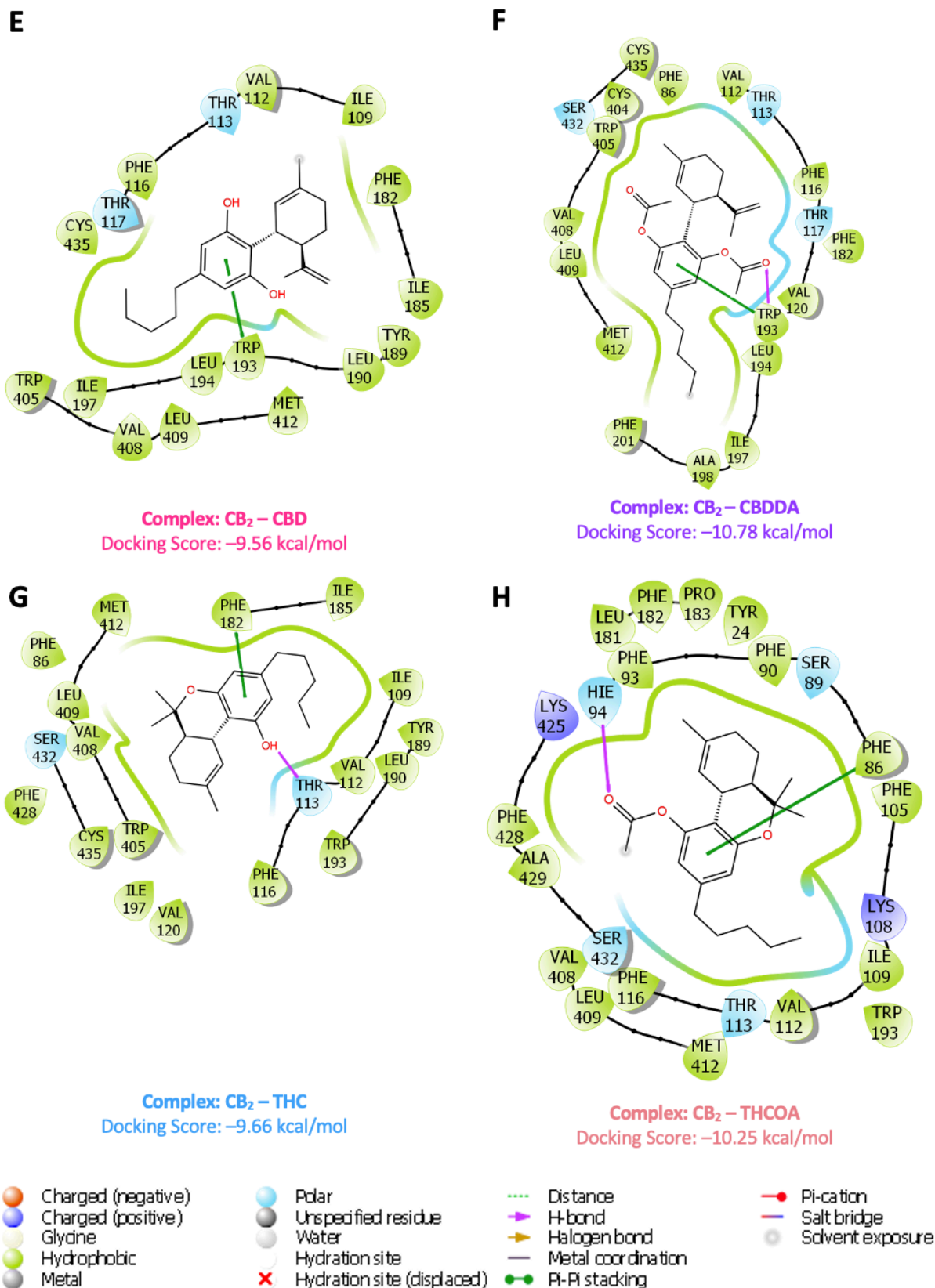


Figure 5.2: Induced-fit docking interactions and scores of ligands with p-CBRs. This figure illustrates the 2D interaction patterns of phytocannabinoids and their acetylated forms with CB₁ and CB₂ receptors, annotated with their respective docking scores. A – D depicts interactions with CB₁ and E – G and CB₂, Green lines represent π - π stacking interactions, with pink lines indicating H-bonding. The surrounding amino acids are predominantly

hydrophobic. The docking scores, given in kcal/mol, suggest the binding affinity of each compound to the respective receptor, with more negative values indicating stronger predicted interactions. Generated from Maestro Schrödinger Suite.

3.2 Energy Properties of MD Simulations

The energy properties from the simulation quality analysis of CB₁ and CB₂ systems reveal the receptor-ligand complexes thermodynamic stability and structural characteristics over a simulation period of 500 ns. For CB₁ systems (Table 5.1), the average total energy—which includes the kinetic and potential energy—ranges from −116188.16 to −124910.58 kcal/mol, with the Δ^9 -THC complex exhibiting the lowest energy, indicative of a potentially more stable or favourable binding conformation. The potential energy follows a similar trend, suggesting that the interactions within the Δ^9 -THC complex are energetically optimal. CBDDA and Δ^9 -THCOA also show lower average total and potential energies than the apo and CBD systems, suggesting stronger interactions within these complexes. This temperature was maintained consistently around 298 K for all complexes, ensuring a controlled simulation environment. The slight variations in pressure and volume across the complexes suggest minor differences in system density and packing, which could be related to the different ligand sizes and interactions with the receptor.

In CB₂ systems (Table 5.2), the average total energy values are more varied, with the Δ^9 -THC complex showing the lowest average total energy at −145703.94 kcal/mol, potentially indicating the most stable complex among those analysed. The average potential energy is also the lowest for the Δ^9 -THC complex, which is consistent with a stable interaction network. CBDDA exhibits notably lower energy values compared to the apo and CBD systems, similar to the CB₁ systems, which may reflect a more energetically favourable binding mode. Temperature consistency is maintained across the CB₂ systems, and the pressure and volume parameters show slight differences, as observed in the CB₁ systems. Furthermore, the slope values being zero for all complexes indicate a stable system over time, which is critical for the validity of the simulation data. These energy metrics are essential for understanding the binding affinity and stability of different phytocannabinoid ligands to p-CBRs, which has implications for their biological effects and potential therapeutic applications.

Table 5.1: Assessment of energy metrics in CB₁ receptor simulations

Statistical Parameters	Apo	CBD	CBDDA	Δ^9 -THC	Δ^9 -THCOA
Simulation Period (ns)	500.00	500.00	500.00	500.00	500.00
Degrees of Freedom	141853	146648	153190	157256	154021
Number of Atoms	65690	68022	70899	72779	71264
Average Total Energy (kcal/mol)	-116188.16	-121479.15	-122555.85	-124910.58	-122745.65
Average Potential Energy (kcal/mol)	-158195.08	-164908.16	-167915.95	-171473.38	-168351.00
Temperature (K)	298.04	298.05	298.01	298.00	298.00
Pressure (bar)	0.59	1.10	0.74	1.30	0.95
Volume ($\text{\AA}^3$)	638208.45	661670.76	688641.71	706778.20	691726.83
Slope (ps^{-1})	0.000	0.000	0.000	0.000	0.000

Table 5.2: Assessment of energy metrics in CB₂ receptor simulations

Statistical Parameters	Apo	CBD	CBDDA	Δ^9 -THC	Δ^9 -THCOA
Simulation Period (ns)	500.00	500.00	500.00	500.00	500.00
Degrees of Freedom	150195	144228	155040	164292	152757
Number of Atoms	69961	66969	72082	76914	70969
Average Total Energy (kcal/mol)	-128285.07	-119869.69	-135207.76	-145703.94	-126395.25
Average Potential Energy (kcal/mol)	-172768.37	-162572.56	-181119.05	-194369.72	-171631.68
Temperature (K)	298.08	298.05	298.03	298.12	298.04
Pressure (bar)	1.38	1.14	1.29	1.55	1.25
Volume ($\text{\AA}^3$)	682427.29	651763.33	705643.58	752348.36	691001.42
Slope (ps^{-1})	0.000	0.000	0.000	0.000	0.000

3.3 Protein RMSD of Carbon Backbone

The structural dynamics of CBRs are reflected by their C _{α} RMSD over 500 ns (Fig. 5.3.1A), revealing valuable insight into the effects of ligands on the dynamics of the protein. CB₁ in its apo form exhibits moderate C _{α} RMSD fluctuations, indicating a dynamic yet stable conformation. CBD, acting as a modulator of CB₁, inherently exhibits higher dynamics at first, reaching stabilisation at ~ 2.5 $\text{\AA}$, suggestive of maintained receptor integrity. CBDDA shows a marginally higher C _{α} RMSD, implying an increase in dynamic behaviour upon acetylation. Δ^9 -THC stabilises after 50 ns, whereas Δ^9 -THCOA indicates reduced stability only partially stabilising at ~ 200 ns, while presenting greater dynamics and conformational variations,

suggesting that acetylation influences the conformation. Additionally, both natural and modified phytocannabinoids are observed to preserve the stability of CB₁.

Comparing plots on Fig. 5.3.2A from the PCA analysis, both natural CBD and its acetylated counterpart, CBDDA, expand the conformational space of the CB₁ receptor compared to its unbound state. In Fig. 5.3.2A, CBD appears to induce a wide distribution of conformations, as indicated by the broad spread of data points, suggesting that CBD allows the receptor to explore a diverse set of structures. On the other hand, CBDDA, shows a similar extent of spread but with a slightly different pattern, hinting at a distinct set of conformations that CB₁ adopts in response to binding. The variation in the distribution patterns between CBD and CBDDA-bound CB₁ receptors emphasises that even subtle changes in ligand structure, such as acetylation, can impact receptor conformational dynamics. In the PCA plots (Fig. 5.3.2B), the impact of Δ^9 -THC and its acetylated form is examined on the conformational landscape of CB₁. Both Δ^9 -THC and Δ^9 -THCOA show an expanded conformational space relative to the unbound state, with Δ^9 -THC demonstrating a pronounced elongation along the primary principal component axis, which may indicate a predominant conformational state influenced by the ligand. Conversely, Δ^9 -THCOA shows a spread with a different orientation, suggesting that acetylation alters the conformational preference of the receptor. These differences between the natural and acetylated forms of Δ^9 -THC may reflect variations in how these compounds modulate CB₁ receptor function and signalling.

Furthermore, the scree plots display the variance explained by each PC (Fig. 5.3.3). When comparing the interaction of CBD and CBDDA with CB₁, the first component accounts for less than half of the total percentage variance (45.1 %, 33.5 %, and 21.4 %). A significant variance is attributed to CBD and CBDDA binding, implying a conformational change when acetylated. This suggests that these components capture most of the receptor's conformational changes when bound. Similarly, the scree plot for Δ^9 -THC versus Δ^9 -THCOA indicates a significant proportion of variance explained by the PCs, confirming that the observed patterns in plots C and D (Fig 5.3.2) capture key conformational changes induced by these ligands. The scree plots thus provide a quantitative backing to the qualitative observations from the PCA plots, reinforcing the conclusion that ligand binding has a pronounced effect on the conformational dynamics of the CB₁ receptor.

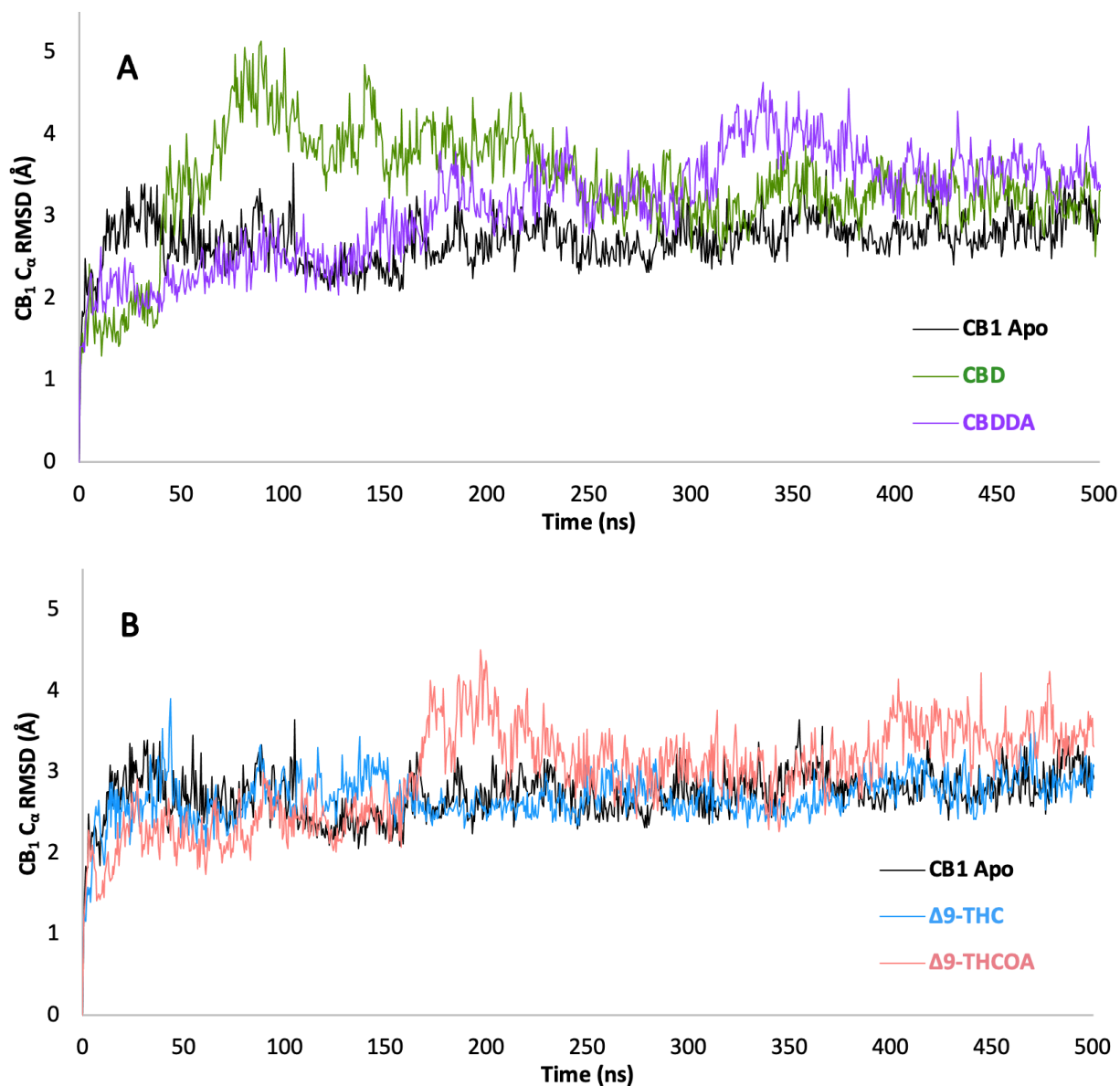


Figure 5.3.1: C α RMSD of CB₁ receptor complexes during MD simulations. A. depicts the C α RMSD for the CB₁ receptor and its complexes with CBD and CBDDA over 500 ns, while B. shows the C α RMSD for the CB₁ receptor with Δ^9 -THC, and Δ^9 -THCOA under the same conditions. The C α RMSD, measured in angstroms (Å), tracks the stability and conformational changes of the receptor-ligand complexes, with lower C α RMSD values indicating greater structural stability over the simulation period. Both acetylated cannabinoids increase C α RMSD when in complex with CB₁ compared to their natural derivatives.

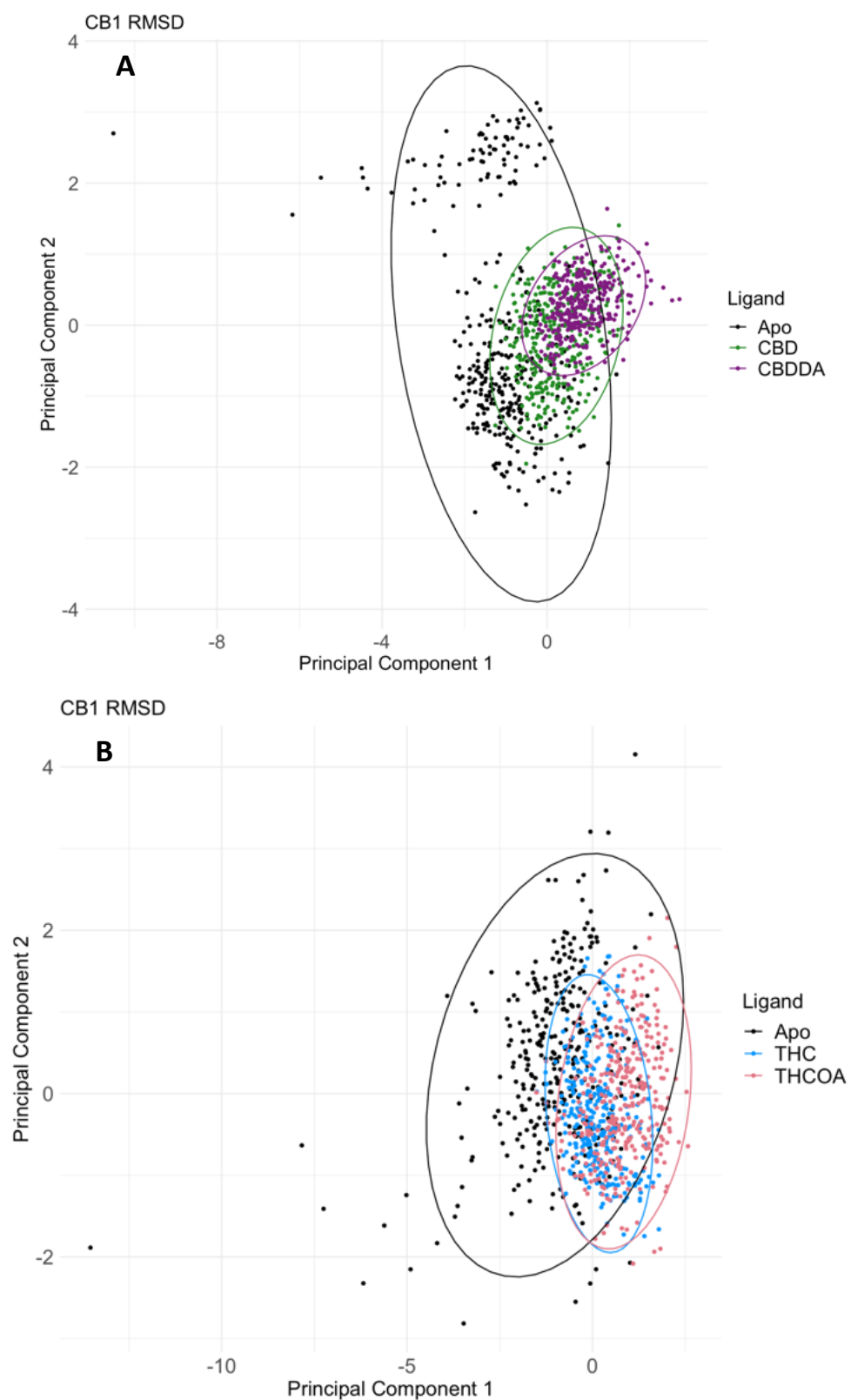


Figure 5.3.2: PCA of C_{α} RMSD for CB₁ in complex with phytocannabinoids. The analysis delineates the conformational distinctions of the CB₁ receptor when bound to natural (CBD, Δ^9 -THC) versus acetylated phytocannabinoids (CBDDA, Δ^9 -THCOA). Each plot corresponds to a specific CB₁-ligand complex: A. CBD and

CBDDA. B. Δ^9 -THC, and Δ^9 -THCOA, illustrating the structural variance of the receptor in response to each ligand. Generated from R Studios.

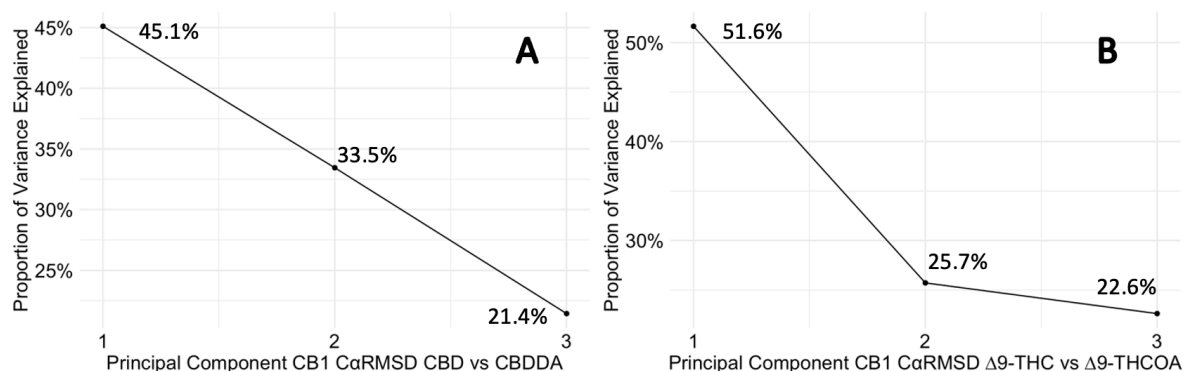


Figure 5.3.3: Scree plots of PCA of CB₁ receptor-ligand C_α RMSD interactions. A. The variance in the conformational space of the CB₁ receptor when bound to CBD as opposed to CBDDA; the first PC accounts for 45.1 % of this variance, followed by 33.5 % for the second PC, and 21.4 % for the third PC, thereby emphasising the distinct conformational shifts triggered by each of these ligands. Similarly, B. of the figure illustrates the variance when the receptor is complexed with Δ^9 -THC versus Δ^9 -THCOA, where the first PC explains a substantial 51.6 % of the variance, the second PC 25.7 %, and the third PC 22.6 %, of which Δ^9 -THCOA alone contributes 22.6 %. Generated from R Studios.

In examining the interaction between CBD and CBDDA with the CB₂ receptor, MD simulations and PCA analyses reveal distinct conformational dynamics and implications for receptor signalling. For CBD, the binding to the CB₂ receptor stabilises at around 5 Å C_α RMSD after 150 nanoseconds (Fig. 5.4.1A), suggesting a well-integrated interaction with the receptor site. This integration is characterised by a broader conformational exploration, as indicated by the diffuse spread in the PCA scatter plot (Fig. 5.4.2A), suggesting that the receptor is flexible in its bound state with CBD. In contrast, CBDDA demonstrates a lower C_α RMSD, indicating a more stable interaction with the CB₂ receptor, as seen in the less spread PCA plots (Fig. 5.4.2A) which could suggest a specific and possibly more rigid receptor conformation. This distinct pattern suggests that the acetylation of CBD alters receptor-ligand interaction dynamics, potentially influencing the signalling pathways of the CB₂ receptor.

When comparing Δ^9 -THC to its acetylated form Δ^9 -THCOA, the C_α RMSD values suggest that acetylation might introduce some elements of instability in the receptor-ligand complex (Fig. 5.4.1B), although both maintain the integrity of the CB₂ receptor. Δ^9 -THC allows for a variety of receptor conformations, as seen in the PCA scatter plot (Fig. 5.4.2B), while the acetylated Δ^9 -THCOA shows a conformational change which might reflect an enhanced dynamic

interaction without leading to receptor destabilisation, as indicated by the Fig. 5.4.1B and PCA scatter plot Fig. 5.4.2B.

The acetylated phytocannabinoids alter their interaction with the CB₂ receptor, potentially modifying their therapeutic effects. The scree plots (Fig. 5.4.3) provide insight into this interaction, with C_α RMSD variances for CBD at 62.6 %, 22.8 %, and 14.6 %, highlighting the principal components of conformational variance. For Δ⁹-THC versus its acetylated analogue Δ⁹-THCOA, the variances are 60.4 %, 23.8 %, and 15.8 %. Both natural ligands indicate a predominant conformational influence alongside notable secondary conformations by their acetylated analogues. This analysis emphasises the dynamic nature of ligand-induced conformational changes and quantitatively captures the influence of both natural and modified cannabinoids on the pharmacological behaviour of the CB₂ receptor.

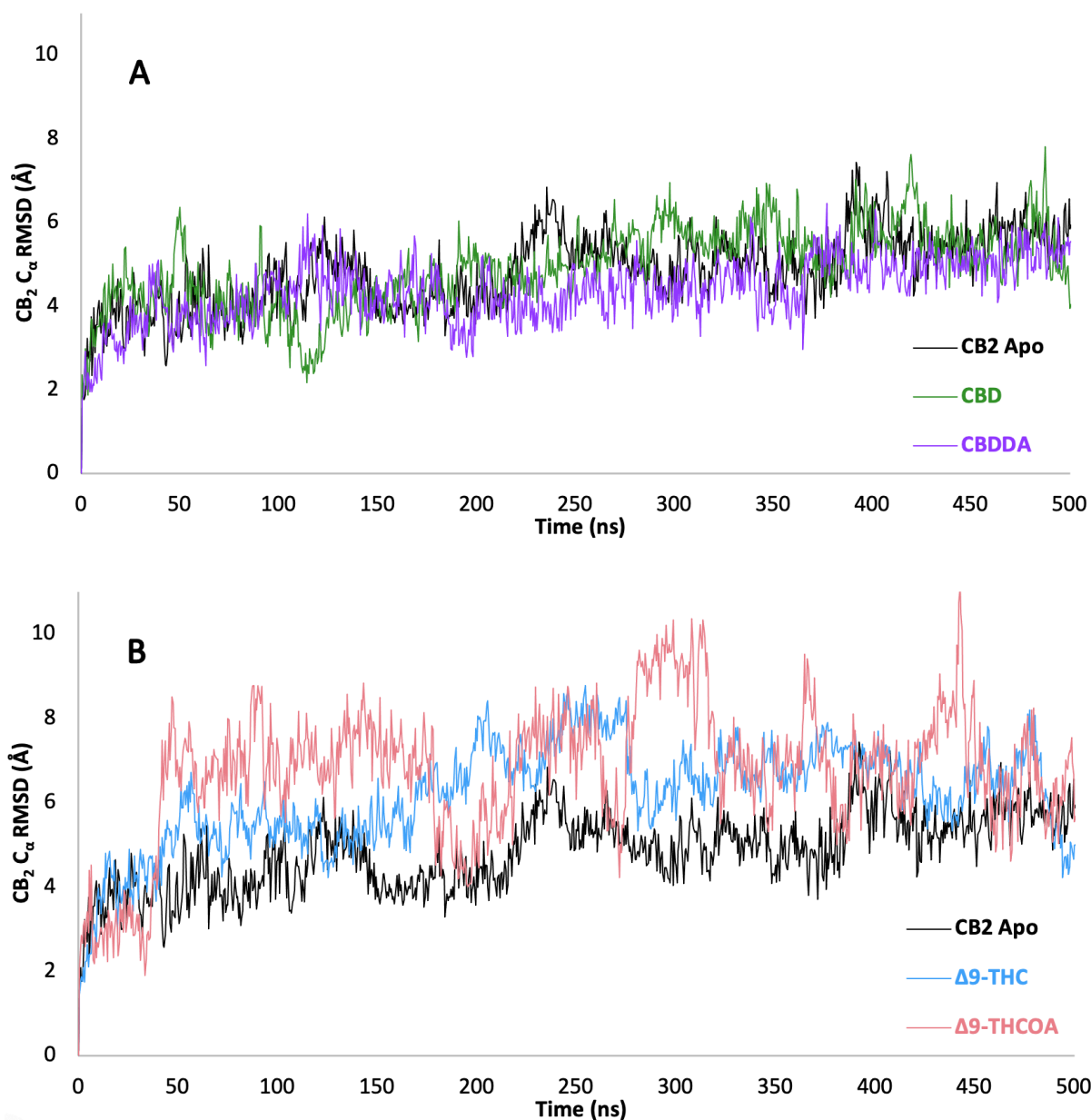


Figure 5.4.1: C_α RMSD of CB₂ receptor-ligand complexes over time during MD simulations. A. Presents the temporal evolution of C_α RMSD for the CB₂ receptor in its apo state and when complexed with CBD and CBDDA across a 500 ns simulation with CBDDA being more stable with a lower C_α RMSD than CBD. B. Illustrates the C_α RMSD trajectories for the CB₂ receptor bound to Δ⁹-THC and Δ⁹-THCOA with the natural cannabinoid being more stable with a lower C_α RMSD than its acetylated derivative.



Figure 5.4.2: PCA of CB₂ receptor dynamics with phytocannabinoids. A. Captures the dynamic behaviour of the CB₂ receptor when complexed with CBD, illustrating the conformational space explored by this natural ligand A. Demonstrates the receptor dynamics with the acetylated phytocannabinoid CBDDA, whereas B. reveals the conformational diversity induced by Δ^9 -THC binding. B. Also presents the receptor dynamics when bound to Δ^9 -

THCOA. Collectively, these plots provide a comparative view of the conformational changes and stability within the CB₂ receptor induced by both natural and acetylated phytocannabinoids. Generated from R Studios.

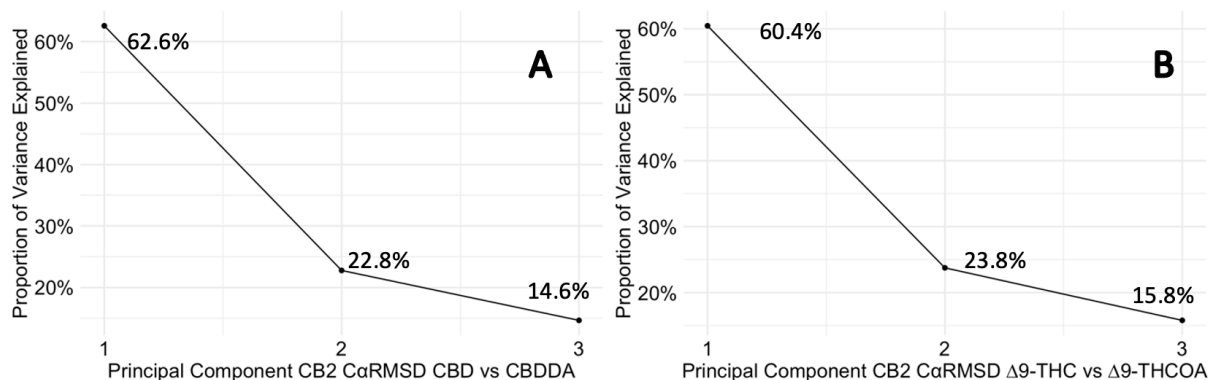


Figure 5.4.3: Variance analysis of CB₂ receptor dynamics. The scree plots depict the proportion of total variance explained by PCs of C_α RMSD for CB₂ with A. comparing CBD and CBDDA and B. comparing Δ⁹-THC and Δ⁹-THCOA. These plots help identify the PCs that contribute most significantly to the overall movement within the receptor structures, with the first PC typically accounting for the largest variance. Generated from R Studios.

3.4 Protein RMSF of Residues

Analysis of the C_α RMSF values from Figure 5.5.1A suggests that the binding of CBD to the CB₁ receptor results in more fluctuations, particularly in regions such as Gln27 and Gly446, which show increased C_α RMSF values in the presence of CBD, indicate enhanced flexibility or dynamic behaviour in these specific parts of the receptor structure. When examining the effects of CBD relative to CBDDA, variations in C_α RMSF values between the two ligands offer insights into their distinct modulation of receptor flexibility. Notably, regions such as Val91, Leu36 and Pro435, where CBDDA elicits higher C_α RMSF values compared to CBD, may reflect altered conformational dynamics, potentially leading to functional discrepancies in the receptor response to each ligand. Moreover, the clustering observed in Fig. 5.5.2 for CBD suggests a more constrained conformational variability, with 82.1 % of the variance accounted for by the first PC, indicating a significant confinement of the receptor's conformations upon CBD binding. In contrast, Fig. 5.5.2 for CBDDA shows a greater spread of data points, reflecting a more expansive conformational space with a reduced variance explained by the first three PCs—82.1 %, 10.91 %, and 6.99 % respectively—highlighting the capacity of CBDDA to induce a wider array of conformations in the CB₁ receptor.

Figure 5.5.1B presents the C_α RMSF profiles for the CB₁ receptor in its apo form and when complexed with Δ⁹-THC and Δ⁹-THCOA. This data accentuates the dynamic behaviour of the receptor at specific residues, particularly noting the pronounced fluctuations at sites like Glu45

and Cys51 when bound to Δ^9 -THCOA. The C_α RMSF profiles indicate that Δ^9 -THCOA increases the flexibility of these regions, suggesting a more malleable receptor conformation compared to the apo state but much less so in comparison to Δ^9 -THC which shows a high degree of flexibility in multiple regions (Fig. 5.5.1B) compared to the apo. Such variances in receptor flexibility may imply shifts in receptor activation states or differences in ligand-receptor interactions, which could manifest as diverse biological effects. Additionally, Fig. 5.5.2 reveals the conformational behaviour of the CB₁ receptor with Δ^9 -THC and Δ^9 -THCOA. Δ^9 -THC presents a tighter cluster accounting for 83.48 % of the variance by the first PC (Fig. 5.3B), suggesting that Δ^9 -THC promotes a relatively homogeneous conformational state of the receptor. On the contrary, the Δ^9 -THCOA interaction depicted in Fig. 5.5.2 displays a broader distribution, with a slightly lower explained variance implies that Δ^9 -THCOA might lead to a greater conformational diversity of the CB₁ receptor with reference to the apo C_α RMSF profile, with potential implications for its biological activity and receptor signalling.

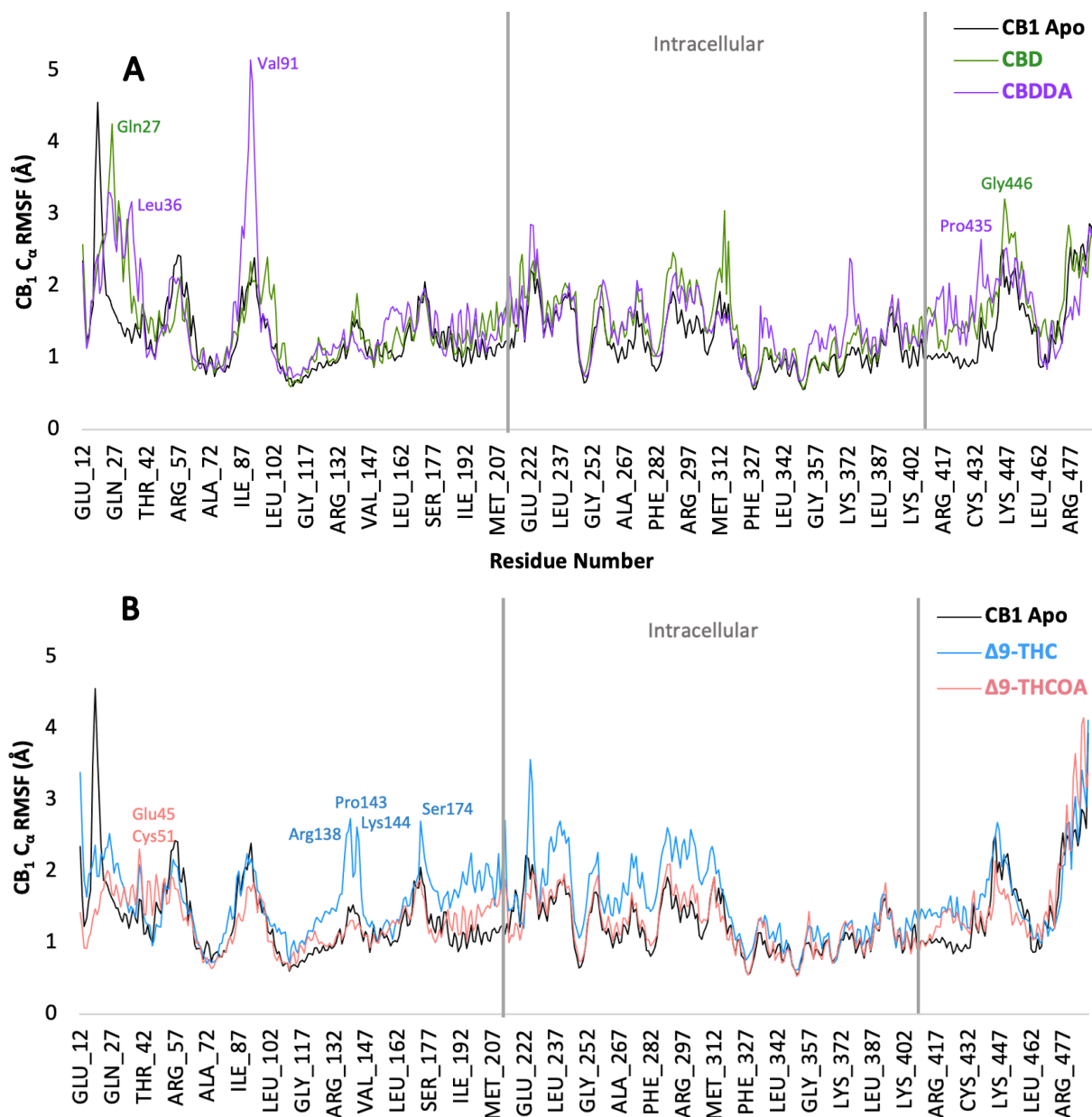


Figure 5.5.1: Residue flexibility profiles in CB₁. A. represents the C_αRMSF of residues in the CB₁ receptor in apo form and when bound to CBD, CBDDA, B. Δ⁹-THC, and Δ⁹-THCOA. Each plot tracks the fluctuation in Å for all residues, providing insight into the local dynamics and stability of receptor-ligand interactions. The portion between the grey regions represent the intracellular region. Both acetylated cannabinoids showed a decrease in C_α RMSF compared to their natural counterparts.

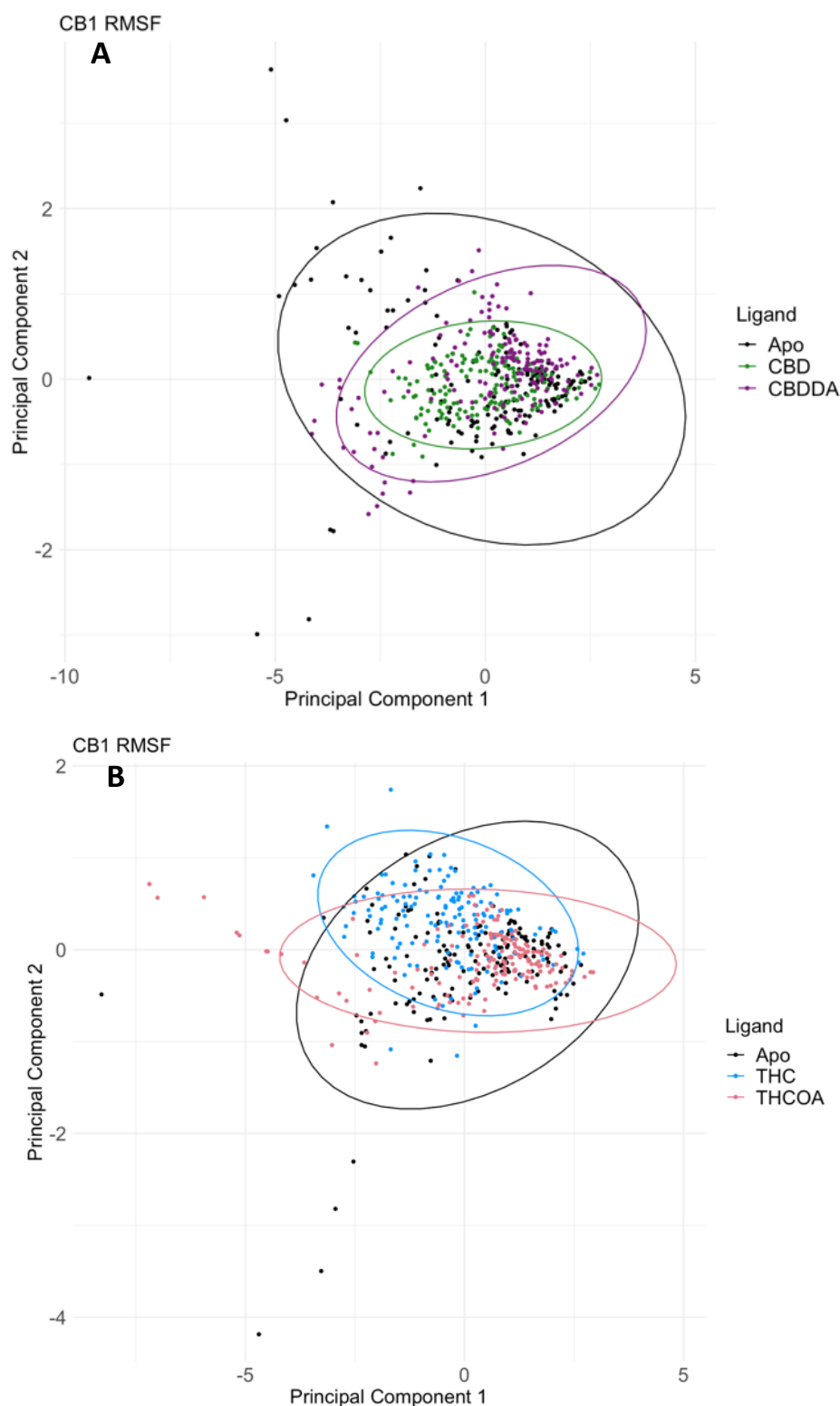


Figure 5.5.2: PCA Plots for CB₁ Receptor-Ligand Complexes. This figure presents the variance in CB₁ receptor conformations as influenced by different ligands, with A. showing the receptor bound to CBD, CBDDA and B. Δ^9 -THC, and Δ^9 -THCOA, respectively. The plots illustrate the dispersion of conformational states, with the spread of points reflecting the diversity of conformations adopted by the receptor in each ligand-bound state. Generated from R Studios.

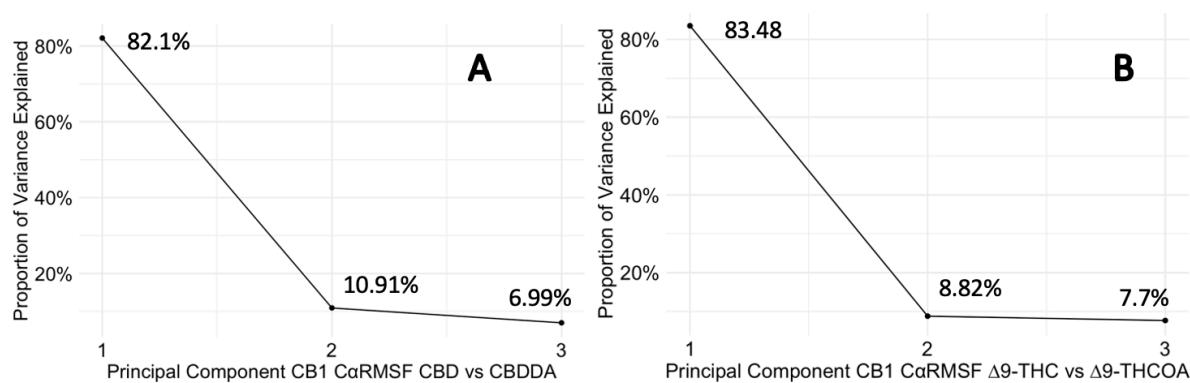


Figure 5.5.3: Variance explained by PCs for CB₁ receptor with ligands. The scree plots show the percentages of total variance captured by the PCs for the CB₁ receptor bound to A. CBD vs. CBDDA and B. Δ⁹-THC vs. Δ⁹-THCOA. Generated from R Studios.

The apo form C_α RMSF plot (Fig. 5.6.1A) establishes the intrinsic flexibility of the CB₂ receptor, serving as a control for assessing the impact of ligand binding. In this analysis, the binding of CBD to the CB₂ receptor generally shows increased C_α RMSF values compared to the apo state, particularly at residues like Lys141, Leu143, Arg176, Leu191, Gly209 and more (Fig. 5.6.1A). Therefore, indicating that CBD binding may enhance the flexibility of these regions within the receptor. This increased flexibility upon CBD binding could suggest a more dynamic interaction between the receptor and CBD, potentially influencing the functional responses of the receptor. Conversely, CBDDA binding appears to result in lower C_α RMSF values. This suggests that CBDDA may confer a more stabilising effect on these regions, potentially restricting the flexibility and, consequently, the range of conformational states accessible to the receptor compared to CBD. This differential modulation of receptor flexibility by CBD and CBDDA could lead to variations in the receptors signalling and regulatory mechanisms upon ligand binding. Furthermore, Fig. 5.6.2A shows the receptor bound to CBD and CBDDA, respectively, indicating distinct conformational spaces as evidenced by the spread of data points in each PCA plot; this is quantitatively supported by the scree plot in Fig. 5.6.3A, where CBD and CBDDA complexation accounts for 89.54 % of the variance in the first principal component, highlighting that both ligands similarly restrict the conformational diversity of the receptor.

Upon binding with Δ⁹-THC (Fig. 5.6.2B), certain residues in CB₂ show a distinct fluctuation pattern which closely follows the baseline established by the apo state. This suggests that Δ⁹-THC maintains a similar degree of flexibility in the receptor as seen in the unbound state. In comparison, the C_α RMSF profile for Δ⁹-THCOA displays more pronounced fluctuations, suggesting that Δ⁹-THCOA imparts increased flexibility to the CB₂ receptor. This is evident

when considering the dynamic behaviour of residues such as Gly29, Gln62, Lys66, Ala103, Ser139, and His218, which already exhibit notable flexibility in the apo state. The acetylated form of Δ^9 -THC seems to amplify the mobility of these residues, potentially leading to significant conformational changes, as highlighted by the marked fluctuations at the C-terminal residue Leu386. Such variations in receptor flexibility, especially in the intracellular region, may signal distinct effects of Δ^9 -THCOA on downstream signalling mechanisms, potentially diverging from the pathways activated by Δ^9 -THC and influencing the overall physiological response of the receptor. Moreover, Figure 5.6.2B present PCA scatter plots for the CB₂ receptor bound to Δ^9 -THC and Δ^9 -THCOA, with the plots suggesting that these ligands may affect the receptor's conformational space differently when compared to the apo, as seen in the spread of PCA data points. The corresponding scree plot in Figure 6.3B reveals that Δ^9 -THC and Δ^9 -THCOA also maintain a high variance explained by the first principal component (89.71 %), suggesting that both ligands, despite their chemical differences, similarly influence the receptor's primary conformational state. The slight differences in variance explained by the subsequent PCs (7.72 % and 2.57 % for Δ^9 -THC and Δ^9 -THCOA, respectively) could indicate subtle variations in the secondary conformational states induced by these two ligands.

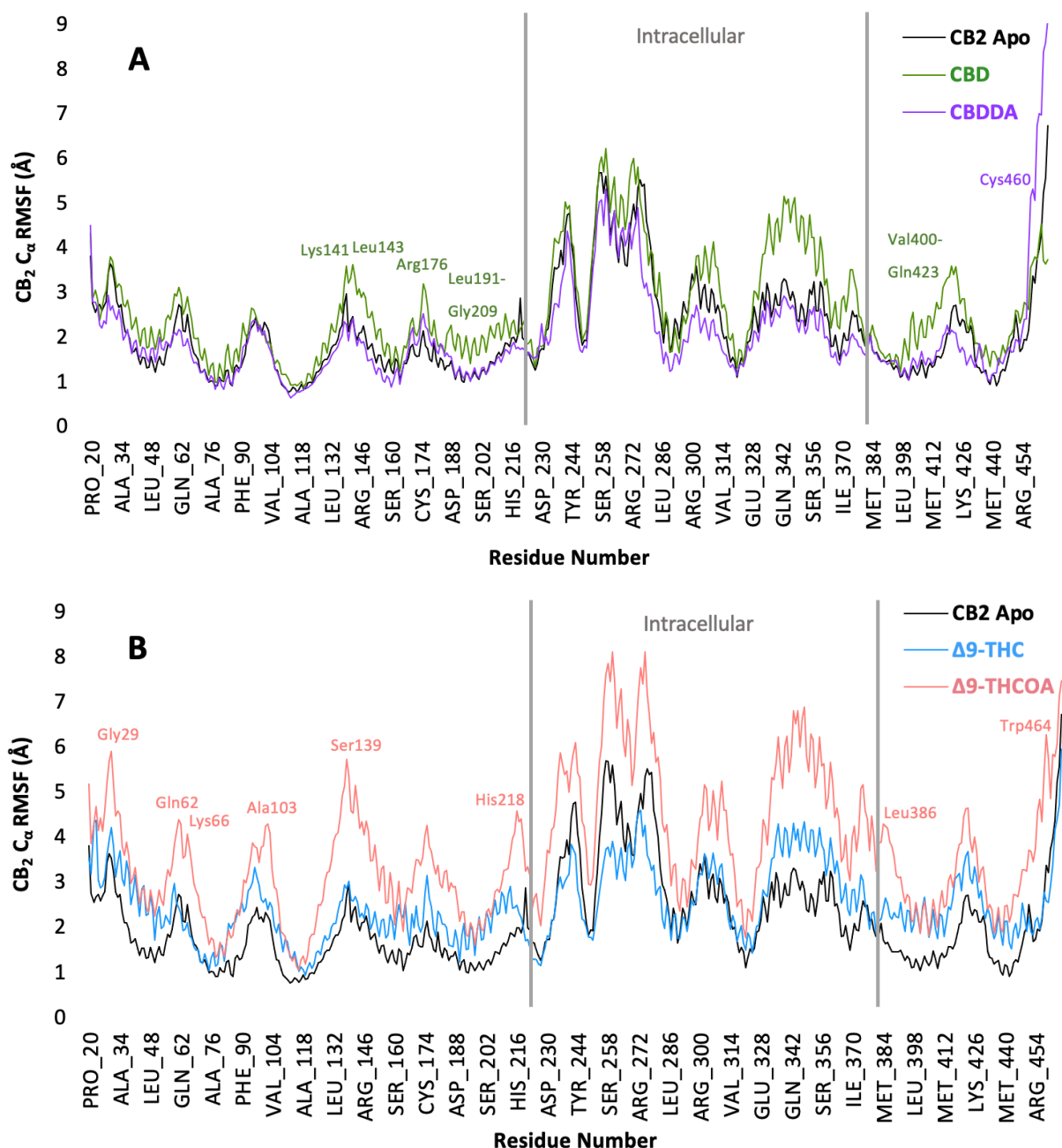


Figure 5.6.1: Residue flexibility profiles in CB_2 receptors. A. Represents the C_α RMSF of residues in the CB_1 receptor in apo form and when bound to CBD, CBDDA. CBDDA shows a more stable interaction with lower C_α RMSF values. B. Shows the C_α RMSF for the CB_2 receptor Δ^9 -THC, and Δ^9 -THCOA. The C_α RMSF values for Δ^9 -THCOA increased compared to Δ^9 -THC. Each plot tracks the fluctuation (Å) for all residues, providing insight into the local dynamics and stability of receptor-ligand interactions. The section between the grey lines denotes the intracellular region.



Figure 5.6.2: PCA scatter plots for CB₂ Receptor with various ligands. A. shows CB₂ bound to CBD, CBDDA and B. shows Δ^9 -THC, and Δ^9 -THCOA, respectively. The scatter plots reveal the conformational variability of the receptor under different ligand-induced states.

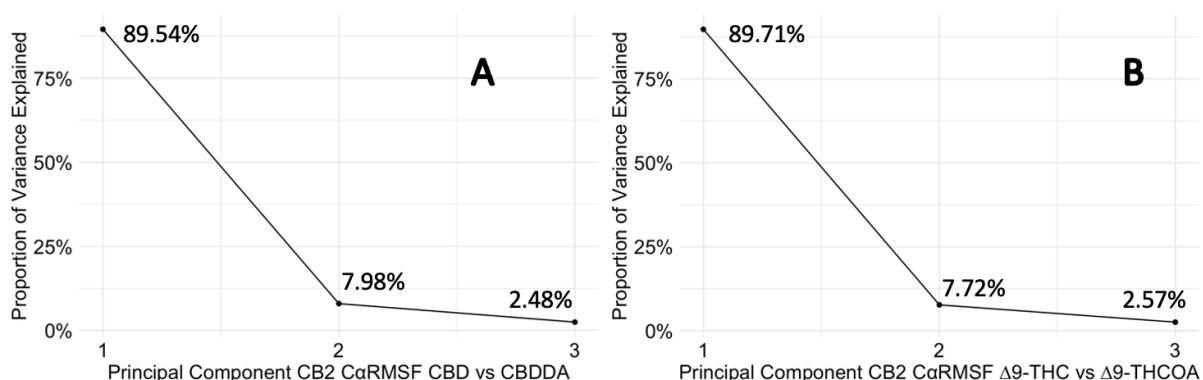


Figure 5.6.3: Distribution of variance in CB₂ receptor C_α fluctuations. A. Displays a scree plot detailing the percentage of total variance attributed to each PC for the C_αRMSF in CB₂ complexed with CBD and CBDDA. B. Shows the equivalent distribution for the CB₂ receptor in complexes with Δ⁹-THC and Δ⁹-THCOA.

3.5 Protein Compactness

The apo form of the CB₁ receptor exhibits a relatively consistent RoG throughout the simulation, punctuated by occasional fluctuations indicating transient conformational changes (Fig. 5.7.1A). Binding with CBD leads to a slightly higher average RoG for the CB₁ receptor, implying a less compact conformation. In contrast, CBDDA is associated with a reduced RoG, suggesting a more compact receptor conformation. While CBD exhibits a more compact conformation according to Fig. 5.7.2A, it demonstrates a longer duration of elevated RoG compared to CBDDA, which shows transient increases in gyrations. This dispersion suggests that each ligand distinctly influences the conformational flexibility of the receptor. Therefore, this could indicate a more stable CB₁-CBDDA interaction, potentially affecting the ligand's efficacy and impact on signal transduction pathways. The scree plot in (Fig. 5.7.3A) further illustrates this by showing a nearly equal distribution of variance across the PCs for the receptor bound to natural and acetylated CBD (PC1: 36.4 %, PC2: 33.8 %, PC3: 29.8 %), highlighting the significance of all three components in contributing to the conformational diversity of the receptor.

Subsequently, the Δ⁹-THC-bound state exhibits considerable RoG fluctuations, suggesting that the natural form of Δ⁹-THC does alter the compactness of the receptor (Fig 5.7.1B). Conversely, the plot for Δ⁹-THCOA, the acetylated version of Δ⁹-THC, shows a trend of reduced RoG, markedly distinct from the Δ⁹-THC profile. This reduction implies acetylation leads to a more compact receptor conformation, potentially signifying a stronger and more stable CB₁ receptor interaction. Figure 5.7.2B corresponds to Δ⁹-THC and Δ⁹-THCOA binding, respectively, and unlike the apo state, these panels show a more diverse conformational space, with each ligand

promoting distinct conformational states. Fig. 5.7.3B analysis of Δ^9 -THC versus Δ^9 -THCOA reveals a consistent variance distribution across PCs (PC1: 35.6 %, PC2: 32.8 %, PC3: 31.6 %), demonstrating that acetylated and non-acetylated forms of Δ^9 -THC influence the gyration of the receptor, thereby affecting signalling.

From these analyses, it can be inferred that the CB₁ receptor exhibits a relatively constrained conformational space when unbound. Binding with natural or acetylated phytocannabinoids significantly increases this space, reflecting the flexibility of the receptor in accommodating various ligand-induced conformations. Notably, the scree plots suggest that both natural and acetylated forms of CBD and Δ^9 -THC contribute to receptor conformational diversity without a single dominant pattern of variance, implying that multiple structural adaptations are significant in the receptor-ligand interactions. This detailed understanding of CB₁ receptor conformational changes is crucial for the development of phytocannabinoid-based therapies, as it provides insights into how ligand binding could influence receptor function and downstream signalling.

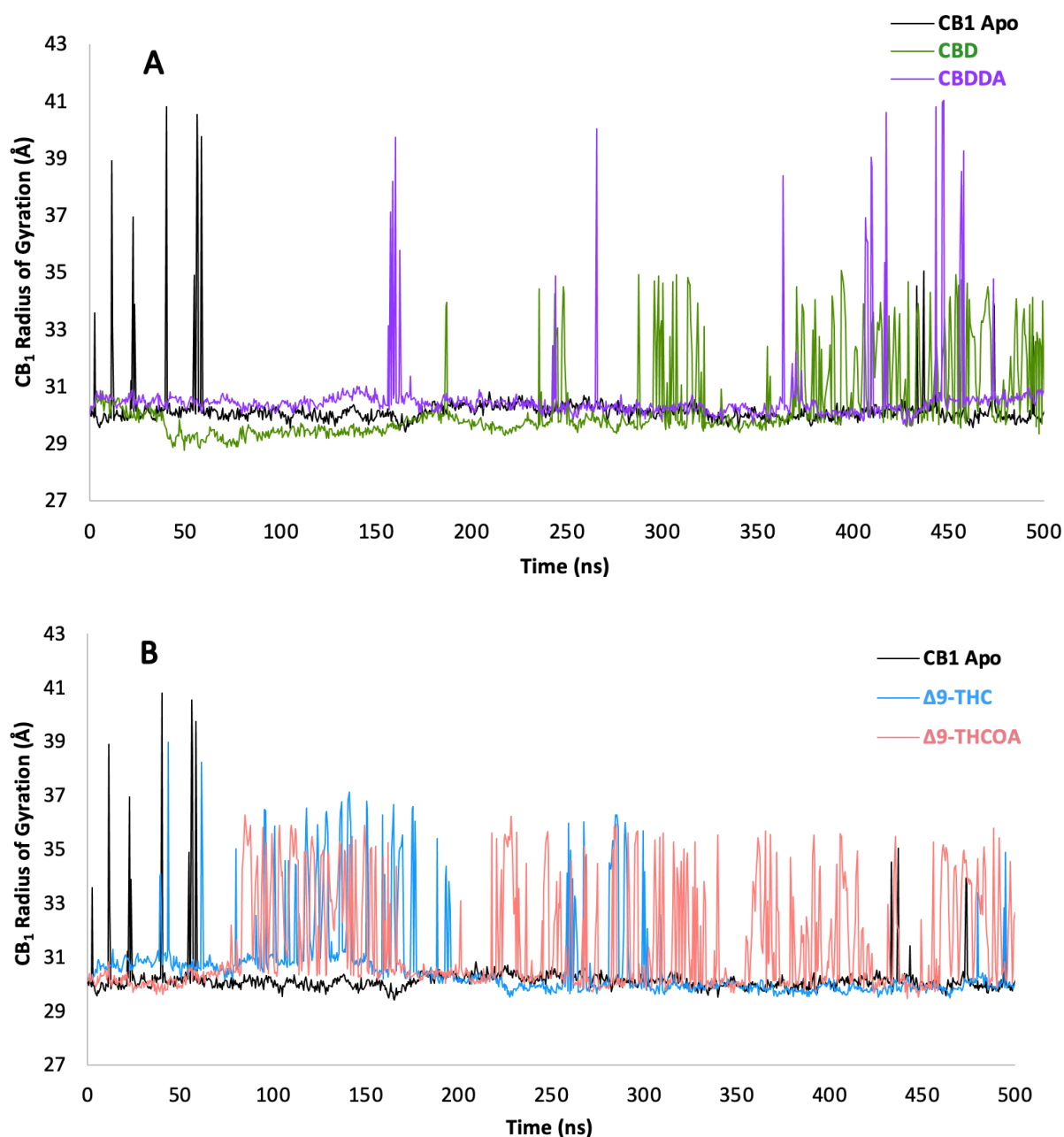


Figure 5.7.1: Temporal analysis of receptor compactness. A. displays the RoG for CB₁ and its complexes with CBD, CBDDA over a 500 ns simulation. B. Similarly depicts the RoG for CB₁, Δ⁹-THC, and Δ⁹-THCOA. RoG is a measure of the structural compactness of a protein, with lower values indicating tighter folding. Both acetylated cannabinoids show an increase in RoG when bound to CB₁ indicating a decrease in the compactness of the protein.

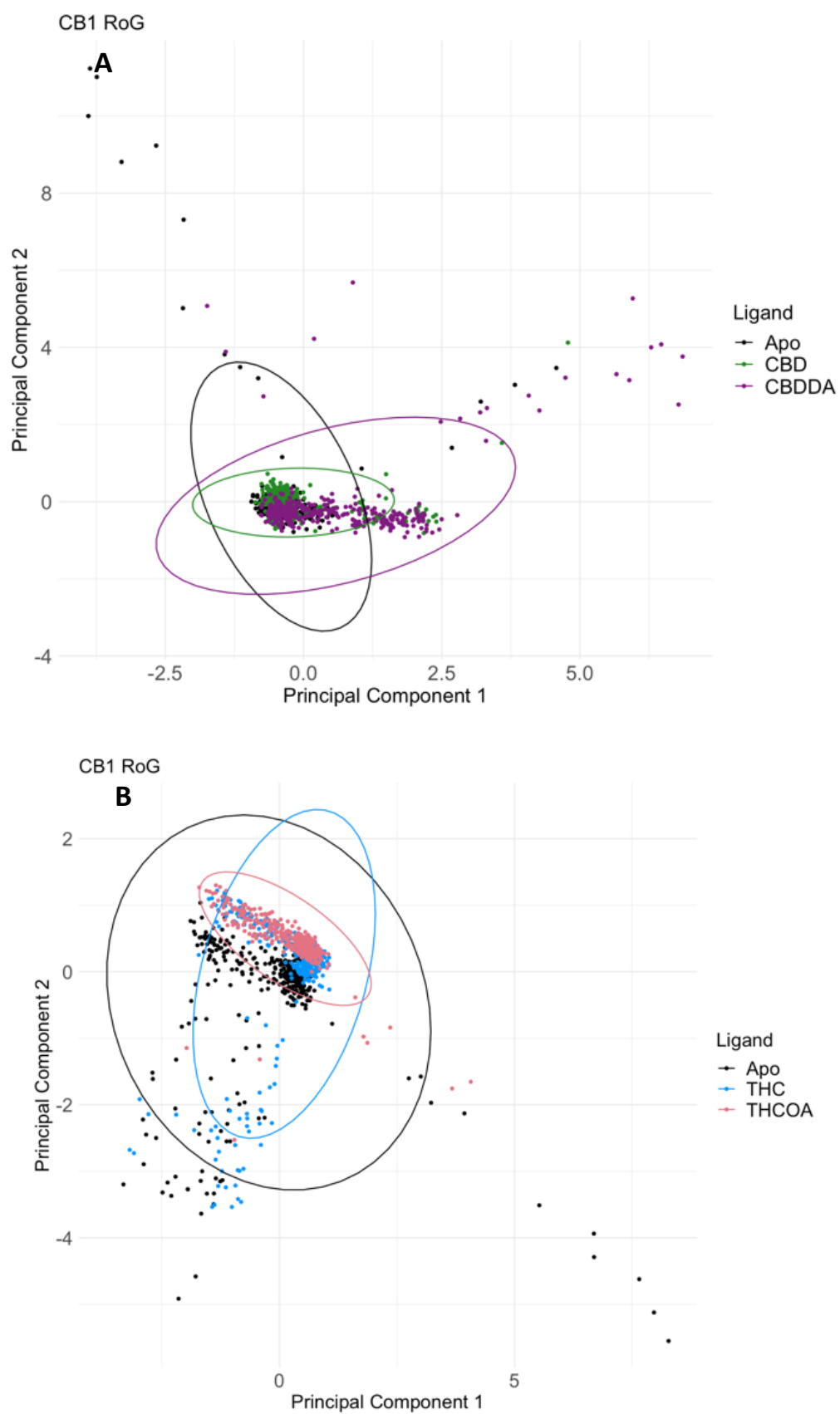


Figure 5.7.2: PCA of the RoG for CBRs. The PCA scatter plots for CB₁ receptor when complexed with A. CBD, B. CBDDA, C. Δ^9 -THC, and D. Δ^9 -THCOA.

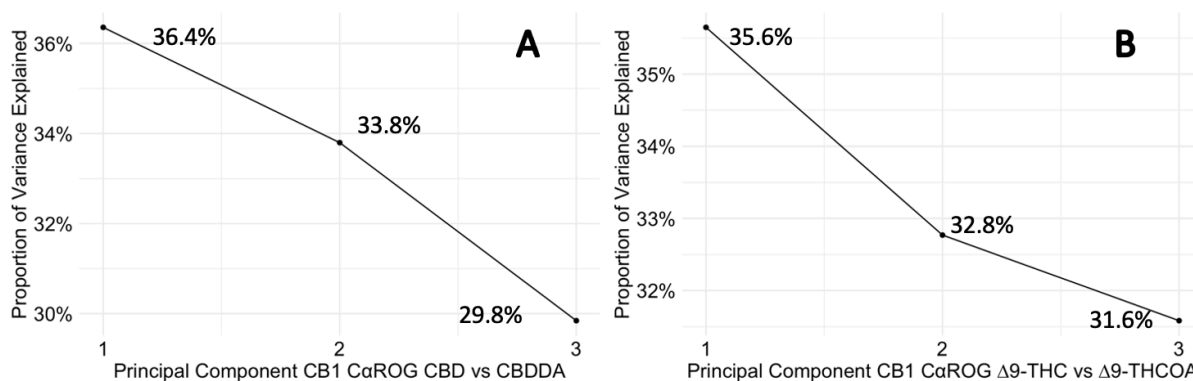


Figure 5.7.3: Scree plots depicting the proportion of total variance explained by PCs in the gyration of CB₁ receptors. A. compares the variance explained by PCs when evaluating natural CBD versus acetylated CBD, revealing the following percentages for PCs: PC1 (36.4 %), PC2 (33.8 %), and PC3 (29.8 %). B. Depicts a similar analysis is conducted for Δ^9 -THC versus Δ^9 -THCOA, yielding percentages of PC1 (35.6 %), PC2 (32.8 %), and PC3 (31.6 %).

In assessing the RoG profile for the CB₂-CBD complex, it is observed that the reduced fluctuations and narrower distribution, as depicted in Figure 5.8.1A, imply that CBD confers greater conformational stability on the receptor. However, the more variable and expansive RoG observed with CBDDA suggests it may induce a higher degree of conformational flexibility within the receptor. In addition, there is a notable difference in the spread of the data points in the PCA plots. The plot for CBD (Fig. 5.8.2A) shows a greater spread along both PC axes compared to the plot for CBDDA. This suggests that the CB₂ receptor exhibits a greater diversity of conformations when bound to CBD compared to CBDDA when compared to the apo, which may imply a more flexible interaction with CBD. The scree plot for CB₂ shows that PCs explain 36.9 %, 32.8 %, and 30.3 % of the variance, respectively. This indicates a certain level of conformational diversity with a more significant variance explained in the apo state. When the CB₂ receptor is complexed with the natural phytocannabinoid CBD, the variance explained decreases but remains relatively high, suggesting that CBD maintains a considerable influence on the conformational space of the receptor. However, with the acetylated version, CBDDA, the variance explained by the PCs further decreases, pointing to an increased conformational diversity yet potentially has specific interactions with the receptor.

When comparing the interactions of the CB₂ receptor with Δ^9 -THC and its acylated derivative Δ^9 -THCOA using the RoG, a steadier RoG with less variability would imply that Δ^9 -THC constrains the receptor to a more limited array of conformations, indicating a more specific interaction, whereas a RoG trace for Δ^9 -THCOA marked by greater fluctuations and potentially higher values would suggest a more dynamics and less restricted interaction, allowing the

receptor to explore a wider variety of conformational states, thus reflecting the distinct conformational influences of the acylated versus the non-acylated form of the molecule. In contrast, when comparing the receptor complexed with Δ^9 -THC to Δ^9 -THCOA, both plots (Fig. 5.8.2B) show a wide dispersion of data points, although the plot for Δ^9 -THCOA shows a slightly more compact clustering than Δ^9 -THC. This could indicate that while both ligands induce flexibility in the conformation of the receptor, Δ^9 -THCOA may lead to a slightly more constrained set of conformations compared to Δ^9 -THC when compared to the RoG of the apo. Similarly, the scree plot for CB₂ Δ^9 -THC shows that the apo state explains 45.9 % of the variance, indicating a dominant conformational state. Upon binding with the natural cannabinoid Δ^9 -THC, the explained variance reduces to 32.2 %, which still signifies a strong influence on the receptor conformational dynamics. Yet, with the acetylated version Δ^9 -THCOA, the variance explained diminishes drastically to 21.9 %, signifying a much broader range of conformations that the receptor can adopt. This implies that Δ^9 -THCOA induces a distinct array of conformational changes in the receptor than the natural form, Δ^9 -THC.

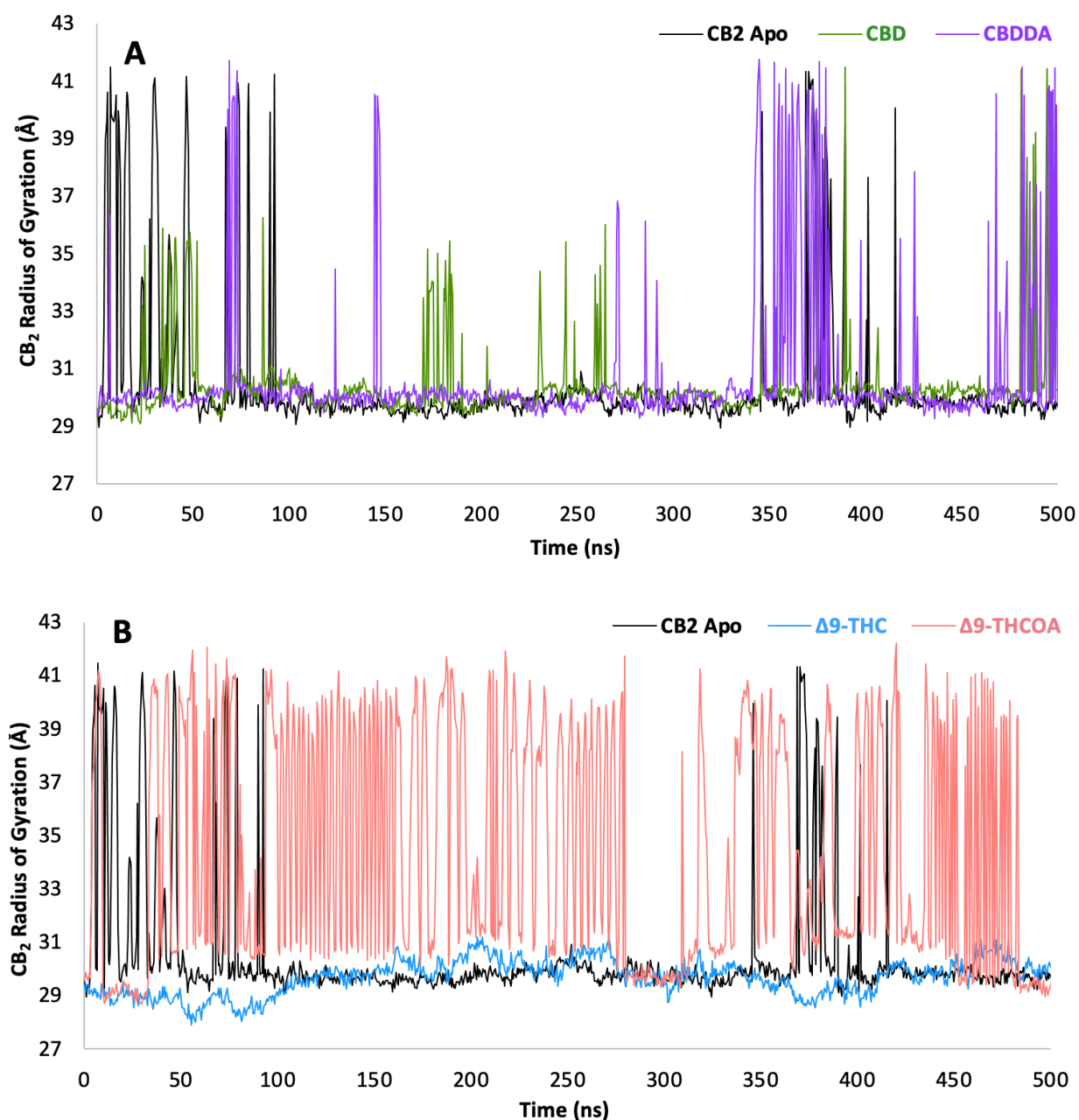


Figure 5.8.1: Temporal analysis of receptor compactness. Displays the RoG for CB₂ and its complexes with A. CBD, CBDDA, B. Δ⁹-THC, and Δ⁹-THCOA over a 500 ns simulation. When bound to CB₂, both acetylated cannabinoids increase the RoG compared to the natural cannabinoids.

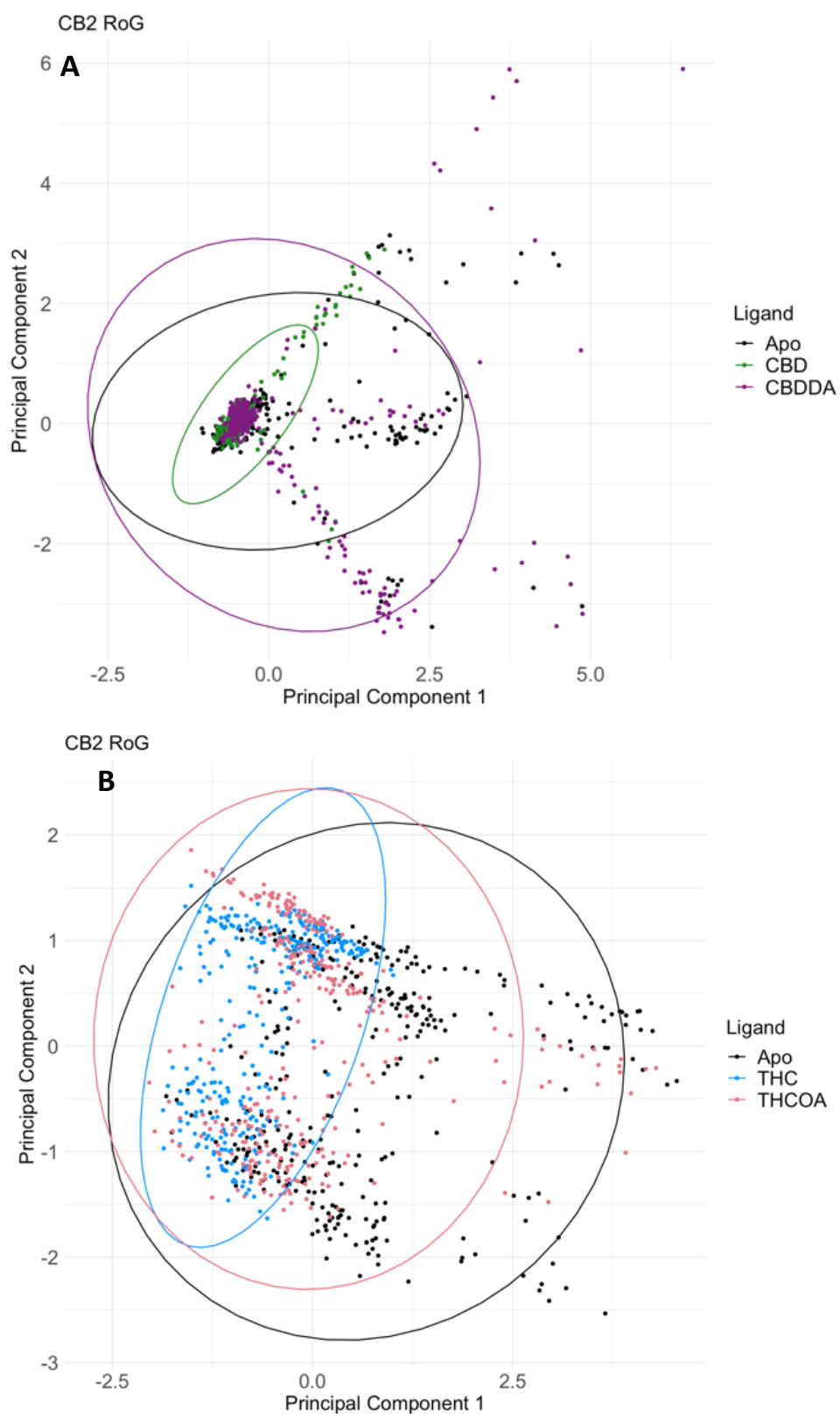


Figure 5.8.2: PCA of the RoG for CB₂. A. illustrate the PCA scatter plots for CB₂ receptor in its apo form and when complexed with A. CBD, CBDDA and B. Δ^9 -THC, Δ^9 -THCOA.

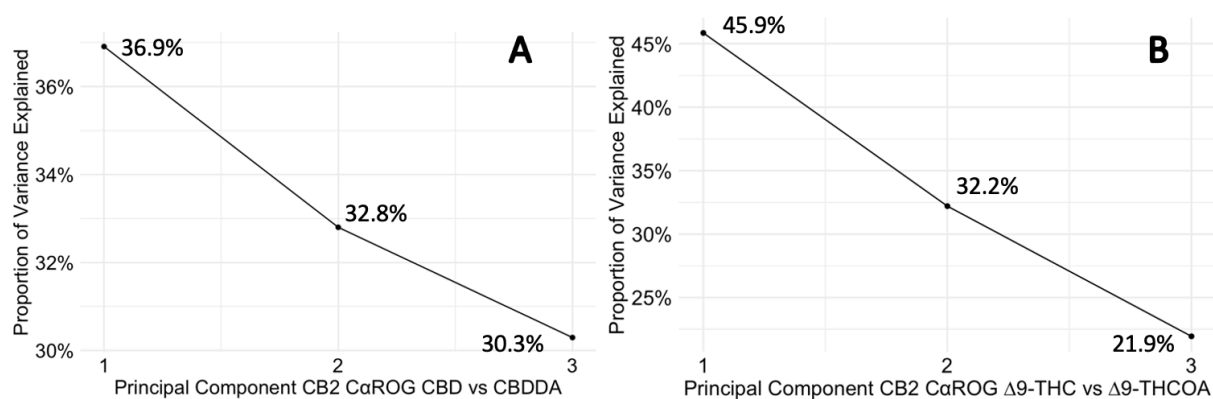


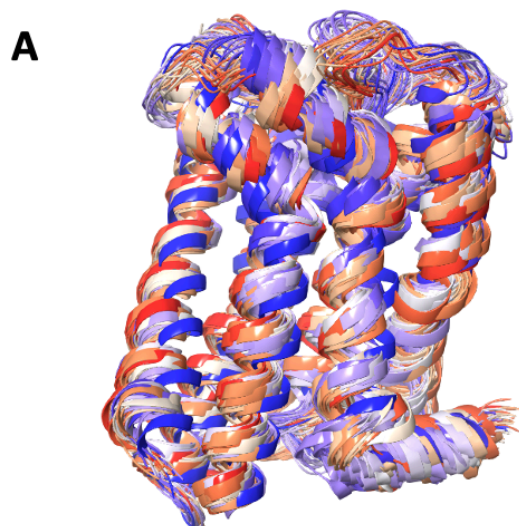
Figure 5.8.3: Variance explained by PCs and CB₂ receptors gyration. A. Illustrates the variance for the CB₂ receptor complexed with CBD versus CBDDA, and B. compares the variance for complexes with Δ⁹-THC versus Δ⁹-THCOA, highlighting the differences in conformational space explored by the receptor with each ligand.

3.6 Simulation Snapshots

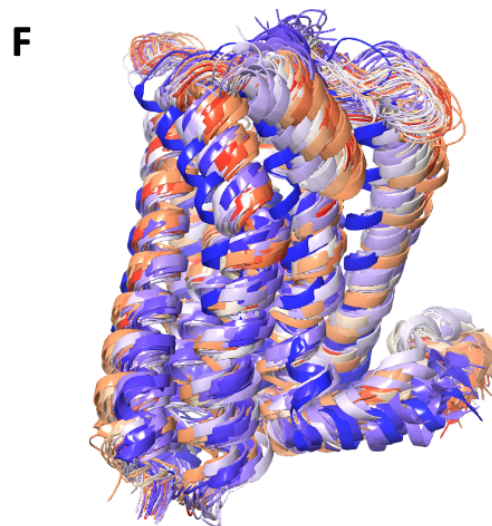
Snapshots of CBRs (Fig. 5.9) visually capture the conformational changes of the membrane-bound portion of p-CBRs throughout a 500 ns molecular dynamics simulation. This sequence of structural snapshots highlights the inherent flexibility of both receptors, shown in their apo forms and when bound to their natural ligands CBD and Δ⁹-THC, as well as their acetylated derivatives, CBDDA and Δ⁹-THCOA. The apo form of CB₁ displays a wide range of conformations, accentuating the receptor's intrinsic flexibility. Interaction with CBD causes the CB₁ receptor to adopt a distinct set of conformations, which are subtly modified upon binding with the acetylated version, CBDDA. This modification suggests that acetylation might amplify the ligand-induced conformational diversity, potentially uniquely modulating the activity of the receptors. Δ⁹-THC binding also results in specific conformational changes within CB₁, indicative of altered activation pathways or signalling mechanisms. In contrast, Δ⁹-THCOA binding appears to stabilise the receptor, as evidenced by fewer conformational changes in the snapshot series compared to the apo state.

The apo snapshots for CB₂ reveal its inherent conformational variability (Fig. 5.9). The interaction with CBD maintains a similar conformational profile to the apo receptor throughout the simulation, suggesting that CBD binds in a way that preserves the native inactive state of the receptor, yet introduces dynamic fluctuations. In contrast, the acetylated analogue CBDDA prompts more substantial conformational shifts in CB₂, hinting at a more robust or distinct receptor interaction. The binding of Δ⁹-THC to CB₂ shows a unique set of conformations, which is further altered by the acetylated Δ⁹-THCOA, implying that acetylation may amplify the ligand impact on receptor dynamics and potentially elicit stronger or altered physiological

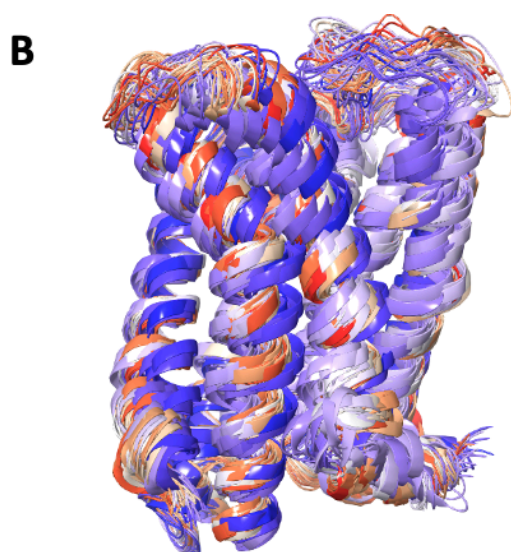
effects. These snapshots highlight the conformational elasticity of CBRs and their ability to respond to various ligand-induced stimuli. The comparison between the natural and acetylated variants highlights the impact of chemical modifications on receptor dynamics, which is fundamental to understanding the pharmacological and therapeutic potential of these compounds.



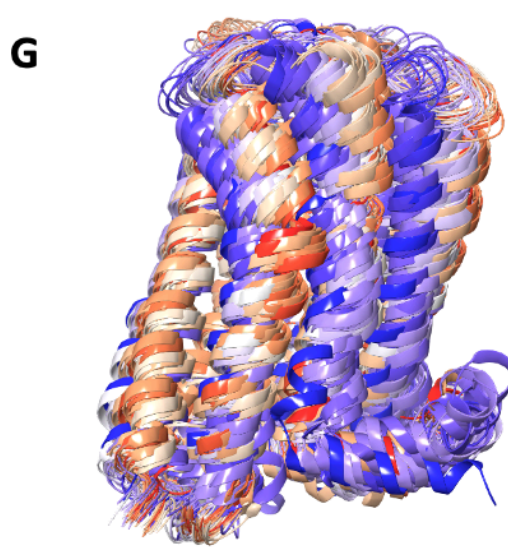
Complex: CB₁ – Apo



Complex: CB₂ – Apo



Complex: CB₁ – CBD



Complex: CB₂ – CBD

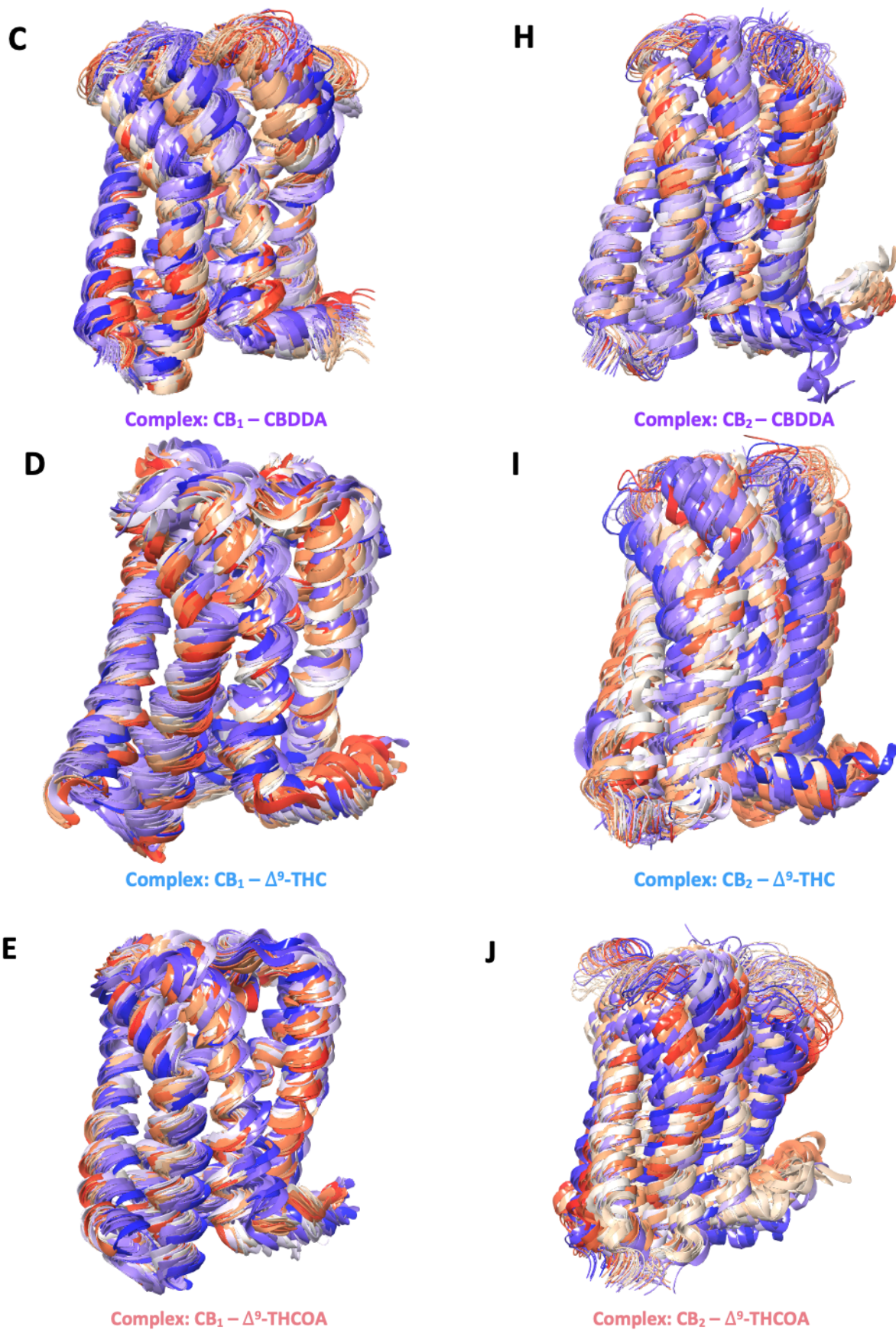
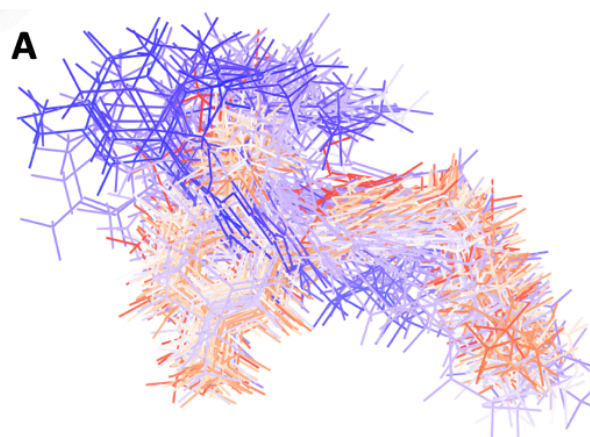


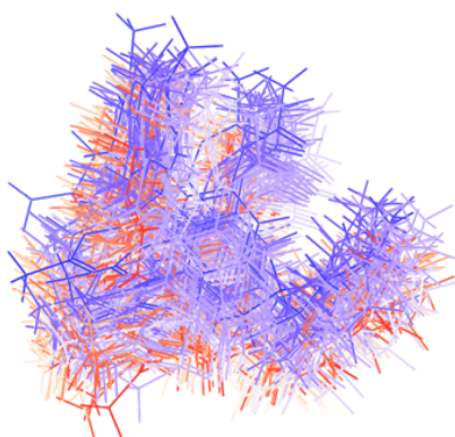
Figure 5.9: Ensemble of conformational states for CB₁ and CB₂ receptors during MD simulations when interacting with various ligands. This collection of molecular models showcases the range of conformations adopted by the

CB₁ (A – E) and CB₂ (F – J) receptors in their apo forms and when bound to CBD, CBDDA, Δ^9 -THC, and Δ^9 -THCOA. Each ensemble represents the dynamic structural shifts observed over the simulation period, with the diversity of receptor shapes reflecting the influence of each ligand on the conformational landscape of the receptor with the beginning of the simulation snapshots starting from blue and ending with red. Generated from Maestro Schrödinger Suite.

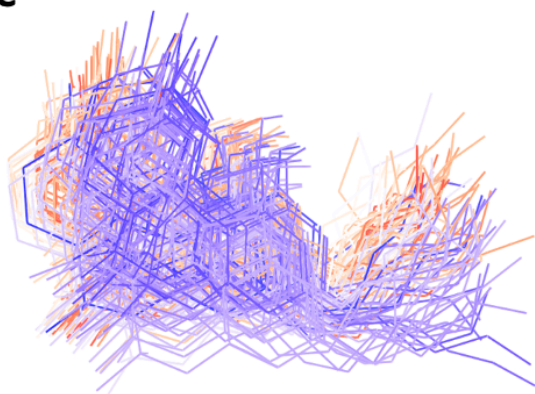
The dynamic conformational behaviour of phytocannabinoid ligands within CBRs binding sites were captured over a 500 ns MD simulation (Fig. 5.10). These visualisations emphasise the inherent flexibility of the ligands and the variety of conformations they can adopt within the protein. In CB₁, the ligands demonstrate a tendency to adopt a compact set of conformations, suggesting a stable and consistent interaction within the binding site. CBD shows a predominant conformation within the binding pocket, with some variability observed at the onset of the simulation. CBDDA also exhibits a primary orientation, with occasional variations indicating a slightly different interaction pattern. Δ^9 -THC and its acetylated variant, Δ^9 -THCOA, display distinct binding conformations, which may indicate different modes of receptor engagement and potential signalling outcomes. Within CB₂, a diverse array of ligand conformations is observed. Notably, CBDDA showcases significant conformational diversity, suggesting a dynamic interaction that could potentially lead to different signalling outcomes in CB₂. Conversely, Δ^9 -THCOA presents a more consistent binding conformation, hinting at a potentially more stable interaction within the receptors binding site. The diversity of conformations also raises questions about the predictability of these responses and the potential for off-target effects (Xie et al., 2011). On the other hand, the more stable conformation observed with Δ^9 -THCOA may suggest a more predictable and perhaps more targeted therapeutic profile, although the potential for increased potency must be carefully evaluated.



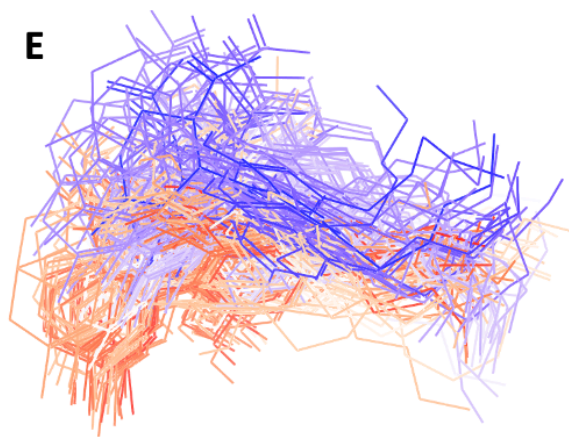
B Complex: CB₁ – CBD



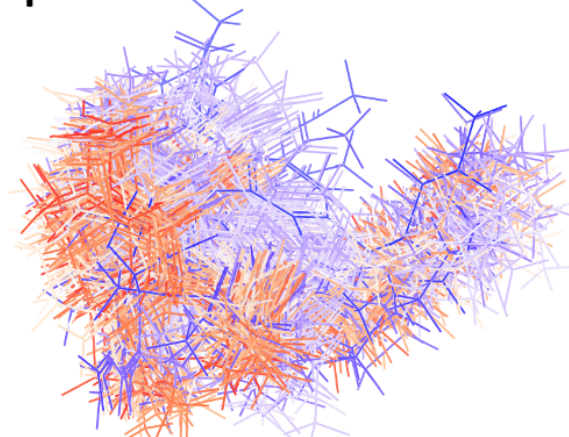
C Complex: CB₁ – CBDDA



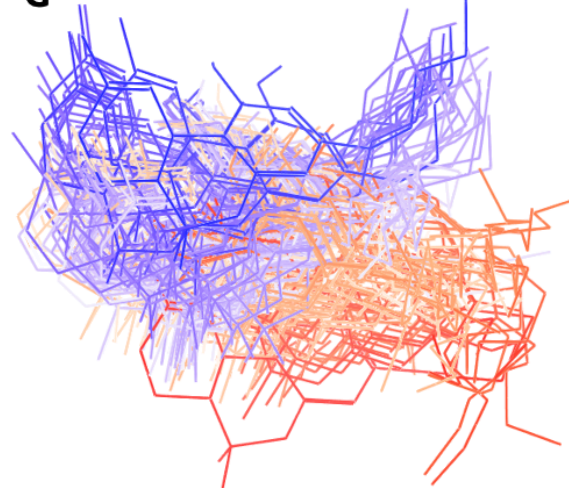
Complex: CB₁ – Δ⁹-THC



F Complex: CB₂ – CBD



G Complex: CB₂ – CBDDA



Complex: CB₂ – Δ⁹-THC

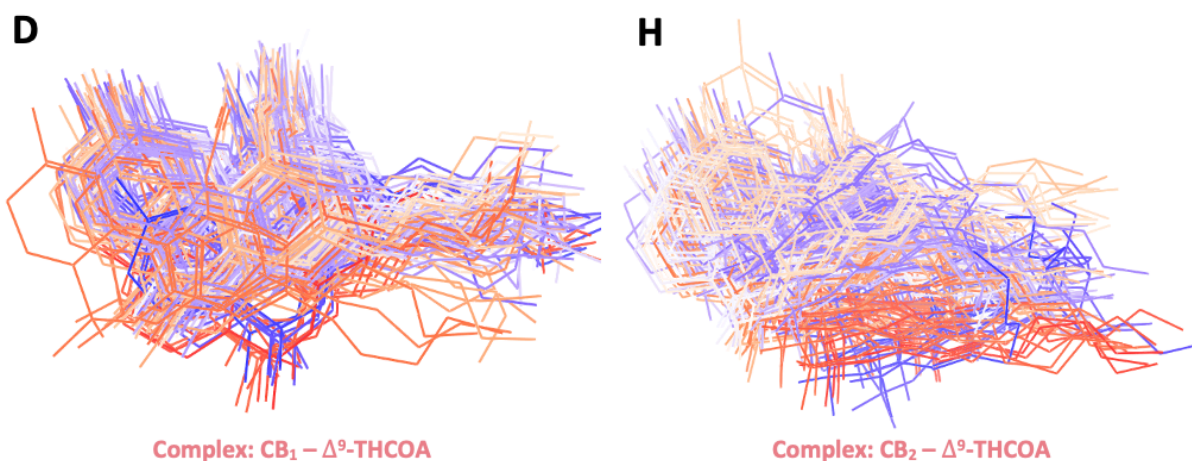


Figure 5.10: Dynamic ligand binding conformations in CB₁ and CB₂. Overlaid snapshots capture the array of conformations exhibited by CBD, CBDDA, Δ⁹-THC, and Δ⁹-THCOA within the CB₁ (A – D) and CB₂ (E – H) binding sites over a 500 ns MD simulation beginning with blue and ending with red. These visualisations illustrate the flexibility and range of interactions that each ligand can engage in with the respective CBRs, offering insights into the structural adaptability of the ligands within the binding site of the receptor. Generated from Maestro Schrödinger Suite.

3.7 Ligand Interactions with p-CBRs

The lig-RMSD trajectories for CBRs are presented over a 500 ns MD simulation (Fig. 5.11). Lig-RMSD values provide a quantitative measure of the stability of the ligand position within the binding site over time, and a low lig-RMSD indicates a stable interaction with the receptor. For CB₁, CBD shows a relatively stable lig-RMSD, plateauing at ~1.8 Å around 180 ns, implying a stable interaction with the receptor while retaining some flexibility and dynamism. CBDDA and Δ⁹-THC display similar patterns in their RMSD trajectories, with both stabilising at ~100 ns and maintaining an RMSD between 0.5–1 Å, suggesting a secure and consistent interaction within the CB₁ binding site. In the case of CB₂, CBDDA quickly stabilises at around 25 ns, indicating a rapid attainment of a stable binding conformation. In contrast, the other ligands, including CBD, Δ⁹-THC, and Δ⁹-THCOA, exhibit greater fluctuations throughout the simulation, reflecting a more dynamic and less predictable interaction within the CB₂ binding pocket. These lig-RMSD findings correlate with the observed ligand conformations in Figure 10, where distinct rotational and positional changes of the ligands within the CB₁ binding pocket are evident. This suggests each ligand attains a stable conformation over time. For CB₂, the increased fluctuation in the lig-RMSD implies more diverse rotational and conformational changes, pointing to a less consistent binding pattern and potentially different activation or signalling mechanisms triggered by these ligands. This detailed analysis of lig-RMSD trajectories highlights the importance of considering both the stability and the dynamic nature

of ligand-receptor interactions, which are critical factors in the development of cannabinoid-based therapeutics. The conformational behaviour of ligands within the binding site is essential for understanding their pharmacological profiles and optimising their therapeutic efficacy and safety.

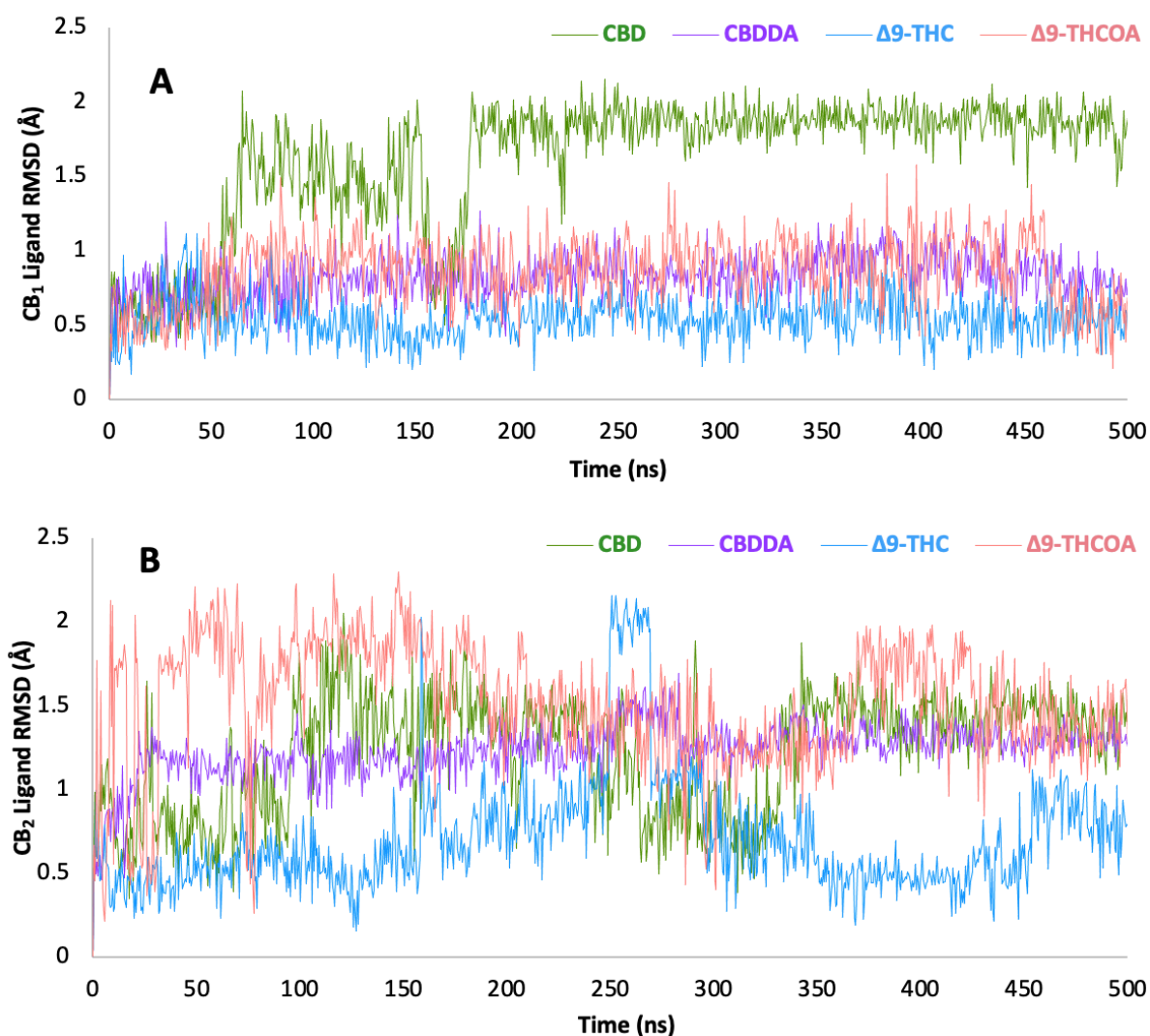
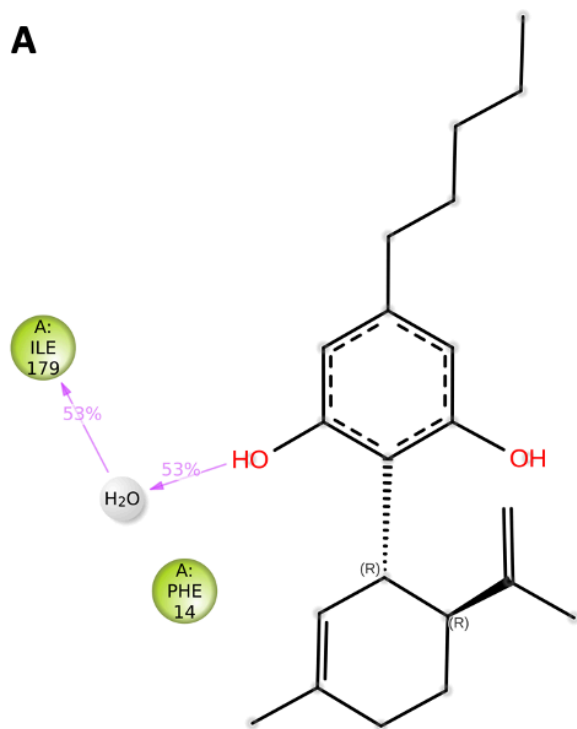
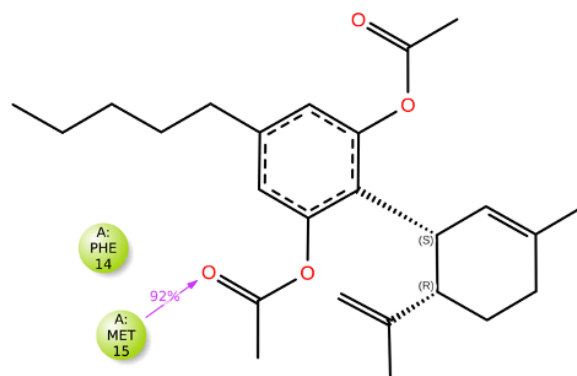
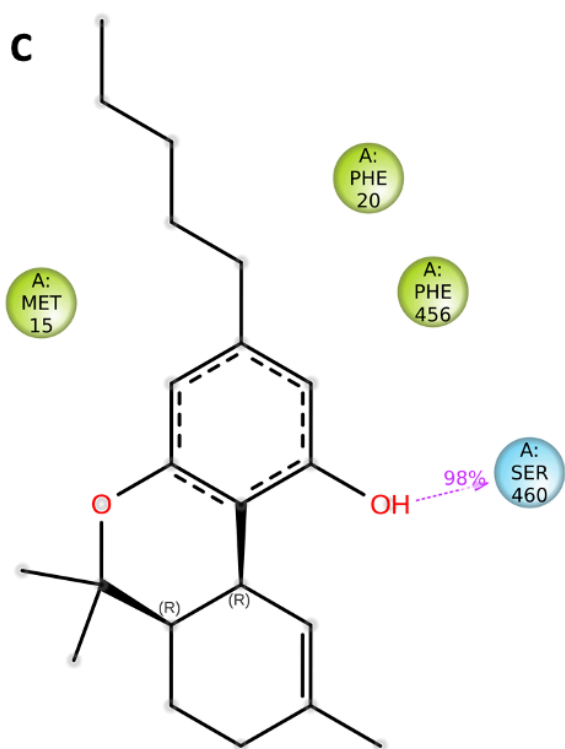
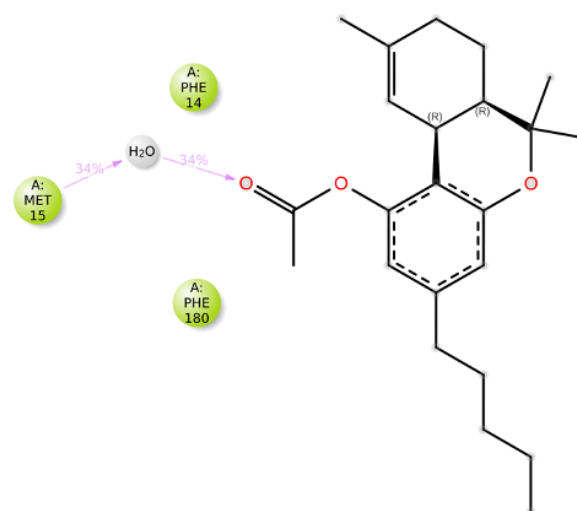


Figure 5.11: RMSD of Ligands in CB₁ and CB₂ Receptor Complexes. A. tracks the RMSD of CBD, CBDDA, Δ⁹-THC, and Δ⁹-THCOA when bound to the CB₁ receptor throughout a 500 ns simulation. B. depicts the ligand-RMSD for the same ligands in complex with the CB₂ receptor. Lig-RMSD measures the positional stability of each ligand within the binding site, with lower and more consistent lig-RMSD values indicating a stable interaction, and higher or more variable lig-RMSD values reflecting dynamic binding and conformational flexibility. These trajectories provide insights into the comparative stability of ligand-receptor interactions over time.

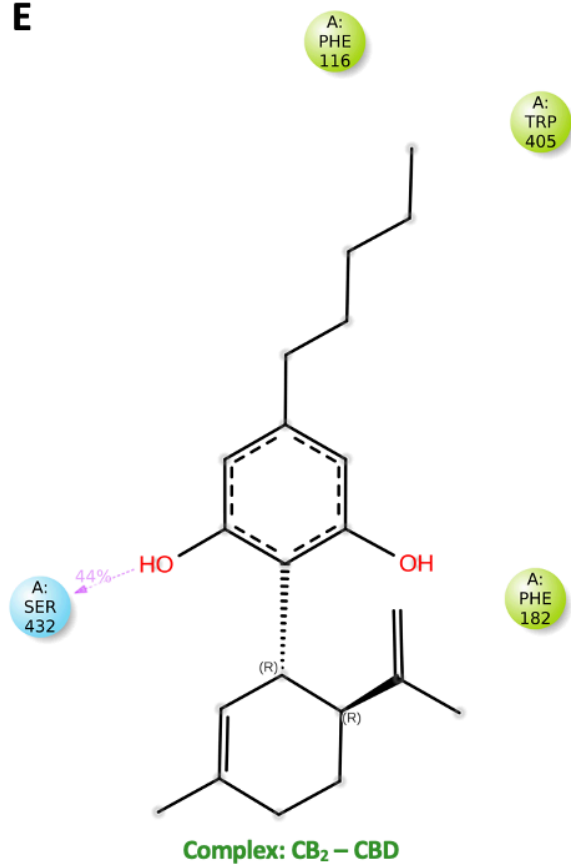
The contact diagrams illustrated in Fig. 5.12 meticulously detail the specific interactions between ligands and CB₁, highlighting the implicated amino acid residues and the persistence

or intensity of these contacts throughout the simulation. For CBD, a prominent interaction involves a water molecule which consistently forms a bridge to the OH group for 53 % of the simulation time, connecting to Ile179. This interaction is particularly relevant considering CBDs role as a modulator of the CB₁ receptor, where it does not rigidly lock the receptor into an active or inactive conformation but rather appears to maintain a state that allows for receptor flexibility. In the CB₁-CBDDA complex, there is a notable hydrophobic interaction with Met15, engaging with an O atom on the ligand for 93 % of the simulation duration. This persistent interaction aligns with the IFD results, suggesting that Met15 is a critical residue for ligand binding. This is consistent across THC analogues as well, where Δ^9 -THCOA exhibits a water-mediated interaction involving an O atom for 34 % of the simulation, potentially affecting the ligands conformational stability and receptor activity. Additionally, Ser460 is identified as a significant hydrophilic residue for ligand interaction, particularly with an OH group of Δ^9 -THC, maintaining this interaction for 98 % of the simulation time. This observation further corroborates the IFD findings and emphasises Ser460 potential role in stabilising the ligand within the CB₁ binding pocket. For CB₂, the interaction profile with CBD highlights a significant hydrophilic interaction, predominantly featuring Ser432 which engages with the ligands OH group for 44 % of the simulation. This affinity for hydrophilic serine residues underlines a preference for polar interactions within the binding pocket, facilitating the binding stability of CBD to CB₂. Additionally, the enduring presence of hydrophobic phenylalanine residues at the binding site for both CBRs supports a stable ligand-receptor complex formation through consistent hydrophobic interactions. Concerning the acetylated variant Δ^9 -THCOA, a notable feature within the CB₂ complex is the ligand's π - π stacking interactions with Phe182 and Phe86, occurring for 35 % and 56 % of the simulation time, respectively. These persistent π - π stacking interactions are indicative of Δ^9 -THCOA strong binding affinity and specific orientation within the binding site of the CB₂ receptor, potentially influencing receptor activation, and signalling pathways.

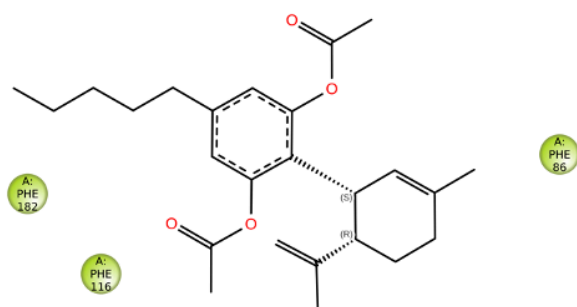
These insights into the ligand-receptor interactions within the CB₂ binding pocket highlight the complex interplay of hydrophilic and hydrophobic forces that govern ligand affinity and receptor dynamics, and how modifications like acetylation can modulate these interactions. Understanding these detailed binding mechanisms is critical for the rational design of phytocannabinoid-based therapeutics with targeted efficacy and minimised adverse effects.

A**Complex: CB₁ – CBD****B****Complex: CB₁ – CBDDA****C****Complex: CB₁ – Δ⁹-THC****D****Complex: CB₁ – Δ⁹-THCOA**

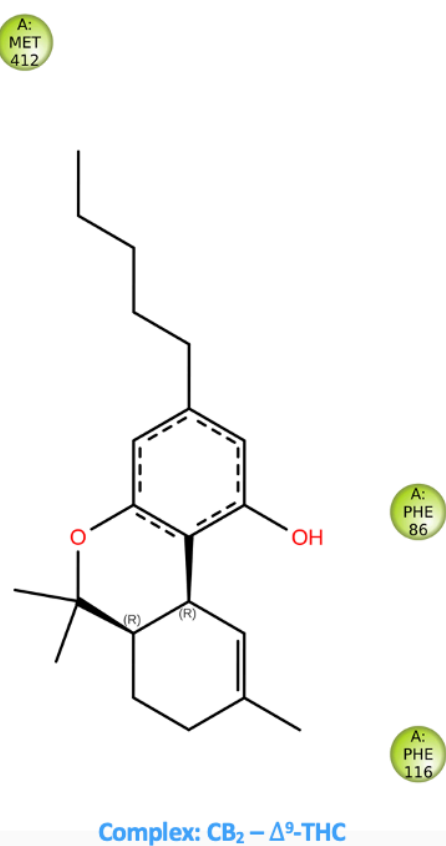
E



F



G



H

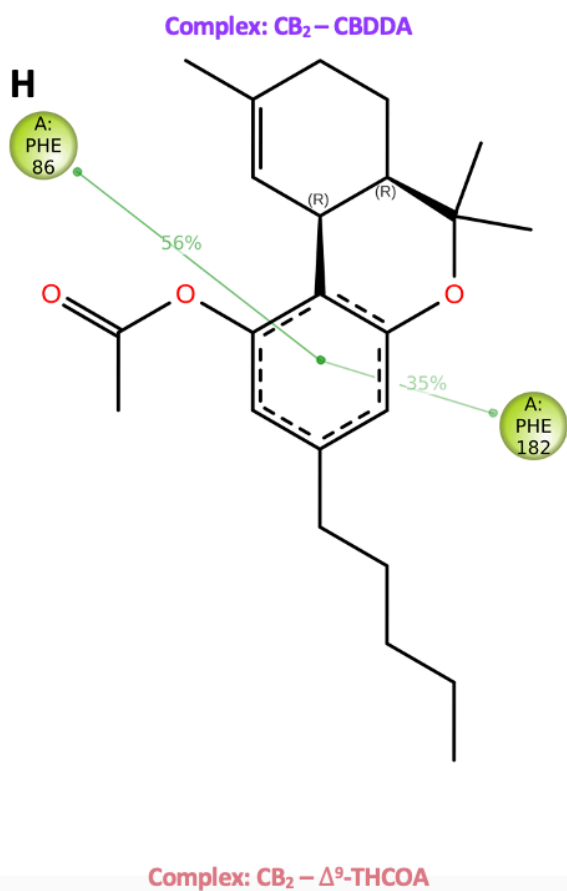




Figure 5.12: Interaction Maps of Ligands with CB₁ and CB₂ Receptors Post-Molecular Dynamics Simulations. A – D detail the interaction of CBD, CBDDA, Δ^9 -THC, and Δ^9 -THCOA with the CB₁ receptor, while panels E – H replicate these mappings with the CB₂ receptor. The illustrations highlight key residues within the receptors that maintain persistent contact with the ligands for at least 30 % of the simulation time, as denoted by the proximity lines. These contact summaries provide insights into the stability and specificity of ligand binding, reflecting the conserved and distinct interaction patterns that emerge from the dynamic simulation environment, which may underlie the functional outcomes of ligand-receptor binding. Generated from Maestro Schrödinger Suite.

3.8 Water Measurement Analysis

The water molecule (11077) present in CB₁-CBD complex shows a relatively steady pattern of fluctuation in the radius distance measurements (Fig. 5.13), with fewer peaks and troughs, suggesting that the interaction between the water molecule and the CB₁-CBD complex is comparatively stable. This could imply that Water-11077 forms a persistent hydration shell or network that maintains consistent contact with the receptor-ligand complex, potentially contributing to a stable structural conformation of the CBD bound to CB₁. The CB₁- Δ^9 -THCOA complex presents a more erratic and pronounced fluctuation, with distance peaks suggesting occasional loss of interaction and troughs indicating closer contact or formation of transient stabilising interactions such as H bonds. The greater variability in the distance measurements for Water 9938 could be indicative of a more flexible and dynamic hydration environment around the Δ^9 -THCOA complex, which may affect the binding affinity and efficacy of the ligand interaction with the CB₁ receptor. The contrasting interaction patterns observed for the two complexes might reflect intrinsic differences in the molecular structure and properties of CBD and Δ^9 -THCOA, such as their hydrophobicity, electronic distribution, or molecular geometry, which in turn modulate the behaviour of surrounding water molecules. These interactions are critical as they can influence the pharmacodynamics of the ligands, affecting how they activate or inhibit the CB₁ receptor, with possible implications for their therapeutic profiles and efficacy.

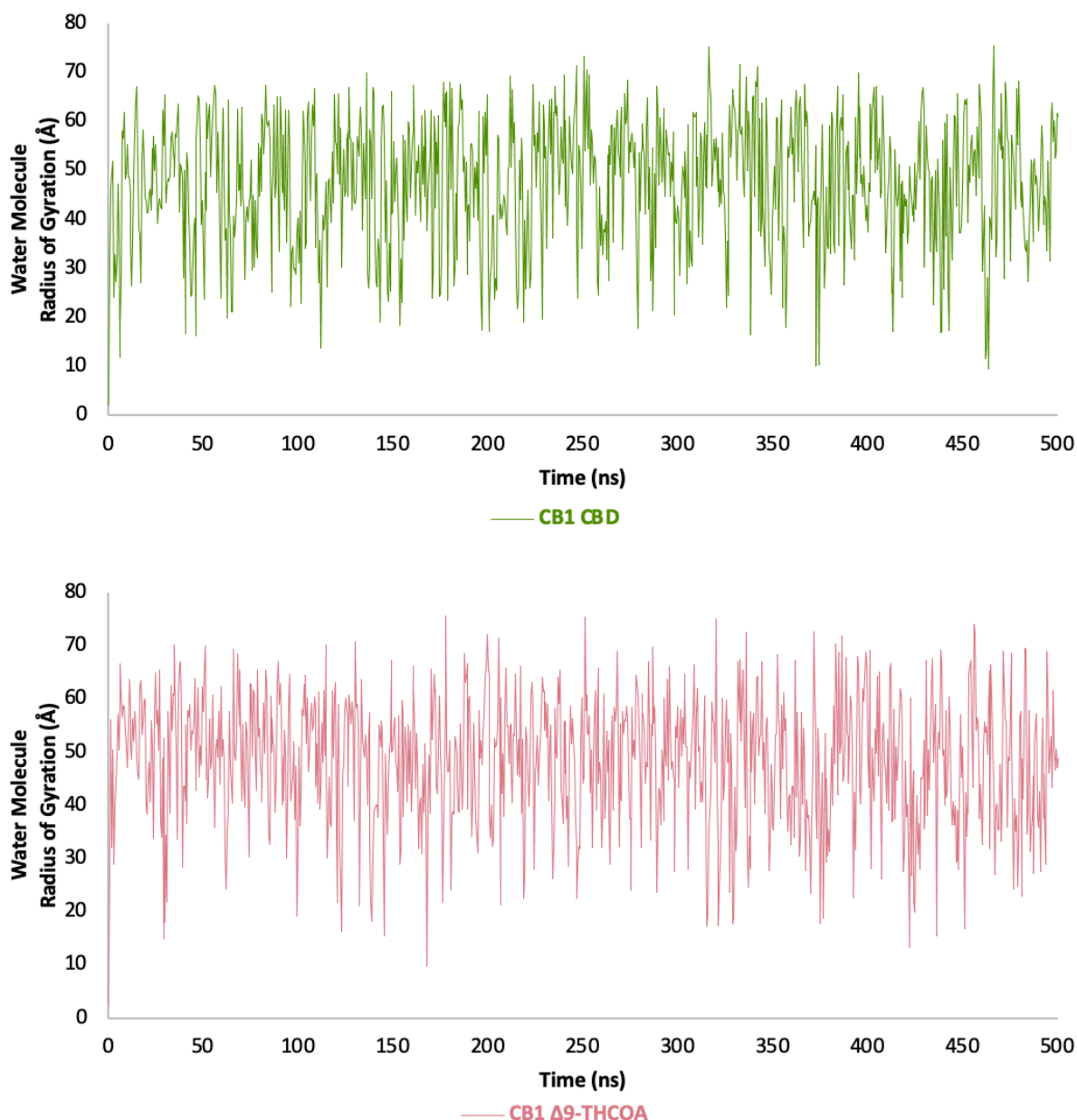


Figure 5.13: Fluctuation analysis of water molecule interactions with CB₁ receptor complexes. The graph displays the dynamic distance changes (Å) of water molecules in proximity to the CB₁ receptor when bound to A. CBD (Water 11077) and B. Δ^9 -THCOA (Water 9938) over the course of a 500 ps simulation. The plot shows the temporal behaviour of these water molecules, indicating their role in the stability and conformational changes within the active site of the receptor throughout the simulation period.

3.9 Effects of Acetylation

After analysing the effects of natural and acetylated phytocannabinoids for CB₁ (Fig. 5.14A). Both CBD and CBDDA increase the C _{α} RMSD of the apo protein, with CBDDA causing a more variable C _{α} RMSD than natural CBD. This suggests that both compounds might induce conformational changes in the CB₁ receptor, although the modification in CBD could lead to

greater variability in receptor conformation. In terms of C_{α} RMSF, both CBD and CBDDA decrease this measure, indicating that they may stabilise certain regions of the CB₁ receptor. However, CBD decreases the C_{α} RMSF more than CBDDA, suggesting that CBD may confer a greater degree of stability to the dynamic regions of the receptor compared to CBDDA. CBD slightly decreases the RoG, while CBDDA slightly increases it. This subtle difference hints that CBD may induce a more compact receptor conformation than CBDDA. The RoG is an indicator of the overall protein compactness, thus, a decrease in RoG could correlate with a more tightly packed, potentially less flexible receptor structure. Regarding lig-RMSD, CBD has a higher value compared to CBDDA, suggesting that CBD might interact with the receptor in a more dynamic and perhaps less consistent manner than CBDDA.

Δ^9 -THC has a similar C_{α} RMSD to the CB₁ apo protein, indicating minimal disruption to the overall receptor conformation. In contrast, Δ^9 -THCOA increases the C_{α} RMSD, which could suggest a greater potential for inducing conformational changes within the CB₁ receptor. When it comes to C_{α} RMSF, Δ^9 -THC slightly increases it, suggesting it introduces more flexibility within the receptor. Δ^9 -THCOA decreases the C_{α} RMSF compared to the apo protein, which might indicate a stabilising effect on certain dynamic regions of the receptor. Both Δ^9 -THC and Δ^9 -THCOA increase the RoG, with Δ^9 -THCOA causing a higher RoG compared to the apo protein and Δ^9 -THC. This indicates that Δ^9 -THCOA may promote a more open or expanded conformation of the CB₁ receptor compared to Δ^9 -THC, which might affect the receptor's signalling mechanisms. Δ^9 -THC has a lower lig-RMSD compared to Δ^9 -THCOA, suggesting that Δ^9 -THC may have a more consistent binding conformation and potentially a more predictable interaction with the CB₁ receptor than Δ^9 -THCOA, which might bind in a more variable and less precise manner.

The comparative analysis of the effects of CBD, CBDDA, Δ^9 -THC, and Δ^9 -THCOA on the CB₁ receptor shows the intricate balance between receptor conformation, stability, and signalling potential. CBD and CBDDA both appear to stabilise the receptor, yet they do so with varying degrees of impact on receptor dynamics. CBD's potential for a more dynamic interaction may translate into a broader range of therapeutic outcomes or varying effects based on the receptor's state or associated signalling complexes. The differences in effects between Δ^9 -THC and Δ^9 -THCOA are particularly noteworthy. The tendency of Δ^9 -THCOA to increase both the C_{α} RMSD and RoG significantly more than Δ^9 -THC suggests that it may alter the conformation and signalling of the receptor in ways that are distinct from the effects of Δ^9 -THC. This could

have implications for the development of novel therapeutics aimed at leveraging the psychoactive effects of Δ^9 -THC while minimising side effects or targeting different signalling pathways for therapeutic benefit. Therefore, understanding these molecular dynamics is vital for the rational design of phytocannabinoid-based therapies. Insights into how these compounds interact with the CB₁ receptor can inform the development of drugs that target specific pathways, providing the efficacy needed for treatment while reducing the potential for adverse effects.

In terms of structural deviation of CB₂ (Fig. 5.14B) indicated by C _{α} RMSD, CBD is similar to the baseline set by the unbound receptor, suggesting a conservative interaction that does not dramatically alter the receptor structure. CBDDA, however, takes this a step further by decreasing C _{α} RMSD, suggesting an even more stable receptor-ligand complex. This decreased C _{α} RMSD with CBDDA could imply a more predictable therapeutic response due to less alteration of the native receptor conformation. As for C _{α} RMSF, the increase caused by CBD to CB₂ binding might facilitate some mobility within the receptor, potentially influencing how the receptor engages with intracellular signalling partners. In contrast, CBDDAs decrease in C _{α} RMSF suggests a restriction of such mobility, which could result in more selective signalling outcomes. The slight increase in RoG for both CBD and CBDDA, relative to the apo receptor, suggests a subtle expansion of the structure of the receptor upon ligand binding. However, since the effect of CBDDA binding on RoG is less than that of CBD implies a tighter, potentially more ordered binding conformation. This could influence the interaction of CBRs with G-proteins or other signalling molecules and may bear implications for the selectivity of the downstream signalling. CB₁ receptors, for instance, interact with G_i proteins and play a critical role in suppressing adenylyl cyclase activity, leading to a decreased concentration of cyclic AMP (cAMP) in the cell. This interaction further modulates ion channels by promoting the opening of potassium channels and inhibiting calcium channels, which ultimately affects neurotransmitter release. Conversely, CB₂ receptors predominantly engage with G_o proteins. Although this interaction similarly curtails adenylyl cyclase activity and reduces cAMP levels, the ensuing effects distinctly target the immune system, resulting in reduced migration of immune cells and pronounced anti-inflammatory responses. Lig-RMSD provides insight into the binding fit. CBDDA has a lower lig-RMSD suggesting a more stable, possibly more efficacious binding. This could mean that CBDDA might engage in fewer off-target interactions compared to CBD, potentially reducing the likelihood of side effects.

Δ^9 -THC and Δ^9 -THCOA both elevate C_α RMSD, but Δ^9 -THCOA does so to a greater extent. This heightened alteration in the receptor's structure could affect not just the binding affinity but also the efficacy and potency of Δ^9 -THCOA. A higher C_α RMSD may also be associated with an increased ability to induce conformational changes in the receptor, which could trigger distinct signalling pathways compared to Δ^9 -THC. When observing C_α RMSF, Δ^9 -THCOA the increase is more pronounced than Δ^9 -THC, suggesting that Δ^9 -THCOA may affect a broader array of conformations within the receptor, potentially leading to a wider spectrum of biological effects. The contrasting effects on RoG stand out as Δ^9 -THCOA has a significant increase suggesting a substantial reorganisation of the receptor's tertiary structure, which could lead to altered receptor activity or even the engagement of different signalling cascades. The minimal decrease in RoG by Δ^9 -THC suggests a more conservative modulation of the receptor's shape. The comparison of lig-RMSD reveals that Δ^9 -THCOA binds in a manner that is less precise than Δ^9 -THC. The higher lig-RMSD for Δ^9 -THCOA might reflect a conformationally diverse interaction that could underlie a broader range of effects - both therapeutic and adverse.

The detailed examination of these phytocannabinoids effects on the CB₂ receptor highlights the complexity of receptor-ligand interactions. The role the CB₂ receptor plays in immune modulation makes these findings particularly pertinent for conditions such as inflammation, autoimmunity, and pain. The differences in C_α RMSD and C_α RMSF between CBD and CBDDA suggest that CBDDA could offer a more stable and perhaps safer profile for therapeutic applications that target the CB₂ receptor. Meanwhile, the substantial differences in the effects caused by binding of Δ^9 -THC and Δ^9 -THCOA on C_α RMSD, C_α RMSF, and RoG hint at the possibility of Δ^9 -THCOA having a distinct pharmacological profile, with potential for both increased efficacy and risk of side effects. Understanding these subtleties provides invaluable insights for drug design, suggesting that minute structural variations in cannabinoids can result in significant differences in how they interact with the CB₂ receptor, and by extension, their therapeutic potential and safety profiles.

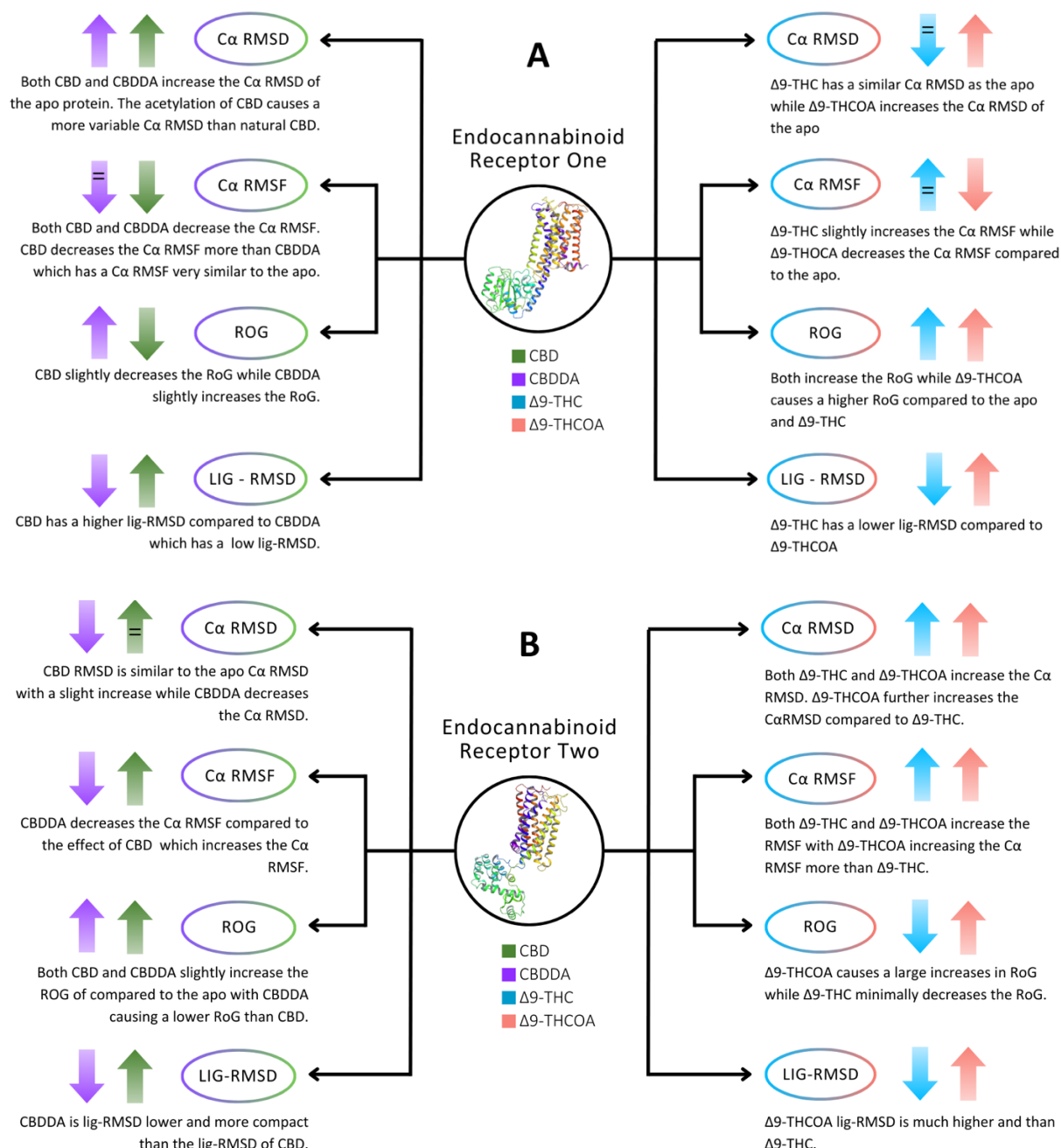


Figure 5.14: A comparison of the structural and dynamical impacts of phytocannabinoids. Analysing effect of CBD, CBDDA, Δ^9 -THC, and Δ^9 -THCOA on A. CB₁ and B. CB₂. Metrics such as C α RMSD, C α RMSF, RoG and lig-RMSD are used to assess changes upon ligand binding compared to the apo state of the receptors. Arrows with equal signs in the middle show that the value was similar to the apo with a slight increase or decrease.

4 DISCUSSION

The conformational dynamics of CB₁ and CB₂ receptors, which are both part of the GPCR family, are fundamental to their function. These receptors are key components in the ECS and are responsible for mediating the effects of cannabinoids, which include both endogenous cannabinoids (produced within the body) and phytocannabinoids (found in plants such as

Cannabis). When phytocannabinoids like CBD or Δ^9 -THC bind to CB₁ or CB₂ receptors, they cause a change in the receptor's conformation. This change is what allows the receptors to interact with intracellular proteins, such as G proteins, and initiate a cascade of signalling events within the cell.

This study is focused on acetylated phytocannabinoids, CBDDA and Δ^9 -THCOA, which are modified forms of CBD and Δ^9 -THC. The acetylation of these molecules could potentially alter their shape, size, charge, and hydrophobicity, leading to different conformational dynamics when they interact with CB₁ and CB₂ receptors compared to their non-acetylated counterparts. Therefore, if acetylated phytocannabinoids induce different conformational changes in CB₁ or CB₂ receptors, this could affect how these receptors couple with G proteins and other signalling molecules. This could result in changes in the strength, duration, or even the type of signal being transmitted inside the cell. Such differences could be significant in terms of therapeutic effects, side effects, and the overall physiological response to these compounds. Therefore, in the context of this study, exploring the interaction between CBRs and acetylated phytocannabinoids versus their natural counterparts involves understanding how these different ligands can alter the conformational dynamics of the receptors and what implications these alterations may have for their signalling capabilities and potential clinical applications.

The aim of this research was to explore the interaction between CBRs and acetylated phytocannabinoids, specifically CBDDA and Δ^9 -THCOA compared to their natural phytocannabinoids derivatives (CBD and Δ^9 -THC, respectively). The focus on these compounds is driven by their emerging presence in the consumer market and the lack of pharmacological data regarding their effects (Bone et al., 2022; Holt et al., 2022; Kruger et al., 2023). After an *in silico* analysis, it is evident that CBDDA and Δ^9 -THCOA exhibit distinct binding dynamics when compared to their natural counterparts, CBD and Δ^9 -THC. These differences, reflected in their altered binding affinities and conformational changes, suggest diverse pharmacological profiles.

In the examination of the CB₁ receptor interaction, the acetylation of phytocannabinoids demonstrates a significant impact on their binding and conformational dynamics. Acetylated phytocannabinoids, CBDDA and Δ^9 -THCOA, present a more stable interaction with the CB₁ receptor as compared to their natural counterparts, CBD, and Δ^9 -THC. The data suggests that acetylation may enhance the binding affinity of the ligands, as seen with the average total and potential energies being notably lower for the acetylated forms. This could be indicative of a

more energetically favourable interaction, which often correlates with increased ligand efficacy (Golan et al., 2011). The structural dynamics, as revealed by the C_{α} RMSD over the 500 ns simulations, show that acetylation leads to an increased stabilisation period for both CBDDA and Δ^9 -THCOA. While Δ^9 -THC exhibits stabilisation after 50 ns, Δ^9 -THCOA takes a longer period, stabilising only after ~ 200 ns and presenting greater dynamics throughout the simulation. This suggests that acetylation may affect the initial binding process and subsequent conformational adjustments within the receptor-ligand complex. Moreover, the acetylated phytocannabinoids tend to induce a more compact receptor conformation, as evidenced by the decreased RoG values. Specifically, CBDDA and Δ^9 -THCOA show a reduced RoG compared to CBD and Δ^9 -THC, suggesting that the acetylated forms may be promoting a tighter, possibly more efficient, receptor-ligand interaction. The stronger binding affinity of CBDDA could indicate more potent interactions with the CB₁ receptor, potentially leading to more pronounced physiological effects. The variations in C_{α} RMSF and RoG suggest that CBDDA might induce different receptor activation states compared to CBD. This could result in a more specific activation pattern of the receptor, which might translate to a more targeted therapeutic effect. The reduced conformational space could also mean a decreased potential for off-target effects, which is a critical consideration in drug design (Xie et al., 2011). In addition, it is essential to recognise that the acetylation of phytocannabinoids does not simply enhance the potency by increasing the receptor binding affinity. Instead, it alters the ligand-receptor interaction dynamics, potentially leading to different therapeutic outcomes and safety profiles (Hauser et al., 2017).

The CB₂ receptor interaction with natural and acetylated phytocannabinoids revealed that CBD binding to the CB₂ receptor allowed the receptor to maintain a dynamic conformation, indicated by the varied C_{α} RMSF values across key residues. In contrast, the acetylated form, CBDDA, showed a more stable interaction, evidenced by a tighter distribution in the PCA and lower C_{α} RMSF values. This suggests that acetylation of CBD leads to a more stable, possibly more rigid receptor-ligand complex, which might have implications for how the CB₂ receptor transduces signals. For Δ^9 -THC and its acetylated form, Δ^9 -THCOA, the simulation data indicated that acetylation might introduce some instability in the receptor-ligand complex, although the receptor maintained its structural integrity with both ligands. Δ^9 -THCOA, was associated with increased C_{α} RMSF across several residues, suggesting that acetylation enhances the mobility and dynamic behaviour of the receptor-ligand complex. This could affect how the CB₂ receptor activates and the overall physiological responses it mediates. The PCA

plots further demonstrated that both natural and acetylated forms of Δ^9 -THC influence the receptors primary conformational state, with subtle differences in secondary states suggested by the variance in the scree plots.

Upon examining the interactions between CB₂ and the phytocannabinoids, the simulations suggest that acetylated cannabinoids like CBDDA and Δ^9 -THCOA present a more stable interaction with the CB₂ receptor, as indicated by lower C _{α} RMSD values, which reflect the dynamic behaviour of the protein-ligand complex. This stability is also evidenced by the lower average total and potential energy values for the acetylated forms compared to their non-acetylated counterparts, suggesting a stronger and more favourable interaction with the receptor. The RoG analysis further supports these findings, showing that CBDDA and Δ^9 -THCOA induce a more compact receptor conformation. This is visually represented in the PCA plots, where acetylated forms exhibit less dispersed data points, indicating a more constrained conformational space. Such a tight conformation could have significant implications for the receptor signal transduction pathways, possibly leading to more pronounced physiological effects. Moreover, the scree plots from the PCA provide quantitative backing to these observations. They show that a large variance in the conformational space of CB₂ is attributed to the binding of the acetylated cannabinoids. This implies that acetylation may lead to a more specific and potentially more potent interaction with the receptor, altering activation states and influencing therapeutic outcomes.

The enhanced potency of acetylated phytocannabinoids, such as CBDDA and Δ^9 -THCOA, is consistent with current anecdotal reports on the effects of Δ^9 -THCOA stating that it seems to be ~2-3 times stronger than Δ^9 -THC. Studies have suggested that the acetylation of phytocannabinoids may facilitate an increased ability to penetrate the BBB, thereby leading to more significant effects (Brown, 1998; Pardridge, 2012). Nonetheless, there is growing concern regarding the potential adverse effects associated with these modified phytocannabinoids. For example, consumers have documented instances of increased anxiety, cognitive impairment, and other psychotropic effects, which may be attributed to the acetylation process (Benowitz et al., 2023; Brown et al., 2021; Holt et al., 2022; Kruger et al., 2023; Sugawara et al., 2023). This chemical modification could potentially enhance the psychoactive properties of phytocannabinoids, resulting in outcomes that parallel those associated with Δ^9 -THC (Kruger et al., 2023; Kruger & Kruger, 2023). Therefore, understanding the role of acetylation in

altering phytocannabinoid activity is crucial, as it may significantly impact both the therapeutic benefits and the risk profile of these compounds (Brown, 1998).

In practical terms, these results suggest that the acetylated phytocannabinoids CBDDA and Δ^9 -THCOA might induce more potent effects than their natural counterparts due to their increased affinity for the receptors. Higher affinity can lead to greater receptor activation, which in turn can amplify the physiological responses (Eyer, 2002). For CBD, which is non-psychoactive and more known for its therapeutic potential, an increased affinity in CBDDA could potentially enhance its beneficial effects such as anti-anxiety or anti-inflammatory properties (Laprairie et al., 2015). For Δ^9 -THC, while its psychoactive properties can be beneficial for some conditions, a stronger binding affinity as seen with Δ^9 -THCOA could lead to more intense and potentially adverse psychoactive effects, such as anxiety, paranoia, or cognitive impairment (Howlett, 2002). Moreover, a higher affinity might result in a longer duration of action, which could be problematic if negative effects occur, as they would persist for extended periods (Golan et al., 2011; Hauser et al., 2017). This could also complicate the management of dosing schedules, potentially leading to a higher likelihood of tolerance development. When the body adapts to the presence of a drug, higher doses may be required to achieve the same effect, which can increase the risk of dependence and withdrawal symptoms (Rang et al., 2011). In addition, a strong ligand-receptor binding can influence receptor desensitisation and downregulation. Continuous activation of receptors by high-affinity ligands can make the receptors less responsive over time or cause them to be internalised by the cell, reducing their number on the cell surface, and leading to diminished efficacy of the ligand (Katzung, 2012). For medical applications, it is crucial to balance the desired therapeutic effects with the potential for adverse reactions. Therefore, while increased affinity can be advantageous in terms of efficacy, it must be carefully controlled to prevent undesirable outcomes, emphasising the importance of precise dosing and monitoring.

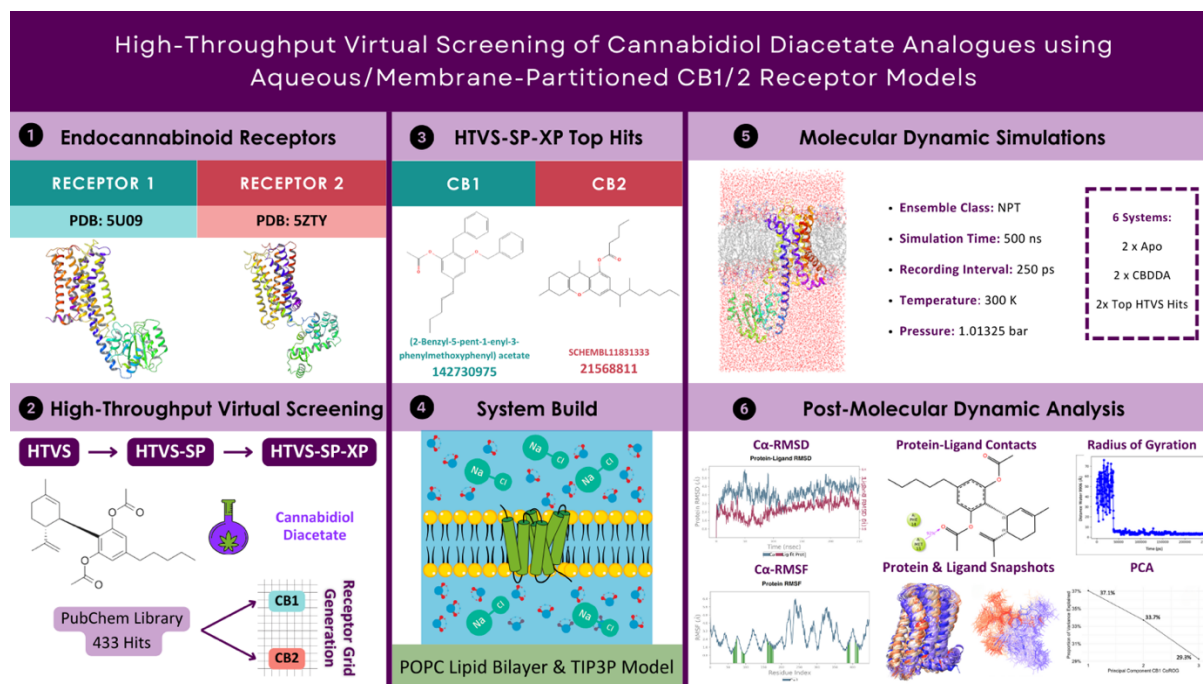
5 CONCLUSION

This study demonstrates that the modification of natural phytocannabinoids through acetylation could lead to increased potency and efficacy, potentially offering therapeutic advantages as a biopharmaceutical agent. However, the increased strength also raises safety concerns. This calls for comprehensive research to evaluate the pharmacokinetics, safety profiles, and therapeutic windows of these modified compounds. Current literature lacks extensive data on the effects and safety profiles of acetylated phytocannabinoids, making these findings both timely and

crucial for guiding future research. As such, rigorous *in vitro* and *in vivo* studies are needed to assess the pharmacodynamics, potential side effects, and interactions with biological systems. These studies should seek to establish appropriate dosages and safety measures for the use of acetylated cannabinoids as therapeutic agents. Given their potency and the ability to cross the BBB effectively, the development and application of these compounds must be approached with caution to maximise their therapeutic potential while mitigating risks.

HIGH-THROUGHPUT VIRTUAL SCREENING OF CANNABIDIOL DIACETATE ANALOGUES USING AQUEOUS/MEMBRANE-PARTITIONED PSEUDO-ENDOCANNABINOID CB_{1/2} RECEPTOR MODELS

GRAPHICAL ABSTRACT



ABSTRACT

This investigates the potential of novel CBDDA analogues as therapeutic agents targeting the CB₁ and CB₂ receptors of the endocannabinoid system (ECS). Utilising high-throughput virtual screening (HTVS) and molecular dynamics (MD) simulations, the study identified ligand 142730975 and 21568811 as top hits for CB₁ and CB₂ receptors, respectively. The HTVS process, employing stages of HTVS-SP and HTVS-SP-XP, progressively refined ligand selection based on predicted affinity, yielding the most favourable ΔG_{Bind} values of -78.35 kcal/mol and -75.67 kcal/mol for these ligands. These values suggest robust and specific binding modes, essential for therapeutic efficacy and potency. The study extends beyond affinity prediction to explore the dynamic interaction landscapes, where ligand 142730975 exhibited a stabilising effect on the CB₁ receptor with compact C $_{\alpha}$ RMSD and RoG values, and ligand 21568811 showing a stabilising effect as well on the CB₂ receptor. These findings highlight the potential of these analogues for the development of potent and selective

cannabinoid-based medications. The structural integrity and stability observed for the ligand-receptor complexes through intricate MD simulations and interaction mapping suggest these candidates may lead to safer, targeted therapies for ECS-related conditions. This research not only enriches the field of cannabinoid science but also sets a foundation for medicine advancements in drug discovery.

1 INTRODUCTION

Cannabidiol has gained significant attention in the medical community due to its wide array of pharmacological effects. It offers anti-inflammatory, anticonvulsant, anxiolytic, and analgesic properties without the psychoactive impact associated with Δ^9 -tetrahydrocannabinol (Δ^9 -THC), making it an attractive therapeutic option (Sholler et al., 2020). Beyond the ECS, CBD interacts with various receptors and cellular pathways, enhancing its potential in treating a multitude of conditions (Lu & Mackie, 2016). The development of CBD derivatives marks a leap in cannabinoid research, aiming to strengthen its therapeutic effectiveness. By modifying the structure of CBD, such as through acetylation to create compounds such as CBDDA, it enhances cell membrane permeability and interaction with cellular targets (He et al., 2018; Holt et al., 2022). These modifications could lead to improved efficacy and longer-lasting effects, improving the pharmacokinetics of CBD and CBDDA for more precise dosing and a broader therapeutic range. CBDDA represents an innovative step in enhancing the lipophilic nature of CBD, by improving its bioavailability and ECS receptor interactions. Such modifications could refine the therapeutic profiles of cannabinoids, allowing for targeted ECS pathway treatments for specific diseases (Lu & Mackie, 2016).

Furthermore, the ECS is a critical cell-signalling network that maintains homeostasis, primarily through CB₁ and CB₂ receptors. CB₁ receptors, mainly in the CNS, regulate pain, mood, and cognition (Graham et al., 2009). CB₂ receptors are more prevalent on immune cells, influencing inflammation and immune responses (Bie et al., 2018; Dhopeswarkar & Mackie, 2014). The interaction of cannabinoids with these receptors presents opportunities for treating a variety of medical conditions. The exploration and synthesis of CBD derivatives are driven by the desire to maximise the therapeutic properties of CBD. By enhancing the drug-like characteristics of the molecule, researchers aim to provide more effective and sustained relief from various ailments, expanding the potential for cannabinoid-based treatments. Anxiety disorders, often stemming from complex CNS dysfunctions, may be mitigated by the anxiolytic actions of cannabinoids via the ECS. The immunomodulatory capabilities of cannabinoids also present

new pathways for treating immune-mediated ailments, such as rheumatoid arthritis and inflammatory bowel disease, potentially offering anti-inflammatory benefits without the drawbacks common to standard anti-inflammatory drugs (Graham et al., 2009; Mackie, 2008).

The potential of cannabinoids in therapy lies in the precision and effectiveness of their interactions with ECS receptors. This precision encompasses receptor specificity, tissue distribution, and the resulting physiological outcomes. Thus, the current focus in cannabinoid research is on identifying and crafting compounds with optimal binding properties to CBRs. These compounds should not only bind effectively to receptors but also demonstrate favourable pharmacokinetics and dynamics, maintaining stability and effectiveness in the body. HTVS is a cutting-edge method in drug discovery, pivotal in the hunt for synthetic cannabinoid derivatives. It enables rapid, efficient *in silico* evaluations of large compound libraries against biological targets (Zhang, 2022). Particularly useful in analysing CBD diacetate analogues, HTVS and MD simulations are fast and cost-effective, which facilitates prompt predictions of compound interactions with CBRs (Willems et al., 2020). Moreover, they offer an in-depth examination of the potential therapeutic interplay within the ECS by utilising advanced computational models that simulate complex receptor environments, both in aqueous solutions and within membrane contexts, to identify the most promising therapeutic compounds.

This study uses HTVS to examine a specially curated library of compounds analogous to CBDDA. Utilising advanced computational modelling, to identify compounds that demonstrate a high affinity for CBRs, as well as favourable pharmacokinetic and pharmacodynamic properties for medicinal use. This investigative approach sheds light on the binding efficiency, stability, and dynamic behaviour of these analogues, offering a detailed perspective on their potential effectiveness and modes of action within the binding site of the receptor. The research seeks to provide an in-depth understanding of these compounds, potentially guiding the development of more efficacious cannabinoid therapies.

2 MATERIALS AND METHODS

Appendix C lists the details of the hardware and software utilised during the testing processes with Maestro software.

2.1 Ligand Library Preparation

A library of compounds for HTVS studies was prepared, starting with the identification of the parent compound. CBDDA was identified within the PubChem database (CID: 15139512) (Information, 2024). The “Cannabidiol Diacetate” identifier served as the input for the search bar, initiating the search process. Subsequently, a similarity search was conducted, employing the structural details of cannabidiol diacetate as the reference. The LigPrep algorithm within the database generated a list of compounds with structural characteristics similar to the parent compound. This search strategy utilised the molecular structure of CBDDA to identify analogues that might exhibit comparable biological activities. From the generated list, 433 compounds were selected based on their structural similarity to CBDDA. The selection of these compounds was critical, as it focused on those most likely to demonstrate similar pharmacological properties, thus enhancing the efficiency of the subsequent screening process. Finally, the 2D structures of the selected compounds were downloaded in the Structure-Data File (SDF) format. The SDF is a commonly used file format that records 2D chemical structures along with associated metadata, such as compound names and molecular weights. This format is notably compatible with a variety of cheminformatics software, which aids in the further analysis and screening of the compounds.

Each compound (Fig. 6.1) was subjected to a preparation process utilising the LigPrep utility in the Maestro software suite. This process employed the OPLS2005 force field for molecular mechanics calculations. The role of LigPrep was to generate various tautomeric and stereoisomeric forms, as well as low-energy spatial arrangements of the molecules, simultaneously optimising their ionisation states within a pH range of 7.0 ± 2.0 , leveraging the capabilities of Epik (Bochevarov et al., 2016; Johnston et al., 2023; Jorgensen et al., 1996; Shelley et al., 2007). The objective of this procedure was to refine the molecules into configurations that are suitable for accurate and reliable docking studies.

2.2 Crystal Structure Protein Preparation

Initial structural information for both p-CBRs was obtained from the UniProtKB database, with entries P21554 and P34972, respectively (UniProtKB, 2023). These structures were complemented by additional data from the Protein Data Bank (PDB), specifically under the accession codes 5U09 for CB₁ and 5ZTY for CB₂ (Li et al., 2019; Shao et al., 2016). For regions not resolved in the crystallographic data, the SWISS-MODEL was employed to predict the

structure through homology modelling (Waterhouse et al., 2018). Subsequent to the modelling, the protein structures underwent a series of refinements using the Protein Preparation Wizard in Maestro Software (Madhavi Sastry et al., 2013). These refinements included the addition of H atoms, reconstruction of missing side chains via Prime, assignment of bond orders from the CCD database, and optimisation of the side chain conformations (Jacobson et al., 2002; Jacobson et al., 2004). Such refinements are crucial for enhancing the accuracy of the computational models by resolving the uncertainties often present in the experimental data. Finally, the apo forms of the proteins were prepared ensuring that the proteins were in an optimal state for detailed interaction and movement analysis.

2.3 Receptor Grid Generation

The receptor grid generation for the prepared CBRs was performed utilising the Maestro software (Schrodinger, 2015). This process began by defining the ligand-binding sites of the proteins using the centroid of selected residues from the co-crystal ligand. An enclosing box was created to accommodate docking ligands, with dimensions set to 46 Å to ensure ample space for the ligands to be properly positioned within the active site. During the grid generation, the van der Waals radius scaling factor was set to 1.0, which is essential for accurately determining the steric interactions between the ligand and the protein. Additionally, a partial charge cut-off of 0.25 was implemented, allowing for the distinction of significant electrostatic interactions while discarding those below the threshold, which contributes to the efficiency of the virtual screening docking process (Sahayarayan et al., 2021). With these parameters, the grid was created to represent the physicochemical environment of the binding site. This precise configuration is crucial for the subsequent docking simulations, as it influences the accuracy of the ligand-binding predictions and the reliability of the screening outcomes. The generated grids for CB₁ and CB₂ were used as the basis for docking the library of ligands to assess their interaction potential and binding affinities.

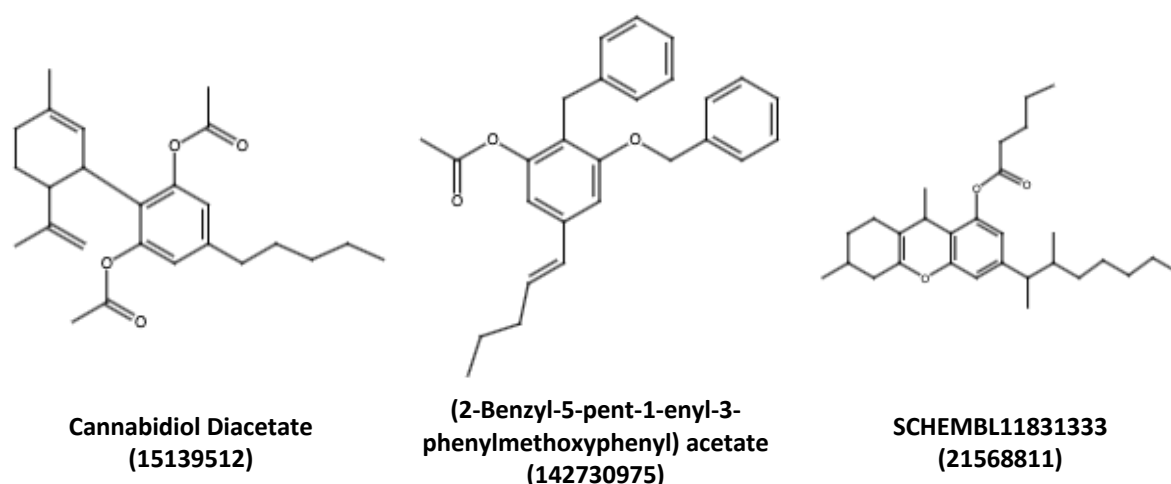


Figure 6.1: Structures of Cannabidiol Diacetate and structurally similar HTVS top hit ligands. On the left, the molecular configuration of Cannabidiol Diacetate (CBDDA, Compound ID: 15139512) is displayed, notable for its two acetate groups. The middle structure is the HTVS top hit for the CB₁ receptor containing a benzyl-pentenyl-phenylmethoxy motif (Compound ID: 142730975). The middle structure represents the top hit ligand for the CB₁ receptor, while the right structure shows the top hit ligand for the CB₂ receptor (Compound ID: 21568811, SCHEMBL11831333), which is distinguished by a single acetate group and a benzyl-alkylated phenyl arrangement. These structures were drawn using MarvinJS (Cherinka et al., 2019).

2.4 Ligand Library Screening

The methodology for conducting HTVS on CB₁ and CB₂ receptor grids via the virtual screening workflow (VSW) in Maestro involved a systematic approach starting with ligand library preparation. This process curated a comprehensive library of 433 ligands, each subjected to a preliminary property assessment using QikProp to evaluate adherence to Lipinski's Rule of Five (Lipinski, 2000; Schrödinger, 2024). This pre-screening effectively filtered out compounds that did not possess drug-like properties. Following this, the ligands were prepared using the Epik tool within the VSW to minimise instances of high-energy ionisation and tautomeric states, ensuring the ligands were in a biologically relevant form for docking (Farid et al., 2006; Friesner et al., 2004; Friesner et al., 2006; Halgren et al., 2004; Sherman, Beard, & Farid, 2006; Sherman, Day, et al., 2006; Yang et al., 2021). Epik also played a crucial role in generating low-energy ring conformations, thereby optimising the ligands for more accurate docking during the virtual screening process.

Subsequently, HTVS was initiated using the virtual screening workflow (VWS). The protocol was set to incorporate Epik state penalties during the docking process, ensuring that the penalties for ligand ionisation and tautomerisation were accurately accounted for in the scoring

function. Initially, ligands were docked using the Glide HTVS mode, which provides a rapid and efficient means of evaluating ligand-receptor interactions (Repasky et al., 2007). After the initial HTVS, the top-performing ligands were subjected to further docking using Glide SP mode. This was followed by an even more accurate and computationally intensive docking using the XP mode from Glide. This iterative docking strategy served to refine the selection of candidate molecules. By increasing the precision with each successive round of docking, the methodology ensured that the top hits were not only potent in their interaction with the receptor but also likely to be true binders as opposed to false positives that might be identified in less stringent screening protocols (Repasky et al., 2007).

Upon completion of the HTVS-SP-XP, a post-process Prime MM- calculation was performed. This post-docking analysis provided an in-depth evaluation of the binding free energy of the ligands, offering further insights into the stability of the ligand-receptor complexes (Genheden & Ryde, 2015). This comprehensive approach to HTVS, combining the speed of Glide HTVS with the accuracy of Prime MM-GBSA calculations and the rigorous refinement of Glide SP and XP docking, was designed to yield a set of the most promising ligands for further computational analysis.

2.5 Aqueous Membrane-Partition Preparation

Upon determining the most promising candidate from the HTVS, the respective protein-ligand complexes were set up for MD simulations. The System Builder feature of the Maestro software was employed to establish a POPC system encased in a TIP3P solvent box, ensuring at least 10 Å of separation from the system boundaries to prevent boundary artefacts (Fig. C1). To simulate a physiological environment, the system was balanced with sodium and chloride ions to reach an ionic strength comparable to 0.15 M, thereby maintaining electrical neutrality (Böckmann et al., 2003). The preparation was designed to reproduce the environment of a natural biological membrane, thus setting the stage for an authentic simulation of the protein-ligand interaction dynamics.

2.6 Molecular Dynamic Simulations

The Desmond module within the Maestro software suite facilitated the execution of MD simulations over a 500 ns time frame to analyse the dynamic behaviour of the receptor-ligand interactions. The simulation protocol was initiated with solvation and ionisation within a comprehensive solvent model, followed by a structured series of eight distinct stages aimed at

equilibrating the system. The initial seven stages involved gradually extending simulation times, culminating in the final 500 ns production run. The process began with setting the system's initial conditions, followed by a 100 ps equilibration at a reduced temperature of 10 K, applying restraints to the heavy atoms of the solute in an NVT ensemble using Brownian dynamics. The third stage extended the NVT conditions for an additional 12 ps. Stages four through seven, with the exception of pocket solvation in the fifth stage, comprised brief NPT ensemble simulations at intervals of 12 ps and 24 ps. These stages were strategically designed to incrementally elevate the system's temperature and systematically apply and then remove atomic restraints. Concluding the preparatory steps, the final stage lifted all restraints on heavy atoms, paving the way for the comprehensive 500 ns simulation under stable NPT conditions at 300 K, thus offering a detailed exploration of the interactions between the ligand and the receptor.

2.7 Post-Molecular Dynamic Analysis

The conformational stability of the protein was assessed by examining the root-mean-square deviation (RMSD) of the protein's alpha carbon (C_α). The flexibility of individual residues and significant structural shifts were determined through evaluations of the protein backbone fluctuations. PCA to analyse the C_α metrics was conducted using RStudio, supplied by Posit Software, PBC (version 2023.09.1+494), and utilised the ggfortify package (RStudio, 2020; Team, 2010). Scree plots generated within R provided a visual representation and interpretation of the variance contributions by the principal components. In addition, the stability of the ligand in its binding pocket was gauged by calculating the lig-RMSD. Furthermore, the MD snapshots of the protein and ligand were analysed, and ligand-contact diagrams were determined for key residues that were consistently present for over 30 % of the simulation time. Collectively, these metrics yield an exhaustive theoretical analysis of the behaviour of the ligand-receptor complex throughout the 500 ns simulation period.

3 RESULTS

3.1 Free Binding Energy

In computational drug discovery, binding energy—specifically ΔG_{Bind} —plays an essential role in understanding the stability of a ligand-receptor complex. ΔG_{Bind} is the energy change associated with the formation of a complex between a ligand and a protein, influencing the energies of the free receptor and the ligand (Hou et al., 2011; Mulakala & Viswanadhan, 2013).

A negative ΔG_{Bind} value indicates a spontaneous and stable interaction, as the system releases energy upon binding. When analysing Table 6.1, the HTVS process output for the CB₁ receptor, a multi-stage screening process is observed by starting with HTVS, followed by screening with HTVS-SP, and ultimately with HTVS-SP-XP. The goal of these stages is to refine the selection of potential ligands based on their predicted affinity for CBRs, which is quantitatively estimated using docking scores and the MM-GBSA score (Genheden & Ryde, 2015).

The top hit for CB₁ across all three screening stages is ligand 142730975, which consistently shows a negative ΔG_{Bind} , with the most favourable being -91.39 kcal/mol during the initial HTVS. This score signifies a strong interaction and high affinity between the compound and the receptor. As the screening precision intensifies, the ΔG_{Bind} values become less negative, registering -78.61 kcal/mol and -78.35 kcal/mol for HTVS-SP and HTVS-SP-XP respectively. The decrement in these values may be attributed to the rigorous nature of the SP and XP protocols, which consider a more detailed representation of the receptor-ligand interactions, including the entropic contributions and potential desolvation effects that are not as thoroughly accounted for in the initial HTVS (Mulakala & Viswanadhan, 2013).

The sustained ranking of compound 142730975 as the top hit throughout the increasingly stringent screening stages suggests a robust and specific binding mode to the CB₁ receptor, reinforcing its potential as a candidate for further experimental investigation. This is especially critical since a stable ΔG_{bind} is correlated with the potential efficacy and potency of a ligand, qualities that are highly sought after in therapeutic drug development. In contrast, other compounds exhibit variability in their positions as the precision level escalates. Such fluctuations are typical in virtual screening as more precise stages can discern subtle energetic differences, bringing to light stabilising interactions or penalising unfavourable contacts not detected in the broader initial screening. In essence, Table 6.1 showcases the effectiveness of multi-tiered virtual screening in narrowing down a list of potential ligands for drug development. It emphasises the importance of ΔG_{Bind} in confirming the viability of ligand-receptor interactions and sets a precedent for utilising such hierarchical screening methods to streamline the discovery and optimisation of novel therapeutic agents.

Table 6.1: High-throughput virtual screening precision process output for CB₁ receptor

TOP HIT	HTVS			HTVS – SP			HTVS – SP – XP		
Position	Docking Score (kcal/mol)	PubChem CID	MM-GBSA Score (kcal/mol)	Docking Score (kcal/mol)	PubChem CID	MM-GBSA Score (kcal/mol)	Docking Score (kcal/mol)	PubChem CID	MM-GBSA Score (kcal/mol)
1 st	-13.11	142730975	-91.39	-11.92	142730975	-78.61	-13.11	142730975	-78.35
2 nd	-12.13	72980313	-78.02	-11.30	142730975	-91.53			
3 rd	-12.13	11486597	-78.02	-10.85	146411227	-66.01			
4 th	-11.99	70638861	-45.03	-10.80	72980313	-76.72			

Table 6.2 portrays the results of a hierarchical virtual screening process aimed at identifying potential ligands for the CB₂ receptor. In this virtual screening, compounds are ranked by their predicted binding affinities to the receptor, calculated using docking scores and MM-GBSA scores. Across the precision levels, compound 21568811 emerges as the top hit in both the HTVS-SP and HTVS-SP-XP phases, with strong negative MM-GBSA scores of -85.59 kcal/mol and -75.67 kcal/mol, respectively. Interestingly, this compound does not appear as the top hit in the initial HTVS phase but progresses to the top position with the refinement of the screening process. The shift in the ranking of 21568811 highlights the importance of employing multiple precision levels in virtual screening to capture the true binding affinity of ligands, which may not be evident in a less precise, high-throughput approach. The docking scores show a similar trend of increased affinity with more precise screening, moving from -11.08 kcal/mol in the initial HTVS to -11.57 kcal/mol in HTVS-SP-XP. The consistency of 21568811 as the top hit in the latter stages of screening suggests that it has a robust interaction with the CB₂ receptor, warranting further experimental validation.

Moreover, the MM-GBSA score, which provides a view of the binding energy by considering solvation effects, demonstrates that 21568811 maintains strong interaction energies with the CB₂ receptor across different levels of screening precision. This indicates that the binding mode of the ligand is not only stable but also energetically favourable, which is a desirable trait for potential therapeutic agents. The other compounds listed in the table exhibit fluctuations in their rankings as the screening precision changes. Compounds that rank highly in the initial HTVS may be outperformed by others upon closer scrutiny in the SP and XP stages, which can reveal additional stabilising interactions or penalise unfavourable ones not previously accounted for. The data from Table 6.2 exemplifies the efficacy of a multi-tiered virtual screening strategy in drug discovery, highlighting how increasing the stringency of computational analyses can refine the selection of potential ligands. The highlighted compound, 21568811, demonstrates

significant promise due to its stable and favourable interaction profile with the CB₂ receptor, emphasising the compound as a candidate for subsequent stages of drug development.

Table 6.2: High-throughput virtual screening precision process output for CB₂ Receptor

TOP HIT	HTVS			HTVS – SP			HTVS – SP – XP		
	Docking Score (kcal/mol)	PubChem CID	MM-GBSA Score (kcal/mol)	Docking Score (kcal/mol)	PubChem CID	MM-GBSA Score (kcal/mol)	Docking Score (kcal/mol)	PubChem CID	MM-GBSA Score (kcal/mol)
1 st	–11.57	68127891	–76.20	–9.73	21568811	–85.59	–11.57	21568811	–75.67
2 nd	–11.34	163979073	–72.11	–9.66	20326694	–81.64			
3 rd	–11.22	20326694	–85.65	–9.66	21568782	–76.27			
4 th	–11.08	21568811	–47.95	–9.63	20326694	–84.50			

The interaction data depicted in Fig. 6.2 captures how the top hit ligands interact with CB₁ and CB₂ receptors. These findings, derived from HTVS-SP-XP, are vital for predicting how new drug candidates might engage with their biological targets, an essential step in pharmaceutical development.

Focusing on the CB₁ receptor, ligand 142730975 engages in a variety of binding interactions with a docking score of –13.11 kcal/mol. The docking score is indicative of a strong binding affinity between the ligand and the receptor, as lower scores correlate with a higher binding affinity. A docking score in the range of –13.11 kcal/mol reflects a significant level of interaction that is likely to be energetically favourable and stable. Ligand 142730975 forms a direct H-bond with the Met15 residue. H-bonds are particularly significant as they can lock the molecule in a position that may affect signalling. Additionally, ligand 142730975 exhibits a π - π stacking interaction with the Phe82 residue, a type of interaction that provides stability due to the attractive forces between aromatic ring structures. In this complex, the presence of H-bonds, hydrophobic interactions, and π - π stacking interactions all contribute to the overall docking score, illustrating a tightly bound state that may effectively activate or inhibit receptor function.

In the case of the CB₂ receptor, the molecule identified as 21568811 displays a distinct pattern of binding with a docking score of –11.57 kcal/mol. While this score is also negative, suggesting a stable interaction, it is slightly less negative than the docking score of the CB₁-ligand complex. The π - π stacking interactions influence this docking score. The docking profile of 21568811 is particularly characterised by π - π stacking interactions with the residues Phe86 and Phe182. These interactions are indicative of critical contact points, using the aromatic side chains of these residues for ligand anchorage within the binding site of the receptor. Such π - π stacking is

recognised for adding stability to the ligand-receptor complex due to the attractive forces between the planar rings of both the ligand and the amino acids involved (Cabaleiro-Lago & Rodriguez-Otero, 2018).

The docking scores are a direct reflection of ligand affinity for their respective receptors, capturing the sum of all favourable and unfavourable interactions that occur in the binding site. These scores are paramount in the initial stages of drug development as they provide a quantitative estimate of how well a ligand can bind to its target receptor, which is crucial for selecting candidates for further biological testing. The data suggest that both ligands, 142730975 and 21568811, have high affinity for their respective receptors, with compound 142730975 showing a particularly strong interaction with the CB₁ receptor, as evidenced by its lower docking score.

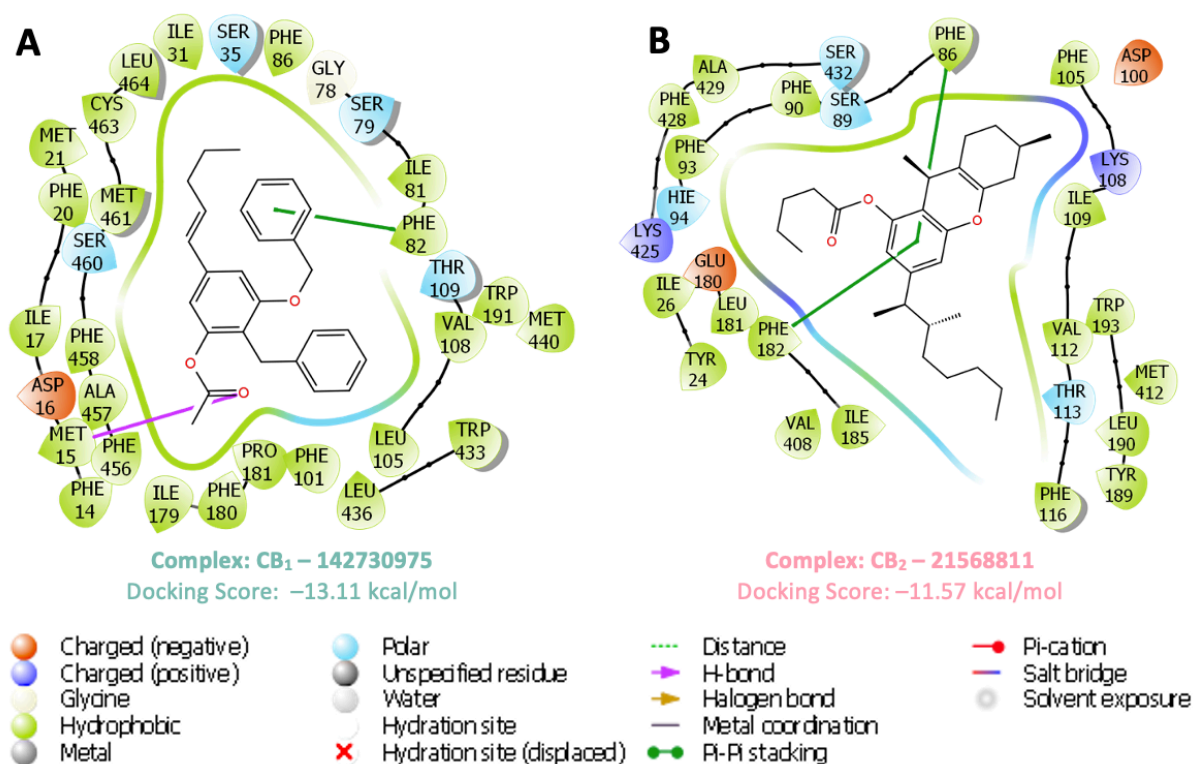


Figure 6.2: Ligand Interactions of CB₁ and CB₂ with their relative HTVS-SP-XP top hits. A. Displays CB₁ receptor interactions with ligand 142730975, featuring a H-bond with Met15 and π - π stacking with Phe82, with a docking score of –13.11 kcal/mol. B. Shows CB₂ receptor interactions with ligand 21568811, characterised by π - π stacking with Phe86 and Phe182, with a docking score of –11.57 kcal/mol, implying stable interactions with nuanced energetic profiles. Generated from Maestro Schrödinger Suite.

3.2 Energy Properties of MD Simulations

The MD simulations energy calculations (Table 6.3) for CB₁ revealed important insights into the interaction dynamics with CBDDA and ligand 142730975. The MD simulations, conducted over a period of 500 ns, show that both ligands lead to an increase in the complexity of the system, as indicated by the rise in degrees of freedom and the number of atoms. This complexity possibly stems from the ligands and additional system components such as water molecules or ions present during the simulations. Specifically, for the CB₁ receptor, ligand 142730975 exhibits a greater stabilising effect compared to CBDDA, as inferred from its more negative average total and potential energy values. This suggests that 142730975 forms a more energetically favourable interaction with the CB₁ receptor. The simulations maintained a constant temperature close to physiological conditions and displayed minimal pressure variations, signifying that the systems were well equilibrated. Moreover, the increase in system volume in the presence of each ligand is indicative of structural adaptations in the receptor, possibly due to an induced fit mechanism upon ligand binding. The stability of the systems is further confirmed by the zero slope in the energy plots, implying that equilibrium was achieved and sustained throughout the simulations. While both CBDDA and 142730975 interact with the CB₁ receptor to increase stability and induce conformational changes, ligand 142730975 appears to form a more stable complex, potentially offering higher affinity binding and more significant receptor modulation.

Table 6.3: Evaluation of Energy Parameters in CB₁ Receptor Simulations

Statistical Parameters	Apo	CBDDA	142730975
Simulation Period (ns)	500.00	500.00	500.00
Degrees of Freedom	141853	153190	157457
Number of Atoms	65690	70899	72979
Average Total Energy (kcal/mol)	-116188.16	-122555.85	-127305.23
Average Potential Energy (kcal/mol)	-158195.08	-167915.95	-173930.69
Temperature (K)	298.04	298.01	298.02
Pressure (bar)	0.59	0.74	1.00
Volume (Å ³)	638208.45	688641.71	709413.74
Slope (ps⁻¹)	0.000	0.000	0.000

The interactions of ligand CBDDA and ligand 21568811 (Table. 6.4) with the CB₂ receptor reveal distinct energy profiles. While both ligands lead to an increase in the number of atoms, reflecting the added complexity of the system due to the presence of the ligands, it is the energy parameters that provide a deeper insight into the stability of the ligand-receptor complexes. CBDDA shows a notably more negative average total and potential energy compared to the apo state, implying a highly stable interaction with the CB₂ receptor. In contrast, the energy values

for ligand 21568811 are much closer to those of the unbound receptor, suggesting a less stable interaction and potentially a lower affinity for the receptor than CBDDA. The degrees of freedom for the complexes with CBDDA and 21568811 do not show as much variation as observed in the CB₁ receptor simulations, which might indicate that the CB₂ receptor experiences less conformational change upon ligand binding. Additionally, the temperature, pressure, and volume parameters across all simulations are consistent and in line with physiological conditions, supporting the equilibrium state of the systems. A key observation is the zero slope in the energy plots for both ligands, indicating that the energy landscapes reached stability during the simulations. This points to the reliability of the simulation data in reflecting the true nature of the receptor-ligand interactions over the extended simulation period.

Table 6.4: Evaluation of energy parameters in CB₂ receptor simulations

Statistical Parameters	Apo	CBDDA	21568811
Simulation Period (ns)	500.00	500.00	500.00
Degrees of Freedom	150195	155040	151329
Number of Atoms	69961	72082	70408
Average Total Energy (kcal/mol)	-128285.07	-135207.76	-127421.81
Average Potential Energy (kcal/mol)	-172768.37	-181119.05	-172239.16
Temperature (K)	298.08	298.03	298.07
Pressure (bar)	1.38	1.29	1.08
Volume (Å ³)	682427.29	705643.58	686253.33
Slope (ps⁻¹)	0.000	0.000	0.000

3.3 Effects on C_α RMSD

The C_α RMSD trajectories (Fig. 6.3.1) reveals the structural dynamics of the CB₁ receptor (Fig. 6.3.1A) interacting with its apo form, CBDDA, and the top hit ligand 142730975 over an extensive 500 ns period. The apo state maintains a stable conformation with C_α RMSD values oscillating between 2 and 4 Å, suggesting little deviation from its initial structure throughout the simulation. The CB₁-CBDDA complex presents a marginally higher C_α RMSD, peaking at around 4.5 Å, indicative of some induced conformational shifts while retaining overall structural integrity. Notably, the ligand 142730975 demonstrates a tightening in C_α RMSD values, with fluctuations similar to or slightly lower than the apo form, pointing to a reduced level of structural deviation. This observation implies that ligand 142730975 may confer a stabilising influence on the receptor, potentially lowering C_α RMSD and suggesting a stronger affinity and tighter control over the receptor conformation compared to CBDDA.

Additionally, the PCA plots visualising the C_α RMSD (Fig. 6.3.2) support this data by offering a comparative view of the conformational behaviour of CB₁ in the presence of different ligands. Fig. 6.3.2A captures the variance in receptor structure when complexed with CBDDA,

revealing a broad dispersion of data points particularly along the PC1 axis. This wide distribution is indicative of a diverse array of conformations that the receptor adopts in response to CBDDA binding. In contrast, PCA for the CB₁ receptor bound to ligand 142730975, shows a noticeably tighter clustering of points. This reduced dispersion suggests that ligand 142730975 restricts the receptor to a smaller conformational space, potentially leading to a more specific ligand-receptor interaction. The tighter clustering might also reflect a more stabilised receptor conformation, suggesting that 142730975 could enforce a degree of rigidity upon binding to the CB₁ receptor. This comparison between the two ligands via PCA highlights the potential for ligand 142730975 to provide more precise modulation of CB₁ due to its ability to limit receptor conformational dynamics. The scree plot (Fig. 6.3.3A) for the CB₁ receptor shows that the first PC alone captures nearly half of the total variance, suggesting that it is a significant contributor to receptor conformational changes. The second and third components also contribute meaningfully, indicating that they capture additional aspects of the conformational space explored by the receptor-ligand interactions which can affect signalling outcomes.

Figure 6.3.1B represents the C_α RMSD trajectories of the CB₂ receptor, providing insights into its conformational stability when unbound and when interacting with the ligands CBDDA and 21568811. The apo state of the CB₂ receptor displays a foundational level of structural fluctuation, indicative of its inherent flexibility. Upon binding to CBDDA, the C_α RMSD values largely span between 4 to 5 Å, pointing to increased variability in receptor conformation. This increased C_α RMSD range suggests that while CBDDA may induce some structural changes, these do not lead to a state of greater conformational stability compared to the unbound receptor. In contrast, ligand 21568811 initially introduces a more erratic behaviour in the receptor, with C_α RMSD peaks peaking at ~8 Å. This could be reflective of an initial adjustment phase where the receptor accommodates the ligand, potentially through significant conformational rearrangements. Notably, as the simulation progresses, the C_α RMSD for the CB₂-21568811 complex stabilises but still deviates more than the CB₂-CBDDA complex, consistently hovering around 3 Å. This suggests that ligand 21568811, after an initial period of accommodation, still forms a stable complex with the receptor, potentially indicative of a robust binding interaction that could have implications for the efficacy of the ligand in modulating receptor activity. In the PCA plots concerning the CB₂ receptor (Fig. 6.3.2B) showcases receptor behaviour when bound to the ligand CBDDA, revealing a wide spread of data points while the receptor bound to ligand 21568811, shows a slightly more concentrated clustering of points, indicating a

narrower conformational variability. This restrained spread could signify that ligand 21568811 keeps CB₂ receptor in a more limited set of conformations, potentially indicative of a more selective and stable ligand-receptor interaction. This comparison between the two plots highlights the distinct influence that different ligands have on the dynamic conformational landscape of the CB₂ receptor. Similarly, the scree plot for the CB₂ receptor reveals that the first PC accounts for more than half of the total variance, which is even more pronounced than in the CB₁ analysis. The second PC is also substantial, while the contribution by the third component drops significantly compared to the first two indicating that ligand 21568811 had a lower contribution to structural changes.

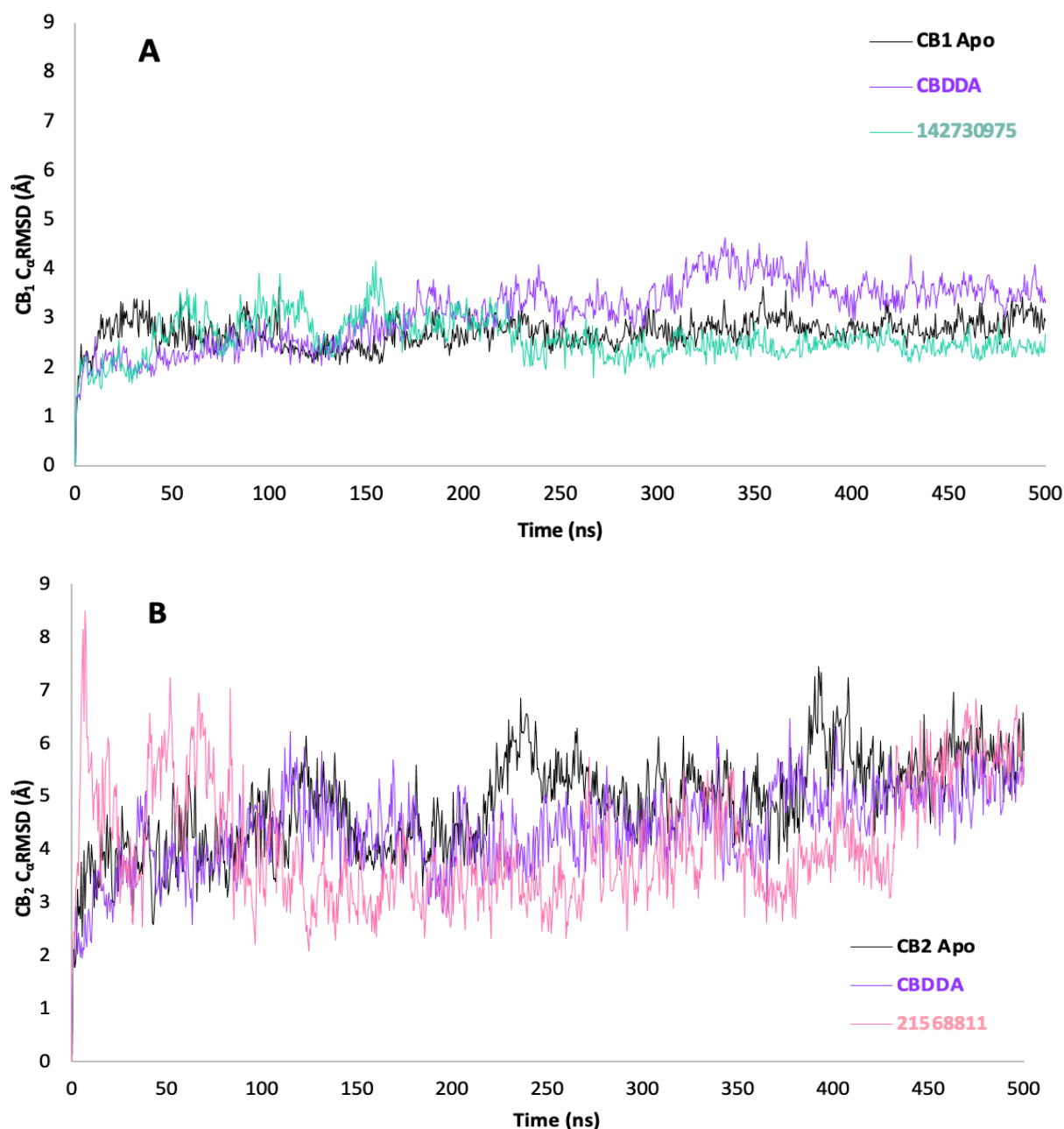


Figure 6.3.1: C_α RMSD for CBRs with CBDDA and its analogue. A. Tracks the C_α RMSD over a 500 ns MD simulation for the CB_1 receptor in its apo form and bound to CBDDA and 142730975. B. displays similar C_α RMSD data for the CB_2 receptor with its apo form, CBDDA, and 21568811. These plots illustrate the stability and conformational changes of each receptor-ligand complex over time.

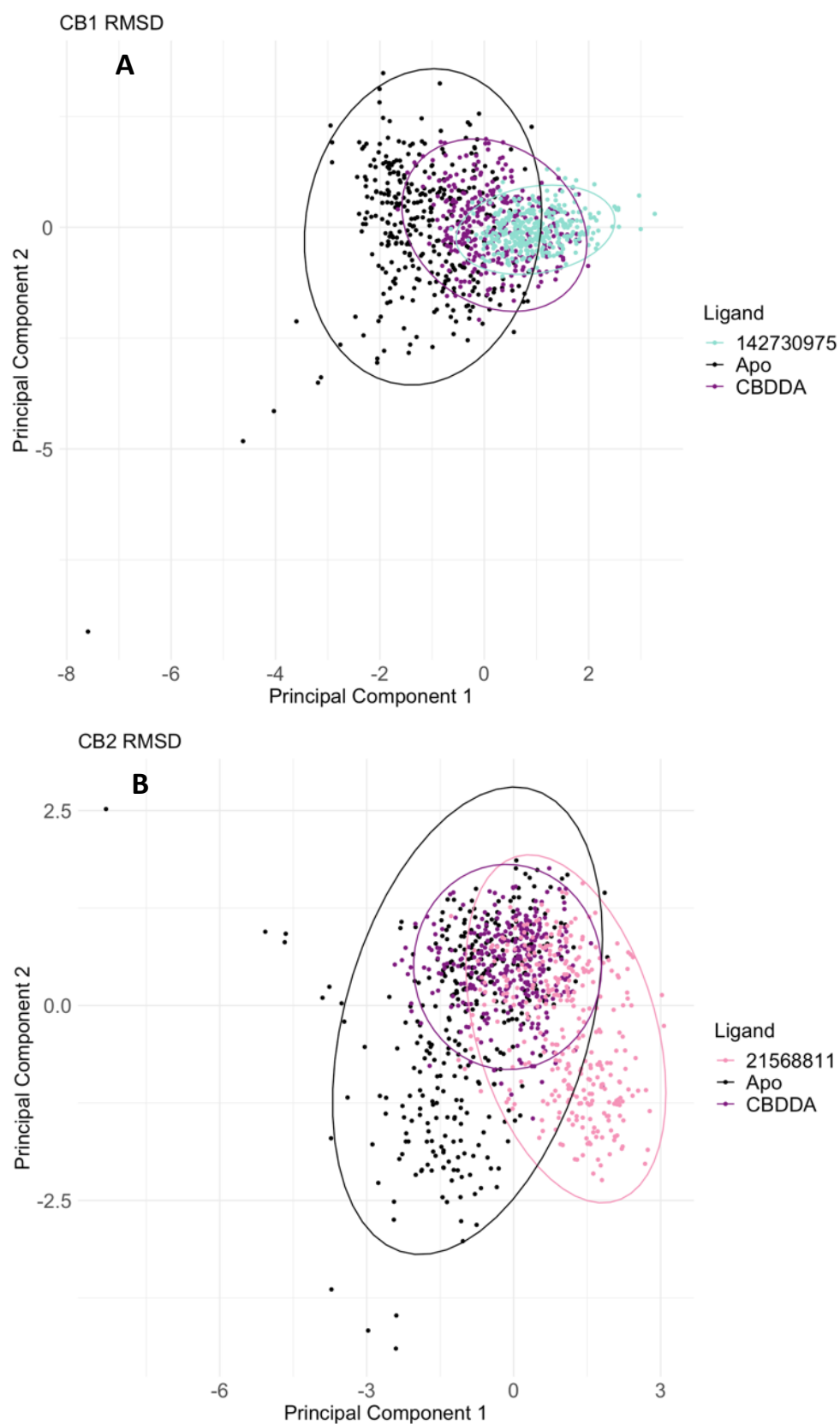


Figure 6.3.2: PCA of C_{α} RMSD for cannabinoid receptors. A. and B. Displays the CB₁ and CB₂ receptor complexes with CBDDA, complexes with the top hits (142730975 for A. CB₁ and 21568811 for B. CB₂). The scatter plots illustrate the conformational space explored during MD simulations.

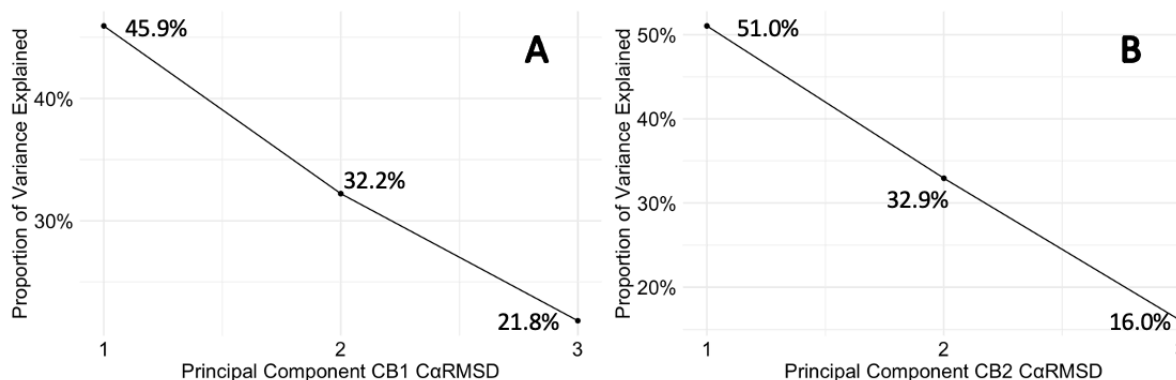


Figure 6.3.3: Scree plots displaying the variance explained by the PCs of C_{α} RMSD during MD simulations of the CB₁ and CB₂ receptors. A. Corresponds to the CB₁ receptor, with PCs accounting for 45.9 %, 32.2 %, and 21.8 % of the variance, respectively. B. Relates to the CB₂ receptor, with PCs explaining 51.0 %, 32.9 %, and 16.0 % of the variance. These plots help in understanding the dominant motions and conformational changes of the receptors upon ligand binding.

3.4 Effects on C_{α} RMSF

The C_{α} RMSF profiles for the CB₁ receptor, illustrated in Figure 6.4.1A, provide insights into receptor dynamics in its apo form as well as its interactions with two distinct ligands, CBDDA and ligand 142730975. The alignment of C_{α} RMSF values between the apo and CBDDA-bound CB₁ receptor suggests that CBDDA does not significantly alter the behaviour of the receptor. Notably, distinct peaks at Ala30, Val91, and within the Met414-Val441 region emerge upon CBDDA binding. Similarly, ligand 142730975 induces specific fluctuations, particularly between Cys169-Leu183 residues. These fluctuations indicate that the binding of CBDDA and 142730975 could lead to regions of increased flexibility or unique conformational changes that diverge from the apo state. Such differential flexibility patterns are suggestive of potential impacts on the functional activity of the receptor.

Further insights were gathered from the PCA plots of the CB₁ receptor, where distinct clustering patterns emerge when the receptor is bound to CBDDA (Fig. 6.4.2A) and ligand 142730975. The broad distribution of CBDDA data points in Fig. 6.4.2A points to a variety of conformations, whereas the tighter clustering with ligand 142730975 implies a more confined conformational range, suggesting a more precise ligand-receptor interaction. Complementing this analysis, the scree plots for the CB₁ receptor, referred to as Fig. 6.5.3A, quantify the variance in fluctuations of the receptor. The dominance of the first PC, capturing 79.53 % of the total variance, signifies that it encapsulates the majority of the dynamic behaviour of the receptor. The subsequent components, accounting for 11.78 % and 8.69 % of the variance,

respectively, denote additional movements within the receptor that, while less dominant, are still an integral part of the receptor-ligand interaction landscape.

Panel B of Fig. 6.4.2 outlines the C_{α} RMSF profile for the CB₂ receptor, comparing its apo form with states bound to CBDDA and the top hit ligand 21568811. The profile reveals similar flexibility between the apo state and its CBDDA-bound state, indicating that CBDDA does not significantly increase or modify dynamic behaviour of the receptor. Conversely, binding with ligand 21568811 displays pronounced fluctuations at residues such as Pro30, Val95-Gly98, Ala124, and Arg332, suggesting these regions may experience notable changes in flexibility, which could signify a stabilising influence or significant conformational alterations by the top hit ligand, potentially affecting receptor signalling and function. The PCA plots for the CB₂ receptor (Fig. 6.4.2B) further illustrate that the CBDDA-bound state shows a broad range of conformational states. However, the ligand 21568811 appears to limit the receptor to a narrower conformational range, suggesting a more selective ligand-receptor interaction. The variance of the first PC (Fig. 6.4.3B) in the scree plots, accounting for 91.8 % of the variance in the CB₂ receptor, suggests that receptor behaviour is primarily governed by a single mode of fluctuation. The lesser contribution of subsequent components reinforces the presence of a predominant fluctuation pattern.

The collective analysis from PCA and scree plots indicates that the receptors have a primary mode of fluctuation that is significantly influenced by ligand binding. The top hit ligands seem to restrict the conformational diversity of the receptors, as shown by tighter clustering in the PCA plots when compared to CBDDA. This restriction could reflect more specific binding and a more pronounced impact on receptor function. The scree plots emphasise the dominance of the first PC in the CB₂ receptor, suggesting that a major, characteristic motion or structural change defines the receptor dynamics upon ligand binding while the other two PCs induce less variation. These analyses offer valuable insights into the dynamics of CBRs in response to ligand binding. The reduced conformational space for the top hit ligands may be beneficial for developing drugs with precise effects and minimal side profiles. The significant presence of characteristic fluctuations, could be crucial for receptor function and the specificity of ligand interactions, aiding in the design of targeted therapeutics.

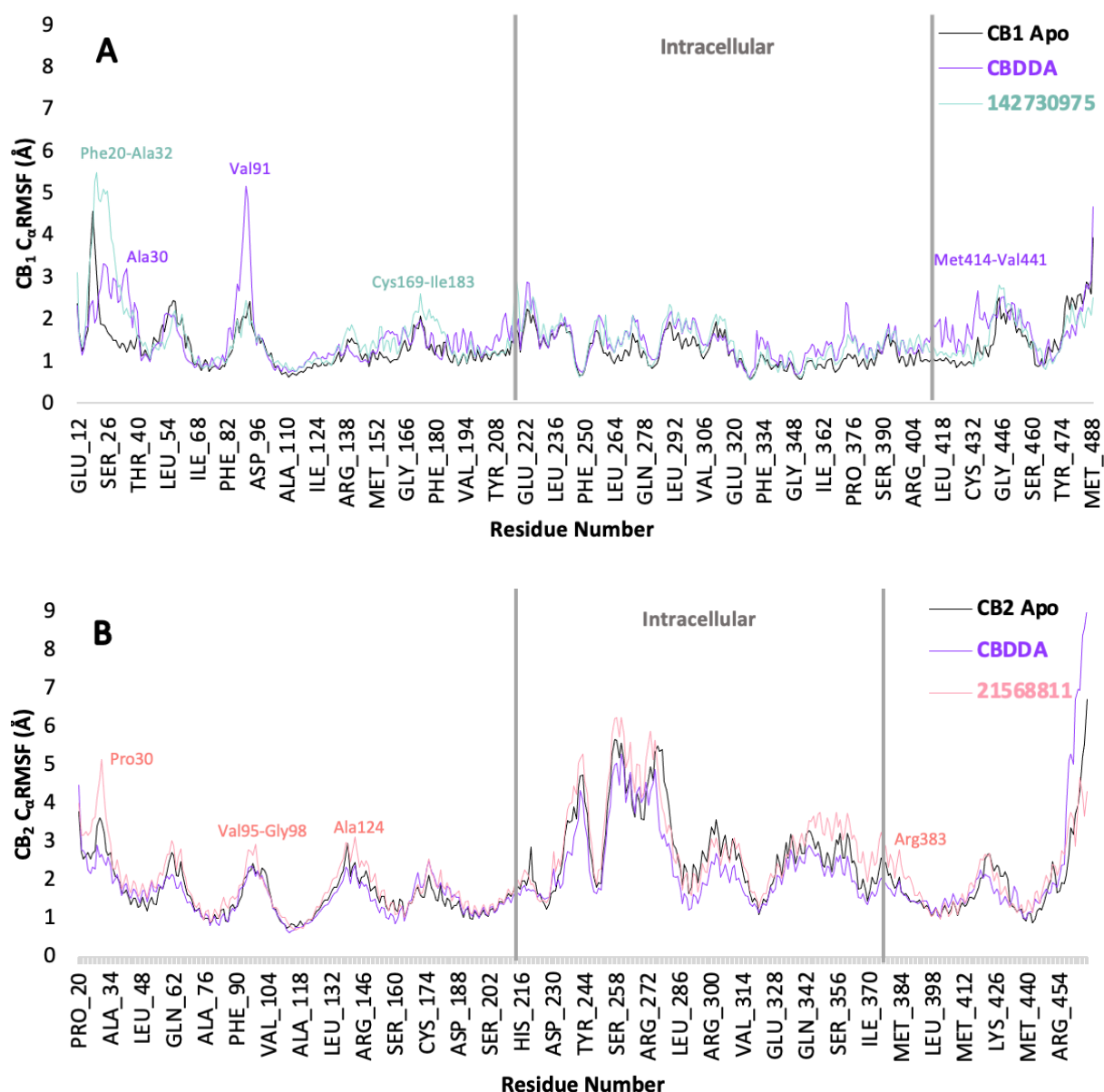


Figure 6.4.1: Residue Flexibility Profiles in CB₁ and CB₂ Receptors. A. Depicts the flexibility of amino acid residues within the CB₁ receptor in its apo state, in complex with CBDDA, and with the CB₁ top hit ligand 142730975. B. Shows the corresponding flexibilities within the CB₂ receptor for its apo state, in complex with CBDDA, and with the top hit ligand 21568811. Peaks indicate areas of higher flexibility, with notable residues highlighted along the receptor sequences, suggesting potential interaction sites or conformational changes upon ligand binding.

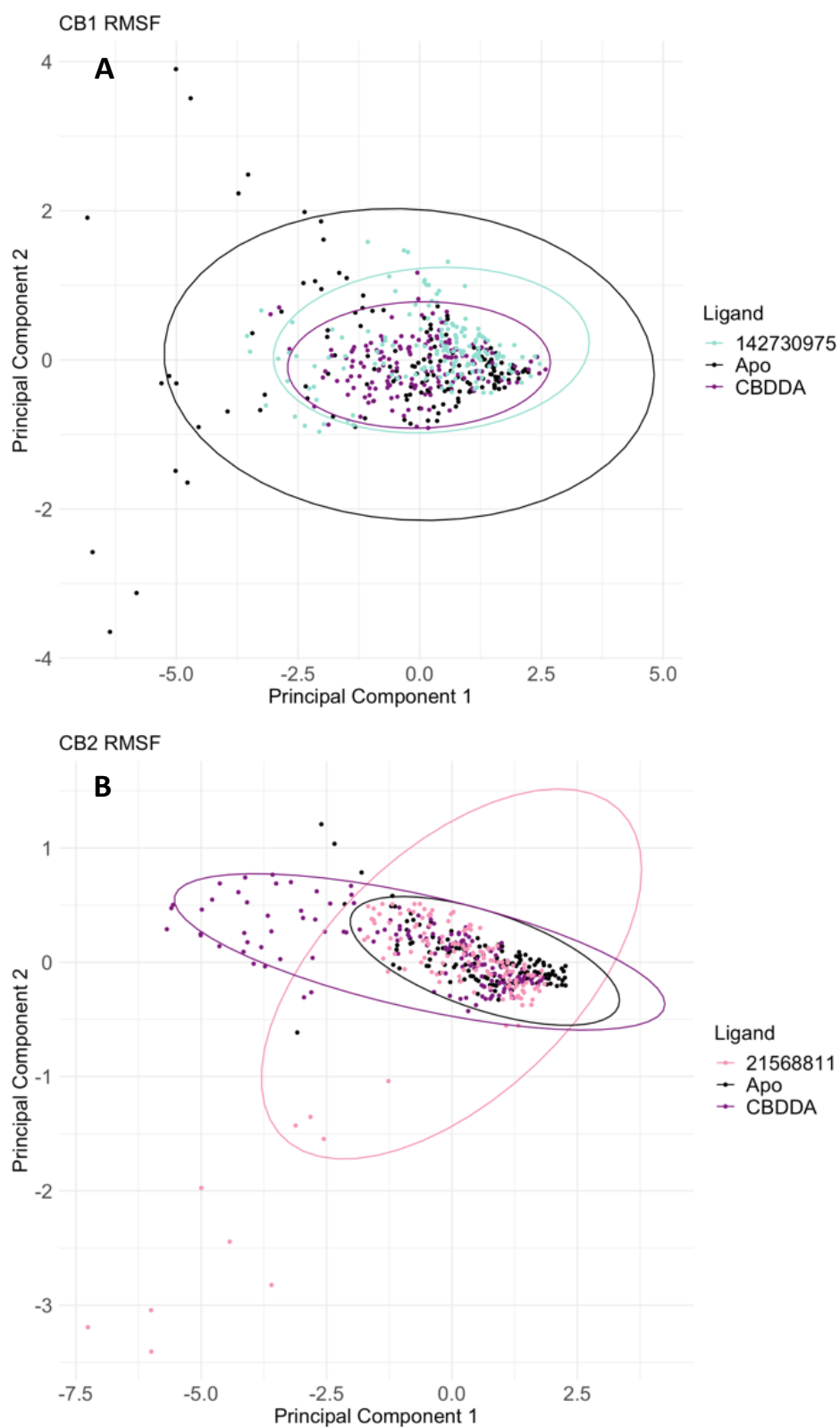


Figure 6.4.2: PCA showing residue flexibility of Ligand-Bound CBRs. This figure presents the PCA plots reflecting the conformational space explored by the CBRs when bound to different ligands. A. Illustrates the PCA distribution for the CB₁ receptor bound to CBDDA and the PCA for the CB₁ receptor bound to the top hit ligand 142730975. B. Represents the PCA for the CB₂ receptor bound to CBDDA, and for the CB₂ receptor bound to the top hit ligand 21568811.

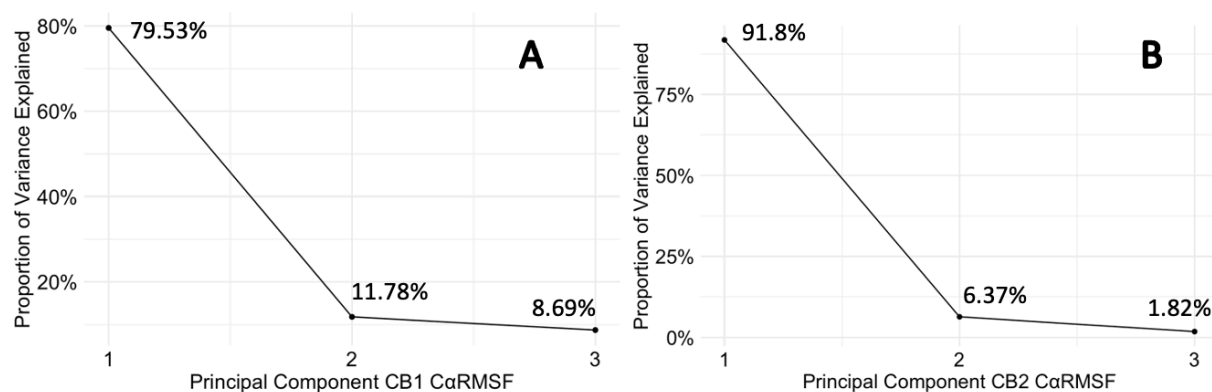


Figure 6.4.3 Presents scree plots for the CB₁ and CB₂ receptors, quantifying the variance in receptor dynamics accounted for by their respective PCs. A. Shows that 79.53 % of CB₁ receptor conformational variance is captured by the first PC, with the second and third components explaining 11.78 % and 8.69 %, respectively. B. The CB₂ receptor demonstrates an even greater dominance of the first PC, which accounts for 91.8 % of the variance, and significantly smaller contributions from the second and third components, at 6.37% and 1.82 %, indicating a highly centralised pattern of receptor fluctuations.

3.5 Protein RoG of Carbon Backbone

The RoG analysis for CB₁, depicted in Fig. 6.5.1A, examines the structural compactness across three different states: the unbound receptor, the receptor bound to CBDDA, and the receptor complexed with the top hit ligand 142730975. For the apo state, variability in its compactness is observed, as indicated by several peaks in the graph, suggesting transient expansions in receptor structure. Binding with CBDDA appears to moderate these fluctuations, suggesting that CBDDA may promote a greater degree of structural consistency in the receptor. The top hit ligand 142730975 presents a profile that is comparable to that of CBDDA, but with a reduced occurrence of large fluctuations, potentially pointing to a more stable or uniformly compact configuration of the receptor-ligand complex. In terms of the CB₁ receptors conformational changes, (Fig. 6.5.2A) for CBDDA and 142730975 complexes, respectively, reveal diverse patterns of distribution along the PC. This variation suggests that each ligand distinctively impacts the structure of the receptor. The CB₁-142730975 complex shows a more compact congregation of data points, implying a more uniform influence on receptor conformation and density. When analysing the variance in CB₁ receptor conformational behaviour, it is distributed relatively evenly across the three PCs, with the first, second, and third components accounting for 37.1 %, 33.7 %, and 29.3 % of the variance, respectively. This distribution signifies that a variety of movement modes or structural rearrangements contribute in comparable measures to receptor overall conformational dynamics.

In the analysis of CB₂ receptor RoG, Fig. 6.5.1B shows distinct behaviours across the unbound receptor, when bound to CBDDA, and when engaged with the top hit ligand 21568811. The unbound receptor exhibits significant fluctuations in compactness, with pronounced spikes indicating intermittent expansions of its structure. These expansions become less frequent and intense upon CBDDA binding, implying the role of CBDDA in stabilising receptor structure. The interaction with ligand 21568811 further reduces these fluctuations, suggesting an even more consistent compactness and possibly a more precise or encompassing binding mode that maintains receptor stability. In the corresponding PCA plots (Fig. 6.5.2B), CBDDA and 21568811 for the CB₂ receptor show varying distributions. The compact clustering seen with the top hit ligand 21568811 suggests a more uniform effect on receptor compactness compared to the wider spread observed with CBDDA. This could indicate that the top hit ligand fosters a more standardised receptor conformation. Mirroring the CB₁ receptor, CB₂ receptor variance is relatively balanced across the three PCs, with 36.4 %, 32.7 %, and 30.9 % contributions, respectively. This distribution implies that receptor conformational changes are not dominated by a single pattern of movement but are rather characterised by multiple significant modes. Together, these analyses suggest that while the top hit ligands may encourage more consistent receptor conformations, as seen by the tighter PCA plot clustering, both receptors display a multitude of conformational changes throughout the simulations, with no single mode of movement predominating.

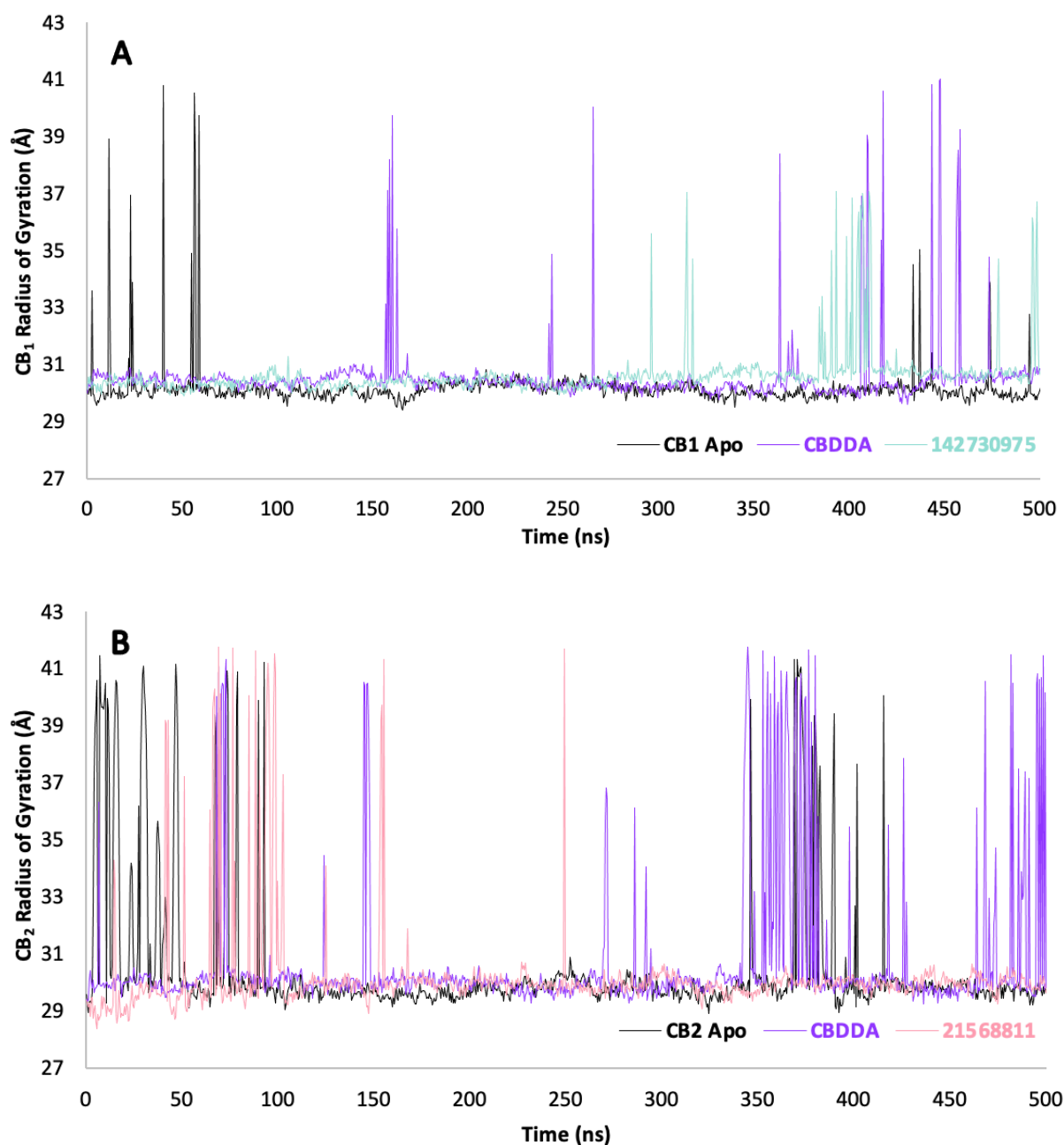


Figure 6.5.1: Temporal Analysis of Receptor Compactness in CB₁ and CB₂. A. Shows the RoG over 500 ns for the CB₁ receptor in its apo form and when bound to CBDDA and 142730975. B. Shows the same for the CB₂ receptor with CBDDA and 21568811. The RoG provides insight into the overall shape and volume occupied by the receptor-ligand complexes over time.

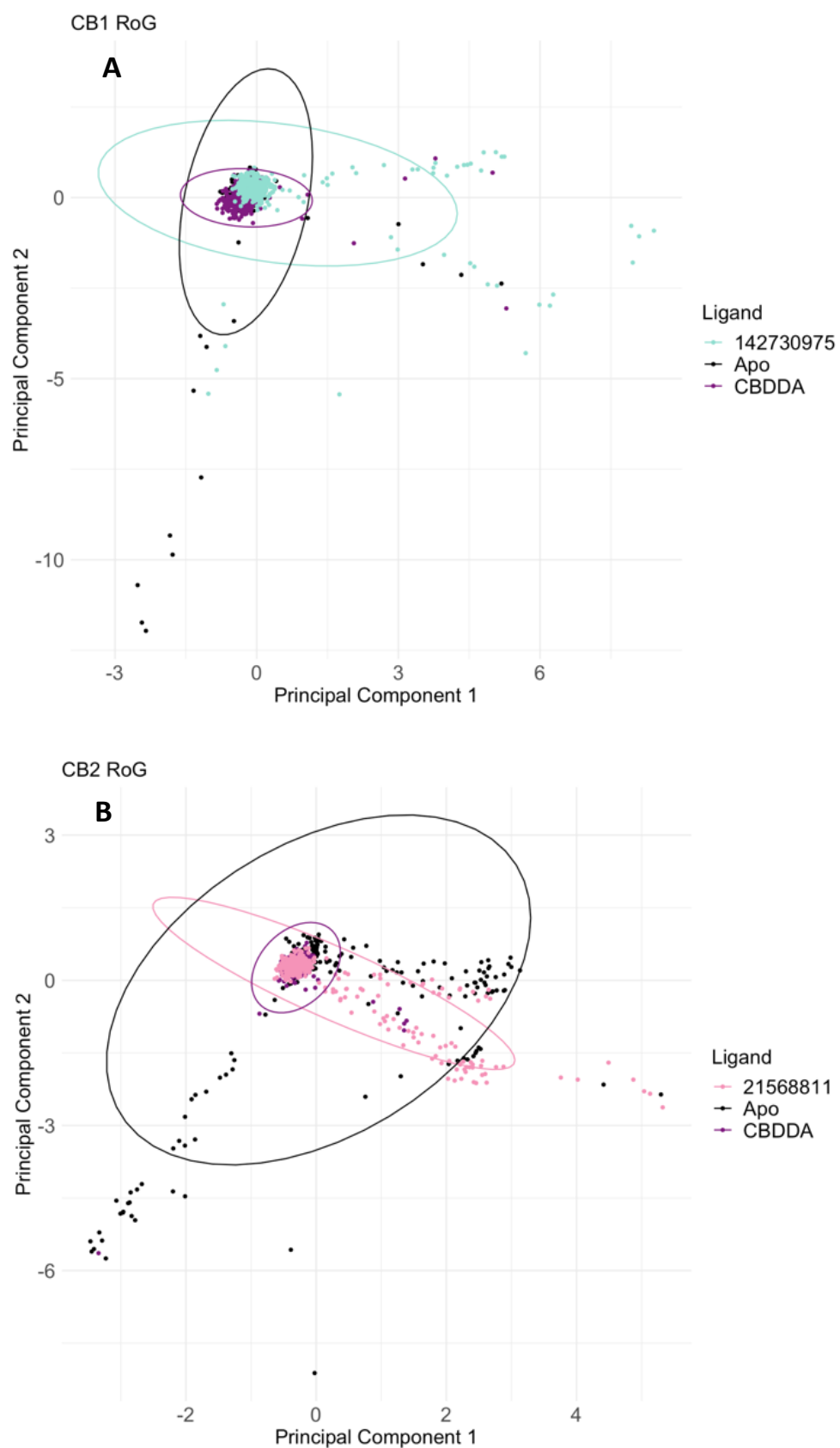


Figure 6.5.2: PCA of the RoG for CBRs. The scatter plots track the conformational changes of CB₁ A. and CB₂ B. receptors when bound to ligands CBDDA and their respective top hits (142730975 for CB₁ and 21568811 for CB₂) over the simulation time frame.

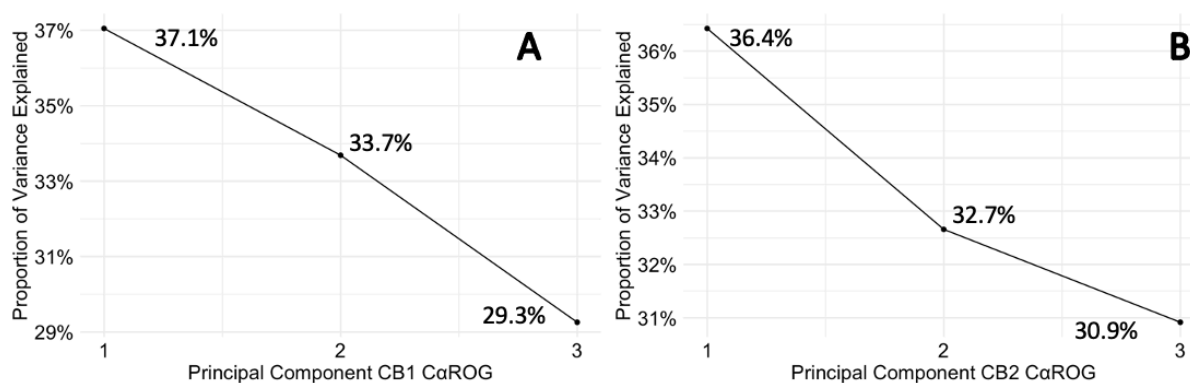


Figure 6.5.3: Scree Plots of PC Contributions to RoG Variance in CB₁ and CB₂. A. Shows the percentage of total variance explained by the three PCs for the CB₁ receptor RoG, with values at 37.1 %, 33.7 %, and 29.3 %. B. Displays the same for the CB₂ receptor RoG, with PCs accounting for 36.4 %, 32.7 %, and 30.9 % of the variance, respectively.

3.6 Simulation Snapshots

The simulation snapshots (Fig. 6.6) offer a comparative analysis of CBRs in three distinct states. They illustrate the unbound apo form, the form bound to CBDDA, and the form bound to their respective top hit ligands, 142730975 for CB₁ and 21568811 for CB₂. The colour coding from blue at the start of the simulation to red at the end visually represents the dynamic structural changes throughout the simulation period. When bound to CBDDA, the CB₁ receptor (Fig. 6.6B) shows a reduction in conformational diversity, suggesting that CBDDA binding restricts receptor movement to a degree. However, this restriction is not as prominent as that imposed by ligand 142730975 (Fig. 6.6C), which seems to significantly limit the CB₁ receptor to a smaller set of conformations. This suggests that the receptor predominantly remains in these few conformations throughout the simulation period. The CB₂ receptor exhibits similar trends. The CBDDA-bound state shows a reduction in conformational variability compared to the apo form (Fig. 6.6D, E), but still retains a mix of conformations. In contrast, the receptor bound to the top hit ligand 21568811 (Fig. 6.6F) reveals a more constricted conformational range, which could indicate a more robust and stable receptor-ligand interaction. These observations suggest that while both CB₁ and CB₂ receptors explore a wide conformational landscape in their unbound states, ligand binding narrows down this landscape. Notably, the top hit ligands appear to confine the receptors to a more limited set of conformations than CBDDA, which could have implications for the specificity of the signalling pathways they initiate.

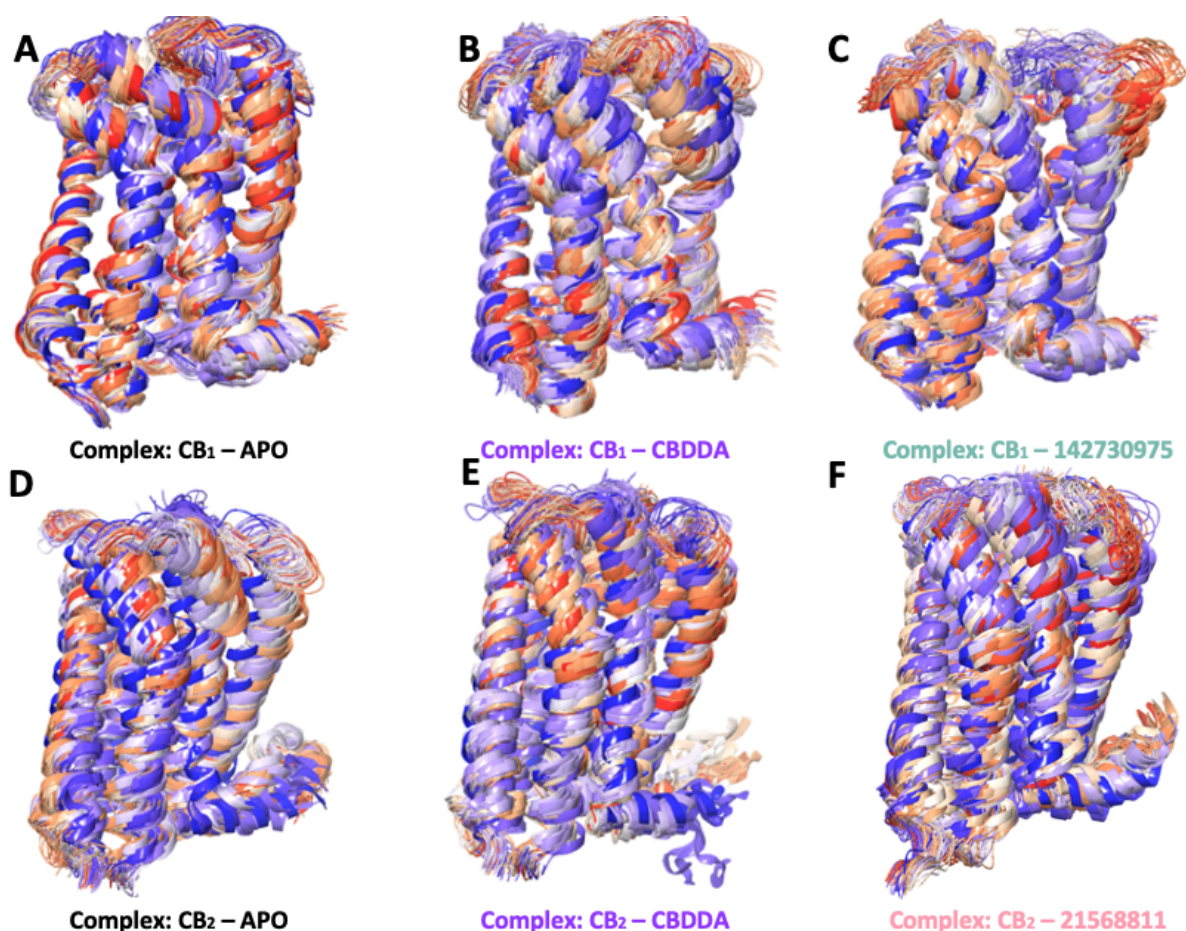


Figure 6.6: Dynamic Snapshots of CB₁ and CB₂ Receptors during MD simulations. Panels A–C depict the CB₁ receptor in its apo form, bound to CBDDBA, and bound to ligand 142730975, respectively. Panels D–F show the CB₂ receptor similarly in apo form, bound to CBDDBA, and bound to ligand 21568811. Each snapshot illustrates the progression of the simulation from start (blue) to finish (red), visualising the conformational changes within the membrane-bound regions of the receptors over time. Generated from Maestro Schrödinger Suite.

The dynamic nature of ligand binding to CB₁ and CB₂ receptors through a series of overlaid snapshots taken over a 500 ns MD simulation is showcased by Fig. 6.7. The progression from blue to red in the visualisations represents the temporal sequence of conformations, with blue indicating the initial binding conformation and red representing the final state. Fig. 6.7A and C illustrate the binding of CBDDBA to CB₁ and CB₂, respectively, while Fig. 6.7B and D depict the interaction of ligands 142730975 and 21568811, with CB₁ and CB₂. In the simulation of the CB₁ receptor complexed with CBDDBA (Fig. 6.7A) and ligand 142730975 (Fig. 6.7B), a predominant conformation is maintained throughout, suggesting a preferred mode of binding. In contrast, the receptor with ligand 142730975 displays two main conformations, with the simulation splitting halfway between these states, indicating a conformational shift occurs mid-simulation. This could mean that the ligand 142730975 allows the CB₁ receptor to transition

between two stable modes, potentially reflecting versatility in how this ligand influences receptor activity.

For the CB₂ receptor, the CBDDA-bound complex also adopts one primary conformation, but with slightly more flexibility and variability than observed in the CB₁-CBDDA complex. The CB₂ receptor bound to ligand 21568811 shows a similar pattern, yet with even more flexibility compared to the CB₂-CBDDA complex. This suggests that while both ligands stabilise specific conformations, ligand 21568811 permits a greater degree of conformational freedom. The implications of these observations are significant for understanding the dynamic interactions between these receptors and their ligands, which could influence the development of cannabinoid-based therapeutics with precise effects on receptor signalling.

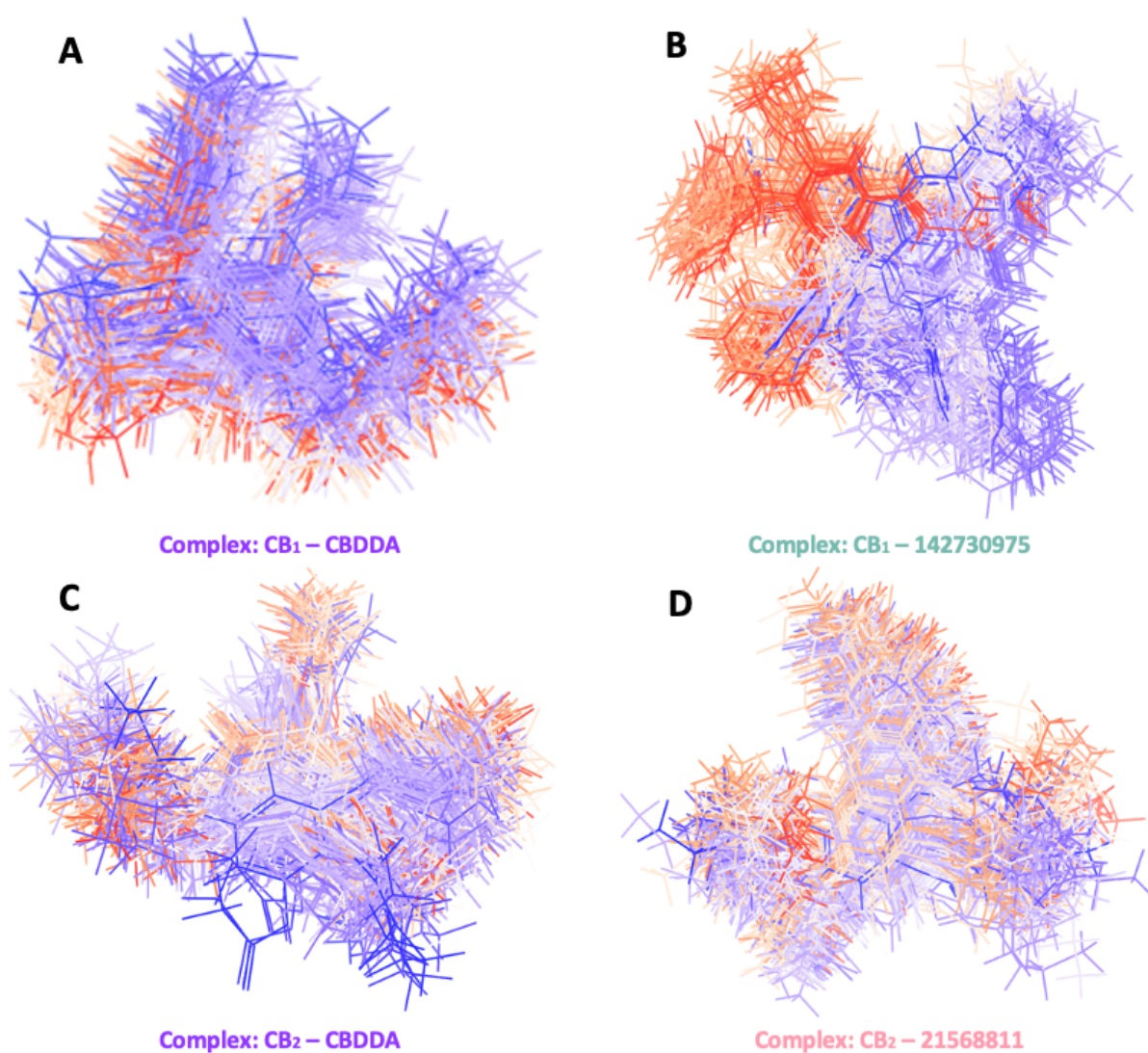


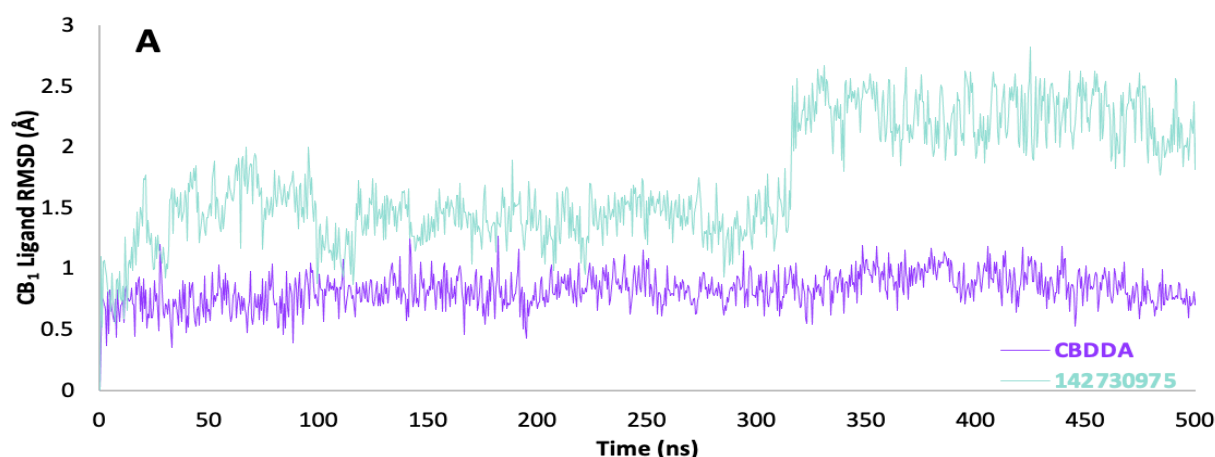
Figure 6.7: Temporal Binding Conformation Ensemble of CB₁ and CB₂ Receptors during MD simulations. Panels A and B show the CB₁ receptor with CBDDA and ligand 142730975 respectively, while panels C and D display the CB₂ receptor with CBDDA and ligand 21568811. The progression from blue to red represents the

conformational evolution during a 500 ns molecular dynamics simulation, highlighting the structural flexibility and interaction diversity of the receptors with various ligands. Generated from Maestro Schrödinger Suite.

3.7 Ligand Interactions with CBRs

Figure 6.8 displays the RMSD trajectories of ligands in their respective cannabinoid receptor (CBR) complexes across a 500 ns MD simulation, offering a measure of the stability of the interactions between the ligands and receptors. Fig. 6.8A showcases the RMSD for the CB₁ receptor complexed with CBDDA and ligand 142730975. CBDDA exhibits a stable RMSD throughout the simulation, indicating a consistent interaction within the receptor binding pocket. Conversely, ligand 142730975 shows a significant fluctuation at ~300 ns, which correlates with the snapshots presented in Fig. 6.7B, where this ligand displays two distinct conformations in the simulation which can be associated with changes in lig-RMSD. The RMSD for ligand 142730975 is also higher than that of CBDDA, suggesting that its interaction with the receptor may be less stable.

Figure 6.8B illustrates the RMSD trajectories for the ligands CBDDA and ligand 21568811 when complexed with the CB₂ receptor. CBDDA shows a stable RMSD throughout the simulation, suggesting a consistent and stable interaction within the receptor binding site. In contrast, ligand 21568811 displays more variability in its RMSD, indicating a greater degree of flexibility and deviation within the binding pocket. This observation aligns with the results depicted in Fig. 6.7, where both ligands maintain relatively stable conformations throughout the simulation. However, ligand 21568811 increased flexibility implies it may adopt a range of conformations, suggesting potential for diverse interactions within the binding site of the CB₂ receptor.



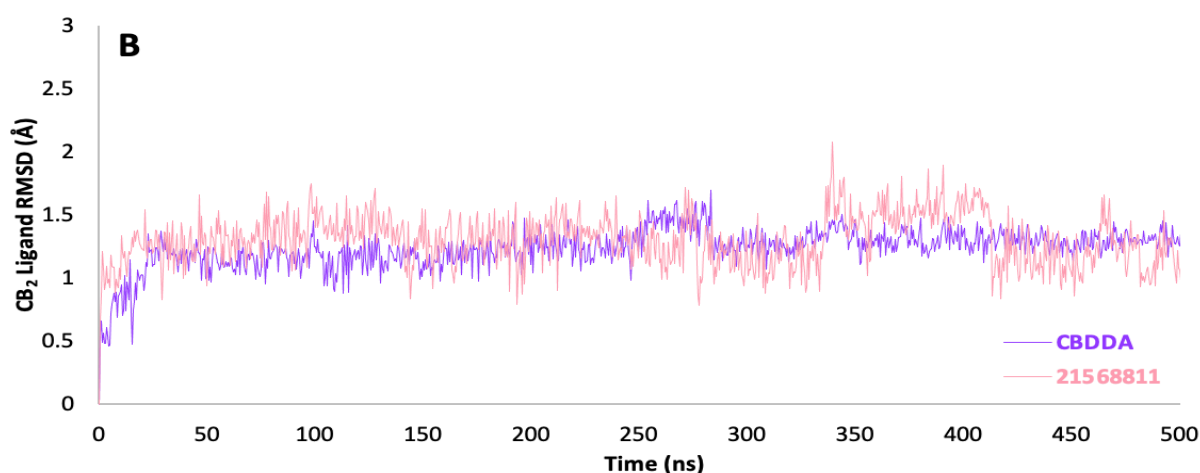


Figure 6.8: RMSD of Ligands in CB₁ and CB₂ Receptor Complexes. when bound to the CB₁ receptor throughout a 500 ns simulation. Panel B depicts the lig-RMSD for the same ligands in complex with the CB₂ receptor. Lig-RMSD measures the positional stability of each ligand within the binding site, with lower and more consistent lig-RMSD values indicating a stable interaction, and higher or more variable lig-RMSD values reflecting dynamic binding and conformational flexibility. These trajectories provide insights into the comparative stability of ligand-receptor interactions over time.

Interaction maps that summarise the binding contacts between ligands and the CB₁ and CB₂ receptors, as derived from post-MD simulations. Each map points out key residues within the receptors that form persistent contacts for more than 30 % of the simulation time with the ligands, which are indicative of the interactions that contribute to the stability and specificity of the ligand-receptor complex. The interaction map provided for the CB₁ receptor complexed with the ligand CBDDA (Fig. 6.9A) highlights the crucial involvement of residues Phe14 and Met15 in ligand binding. The map indicates a durable H-bond between the ligand and Met15, which persists for 92 % of the simulation time. This specific interaction is driven by the O group on the ligand, which has a partial negative charge, making it attractive to the slightly positive sulphur atom present in the side chain of methionine. The sulfur atom can act as a weak H-bond acceptor, and this interaction would contribute significantly to the stability and specificity of the ligand within the binding pocket of the receptor. Similarly, in the interaction map for the CB₁ receptor bound to ligand 142730975 (Fig. 6.9B), a distinct pattern of contacts is observed involving Val108 and Phe180, in addition to a shared interaction with Met15. Met15 interacts with an O group on the ligand for 48 % of the simulation time, similarly to its interaction with CBDDA, although with less frequency. H-bonding is a directional and relatively strong interaction, which can significantly influence ligand orientation and its subsequent signalling effect. The prevalence of this H bond suggests that Met15 is a critical residue in the binding site, potentially acting as an anchor point that defines ligand conformation and influences the

receptor conformational state. This interaction could also have implications for the potency and selectivity of ligands for the CB₁ receptor, as it may enhance ligand binding affinity and its ability to activate or inhibit receptor signalling.

The interaction map of the CB₂ receptor with CBDDA (Fig. 6.9C) highlights key residues such as Phe86, Phe182, and Phe116, which play a significant role in ligand binding. Phenylalanine residues are generally involved in ligand interactions due to their aromatic side chains, which can engage in hydrophobic interactions and π - π stacking with ligands that have compatible aromatic or electron-rich regions. The interactions between CBDDA and these Phe residues suggest that the CBDDA structure includes regions that can participate in these aromatic interactions, stabilising the ligand within the CB₂ receptor binding pocket.

In contrast, the interaction of the CB₂ receptor with the ligand 21568811 (Fig. 6.9D) demonstrates direct π - π stacking contacts with Phe86 and Phe182 for 37 % and 46 % of the simulation time, respectively. This type of interaction is characterised by the alignment of the aromatic rings of the phenylalanine side chains with aromatic rings on the ligand, which is a strong stabilising force due to the overlapping electron clouds of these rings. Additionally, the CB₂-21568811 complex features a direct H-bond with Ser432. The side chain of serine is characterised by a OH group which is polar and can act both as a H-bond donor and acceptor. This versatility allows Ser432 to form an H-bond with polar or charged groups on ligands, such as O groups, which often carry a partial negative charge. The H-bond observed for 53 % of the simulation time suggests that the interaction with Ser432 is a significant component of the stability and specificity of ligand binding to the CB₂ receptor. These interactions are critical for understanding how the ligands may influence receptor signalling and are essential considerations in the design of new cannabinoid-based therapeutics that target the CB₂ receptor with high affinity and desired pharmacological effects.

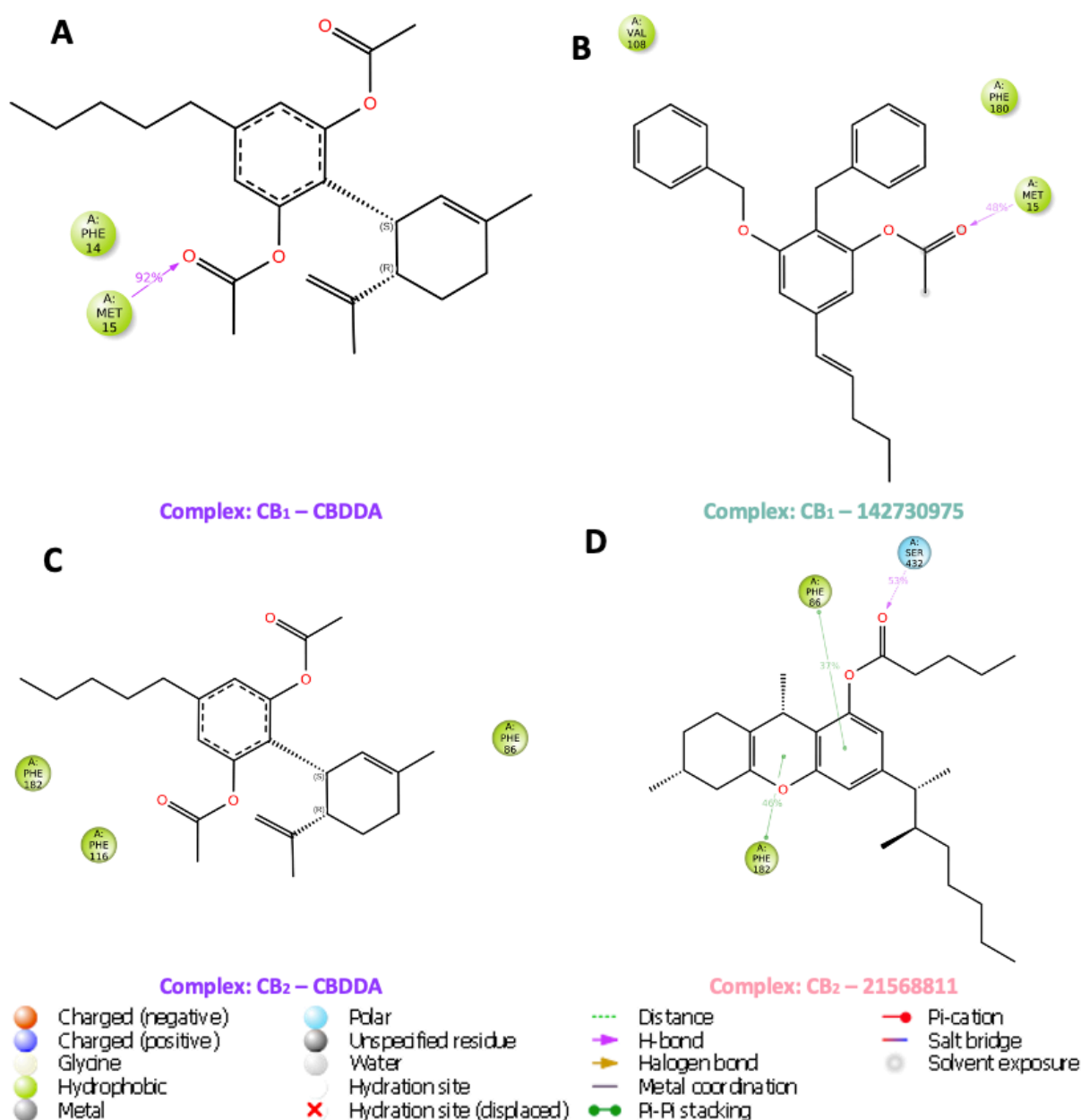


Figure 6.9: Detailed Binding Interactions of CB₁ and CB₂ with Ligands. A and C show the interactions of the CB₁ receptor with CBDDA and ligand 142730975, respectively, while B and D illustrate the interactions of the CB₂ receptor with CBDDA and ligand 21568811. The diagrams indicate persistent contacts with key residues, highlighting the specific binding interactions that contribute to the stability of the ligand-receptor complexes over the course of the simulations. Generated from Maestro Schrödinger Suite.

4 DISCUSSION

The exploration of CBDDA analogues through HTVS contributes to cannabinoid research, particularly in the quest for novel therapeutic agents for the treatment of conditions modulated by the ECS. This includes a range of disorders such as chronic pain, epilepsy, multiple sclerosis, anxiety, depression, and potentially even some cancer types (Dhopeshwarkar & Mackie, 2014;

Mackie, 2008). By targeting specific receptors within the ECS with tailored analogues, there is potential to harness the therapeutic benefits of phytocannabinoids without the psychoactive effects typically associated with *Cannabis* use. CBDDA analogues could lead to significant advancements in the efficacy and safety of cannabinoid-based medications, making them a valuable addition to modern pharmacotherapy (Holt et al., 2022).

This study meticulously investigates the interaction of energetically favourable CBDDA analogues with p-CBRs, using computational systems to predict their affinity and efficacy. Through the use of MD simulations, the research delves into the structural nuances that govern ligand-receptor interactions, providing insights into the pharmacokinetic and pharmacodynamic properties essential for drug development (Zhang, 2022). This comprehensive approach not only highlights the potential of CBDDA analogues as promising therapeutic candidates but also shows the importance of structural integrity in enhancing receptor affinity and activity modulation. The findings suggest a path forward for the development of cannabinoid-based medications with improved safety and efficacy profiles, potentially transforming treatment models for diseases influenced by the ECS (Mackie, 2008; Morales et al., 2022). In essence, the study embodies a pioneering effort in the intersection of computational chemistry and cannabinoid science, offering valuable perspectives on drug discovery and development. Its implications extend beyond the immediate findings, suggesting a broader impact on personalised medicine and the ongoing search for novel pharmacological interventions (Zhang, 2022).

In the analysis of the CB₁ receptor, a clear distinction was observed between the interactions, binding energies, and MD simulation results for the top hit ligand 142730975, CBDDA, and the apo form. Binding energy, specifically the free binding energy change (ΔG_{Bind}), is fundamental in assessing the stability of a ligand-receptor complex. For a ligand to be considered as having a strong and stable interaction with a receptor, a negative ΔG_{Bind} is desirable, as it signifies that the system releases energy upon binding, indicating a spontaneous interaction. In the initial HTVS, ligand 142730975 exhibited the most favourable ΔG_{Bind} of -91.39 kcal/mol with the CB₁ receptor. This value reflects a potent affinity and a strong interaction, suggestive of high receptor-ligand binding specificity. Docking scores provide another layer of quantification, where a lower docking score correlates with higher ligand affinity. Ligand 142730975 presented a docking score of -13.11 kcal/mol, its significant binding interaction with the CB₁ receptor. This score is considered good in virtual screening

protocols, as it is substantially lower than the typical range for compounds with good affinity, which can be between ~ -9 kcal/mol. Docking and MM-BGSA scores are critical in the early phases of drug development, serving as a preliminary indicator of ligand efficacy. They encompass the cumulative effect of all favourable and unfavourable interactions within the binding pocket. Ligand 142730975 had a particularly low docking score, compared to CBDDA, is indicative of strong ligand-receptor interactions, driven in part by specific contact points such as H-bonds with Met15 and π - π stacking interactions with Phe82. These types of interactions are crucial as they can provide stability and specificity to the ligand-receptor complex, influencing signalling pathways.

The MD simulation results for CBDDA, and the ligand revealed 142730975 various effects on C_α RMSD, C_α RMSF, and RoG, which measured increased structural stability, decreased flexibility, and increased compactness, respectively. CBDDA exhibited increased C_α RMSD values, suggesting some induced conformational shifts. In contrast, the top hit ligand 142730975 showed a tightening of these values for C_α RMSD and ROG, which could imply a stabilising effect and possibly a stronger affinity and tighter control over the receptor conformation. The ligand contacts and main residues identified from the interaction maps showed that CBDDA had a significant H-bond with Met15, which was maintained for a large portion of the simulation time. On the other hand, ligand 142730975 had additional contacts, suggesting a more complex interaction pattern compared to CBDDA, potentially contributing to a more stable ligand-receptor complex. Lig-RMSD and ligand snapshots linked these structural dynamics to visual representations, illustrating how the ligand maintains its position within the binding site throughout the simulation. The stability and changes in ligand-receptor contacts over time provided further insights into the dynamic nature of these interactions and the potential impact on receptor signalling. The implications of this work in targeting the CB₁ receptor with CBDDA and 142730975 show that ligand 142730975 offers the potential for more efficacious treatments by virtue of its enhanced receptor affinity and modulatory capabilities, as shown through rigorous computational analyses. The ability to design ligands that can provide a more stable interaction with CB₁ could lead to therapeutics with higher potency, selectivity, and possibly a better safety profile than currently available cannabinoid-based therapies (Mackie, 2008; Willems et al., 2020). These findings open possibilities for treating a range of ECS-related conditions, from neurological disorders to inflammatory diseases, with a new generation of cannabinoid derivatives.

The detailed analysis of the CB₂ receptor has provided valuable insights into the binding dynamics of CBDDA and its analogue, ligand 21568811. Upon interacting with the CB₂ receptor, ligand 21568811 demonstrated significant π - π stacking interactions, specifically with the Phe86 and Phe182 residues. The docking score for the ligand 21568811-CB₂ complex was determined to be -11.57 kcal/mol, and the MM-GBSA score of -75.67 kcal/mol suggests robust interaction energies. Generally, negative MM-GBSA scores indicate strong binding energies, which in this case, imply that ligand 21568811 binds favourably and forms a stable complex with the CB₂ receptor (Ji et al., 2020). These energies offer a comprehensive view of the ligand's interaction with the receptor, taking into account factors such as van der Waals forces, electrostatic contributions, and both polar and nonpolar components of solvation (Genheden & Ryde, 2015; Mulakala & Viswanadhan, 2013). The combination of a favourable docking score and MM-GBSA scores highlights the therapeutic potential of ligand 21568811, positioning it as a strong candidate for further drug development aimed at targeting the CB₂ receptor. This is of significant importance, given the role of the CB₂ receptor in various physiological and pathophysiological processes. Modulation of this receptor by ligand 21568811 could pave the way for novel treatments for conditions like pain, inflammation, and autoimmune diseases (Morales et al., 2022).

Analysing the MD results for CB₂, the C α RMSD analysis revealed that ligand 21568811 exhibits greater variability and with lower C α RMSD Å values for the compared to CBDDA, indicating a broader range of conformations and a dynamic binding nature within the CB₂ receptor. This suggests an enhanced ability of ligand 21568811 to explore different conformations, potentially leading to more effective receptor interactions. Furthermore, the C α RMSF analysis showing the increase in the dynamic behaviour of the receptor, ligand 21568811 induces noticeable fluctuations at specific residues. These fluctuations suggest changes in receptor flexibility and function, possibly affecting the biological activity of the CB₂ receptor. Lastly, the RoG analysis demonstrated that both ligands contribute to a more compact receptor structure relative to the apo form, with ligand 21568811 having a slightly more pronounced effect. This suggests that ligand 21568811 not only promotes tighter binding but also enhances receptor stability. The collective findings from these analyses highlight the dynamic and structural implications of ligand binding on the CB₂ receptor, emphasising the importance of such considerations in the development of therapeutic agents targeting this receptor.

Moreover, the visual snapshots from MD simulations offer a comparative analysis of the structural dynamics between the two ligands interacting with the CB₂ receptor. Ligand 21568811 appears to limit the receptor to a reduced number of conformations compared to CBDDA, a fact that is apparent from the encoded structural snapshots. The interaction map derived from these simulations accentuates key residues within the CB₂ receptor, such as the π - π stacking interactions with Phe86 and Phe182, as well as a H bond with Ser432. These interactions with ligand 21568811 are stable over a substantial portion of the simulation time, contributing to the specificity and stability of the ligand-receptor complex. The identification of these critical interactions is pivotal, as they offer a deeper understanding of the binding mechanics that may underpin the ligand's therapeutic efficacy.

This research illustrates the ability of HTVS and MD simulations in the drug discovery process, allowing for the prediction of ligand efficacy and stability before clinical trials. By providing a detailed mechanistic understanding of ligand-receptor interactions, these computational techniques can streamline the development of targeted therapies, potentially reducing the time and cost associated with bringing new drugs to market (Willems et al., 2020; Zhang, 2022). The insights gleaned from these analyses are invaluable, offering a pathway to develop cannabinoid-based treatments that are both potent and selective, thereby expanding the therapeutic arsenal for a range of ECS-related disorders.

5 CONCLUSION

This study represents a significant advancement in cannabinoid research, particularly in the development of novel therapeutic agents targeting the ECS. Through the meticulous application of HTVS and MD simulations, the research has identified promising CBDDA analogues that exhibit high affinity and specificity towards the CB₁ and CB₂ receptors. These findings not only contribute to our understanding of ligand-receptor interactions but also pave the way for the development of cannabinoid-based treatments with improved safety and efficacy profiles. By focusing on the structural integrity and interaction dynamics of CBDDA analogues, this research highlights the potential for creating more effective and targeted therapies for conditions modulated by the ECS, potentially transforming treatment paradigms in pain management, neurology, and beyond.

SUMMARY

This research focused on identifying the differences between traditional cannabidiol CBD and its chemically modified analogue, CBDDA interaction between CB₁ and CB₂. Through the application of computational methodologies, the investigation revealed the complex interplay of these compounds with the ECS, significantly contributing to the understanding of CBR interactions.

Employing bioinformatics techniques, molecular docking, and dynamics simulations, the research delineated the binding affinities and interaction patterns of CBD and CBDDA with CB₁ and CB₂ receptors. It was determined that CBDDA exhibited a stronger interaction with both receptors when compared to unmodified CBD. This suggested an enhanced therapeutic potential for the modified compound. The computational approach not only provided a blueprint for the molecular interactions at play but also highlighted the possibilities for cannabinoid-based therapeutic interventions. To determine the intricacies of these CBR interactions, this work meticulously analysed the bioinformatics and evolutionary history of CB₁ and CB₂ receptors. It established which regions of these receptors possess conserved sites critical for their functionality. Pseudo-CBRs were utilised to validate the receptors further, preparing the basis for more in-depth studies of acetylated and natural phytocannabinoids. This validation was essential for confirming the suitability of the receptors for subsequent MD studies.

Furthermore, HTVS-SP-XP docking and Prime MM-GBSA post-processing successfully identified two pivotal compounds for their interactive capabilities with CBRs. ligand 142730975 emerged as the top hit for the CB₁ receptor, and this compound demonstrates the potential for significant therapeutic efficacy. For the CB₂ receptor, ligand 21568811, also known as SCHEMBL11831333, was identified as the top hit, displaying significant binding energy values that suggest a stable receptor interaction. The promising interactions of these compounds, alongside their advantageous energy profiles, indicate their capacity to act as effective and selective cannabinoid-based medications. The observed structural integrity and stability in the ligand-receptor complexes, supported by MD simulations and interaction mapping, reinforce the prospects of these molecules as leading candidates for targeted treatments of endocannabinoid system-related conditions. This study not only highlights the

therapeutic promise of these CBDDA analogues but also enriches cannabinoid research, laying the groundwork for further drug discovery initiatives that necessitate experimental validation and clinical trials to corroborate the computational forecasts.

The achievements in this study, while noteworthy, encounter certain limitations inherent to computational approaches. The primary restriction lies in the transition from theoretical simulations to tangible experimental evidence. Simulations serve as a preliminary but indirect form of validation, leaving a chasm that empirical testing can bridge. Additionally, empirical work with cannabinoids is complicated due to regulatory challenges. The stringent control over *Cannabis* necessitates the procurement of special permits for experimental research, which is a complex and often arduous process.

To advance beyond the current findings, the future work on this topic must undertake the critical task of experimentally validating the computational data. After securing appropriate permits, experimental work could include radioligand or fluorescent assays to elucidate the bonding dynamics between CBD, CBDDA and CBRs, aiming to quantify their interaction efficacy. Furthermore, functional analyses could monitor the activation effects of CBRs, by observing alterations in signalling molecules like cAMP or calcium levels. Cell culture studies, utilising strains that express CBRs, are designed to reveal the cellular effects induced by CBD and its diacetate form, spanning from gene expression variations to impacts on cell viability. *In vivo* experiments with animal models would elucidate the systemic effects of these substances on CBRs, investigating behavioural changes, pain mitigation, or anti-inflammatory benefits. Advanced structural biology techniques could map the 3D configurations of these receptors in conjunction with CBD compounds, offering a detailed perspective of their molecular interactions. Additionally, biochemical assessments would serve to validate theoretical interaction models, possibly by analysing receptor-compound complexes or protein interactions. Pharmacokinetic studies are essential for tracing cannabinoid absorption and metabolism, illuminating their therapeutic potential and metabolic pathways. Altogether, affirming the hypotheses posited by computational *in-silico* studies demands an integrated scientific methodology that encompasses molecular biochemistry, cellular biology, and pharmacology, bridging the gap between laboratory and *in vivo* research to comprehensively demonstrate how CB₁/CB₂ receptors interact with CBD compounds, thereby grounding theoretical *in-silico* predictions in empirical reality.

This pivotal study greatly enhances the knowledge of how cannabinoids interact with the ECS, delving into the molecular interactions of CBD and its acetylated variant, CBDDA, with CB₁ and CB₂ receptors. It not only highlights the enhanced therapeutic potential of acetylated cannabinoids but also lays a foundational blueprint for subsequent inquiries. By addressing the recognised limitations and following through with the recommended further research can leverage these insights. Such endeavours are essential to advancing cannabinoid-based therapeutic development, ensuring that emerging treatments are safe, effective, and in sync with regulatory guidelines. As the field of cannabinoid investigation and utilisation evolves, this research stands as a seminal reference, steering the course toward new frontiers in *Cannabis* studies and its clinical applications.

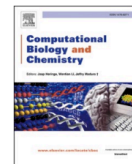
APPENDIX A

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Exploring the evolutionary trajectory and functional landscape of cannabinoid receptors: A comprehensive bioinformatic analysis

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ABSTRACT

The bioinformatic analysis of cannabinoid receptors (CBRs) CB₁ and CB₂ reveals a detailed picture of their structure, evolution, and physiological significance within the endocannabinoid system (ECS). The study highlights the evolutionary conservation of these receptors evidenced by sequence alignments across diverse species including humans, amphibians, and fish. Both CBRs share a structural hallmark of seven transmembrane (TM) helices, characteristic of class A G-protein-coupled receptors (GPCRs), which are critical for their signalling functions. The study reports a similarity of 44.58 % between both CBR sequences, which suggests that while their evolutionary paths and physiological roles may differ, there is considerable conservation in their structures. Pathway databases like KEGG, Reactome, and WikiPathways were employed to determine the involvement of the receptors in various signalling pathways. The pathway analyses integrated within this study offer a detailed view of the CBRs interactions within a complex network of cannabinoid-related signalling pathways. High-resolution crystal structures (PDB ID: 5U09 for CB₁ and 5ZTY for CB₂) provided accurate structural information, showing the binding pocket volume and surface area of the receptors, essential for ligand interaction. The comparison between these receptors' natural sequences and their engineered pseudo-CBRs (p-CBRs) showed a high degree of sequence identity, confirming the validity of using p-CBRs in receptor-ligand interaction studies. This comprehensive analysis enhances the understanding of the structural and functional dynamics of cannabinoid receptors, highlighting their physiological roles and their potential as therapeutic targets within the ECS.

Figure A1: The work from chapter three is published as a manuscript titled 'Exploring the Evolutionary Trajectory and Functional Landscape of Endocannabinoid Receptors: A Comprehensive Bioinformatic Analysis,' published in "Computational Biology and Chemistry journal". DOI: [10.1016/j.compbiolchem.2024.108138](https://doi.org/10.1016/j.compbiolchem.2024.108138).

Peer review status

Comparative Analysis Between Natural and Acetylated Phytocannabinoids Interacting with Pseudo-Endocannabinoid Receptors CB₁/CB₂: Insights from Computational Modelling Studies

- Reviews completed: 0
- Review invitations accepted: 0
- Review invitations sent: 2+

Journal:

Journal of Molecular Graphics and Modelling

Corresponding author:

Ikechukwu Achilonu

First author:

Marushka Sooben

Date of submission:

11th March 2024

Figure A2: Chapter 5 article peer review status for the manuscript 'Comparative Analysis Between Natural and Pseudo-Endocannabinoids Interacting with Acetylated Phytocannabinoid Receptors CB₁/CB₂: Insights from Computational Modelling Studies,' submitted and currently under review in the “Journal of Molecular Graphics and Modelling” Manuscript number: JMGM-D-24-00280.

APPENDIX B

Table B2: Amino acid FASTA sequence of CBRs and p-CBRs

Receptor	Database	FASTA Sequence
CB₁	UniProt P21554	MKSILDGLADTTFRITITDLLYVGSNDIQYEDIKGDMSKLGYPQKFPLTSFRGSPFQE KMTAGDNPQLVPADQVNITEFYNKSLSSFKENEENIQCGENFMDIECFMVLNPSQQL AIAVLSLTGTFTVLENLLVLCVILHSRSLRCRPSYHFIGSLAVADLLGSVIFVYSFIDFHVH RKDSRNVFLFKLGGVTASFTASVGSFLTAIDRYISIHRLPLAYKRIVTRPKAVVAFCLMWT IAIVIAVLPLLGWNCEKLQSVCSDFPHIDETYLMTFWIGVTSVLLLFIVYAYMYILWKAHS HAVRMIQRGTQKSIIHTSEDGKVQVTRPDQARMDIRLAKTLVLILVLIICWGPELLAIM VYDVFGKMNKLIKTVFAFCSMCLLNSTVNPIIYALRSKDLRHAFRSMFSPCEGTAQPLD NSMGSDCLHKHANNAASVHRAESCIKSTVKIAKVTMSVSTDTSAEAL
	PDB	GKENEENIQCGENFMDIECFMVLNPSQQLAIAVLSLTGTFTVLENLLVLCVILHSRSLR CRPSYHFIGSLAVADLLGSVIFVYSFIDFHVHFRKDSRNVFLFKLGGVTASFTASVGSFL AAIDRYISIHRLPLAYKRIVTRPKAVVAFCLMWTIAIVIAVLPLLGWNCEKLQSVCSDFPHI DETYLMFWIGVTSVLLLFIVYAYMYILWKAGIDCSFWNESYLTGSRDERKKSLSKFGM DEGVTFMFIGRFRDQKQGVVDVLLKAIEILSSKKEFQEMRFIIIGKGDPELEGWARSLEEK HGNVKVITEMLSREFVRELYGSVDVIIIPSYFEPFGLVALEAMCLGAIPASAVGGRLDIIT NETGILVKAGDPGELANAILKALELSRDLKSFRENCKKRAMSFSDQARMDIRLAKTLVL ILVLIICWGPELLAIMVYDVFGKMNKLIKTVFAFCSMCLLNSTVNPIIYALRSKDLRHAF RSMFSPCEGTAQPHHHHHHHHHH
CB₂	UniProt P34972	MEECWVTEIANGSKDGLDSNPMKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLIL SSHQLRRKPSYLFIGSLAGADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTA SVGSLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIMWVLSALVSYPLMGWTCCPRPC SELFPLIPNDYLLSWLLFIAFLFSGIITYGHVLWKAHQHVASLSGHQDRQVPGMARMR LDVRLAKTLGLVLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMCLLINSMVNPV IYALRSGEIRSSAHHCLAHWKKCVRGLGSEAKEEAPRSSVTETADGKITPWPDSRDL LSDC
	PDB	MKTIIALSIFCLVFAGAPPMKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSH QLRRKPSYLFIGSLALADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTASVG SLLAAIDRYLCLRYPPSYKALLTRGRALVLLGIMWVLSALVSYPLMGWTCCPRPCSEL FPLIPNDYLLSWLLFIAFLFSGIITYGHVLWKAHQHVASNIFEMLRIDEGLRLKIYKDETEG YYTIGIGHLLTKPSLNAAKSELDAIGRNTNGVITKDEAEKLFNQDVDAAVRGILRNAK LKPVYDSLDAVRRALINMVFMGETGVAGFTNSLRMLQKRWDEAAVNLAWSRW YNQTPNRAKRVITTFRTGTWDAYARMRLDELAKTLGLVLAVLLICWFPVLALMAHSL ATTLSDQVKKAFAFCSMCLLINSMVNPVIYALRSEEIRSSAHHCLAHWKKCVRGLGEFL EVLFQGPHHHHHHHHHHHDYKDDDDK

APPENDIX C

HARDWARE AND SOFTWARE SPECIFICATIONS

In the computational assessment of CBRs interactions, the study employed advanced molecular modelling techniques using two high-performance computing units. The first unit, designated for running the Maestro algorithm, operated on a Windows OS platform, powered by an AMD RYZEN Threadripper 1950X Processor. This setup included an Asus Rog Strix X399-E Gaming Ryzen motherboard, a substantial 4TB internal SSD for extensive data handling, and 64GB DDR4 RAM to ensure smooth computational operations. Graphics processing was managed by an MSI GeForce RTX 2080 Ti graphics card, ensuring detailed visual representations and simulations. The second computing unit focused on Desmond MD simulation algorithms, utilising an Ubuntu OS. It was equipped with an AMD Threadripper 3990X processor, paired with an MSI TRX40 PRO 10G motherboard. The graphical computations of the system were supported by a GeForce RTX 2070 graphics card. Memory and storage were amply provided by 64GB RAM, a 1TB M.2 SSD for rapid data access, and a 4TB HDD for additional storage needs. Across both units, the studies were conducted using Schrodinger Suite 2023-2 for molecular modelling, with Desmond version 7.4 specifically utilised for MD simulations, offering precise and reliable computational analysis of the binding interactions between CBRs and cannabinoids.

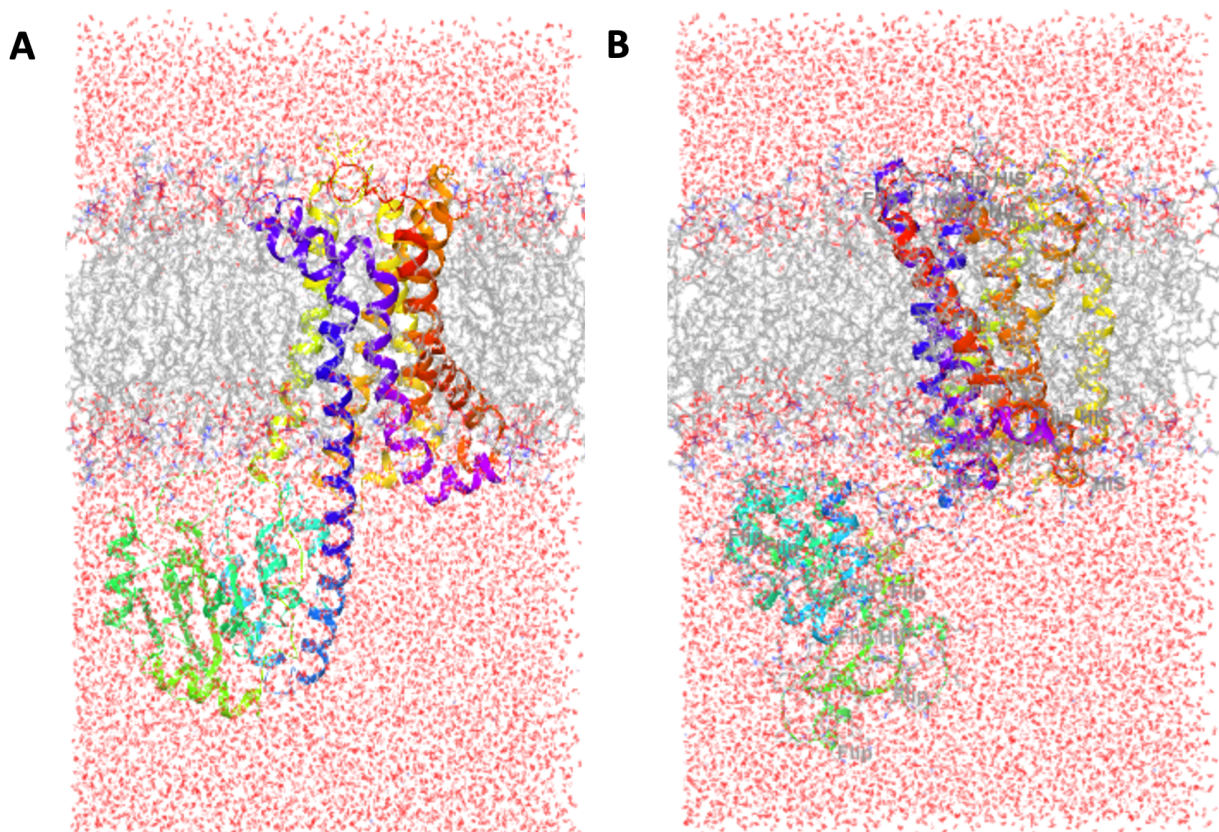


Figure C1: P-CBR systems in POPC lipid bilayer. This figure represents a model of a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) lipid bilayer incorporated with a Transferable Intermolecular Potential with 3 Points (TIP3P) water box. A. represents CB₁ and B. CB₂.

```

1  ENFMDIECFMVLNPSQQLAIAVLSTLTGFTVLENLLVLCVILHSRSLRCRPSYHFIGSLAVADLLGSVIFVYSFIDFHV  80
1  ----PMKDYMI LSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRKPSYLFIGSLALADFLASVVFACSFVNFHV  76

81  FHRKDSRNVFLFKLGGVTASFTASVGSFLAAIDRYISIHRLPAYKRIVTRPKAVVAFCLMWTIAIVIAVLPLLGNCEK  160
77  FHGVDSKAVFLKIGSVTMTFTASVGSLLAAIDRYLCLRYPPSYKALLTRGRALVLLGIMWVLSALVSYLPLMGWTC--  154

161  LQSVCSDFPHIDETYLFWIGVTSVLLLFIYAYMYILWKAGIDCSFWNESYLTGSRDERKKSLLSKFGMDEGVTFMFI  240
155  CPRPCSELFPILPNDYLLSWLLFIAFLFSGIITYYGHVLWKA-----HGHVASNIFEMLRIDEGLR-----  215

241  GRFDRGQKGVDVLLKAIEILSSKKEFQEMRFIIGKGDPELEGWARSLEEKHG---NVKVITEMLSREFVRELYGSVDFV  317
216  -----LKIYKDTEGYTIGIGHLLTKSPSLNAAKSELDKAIGRNTNGVITKDEAEKLFNQVDAAVRGI  279

318  IIPSYFEPF--GLVALEAMCLGAIPASAVGGLRDIITNETGILVKAGDPGELANAILKALELSRSDLKSFRENCKKRAM  395
280  LRNAKLKPVYDSLDAVRRAALINMVFMGETGVAGFTNSLRMLQKRWDEAAVNLAKSRWYNQTPNRAKRVITTFRTGTW  359

396  SFSQDQARMDIRLAKTLVLILVVLICWGPLLAIMVYDVF GKMNKLIKTVFAFCSMCLLLNSTVNPIIYALRSKDLRHA--  473
360  DAYARMRLDVELAKTLGLVLAVLLICWFPVLAALMAHSLATLSDQVKKAFAFCSMCLLLNSMVNPVIYALRSEIIRSSAH  439

474  -----FRSMF  478
440  HCLAHWKCCVRGLG  453

```

Figure C2: P-CBRs alignment of CB₁ and CB₂. This figure illustrates the sequence alignment of the pseudo-endocannabinoid binding receptors CB₁ and CB₂, highlighting the homology and differences between these two protein sequences. Areas of high sequence similarity are indicative of conserved functional domains (Red), essential for receptor binding and activity, while regions of divergence (blue) may suggest differences in ligand specificity and physiological roles. Generated with NCBI BlastP (Altschul et al., 1990).

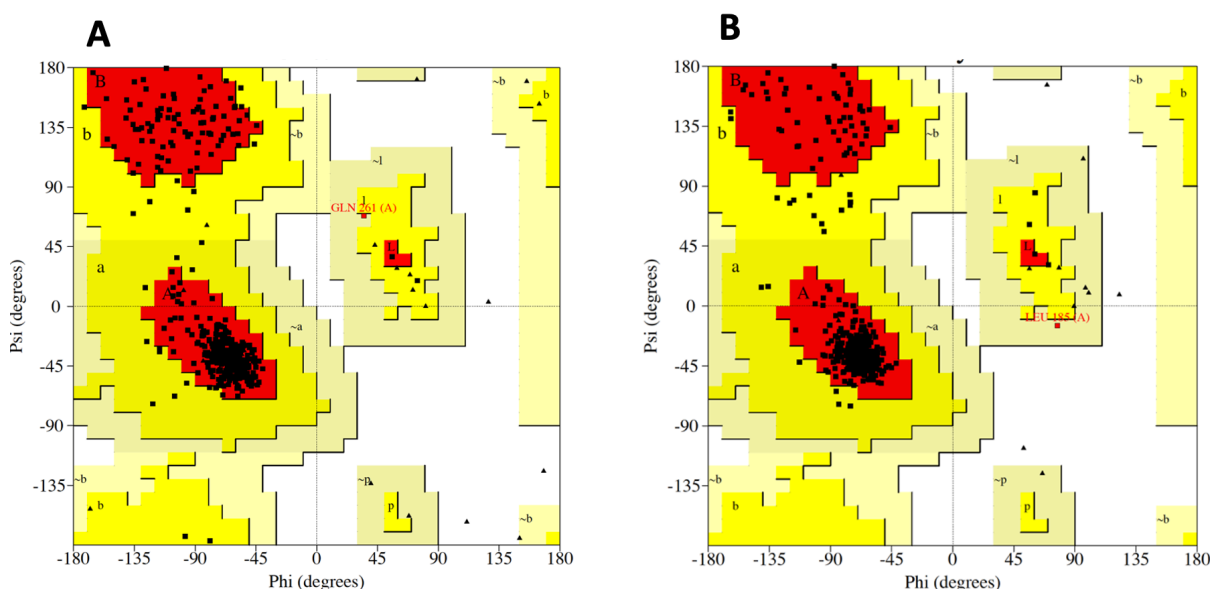


Figure C3: Ramachandran plots of homology modelled endocannabinoid receptors CB₁ and CB₂. Ramachandran plots illustrating the distribution of phi (Φ) and psi (Ψ) torsion angles for backbone amino acids in the homology models of cannabinoid receptors CB₁ and CB₂. Homology modelling was based on the crystallographic templates of PDB entries 5U09 for CB₁ A. and 5ZTY for CB₂. B. Red areas indicate the most favoured regions for amino

acid torsion angles, and yellow to light yellow zones represent additional and generously allowed conformations with white representing disallowed regions. Evaluated with PROCHECK (Chen et al., 2010).

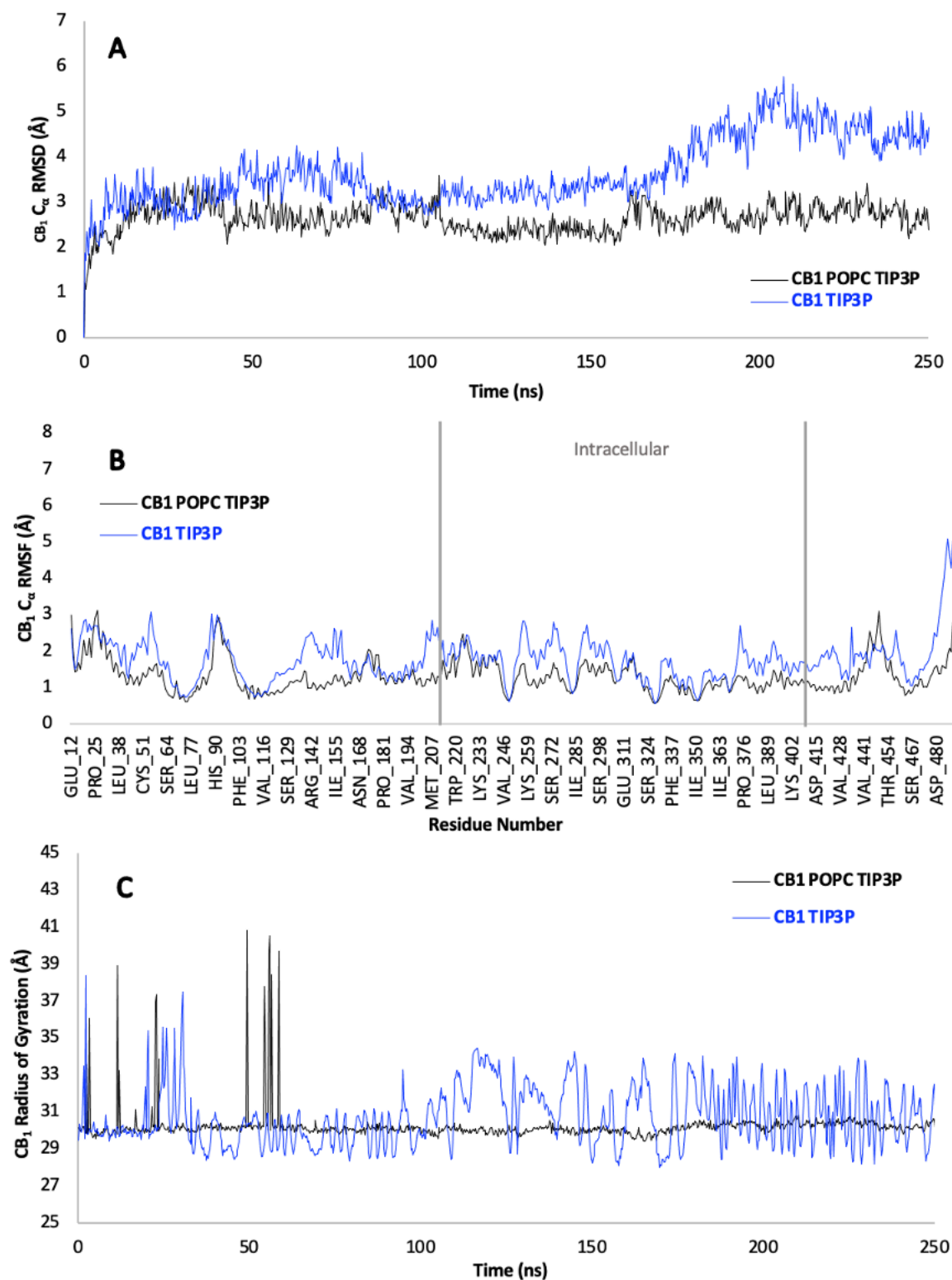


Figure C4: Comparative analysis of CB_1 receptor dynamics in two different environments: A. C_α RMSD of the CB_1 receptor in a membrane (POPC) and solvent system versus a water-only (TIP3P) system over 250 ns, indicating overall structural stability. B. C_α RMSF of CB_1 receptor residues in both systems, highlighting the

flexibility of each amino acid residue. C. Radius of gyration for the CB₁ receptor comparing membrane and water-only systems, reflecting the compactness of the protein structure over time.

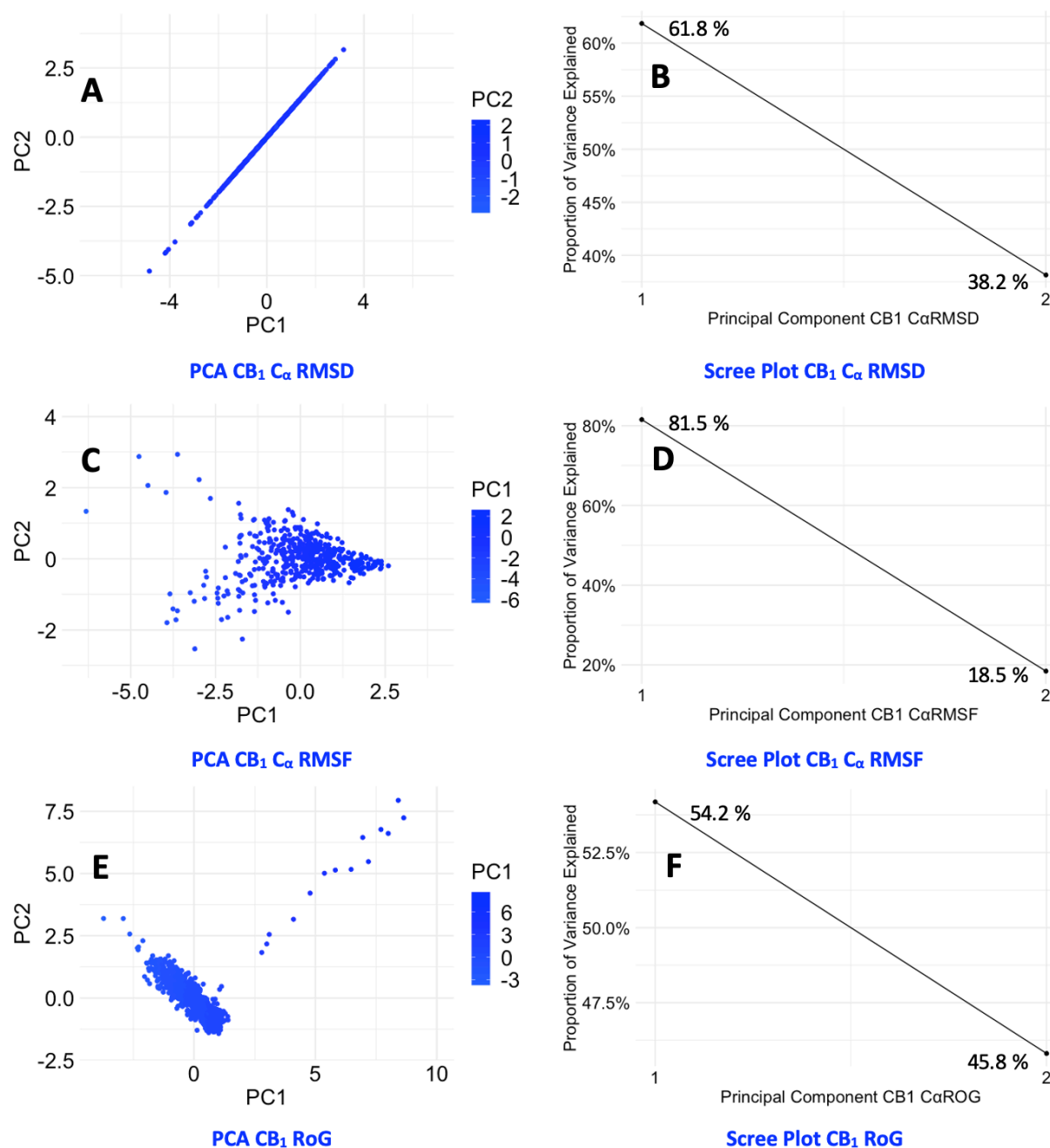


Figure C5: PCA and scree plots showing the molecular dynamics analyses of the CB₁ receptor in different environments. A. C_α RMSD over time, comparing CB₁ in a membrane (POPC) and in a TIP3P system with B. showing the scree plot for C_α RMSD. C. Residue-wise C_α RMSF, highlighting the flexibility of individual amino acids in both systems, with an intracellular region label along with its D. scree plot. E. RoG over time, indicating the compactness of the CB₁ receptor structure in each environment with its corresponding F. scree plot.

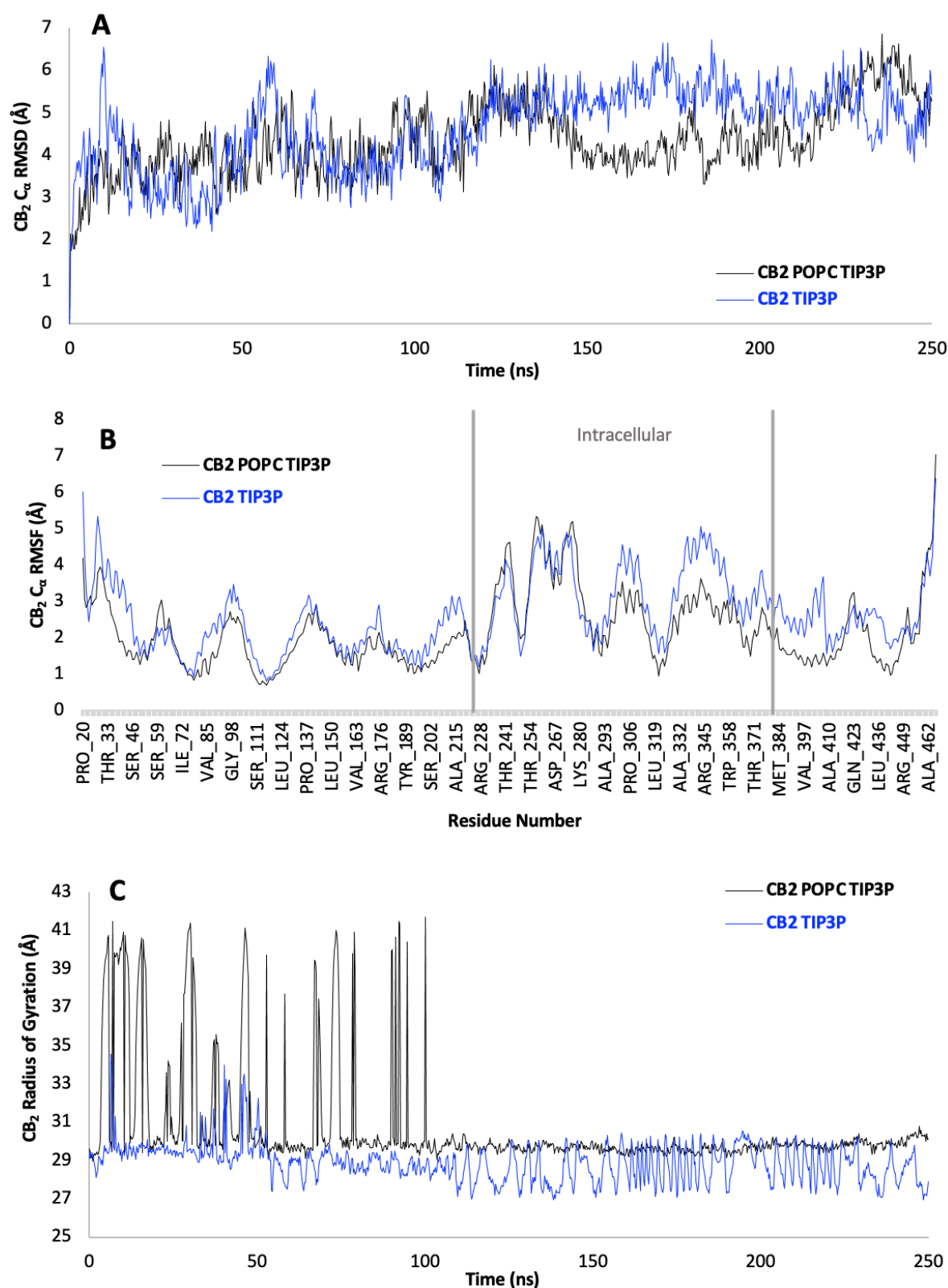


Figure C6: Dynamic properties of the CB_2 receptor in lipid membrane versus aqueous solvent. A. C_α RMSD trends over 250 ns show the structural stability of CB_2 in both environments. B. C_α Residue-specific RMSF profiles reveal regions of flexibility. C. RoG analysis compares the overall shape and compactness of the receptor in the systems.

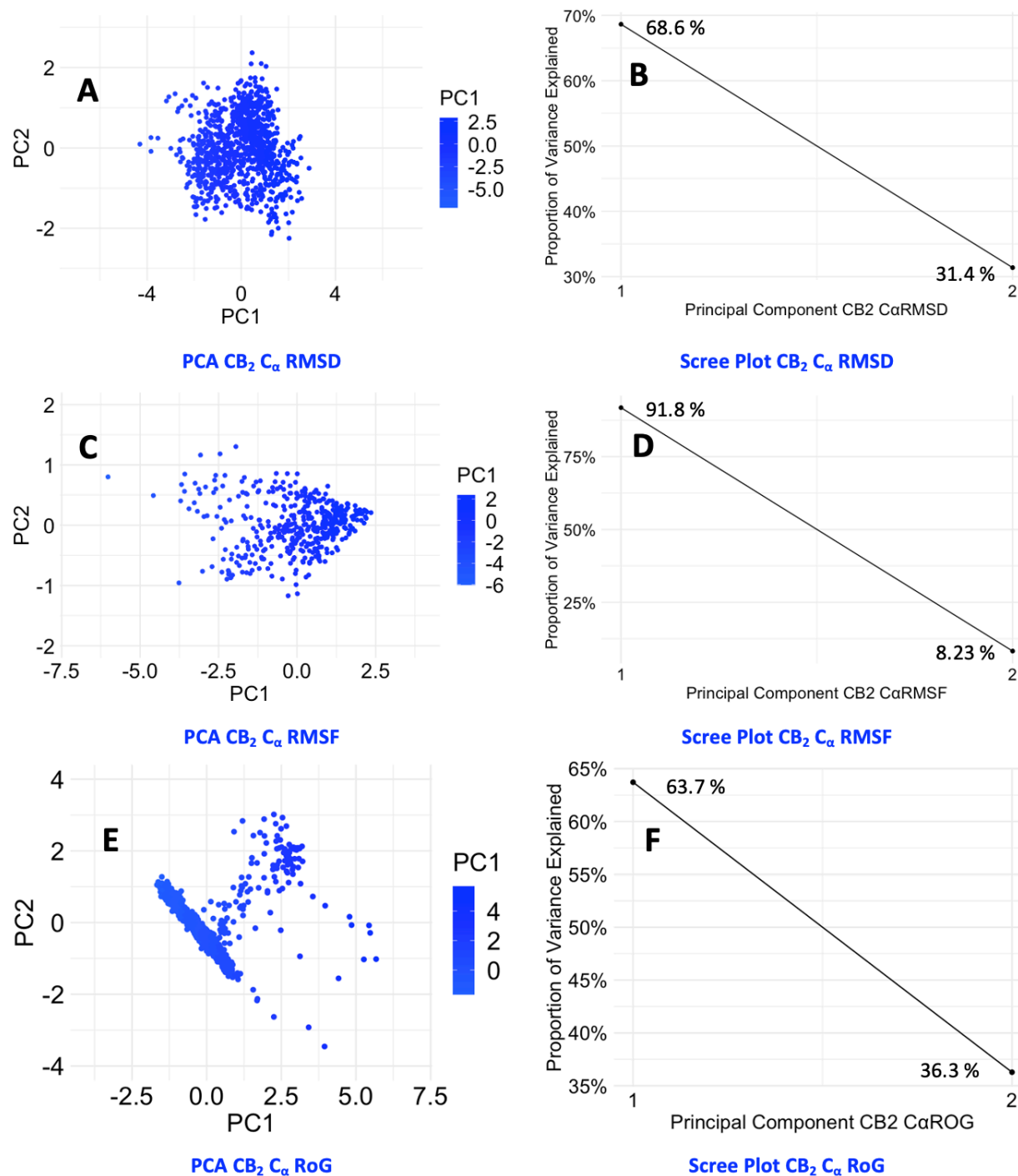


Figure C7: Comparative analysis of CB_1 receptor dynamics in two distinct environments. A. The C_α RMSD of the CB_1 receptor in a membrane (POPC) and solvent system versus a TIP3P system over 250 ns, showing structural stability. C. The C_α RMSF of CB_1 receptor residues in both environments, indicating the dynamic flexibility of the protein. E. The RoG analysis for CB_1 receptor in each system, depicting the protein's compactness throughout the simulation.

APPENDIX D

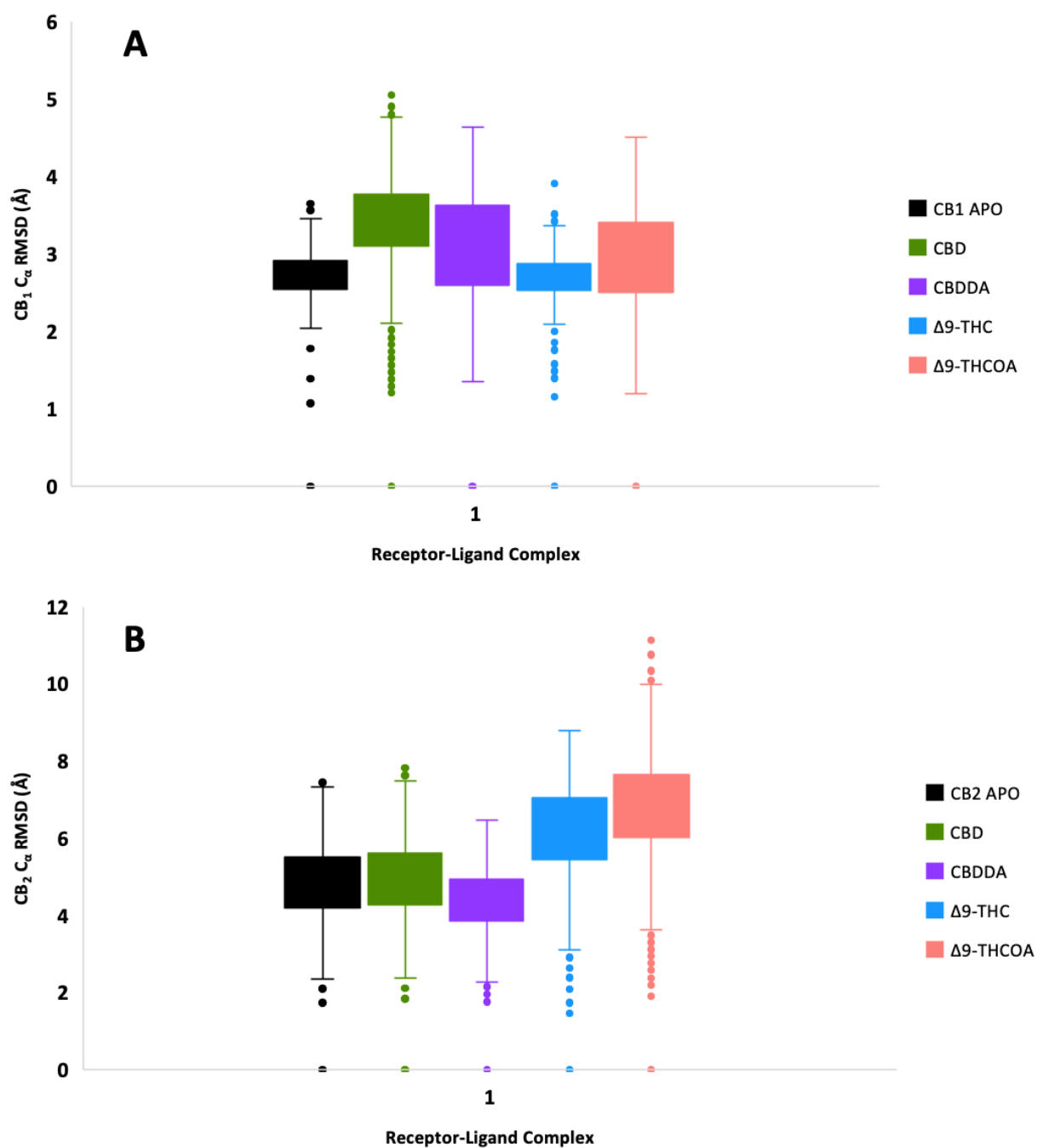


Figure D1: A. and B. Illustrate the C_α RMSD of the CB₁ and CB₂ receptors, respectively, in the absence and presence of various ligands. Each box plot represents the spread of C_α RMSD values for a specific receptor-ligand complex. The black box corresponds to the CB₁/CB₂ receptor in its apo state unbound. The green box shows the C_α RMSD values when bound to CBD, the purple box corresponds to CBDDA, the blue box to Δ⁹-THC, and the pink box to Δ⁹-THCOA. Outliers are indicated by individual points beyond the whiskers. The central line in each box denotes the median C_α RMSD value, and the boxes extend from the first to the third quartiles of the data.

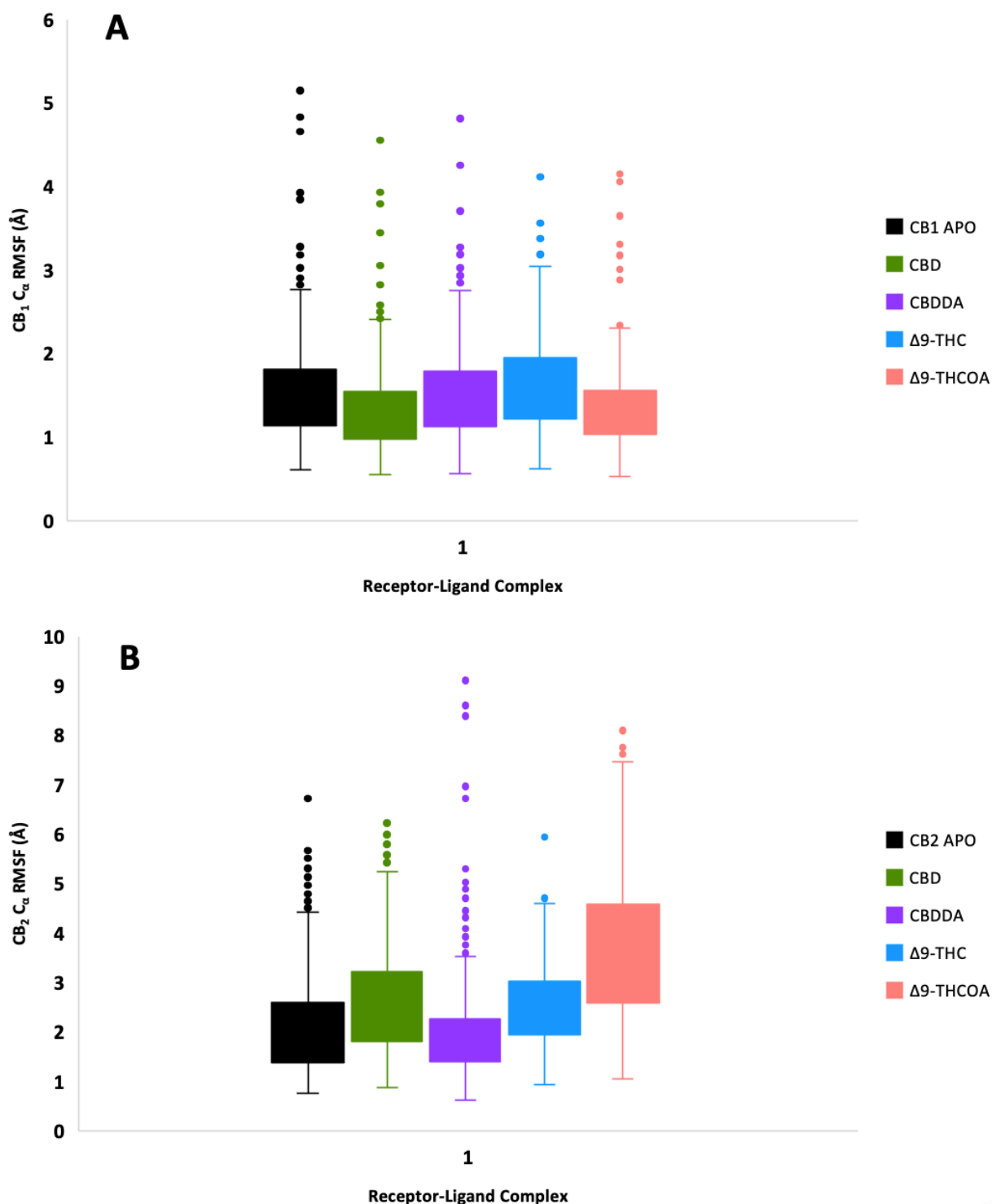


Figure D2: C_α RMSF of CB₁ and CB₂ receptors in various ligand-bound states. A. Represents the C_α RMSF for the CB₁ receptor when unbound and when bound to CBD, CBDDA, Δ⁹-THC, and Δ⁹-THCOA. B. Shows the C_α RMSF for the CB₂ receptor in the same states. C_α RMSF is measured in Å and provides insights into the flexibility of different receptor regions upon ligand binding. The box plots highlight the median C_α RMSF value (central line), the spread of the data (box), and the extent of variation (whiskers); outliers are noted as individual dots.

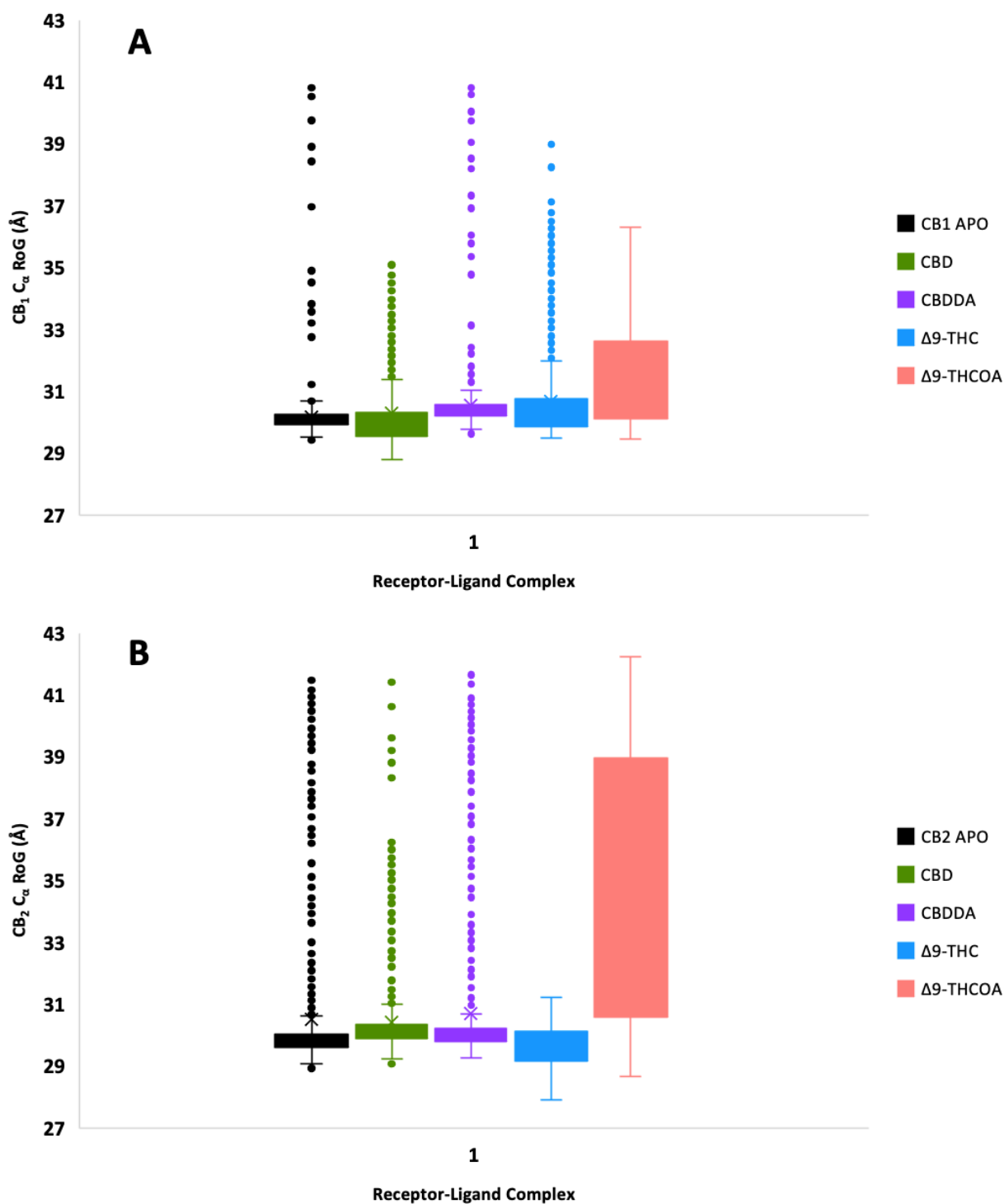


Figure D3: Comparative RoG measurements of CBRs. A. Presents RoG data for CB₁ receptor with CBD, CBDDA, Δ^9 -THC, and Δ^9 -THCOA, alongside its apo form. B. Illustrates RoG values for the CB₂ receptor with the same ligands. RoG is expressed in Å, denoting structural compactness. Box plots indicate median (line), interquartile range (box), and variation (whiskers); points mark outliers.

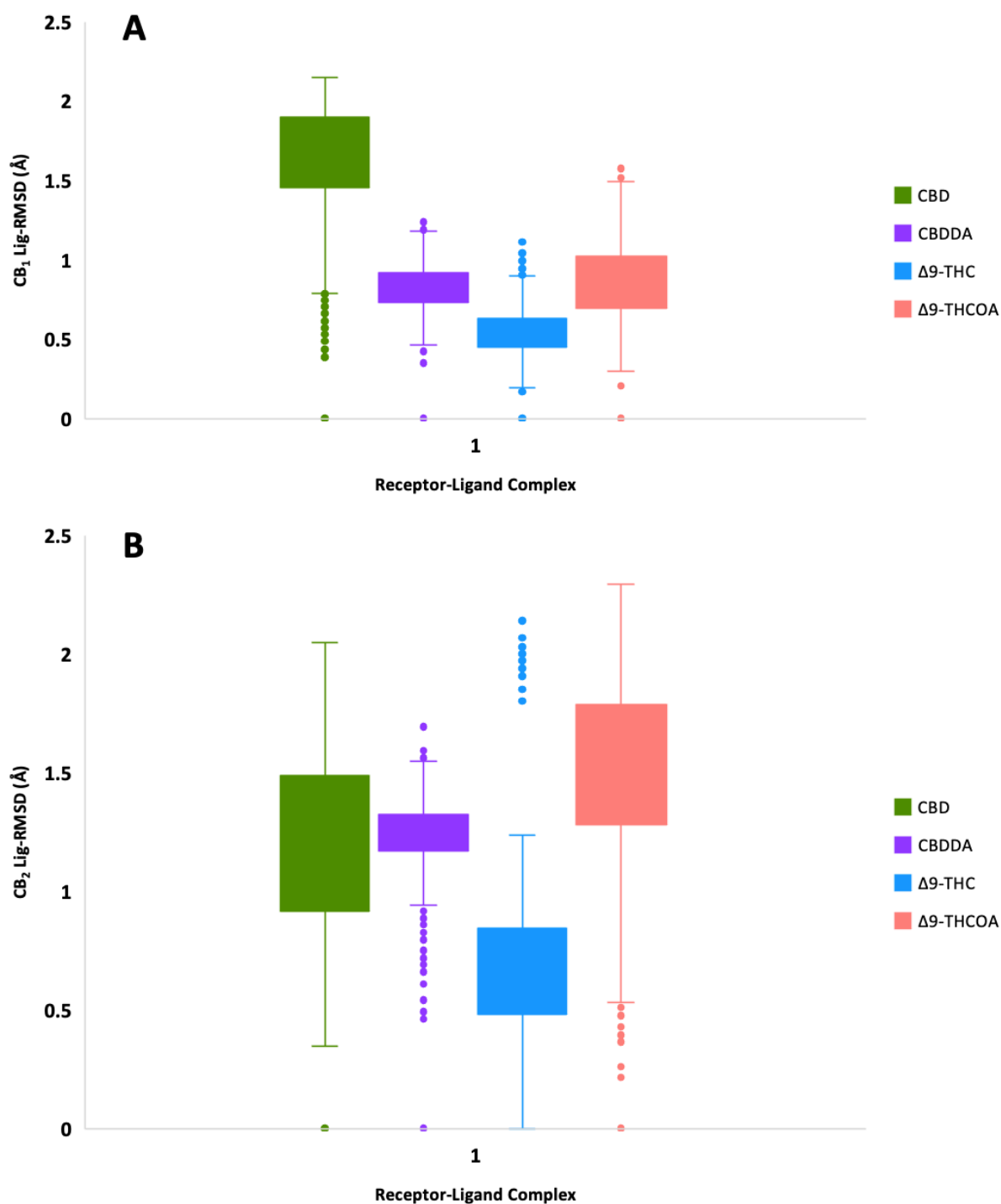


Figure D4: Conformational variability of CB₁ and CB₂ receptors bound to phytocannabinoids. A. Displays the lig-RMSD of CB₁ receptor complexes with CBD, CBDDBA, Δ⁹-THC, and Δ⁹-THCOA. B. Shows the lig-RMSD for CB₂ receptor complexes under the same conditions. Lig-RMSD is reported in Å, reflecting conformational changes upon ligand binding. Box plots represent the median (central line), interquartile range (box), and 1.5x IQR (whiskers); points indicate outliers.

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