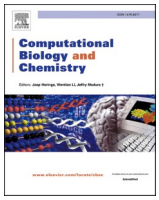
Computational Biology and Chemistry 112 (2024) 108138

Contents lists available at ScienceDirect

Computational Biology and Chemistry

journal homepage: www.elsevier.com/locate/cbac



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

Marushka Soobben, Yasien Sayed, Ikechukwu Achilonu *

Protein Structure-Function Research Unit, School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg 2050, South Africa

ARTICLE INFO

Keywords:
Cannabinoid receptors
Bioinformatics
Evolutionary trajectory
Functional conserved domains

ABSTRACT

The bioinformatic analysis of cannabinoid receptors (CBRs) CB_1 and CB_2 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 CB_1 and 5ZTY for CB_2) 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.

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 endocannabinoid system (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

* Corresponding author.
E-mail address: Ikechukwu.Achilonu@wits.ac.za (I. Achilonu).

https://doi.org/10.1016/j.compbiolchem.2024.108138
Received 7 March 2024; Received in revised form 24 May 2024; Accepted 20 June 2024
Available online 25 June 2024

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 CB_1 and CB_2 receptors are vital for cellular signalling and homeostasis (Graham et al., 2009). CB_1 receptors, predominantly located in the brain and central nervous system (CNS), are critical in modulating neurotransmitter release and neuronal activity. They influence functions such as cognition, memory, and pain perception. CB_2 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, CB_1 and CB_2 receptors are key mediators in the ECS, orchestrating a vast array of physiological processes through their specific signalling pathways.

The significance of CB_1 and CB_2 receptors extends beyond their physiological roles, presenting a frontier for bioinformatics research that holds promise for innovative therapeutic applications. The study of CB_1 and CB_2 receptor bioinformatics is essential for advancing research in areas such as molecular docking and molecular dynamics (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 synthetic cannabinoids (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 is to systematically characterise the bioinformatics profiles of cannabinoid receptors CB_1 and CB_2, 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 is designed to unravel the structural details, evolutionary history, and functional attributes of these receptors. This study seeks to identify conserved domains and patterns within receptor sequences, thereby establishing a comprehensive framework that elucidates their potential physiological impacts when targeted by ligands. By providing a detailed bioinformatics compendium on CBRs, this research will facilitate future investigations 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 National Centre for Biotechnology Information (NCBI) Conserved Domain Search (CD-Search) service (https://blast.ncbi.nlm.nih.gov/) (Lu et al., 2020; Marchler-Bauer et al., 2017; Sayers et al., 2022). The amino acid sequences retrieved from UniProtKB (https://www.uniprot.org/) (CB_1 ID: P21554) (CB_2 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 (PRK), 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 CB_1 receptor, particularly concerning human evolution. The cladogram depicted various mammalian groups, from rodents to primates, highlighting the CB_1 receptors evolutionary significance. 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 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, Reference Sequence Database (RefSeq), and Research Collaboratory for Structural Bioinformatics Protein Data Bank (https://www.rcsb.org/) (RCSB PDB), enhancing the possibility of identifying homologous sequences and providing an evolutionary framework for the receptors (Berman et al., 2000). Sequences of CB_1 and CB_2 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 and 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 NCBIs tools, 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). In order to study and visualise the 2D sequence alignment, PyMOL was used for both the AlphaFold-predicted structures and pseudo-CBRs and the PDB structures of CB_1 and CB_2 (Schrodinger, 2015). Specifically, the 3D structures of these proteins were obtained from AlphaFold (IDs AF-P21554-F1-model_v4 and PDB5U09 for CB_1 and AF-P34972-F1-model_v4 and PDB5ZTYfor CB_2). PyMOL, a molecular visualisation software, was utilised for the structural alignment and visualisation of these proteins.

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 (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR002230/) (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 3D structures of proteins. Interpret Additionally, PTM data obtained from UniPort includes data with ECO codes ECO:0000255 and ECO:0000256 as well as publications. 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 (https://string-db.org/), 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 CB_1 and CB_2 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 Log10(observed/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 CB_1 and CB_2 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

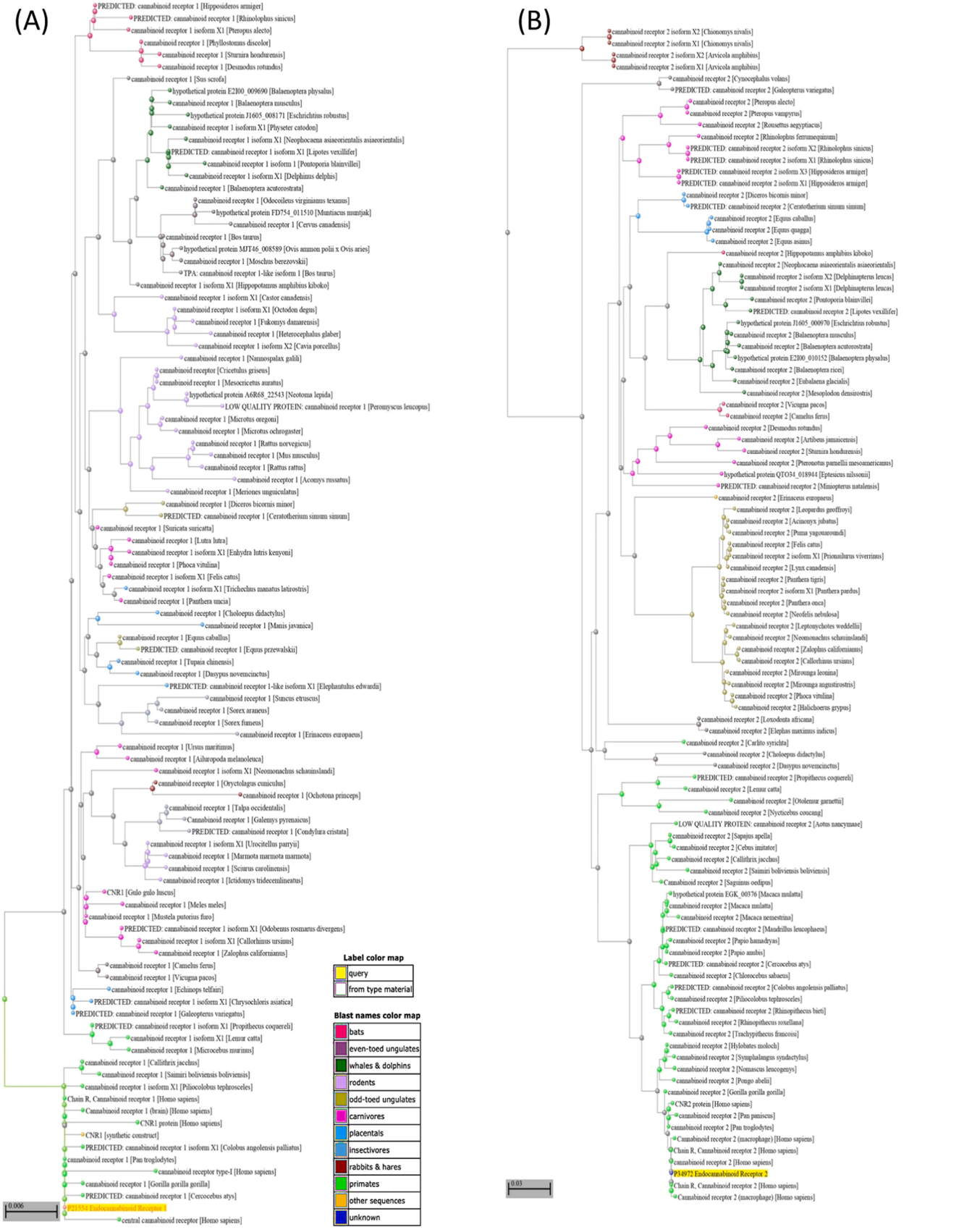
3.1. Evolutionary relationships

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 CB_1 (Fig. 1A), provides an indication into the widespread distribution and evolutionary conservation of CB_1 within the mammalian kingdom. The CB_1 receptors presence in diverse taxa, such as bats, rodents, carnivores, and even-toed ungulates, is 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 eighteen under whales, dolphins, and ungulates signals a considerable degree of research interest and genetic diversity in these species. This diversity highlights the receptors evolutionary adaptability and suggests its pivotal role in the survival and development of these mammals.

Human CB_1 receptors are represented with particular emphasis, denoting a multitude of studies that reflect extensive research interest as there are 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 is 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 CB_1 receptors to those of humans suggests a shared heritage and functional conservation among primates. This trajectory suggests that the receptors evolutionary journey has been integral to the adaptation and survival of mammals, dating back to a common ancestor. Additionally, this cladogram does more than just trace the evolutionary lineage of the CB_1 receptor; it highlights the receptors essential role 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. 1B) traces the evolutionary history of CB_2 across various species, showcasing its divergence and distribution within the animal kingdom (Kitching, 1998). The evolutionary path of the human CB_2 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 CB_2 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 CB_2 receptor within this group, suggesting that while the receptor is conserved, there are differences that have arisen through primate evolution. The human CB_2 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 suggests that the basic structure and function of the CB_2 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 CB_2 receptor has undergone. Notably, the tree indicates that the CB_2 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 CB_2 receptor to its specific ecological niche and physiological needs. Overall, the cladogram illustrates that the CB_2 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 CB_2 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.



(caption on next page)

Fig. 1. The cladogram presented here provides a comprehensive view of the evolutionary relationships of CB_1 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 CB_1 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 CB_1 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 CB_1, 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 CB_2 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. Each nodes colour 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 CB_2 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 CB_2 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

Fig. 2 illustrates the comparative sequence alignment of the amino acid sequences for CB_1 and CB_2 across various species. The CB_1 alignment (Fig. 2A) 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_CB_1 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 CB_1 receptors (Eric W. Sayers et al. 2020; Hanson et al., 2012). Similarly, the CB_2 alignment (Fig. 2B) 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 et al. 2020). The accession number cd15341 in NCBI CDD specifically identifies the conserved domain unique to the CB_2 receptors (Lu et al., 2020; Marchler-Bauer et al., 2017). The alignments for both CB_1 and CB_2 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. 2), labelled as Feature 1 and highlighted in yellow, are 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 CB_1 (Fig. 2A) and pink for CB_2 (Fig. 2B) respectively, define the structure of GPCRs. They traverse the cellular membrane and are essential for the receptor's role in signal transduction from external to internal cellular environments. The evolutionary conservation seen in the alignment (Fig. 2) 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

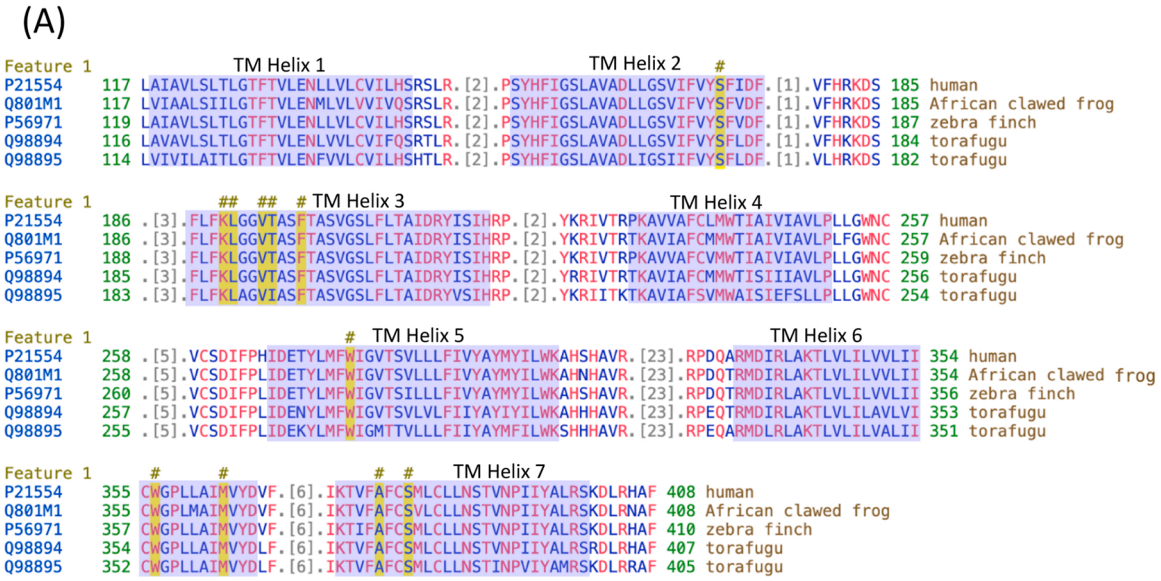


Fig. 2A. This figure displays the conserved domains of the CB_1 receptor as identified by a BLASTp search within the Conserved Protein Domain Family 7tmA_CB_1 (Accession number: cd15340). Highlighted in yellow are the regions representing Feature 1, the putative ligand binding sites, which are predicted 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 receptor's structure, 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 these domains across diverse taxa. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997; Altschul et al., 2005).

Fig. 2B. This figure illustrates the sequence alignment of the CB_2 receptor within the Conserved Protein Domain Family 7tmA_CB_2, (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 TM domain in maintaining receptor activity and integrity. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997, 2005).

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.

The alignment (Fig. 3A) comparing FASTA sequences of both CBRs, as obtained from the UniProtKB database illustrates 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 CB_1 and CB_2 receptor sequences suggests that while they have evolved to potentially have different physiological roles, there is a considerable degree of conservation. This conservation

A

Fig. 3A. A sequence alignment between CB_1 and CB_2, highlighting the conserved and variable regions across the two sequences. Amino acids (red) indicate identical matches between the sequences while residues (blue) represent unidentical 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, 2005).

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.

Figs. 3B and 3C provide sequence alignments for the CB_1 and CB_2 receptors, comparing their FASTA sequences from UniProtKB with their corresponding engineered pseudo-cannabinoid 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 CB_1 and 98.51 % for CB_2, 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-CB_1 and p-CB_2 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.

Fig. 3D presents a detailed sequence alignment of the p-CBRs CB_1 and CB_2, highlighting both conserved and divergent regions between these two proteins. The red-highlighted areas indicate regions of high sequence similarity, representing conserved functional domains crucial for receptor binding and activity. These conserved domains are likely essential for core functionalities such as ligand binding, signal transduction, and maintaining structural stability. In contrast, the blue-highlighted areas denote regions of divergence, suggesting differences in amino acid sequences that could result in varied ligand specificity and physiological functions. Divergent regions might affect how each receptor interacts with its environment, influencing signal transduction pathways and receptor activation mechanisms. This understanding is crucial for designing selective ligands or drugs targeting either p-CB_1 and p-CB_2, thereby minimising off-target effects and enhancing therapeutic efficacy. Additionally, the alignment offers evolutionary insights, helping to trace the divergence of these receptors from a common ancestor and understand the molecular basis of their functional diversification.

The tertiary structure alignments of different representations of CB_1 and CB_2 proteins are illustrated by Fig. 3E and F, highlighting their conserved and divergent regions. Fig. 3E presents the AlphaFold-predicted structures of CB_1 (AF-P21554-F1) and CB_2 (AF-P34972-F1), showcasing their tertiary alignment. These conserved regions are essential for maintaining functional and structural integrity, highlighting crucial functional domains necessary for receptor binding and activity. Fig. 3F displays the tertiary structures of p-CB_1 and p-CB_2, with the red regions denoting conserved areas identified through sequence alignment. These conserved regions are crucial for maintaining the functional and structural integrity of the proteins, as they represent essential domains for receptor activity and binding. The overall alignment offers insights into the similarities and differences between these two p-CBRs, highlighting potential variations in ligand specificity and physiological roles. Both figures emphasise the structural conservation between CB_1 and CB_2, with the red regions marking the conserved residues identified through sequence alignment. These conserved areas are pivotal for the functionality of the receptors, underpinning essential activities such as ligand binding and signal transduction. The differences highlighted in these figures, particularly in the non-conserved regions, suggest variations in ligand specificity and physiological roles between CB_1 and CB_2.

B

Fig. 3B. Sequence alignment of CB_1 and p-CB_1 receptors. This alignment compares the primary amino acid sequences of the UniProtKB (P21554) CB_1 receptor (top) with the PDB (5U09) p-CB_1 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 CB_1 receptor sequence in the p-CB_1 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, 2005).

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1    MEECWVTEIANGSKDGL---DSNPMKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRKPSYLFIGSLA  77
1    MK----TIIALSYIFCLVFAGAPPMKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRKPSYLFIGSLA  76

78   GADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTASVGSLLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIM  157
77   LADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTASVGSLLLAAIDRYLCLRYPPSYKALLTRGRALVLLGIM  156

158  WVLSALVSYLPLMGWTCCPRPCSELFPLIPNDYLLSWLLFIAFLFSGIIYTYGHVLWKAHQHVASL---------------  223
157  WVLSALVSYLPLMGWTCCPRPCSELFPLIPNDYLLSWLLFIAFLFSGIIYTYGHVLWKAHQHVASNIFEMLRIDEGLRLK  236

224  -------------SGHQDRQVPGM-------------------------------------------------------  234
237  IYKDTEGYYTIGIGHLLTKSPSLNAAKSELDKAIGRNTNGVITKDEAEKLFNQDVDAAVRGILRNAKLKPVYDSLDAVRR  316

235  ------------------------------------------------------------------ARMRLDVRLAKTLGL  249
317  AALINMVFQMGETGVAGFTNSLRMLQQKRWDEAAVNLAKSRWYNQTPNRAKRVITTFRTGTWDAYARMRLDVELAKTLGL  396

250  VLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSMVNPVIYALRSGEIRSSAHHCLAHWKKCVRGLGS--E  327
397  VLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSMVNPVIYALRSEEIRSSAHHCLAHWKKCVRGLGEFLE  476

328  AKEEAPRSSVTETEADGKITPWPDSRDLDLSDC  360
477  VLFQGPHHHHHHHHHH--------DYKDDDDK--  500
```

C

Fig. 3C. Sequence alignment of CB_2 (UniProtKB ID: P34972) and p-CB_2 Receptor (PDB ID: 5ZTY). This figure presents an alignment between the FASTA amino acid sequence of the CB_2 receptor, as sourced from UniProt (P34972), and the p-CB_2 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. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997, 2005).

```
1    ENFMDIECFMVLNPSQQLAIAVLSLTLGTFTVLENLLVLCVILHSRSLRCRPSYHFIGSLAVADLLGSVIFVYSFIDFHV  80
1    -----PMKDYMILSGPQKTAVAVLCTLLGLLSALENVAVLYLILSSHQLRRKPSYLFIGSLALADFLASVVFACSFVNFHV  76

81   FHRKDSRNVFLFKLGGVTASFTASVGSLFLAAIDRYISIHRPLAYKRIVTRPKAVVAFCLMWTIAIVIAVLPLLGWNCEK  160
77   FHGVDSKAVFLLKIGSVTMTFTASVGSLLLAAIDRYLCLRYPPSYKALLTRGRALVLLGIMWVLSALVSYLPLMGWTC--  154

161  LQSVCSDIFPHIDETYLMFWIGVTSVLLLFIVYAYMYILWKAGIDCSFWNESYLTGSRDERKKSLLSKFGMDEGVTFMFI  240
155  CPRPCSELFPLIPNDYLLSWLLFIAFLFSGIIYTYGHVLWKA-----------------HQHVASNIFEMLRIDEGLR-----  215

241  GRFDRGQKGVDVLLKAIEILSSKKEFQEMRFIIIGKGDPELEGWARSLEEKHG---NVKVITEMLSREFVRELYGSVDFV  317
216  ------------------LKIYKDTEGYYTIGIGHLLTKSPSLNAAKSELDKAIGRNTNGVITKDEAEKLFNQDVDAAVRGI  279

318  IIPSYFEPF--GLVALEAMCLGAIPIASAVGGLRDIITNETGILVKAGDPGELANAILKALELSRSDLSKFRENCKKRAM  395
280  LRNAKLKPVYDSLDAVRRAALINMVFQMGETGVAGFTNSLRMLQQKRWDEAAVNLAKSRWYNQTPNRAKRVITTFRTGTW  359

396  SFSDQARMDIRLAKTLVLILVVLIICWGPLLAIMVYDVFGKMNKLIKTVFAFCSMLCLLNSTVNPIIYALRSKDLRHA--  473
360  DAYARMRLDVELAKTLGLVLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSMVNPVIYALRSEEIRSSAH  439

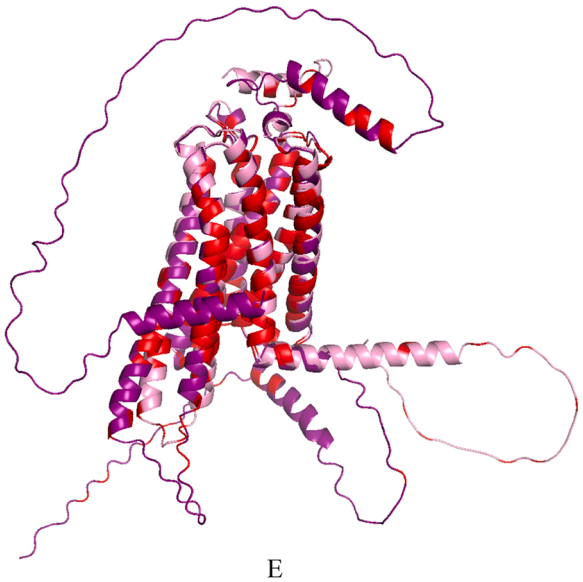
474  ----------FRSMF  478
440  HCLAHWKKCVRGLG  453
```

D

Fig. 3D. Alignment of pseudo-cannabinoid receptors CB_1 and CB_2. This figure showcases the sequence alignment of the p-CBR binding receptors CB_1 and CB_2, emphasising the homology and differences between these two protein sequences. Regions of high sequence similarity are marked in red, indicating conserved functional domains crucial for receptor binding and activity. Conversely, areas of divergence are highlighted in blue, suggesting potential variations in ligand specificity and physiological functions. Generated from NCBI BLASTP 2.15.0+ (Altschul et al., 1997, 2005).

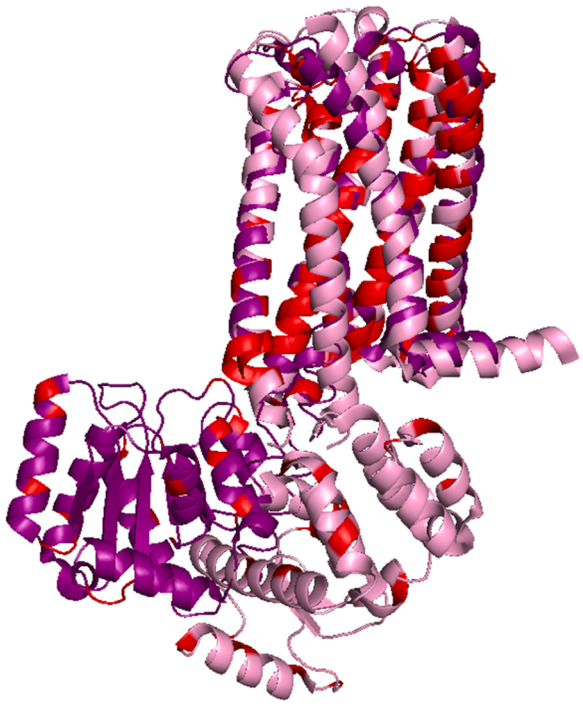
3.3. Characteristic protein features

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), CB_1, CB_2, 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. 4), 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.



E

Fig. 3E. Tertiary Structure Alignment of AlphaFold-Predicted CB_1 and CB_2 Proteins. This figure presents the AlphaFold-predicted structures of CB_1 (AF-P21554-F1, purple) and CB_2 (AF-P34972-F1, pink), showcasing their tertiary alignment. The alignment, indicated in red, highlights the conserved regions identified through sequence alignment, illustrating their spatial correlation within the 3D structure. The conserved regions are essential for maintaining functional and structural integrity, while the overall structural alignment provides insights into the similarities and differences between these two pseudo-endocannabinoid binding receptors. The image was generated using PyMOL, with red areas marking the aligned residues from the 2D sequence alignment.



F

Fig. 3F. Tertiary Structure Alignment of p-CB_1 and p-CB_2 Proteins. This figure displays the tertiary structures of p-CB_1 (purple) and p-CB_2 (pink), highlighting their alignment. The red regions denote conserved areas identified through sequence alignment, demonstrating their spatial relationship within the 3D structures. These conserved regions are crucial for maintaining the functional and structural integrity of the protein. The overall alignment offers insights into the similarities and differences between these two p-CBRs. The image was generated using PyMOL, with red areas indicating the residues aligned from the 2D sequence alignment.

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 CB_1 and CB_2 utilise a toggle switch (Table 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 CB_1, activation involves the synergistic movement of twin toggle switch residues Phe3.36 and Trp6.48, whereas CB_2 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 CB_1 and CB_2, 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 domains contributions to the function of cannabinoid receptors is fundamental to grasping their mechanism of action and developing targeted interventions within the endocannabinoid signalling pathways.

Table 1
Attributes of CB_1 and CB_2.

Attributes	Cannabinoid Receptor One	Cannabinoid Receptor Two
UniProtKB ID	P21554	P34972
Species	*Homo sapiens*	*Homo sapiens*
Protein Type	GPCR	GPCR
Gene Name	CNR1	CNR2
Protein Name	Cannabinoid receptor one (CB_1)	Cannabinoid 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

The crystal structures (PDB ID: 5U09 for CB_1 and 5ZTY for CB_2) 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 CB_1 receptor, illustrated in Fig. 4A, displayed a 3D conformation with seven TM helices, each color-coded for clarity. The N-terminal region was marked in red and the C-terminal in purple, providing a visual demarcation of the extremities of the receptors. Similarly, the CB_2 receptors structure demonstrated the same level of detail, with an

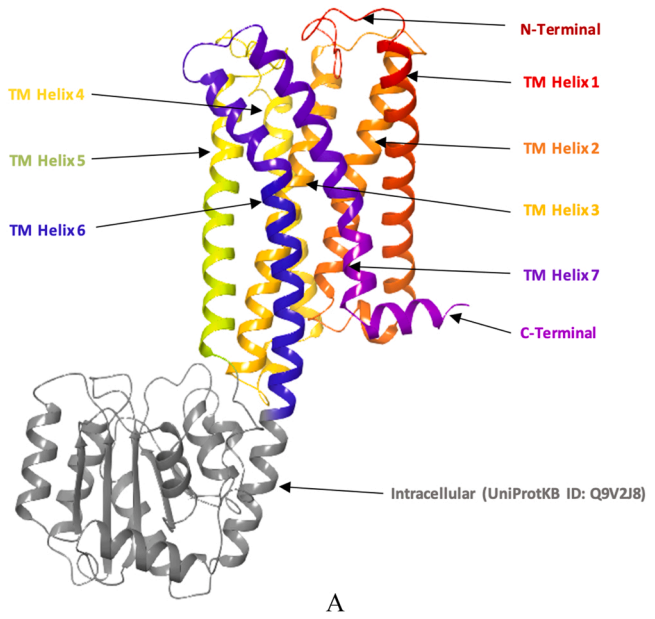


Fig. 4A. 3D Structural Representation of the CB_1 Receptor (PDB ID: 5U09). The receptor is shown with its seven TM helices, color-coded to differentiate each helix. The N-terminal and C-terminal regions are indicated, with the N-terminal shown in red and the C-terminal in purple. An intracellular stabilising region (grey) contributes to the stability of this receptor structure.

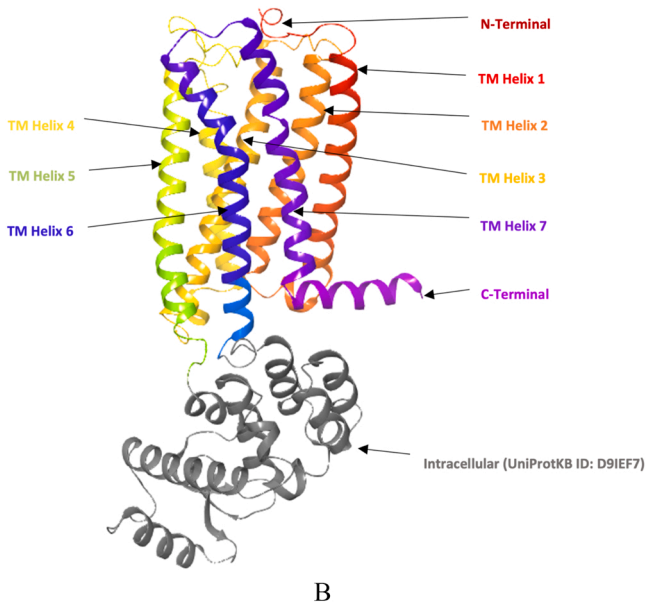


Fig. 4B. 3D Configuration of the CB_2 (PDB ID: 5ZTY). This representation features the seven TM helices that are fundamental to the receptor's structure, each helix presented in a distinct colour for visual clarity. The N-terminal region is shown at the top, transitioning through the helices to the C-terminal 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.

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.

The high-resolution crystal structure of the human CB_1 (Fig. 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 $Å^2$, and its volume of 321.277 $Å^3$, 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 CB_2 is not available on CASTp 3.0, analyses were inferred by analysing the CB_1 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 CB_1 and CB_2, 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).

3.4. Post-translation modifications

For the CB_1 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 receptors transport to the cell membrane, its ability to bind ligands, and its interactions with other cellular proteins. These glycosylation sites on the CB_1 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

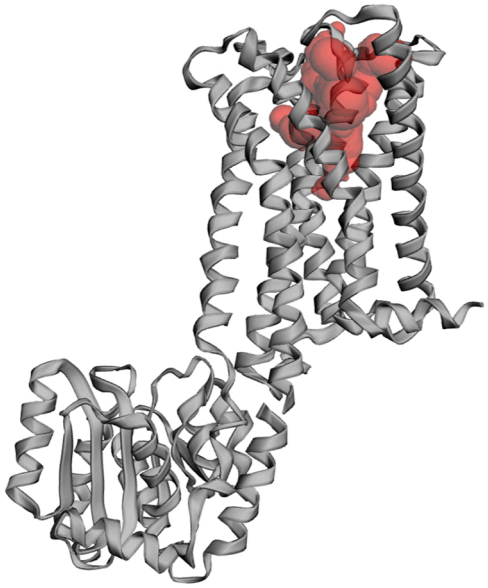


Fig. 5. A high-resolution crystal structure of the human CB_1 receptor, highlighting its binding pocket in red. The binding pocket, as identified by CASTp 3.0 with Pocket ID 1, showcases a surface area of 624.481 $Å^2$ and a volume of 321.277 $Å^3$, providing insights into the potential interactions within the receptors active site. Sourced from CASTp 3.0 (Tian et al#, 2018).

(PXD012174), plays a regulatory role in CB_1 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

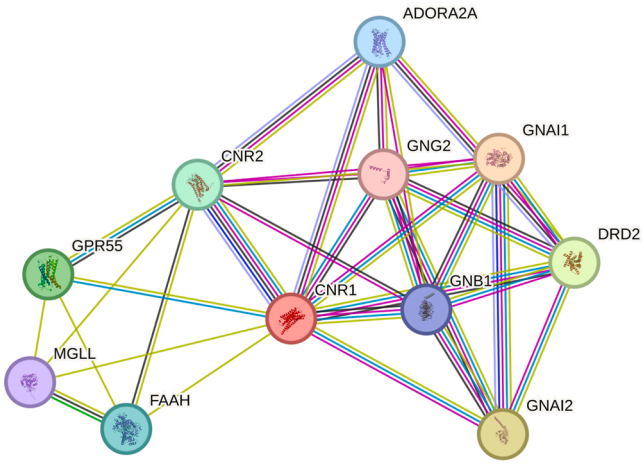
serine residues 425 and 429 is inferred by similarity (UniProtKB: P47746), meaning that analogous phosphorylation events have been observed in related proteins or in homologous proteins across different species such as *Mus musculus* (mouse). Such phosphorylation is presumed to be involved in receptor 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 particular 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 CB_1 receptor, particularly in its ability to localise within the membrane and its capacity for signal transduction (Oddi et al., 2012).

When analysing CB_2, the PTM of glycosylation at asparagine residue 11 is likely part of the extracellular domain of the receptor, which could significantly affect the ability of the receptor 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 CB_2 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 structure of the receptor 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 CB_2 receptor. This phosphorylation is involved in the receptors regulatory mechanisms, 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 CB_2 receptor function and the complex network of intracellular signalling cascades in which it participates.

3.5. Protein-protein interactions

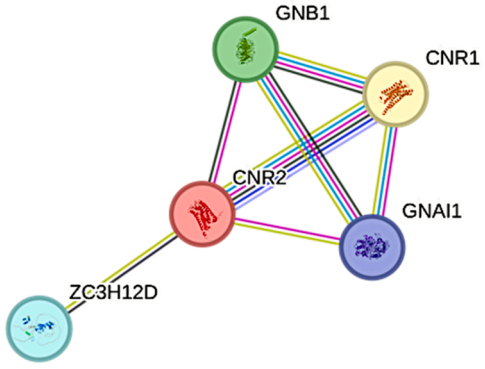
Fig. 6A reveals insights into how CB_1 and its associated proteins contribute to cellular function and how they might be targeted for therapeutic purposes centring around CB_1. This network has 11 nodes, each representing a protein, and 25 edges that show the interactions between them. The filled nodes 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 networks average node 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 (Szklarczyk et al., 2021).



A

Fig. 6A. figure illustrates a protein-protein interaction network with CB_1 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.

Fig. 6B depicts a PPI network. The nodes Guanine Nucleotide Binding Protein, Beta Polypeptide 1 (GNB1), CB_1, CB_2, 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



B

Fig. 6B. This figure presents a protein-protein interaction network with five nodes and seven edges. 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.

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.

3.6. Tissue specificity and isoform expression

The CB_1 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 CB_1 plays in neural development and regulation of neuronal function. The brains high expression levels indicate that CB_1 receptors are crucial for a variety of brain functions, including cognition, memory, pain sensation, and motor control. Interestingly, the expression levels of isoforms 2 and 3 of the CB_1 receptor are significantly lower than those of isoform 1 (Reggio, 2010). Isoform 1 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. The presence of multiple isoforms could reflect a mechanism allowing precise receptor function or a means to diversify the physiological roles of CB_1 across different tissues and developmental stages. The tissue-specific expression of CB_1, particularly its dominance in the brain, highlights the receptors potential as a therapeutic target in neurological conditions, where modulation of CB_1 activity could lead to beneficial outcomes (Mackie, 2008). Understanding the differential expression of its isoforms also provides deeper insight into the nuanced regulation of CB_1 receptor function and its implications in health and disease.

Isoform 2 of the CB_1 receptor, designated as CB1a, represents an intriguing splice variant of the human CB_1 receptor. First identified in 2005, this isoform was characterised by its unique starting sequence (Ryberg et al., 2005). Unlike the canonical CB_1 receptor, CB1a initiates at a downstream AUG codon, resulting in a truncated protein consisting of only 36 amino acids. Additional insights from 1991 explored the molecular cloning of this receptor variant, highlighting its expression in the testis and adding a layer of complexity to its functional role (Gerard et al., 1991). This finding suggests potential specialised roles in reproductive tissues or distinct regulatory mechanisms compared to the canonical CB_1 receptors. Moreover, this variant is described as an amino-terminal variant resulting from alternative splicing (Shire et al., 1995). The differences in the amino-terminal sequence could potentially affect how this isoform interacts with ligands or integrates into cellular signalling pathways. Overall, while the physiological relevance of CB1a remains to be fully elucidated, these studies highlight the complexity of cannabinoid receptor biology and the potential for diverse functional impacts depending on receptor structure and expression context.

Isoform 3 of the CB_1 receptor, known as CB1b, is another novel splice variant identified in the human CBR family. This variant is characterised by a specific deletion within its amino acid sequence, missing residues from positions 22 – 54. This deletion potentially alters the structural configuration of the receptor and may impact its binding affinity and signal transduction capabilities (Ryberg et al., 2005). The discovery of CB1b significantly enriches our understanding of the genetic and functional diversity within the ECS. This variant not only highlights the possibility of differential cannabinoid responses across various tissues and physiological or pathological states but also highlights its specific expression and functionality in extraneural tissues such as β-cells and hepatocytes (González-Mariscal et al., 2016). These tissues are pivotal for glucose metabolism but do not involve brain activities, thus identifying CB1b as a prime therapeutic target. The unique positioning of CB1b enables the development of targeted treatments for metabolic disorders such as diabetes, circumventing the neuropsychiatric side effects that are commonly associated with first-generation CB_1 receptor blockers that influence both central and peripheral receptors. This could pave the way for more precise and safer cannabinoid-based therapies, leveraging the absence of certain segments in CB1b that might affect its localisation on the cell membrane, interactions with G-proteins, or overall stability and expression levels.

The tissue specificity of the CB_2 indicates a preferential expression in cells related to the immune system, with notably higher levels found in B-cells and natura; killer (NK) cells. This expression profile is aligned with the receptors known involvement in modulating immune responses. The presence of CB_2 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 CB_2 expression in normal brain tissue suggests that, unlike CB_1, which is prevalent in the nervous system, CB_2 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 CB_2 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 CB_2 isoforms, resulting from alternative promoter usage and differing only in the 5′ untranslated region (UTR), underscores the functional versatility of the receptor. Notably, isoform CB2A, with its predominant expression in the testis and some regions of the brain, contrasts with the distribution of isoform CB2B in the spleen and leukocytes. This differential regulation and potential functional specialisation of these variants in various tissues suggest intricate regulatory mechanisms that could impact receptor function (Gerard et al., 1991; Liu et al., 2009). Additionally, the recent discovery of the CB2A and CB2B isoforms, where CB2A is expressed higher in the testis and brain and CB2B more so in other peripheral tissues, adds another layer of complexity to the CB_2 expression landscape. These findings on the differential expression and responsiveness of CB_2 isoforms, such as the increase in CB2A expression in the cerebellum after treatment with CBR agonist WIN55212–2, provide much improved insights into the gene structure and the human and rodent variants that should be considered when developing CB_2 receptor based therapeutic agents (Liu et al., 2009). This intricate expression pattern of CB_2, 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. 7) enrich our understanding of CB_1 and CB_2 receptors localisation within cells and their pivotal roles in physiological processes (Aleksander et al., 2023; Binns et al., 2009). For the CB_1 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

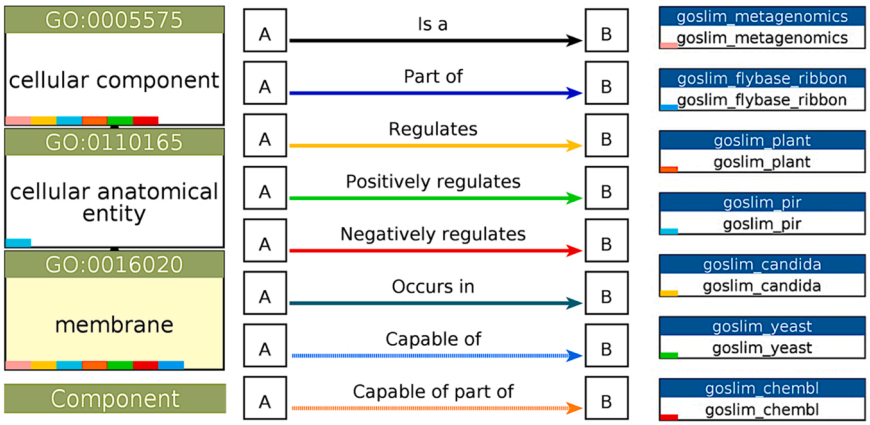


Fig. 7. Hierarchical representation of Gene Ontology (GO) terms associated with the cellular component category for the membrane (GO:0016020) in relation to CB_1 and CB_2 as classified by InterPro. The diagram illustrates membrane classification as a cellular component (green), which is a subset of a cellular anatomical entity within the broader category of cellular components. The membrane serves as the principal location for CB_1 and CB_2 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. Get citation.

projections (GO:0043005), which underlines its involvement in neuron communication and plasticity, essential for neurological functions (Ferreira et al., 2012). Furthermore, the CB_1 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 et al. 2005).

Conversely, the CB_2 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 CB_2 predominantly resides, emphasising its functionality in immune responses.

Combining the subcellular location data with GO analysis, the strategic cellular positioning of both CB_1 and CB_2 receptors becomes apparent, highlighting their critical roles in cellular communication. The subcellular location data for the CB_1 receptor and related proteins, which include Fatty Acid Amide Hydrolase (FAAH), Monoacylglycerol Lipase (MGLL), GNAI2, Guanine Nucleotide Binding Protein, Gamma 2 (GNG2), DRD2, CB_1, CB_2, 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 receptors capacity 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 CB_1 and CB_2 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 CB_1 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 CB_1 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 and Kumar, 2018). The inclusion of the GPR55 receptor alongside the traditional CB_1 and CB_2 receptors highlights a more complex framework of cannabinoid signalling within the ECS than previously recognised. Often classified as an 'orphan receptor,' GPR55 is distinct from the CB_1 and CB_2 receptors in terms of its binding affinities and signalling pathways, which involves the modulation of intracellular calcium levels and potentially influencing various physiological responses (Lauckner et al., 2008). The specific roles of GPR55 in the ECS are still being delineated, with studies suggesting its involvement in pain modulation, bone physiology, and cancer pathophysiology. This nuanced understanding is crucial for the development of targeted therapies aimed at selectively modulating the ECS. By elucidating the biological relevance and molecular interactions of GPR55, researchers can identify new therapeutic targets within the ECS, potentially extending beyond the capabilities of CB_1 and CB_2 receptors alone.

In parallel, the GO term (GO:0004949) for the CB_2 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 CB_2 receptor further emphasising the specificity and biological relevance of this receptor in the ECS. The CB_2 receptors strong association with cannabinoid receptor activity reflects its crucial role in immune system regulation, pain sensation, and inflammation (Zou and Kumar, 2018). It also suggestions possible effects on mood and behaviour, although these are more commonly associated with the CB_1 receptor. The significant FDR coupled with the specificity of the CB_2 receptor's role 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, CB_2 emerges as a promising target for pharmacological intervention in inflammatory and autoimmune diseases, offering therapeutic benefits without the psychoactive side effects often linked to CB_1 activation. Moreover, combining the analyses for CB_1 and CB_2, it is seen that their roles are distinct yet interconnected, with the CB_1 receptor primarily influencing central nervous system processes and the CB_2 receptor more involved in peripheral and immune functions.

The biological processes GO data (Fig. 8) for the CB_1 receptor presents a comprehensive view of the receptors 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 CB_1 and the modulation of cyclic AMP (cAMP) levels (Childers and Deadwyler, 1996; Eldeeb et al., 2016). The pathways strength of 1.94, 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 CB_1 receptor as a significant player in this signalling pathway. This robust association is far from coincidental and highlights the receptor's role in cellular responses to various stimuli, facilitated by cAMP, a pivotal second messenger in cellular signalling.

Additionally, the CB_1 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 receptors involvement 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 and Lupica, 2000). In the context of positive regulation of neurotransmitter transport (GO:0051590), the CB_1 receptor is implicated in the movement of neurotransmitters across cellular membranes, impacting synaptic signalling (Viveros et al., 2007). With a

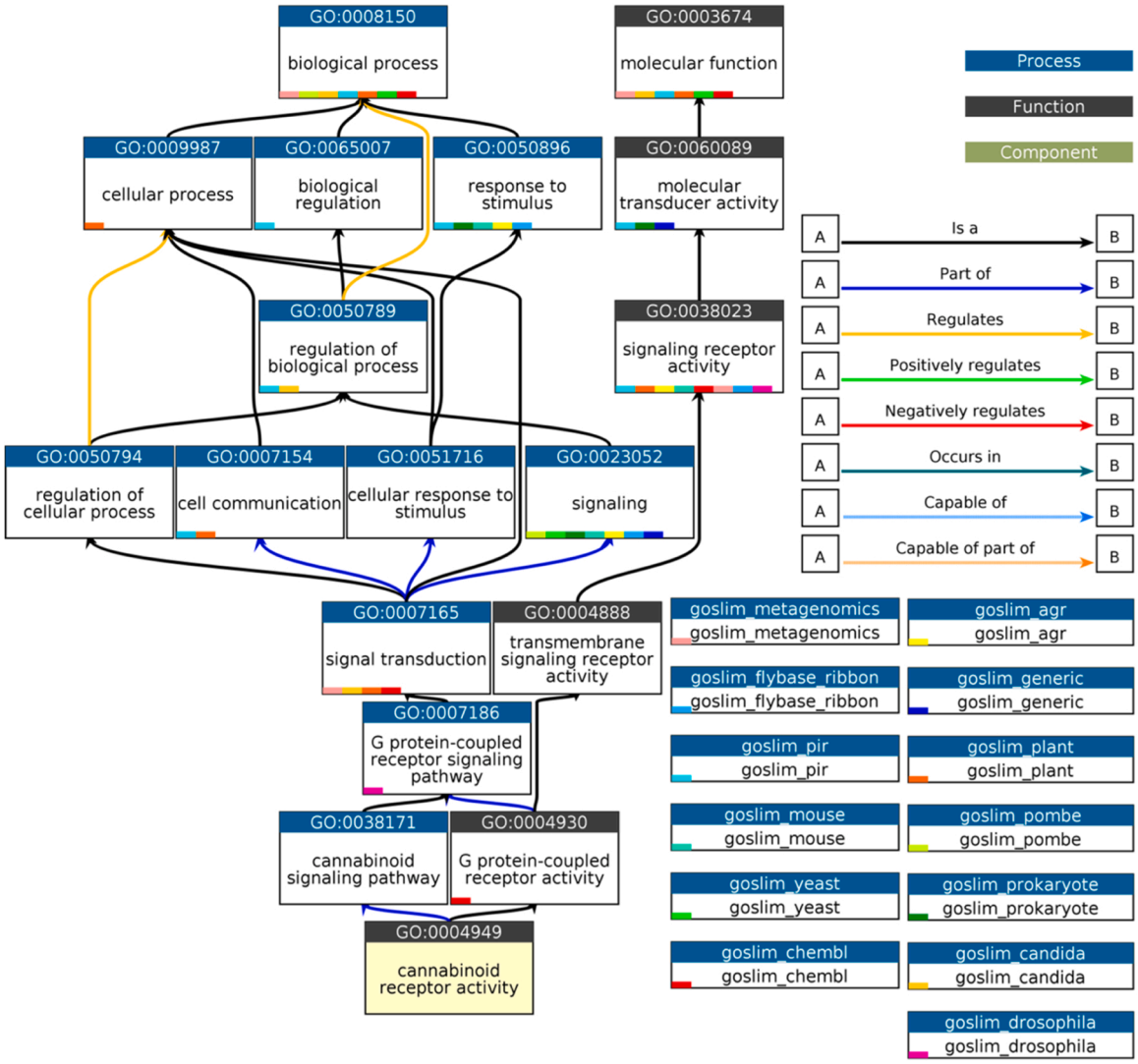


Fig. 8. Hierarchical representation of Gene Ontology (GO) terms associated with the cellular component category for the membrane (GO:0016020) in relation to CB_1 and CB_2 as classified by InterPro. The diagram illustrates the membrane's classification as a cellular component (green), which is a subset of a cellular anatomical entity (grey) within the broader category of cellular components (blue). The membrane serves as the principal location for CB_1 and CB_2 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 color-coded bars at the bottom represent various GO subsets used for annotation. Sourced from EBI QuickGO.

strength of 2.35 and a low FDR of 0.00042, this suggests that the association of the CB_1 receptor in this biological function is statistically significant and not due to random chance. Therefore, this shows the receptors potential impact on synaptic communication and its pivotal role in synaptic plasticity and neurophysiological processes.

The CB_1 receptors participation 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 CB_1 receptors biological relevance 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 CB_1 receptors substantial influence 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 CB_1 receptor regulates synaptic vesicle dynamics (Patzke et al., 2021). The negative regulation of dopamine secretion (GO:0033602) points to a significant role of the CB_1 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 and Björklund 2010; Parsons and Hurd, 2015). The statistical robustness of this association highlights the importance of CB_1 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 suggests the CB_1 receptors key role 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 CB_1 in the network of neurotransmitter systems, providing valuable insights for therapeutic targeting in cognitive disorders.

The GO data pertaining to CB_2 (Fig. 8) provides substantial evidence for the receptor's involvement in a myriad of biological functions. Though it shares similarities with the CB_1 receptor in terms of cellular signalling pathways, it distinguishes itself through unique physiological responses and implications. In the context of the adenylate cyclase-modulating G protein-coupled receptor signalling pathway (GO:0007188), the CB_2 receptors observed gene count of 4 against a background of 232, a strength of 1.83, and an FDR of 0.0016 illustrate its role in modulating adenylate 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 CB_2 receptors integral function in the modulation of immune regulation (Dhopeshwarkar and Mackie, 2016). Furthermore, the adenylate cyclase-activating protein-coupled receptor signalling pathway (GO:0007189), with a strength of 1.91 and an FDR of 0.0145, points to CB_2 direct activation role in enhancing adenylate 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 CB_2 receptor, capable of both upregulating and downregulating crucial signalling pathways.

CB_2 plays several roles in negative regulation such as the negative regulation of action potential (GO:0045759), CB_2 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 CB_2 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 CB_2 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 CB_2 receptors role in controlling nitric oxide production, a mediator integral to inflammation and vasodilation, thus reflecting the receptors potential impact on cardiovascular health and inflammatory responses (McCollum et al., 2007; Shmist et al., 2006). Lastly, the response to organonitrogen compound (GO:0010243) with a strength of 1.21 and an FDR of 0.0435 indicates the receptors broader role in responding to nitrogen-containing biomolecules (Yang et al., 2013). This could encompass a variety of neurotransmitters or pharmacologically active drugs, emphasising the CB_2 receptors relevance in pharmacological interventions.

3.9. Protein function and signalling pathways

The CB_1 receptors intricate involvement in the endocannabinoid signalling pathway is a focal point of study in various bioinformatics analyses, including KEGG, Reactome, and WikiPathways (Szklarczyk et al., 2021). The KEGG pathway analysis shines a light on the CB_1 receptors integral role in the endocannabinoid signalling pathway, especially within the context of retrograde endocannabinoid signalling (HSA-04723) (Leo and Abood, 2021). The exceptionally low false discovery rate (p-value: 1.32e-10) emphasises the receptors significance 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 CB_1 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 receptors presence 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 receptors widespread impact on cellular processes.

Complementing these findings, the Reactome pathway analysis provides a detailed view of the CB_1 receptors role 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 CB_1 receptor's role 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 and 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 CB_1 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 CB_1 receptors signalling network, highlighting 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, CB_1, CB_2, 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 CB_1 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 CB_1 and CB_2, 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 CB_1 receptors multifaceted role 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 CB_1 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 CB_2 receptors function in GPCR signalling. Reactome pathways analysis sheds light on two noteworthy routes involving the CB_2 receptor, G alpha (i) signalling events (HSA-418594) and GPCR ligand binding (HSA-500792). The first pathway demonstrates the CB_2 receptors role in inhibitory G protein (Gi) 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 CB_2 receptors functionality. The second pathway, GPCR ligand binding, spotlights the nascent activation step of the CB_2 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 CB_2 receptors broader implication in diverse GPCR-mediated signalling pathways beyond classic G_i protein interactions (Tuteja, 2009). These pathways collectively emphasise the CB_2 receptors crucial involvement 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 CB_2 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 pathways specificity 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 role of the CB_2 receptor 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 CB_1 and CB_2 receptors through comparative sequence alignment across a variety of species. Moreover, the 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 CB_1 and CB_2 receptors and their alignment were assessed within the broader context of the GPCR superfamily. The comparison between CB_1 and CB_2 receptors reveals a significant level of similarity, indicating a common evolutionary origin and potentially similar structural characteristics of 44.58 % (Munro et al., 1993). In their 1993 study, Munro and co-workers explain the 44.58 % similarity between CB_1 and CB_2 receptors as indicative of a shared evolutionary background, suggesting that both receptors originated from a common ancestral gene. This degree of similarity emphasises the structural and functional parallels between the two, despite their distinct physiological roles in the nervous and immune systems (Munro et al., 1993).

Additionally, he tertiary structure alignments of different representations of CB_1 and CB_2 proteins, illustrated by Figs. 3E and 3F, highlighted their conserved and divergent regions showcasing potential variations in ligand specificity and physiological roles. Both figures emphasise the structural conservation between CB_1 and CB_2, with the red regions marking the conserved residues identified through sequence alignment. These conserved areas are pivotal for the functionality of the receptors, underpinning essential activities such as ligand binding and signal transduction. The differences highlighted in these figures, particularly in the non-conserved regions, suggest variations in ligand specificity and physiological roles between CB_1 and CB_2. 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 CB_1 receptors involvement in the CNS and its impact on cognition, memory, and pain perception, contrast with the prominent role of the CB_2 receptor 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 int the PDB with the current best resolution contain stabilising artifacts 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.

CRediT authorship contribution statement

Marushka Soobben: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Yasien Sayed:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Ikechukwu Achilonu:** Writing – review & editing, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

M.S. wishes to extend her gratitude to the Postgraduate Merit Award Scholarship from the University of the Witwatersrand for financial support.

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