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Residue-specific orientation of arrestin in 5-HTR$_{1B}$ (Serotonin Receptor)-βArrestin-1 interaction

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G protein-coupled receptor

Neurotensin receptor

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90 rotation

Tango assay

ABSTRACT

Physiologically G protein-coupled receptors (GPCRs) are an important class of cell surface proteins capable of sensing the exogenous signals across the cell membrane through G-protein-dependent and independent pathways. Activated GPCRs initiate diverse G-protein-independent signalling through interaction with arrestin. Arrestins comprise a family of four proteins that act as signal regulators of GPCRs. Arrestin specificity and assembly orientation with a particular GPCR depend on the finger loop's residues. Recent cryo-EM structural elucidation of neurotensin receptor-1(NTSR1)-β-arrestin1complex reveals its striking difference from Rhodopsin-visual-Arrestin by a 90˚ rotation of β-Arrestin1 concerning the receptor. Alignment of neurotensin receptor 1(NTSR1)-β-Arrestin1 assembly with 5-HTR$_{1B}$ (Serotonin receptor) structure shows an ionic interaction mediated complex formation between receptor binding cleft and finger loop of arrestin. Mutational analysis of finger loop residues R65, D67, and D69 of β-Arrestin1 by tango assay confirms its possible interaction with an electropositive pocket of K79 and R161 in 5-HTR$_{1B}$.

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1 Introduction

The structural analysis of GPCRs are critical for innovative cell signalling process findings. The 5% of the human genome coding for GPCRs makes it one of the largest families of membrane-bound proteins (Yin et al. 2019). These molecules, being one of their kind, represent a giant class of receptors having more than 850 participants responsible for their multivarious roles in the physiology of the living world. Both endogenous and exogenous signals control these receptors. They are majorly responsive to hormones, metabolites, and even numerous neurotransmitters. GPCRs are controlled by two important regulators, the G-protein and the arrestins. GPCRs with seven membrane-spanning domains are convenient for the ligands to access and are important drug targets due to their functioning through downstream signalling (Yin et al. 2019; Yang et al. 2021). GPCRs signalling may occur via G-proteins or through Arr1 and Arr2. The activated GPCRs, upon initiating the G-proteins downstream pathways subsequently recruit some unique molecules such as G-receptor kinases (GRKs). GRKs add phosphate groups at specific Ser and Thr positions of GPCRs' cytoplasmic loops and tail, allowing them to bind specifically to the arrestins. As the receptors bind to arrestins, their interactions with G-proteins affect their deactivation. Interestingly, different G-protein-independent cellular signalling pathways are directed by the arrestins interaction with the GPCRs (Figure 1). The arrestin-mediated signalling is crucial for GPCR desensitization and recycling (Yang et al. 2021). GPCRs classification suggests the presence of six different classes, segregated by sequence conservation. The biggest one is Class A, represented by Rhodopsin, the smallest one is Class B, such as the Secretin receptor, and others are classified as Class C or glutamate receptor, Class D or fungal mating pheromone receptor, Class E or cyclic AMP receptor and Class F of Frizzled receptors. The last decade witnessed a behemoth paradigm in GPCR structural biology and was considered the 'golden era' of GPCR research. The Class A type GPCR comprises the family of rhodopsin-like receptors with 719 members subdivided into subgroups like sensory receptors, orphan receptors and others including the peptide, protein, lipid, melatonin, nucleotide, alicarboxylic acid, aminergic, etc. which are the most physiologically active and are effective for targeting of drugs (Basith et al. 2018; Yang et al. 2021). The two major pathways, G-protein and arrestin, are found to operate exclusively through biased GPCR-based ligands, which specifically regulate either arrestin or G-protein-mediated pathways (Reiter et al. 2012). The GPCR ligands are biased and are a better alternative than unbiased activators and inhibitors, as they can induce the receptor to promote the binding of a group of selective partners, which might be therapeutically beneficial with negligible side effects. The research is found to progress more towards biased ligands, which turned out to be new-generation drugs targeting and moderating the function of GPCR- (Kenakin 2012). Furthermore, the role of GPCRs in cancer metastasis and angiogenesis via the efficacious crosstalk mechanism with receptor tyrosine kinases has also been thoroughly investigated (Mandal et al. 2021).

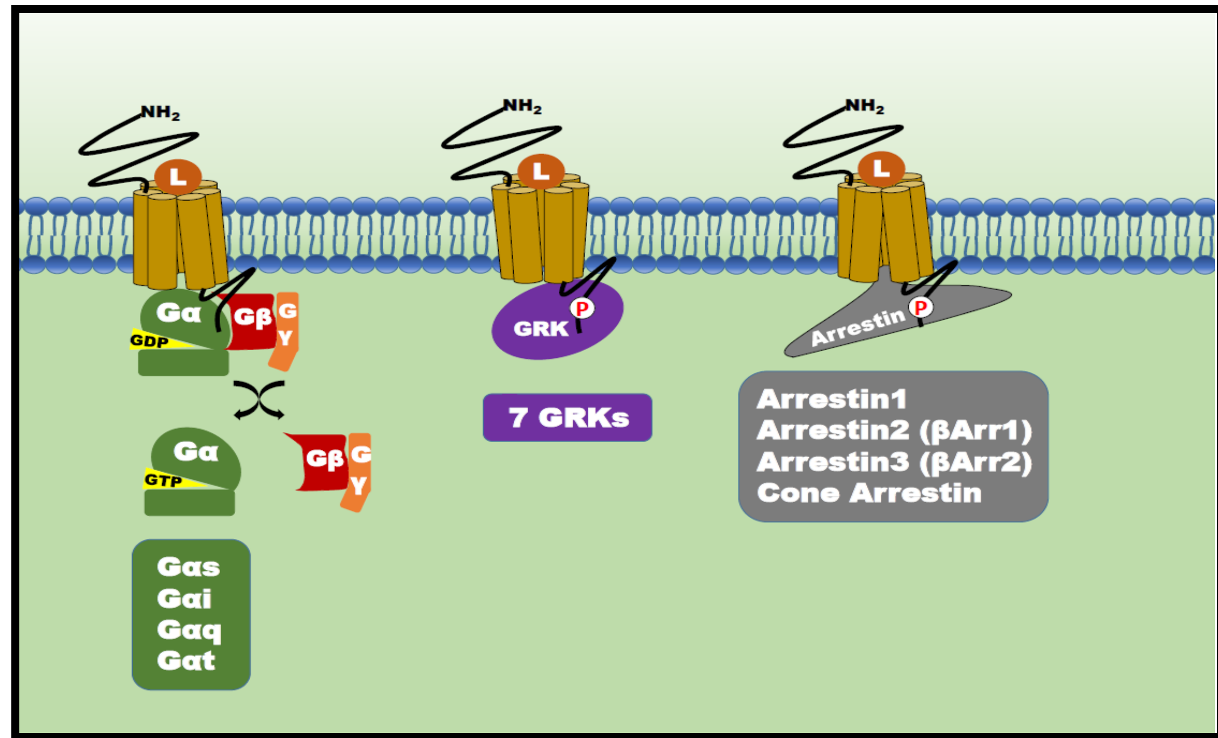


Figure 1 Detailed overview of GPCR Signalling mechanism with different binding partners

1.1 The Serotonin Receptor

The 5-HT (5-hydroxytryptamine) receptors, better known as serotonin receptors, are an important category of receptors that transmit signals across synaptic clefts and are abundant in the neurons. The neurotransmitter serotonin is released from the serotonergic neurons of the brain. It has been observed from various studies that their level rises considerably in neurological tissues that have undergone damage or injury (Sommer 2004). Serotonin and its receptors have been associated with multiple psychiatric and neurological disorders, from regular headaches and migraines to severe and complex consequences like schizophrenia and depression (Berger et al. 2009; McCorvy and Roth 2015). The serotonin receptors modify the secretion of many neurotransmitters, including acetylcholine, dopamine, glutamate, epinephrine/norepinephrine, etc. They are essential mediators of different hormones like oxytocin, vasopressin, prolactin, etc. The Serotonin receptors are the key regulators that influence some of the important neurological processes, and any impairment in its function may lead to disorders like depression, anxiety, cognition loss, etc. Thus, they constitute an important class of drug targets for various types of drugs, contributing to hallucinogens, antidepressants, antipsychotics and antimigraine agents. Being abundant in central and peripheral nervous systems, 14 types of receptors are being discovered, of which 13 are GPCRs targeting almost 40% of the FDA-approved drugs (McCorvy and Roth 2015; Jean-Charles et al. 2017). Due to the stressful lifestyle, people tend to suffer more from migraine and anxiety, leading to more consumption of non-specific medications, which may cause adverse health hazards and have been implicated in causing irreversible damage to the nervous system. A detailed investigation has become the need of the day to explore the structural details of the serotonin receptor- β-arrestin complex. The importance of the serotonin receptor as a potential drug target; hence, it is more likely that this unique structural complex that has not been investigated to date might turn out to be a promising target for drug discovery.

1.2 The negative or positive regulators of GPCRS: Arrestins?

The Arrestins are in the important family of signal-regulating molecules for GPCRs. It includes four types of arrestins viz., Arrestin 1-4. The arrestins 1 and 4 (visual and cone arrestin) are usually more specifically functional to the rhodopsins, while the other two arrestins 2-3 (βArr1 and βArr2) are found to modulate the signalling of many non-visual GPCRs (Yin et al. 2019). GPCRs are cytosolic adaptor proteins previously known to restrain GPCR signalling initiated through G proteins. It has been further biochemically identified that the arrestins do not necessarily 'halt' the signal transduction of GPCRs. Instead, they facilitate endocytosis and kinase activation, which leads to other signalling pathways specific to its position in the endosomes. The signalling promoted by β-arrestins has been revealed as being independent of G-protein activation (Wang et al. 2013). Here, er tried elucidating the serotonin receptor conformation as a drug target through its interaction with β-Arrestin1.

1.3 The Structure of Ergotamine bound 5HTR$_{1B}$

The Serotonin receptor variant in our study is 5-HT$_{1B}$**.** This receptor is mainly found to be abundant in the basal ganglia and lesser numbers in the other regions of the nervous system like the amygdala, cerebral cortex, and hypothalamus. The axon terminals of neurons are sites for 5-HTR$_{1B}$ receptors. This receptor is widely studied for its pivotal role in the development of aggression, cognition, memory, addiction, etc., making it an impeccable drug target. The previously solved structure of 5-HTR$_{1B}$ with Ergotamine (PDB: 4IAQ) in the absence of the third intracellular loop ICL3 shown in Figure 2a was solved and published by Wang

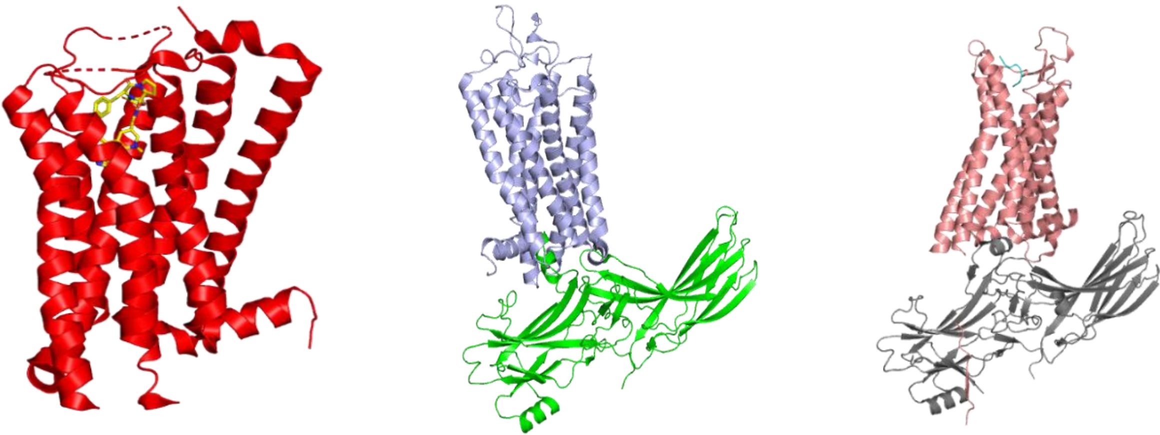


Figure 2 (a) The molecular state of Ergotamine bound 5HTR$_{1B}$, (PDB: 4IAQ), (b) The structural organization of Rhodopsin (Blue)-v-arrestin (Green) complex, (PDB: 4ZWJ), (c) The cryo-EM structure of NTSR-1(Salmon) - β-arrestin1 (Grey) complex, (PDB: 6PWC).

et al. (2013). This was the first structural insight of receptors that became crucial for all future biochemical studies and homology modelling. The structure revealed similar binding modes for the ligands Ergotamine and dihydroergotamine. These ligands were found to occupy orthosteric pockets, which have been created by the conserved residues of the 5-hydroxytryptamine receptor family, that provide evidence in support of family-wide agonist activity of 5-hydroxytryptamine (Wang et al., 2013; Kang et al., 2015).

1.4 The Structural organization of the Rhodopsin-visual Arrestin complex

Another breakthrough in the research of structural GPCR came in 2015 when Yang et al. (2015) published the structural state attained by Rhodopsin with visual arrestin solved by an X-ray laser light source shown in Figure 2b. An attractive feature of this structural complex was that arrestins bind asymmetrically to rhodopsin, hence found to be identical among four molecules in the asymmetric unit and that supports confirmational uniqueness of rhodopsin-arrestin assembly. This work was the first to report the molecular state of a GPCR docked to arrestins, which further supports the investigation of structural plasticity associated with arrestin-biased signalling (Kang et al. 2015)

1.5 The molecular state of NTSR-1- β-arrestin1 Complex

In 2019, the same group unraveled the molecular state of Neurotensin Receptor occupied by β-Arrestin-1 by cryo-EM method for the first time, as shown in Figure 2c. The same group associated with the experimental paper conducted an interesting experiment with the Rhodopsin-v-arrestin complex and NTSR-1-β–arrestin1 complex structures. The two structures were superposed to observe the relative orientation of the two types of arrestins. It showed that the arrestins were positioned at two significantly distinct orientations, which were about 90° rotated around the axis that was vertical to the plasma-membrane layer. Despite being similar kinds of molecules, the complexes revealed a marked difference in the orientation of arrestins to the receptors. The alignment of the transmembrane domains of these two complexes revealed the Arr2 orientation turned by 90° along the visual arrestin axis (Figure 3). Such a facsimile has conferred an overt range of dynamism. Moreover, the interaction of rhodopsin with v-arrestin and NTSR- 1 with β-Arrestin1opened a new avenue for GPCR research and confirmed that specific interaction is a significant aspect of drug designing.

1.6 The structural Alignment of NTSR-1- β-arrestin1 complex with 5HTR_{1B}

A structural alignment of Neurotensin receptor 1(NTSR1)-β-Arrestin1 assembly with Serotonin receptor (5-HTR_{1B}) was designed to understand the interaction between β–arrestin 1 and the serotonin receptor by visualizing in PyMOL as there is no known structure to date. Following the alignment, we deleted the NTSR-1 receptor from the NTSR-1-5HTR_{1B}- β -arrestin1 complex to investigate a closer interaction between 5HTR_{1B} and β -arrestin1

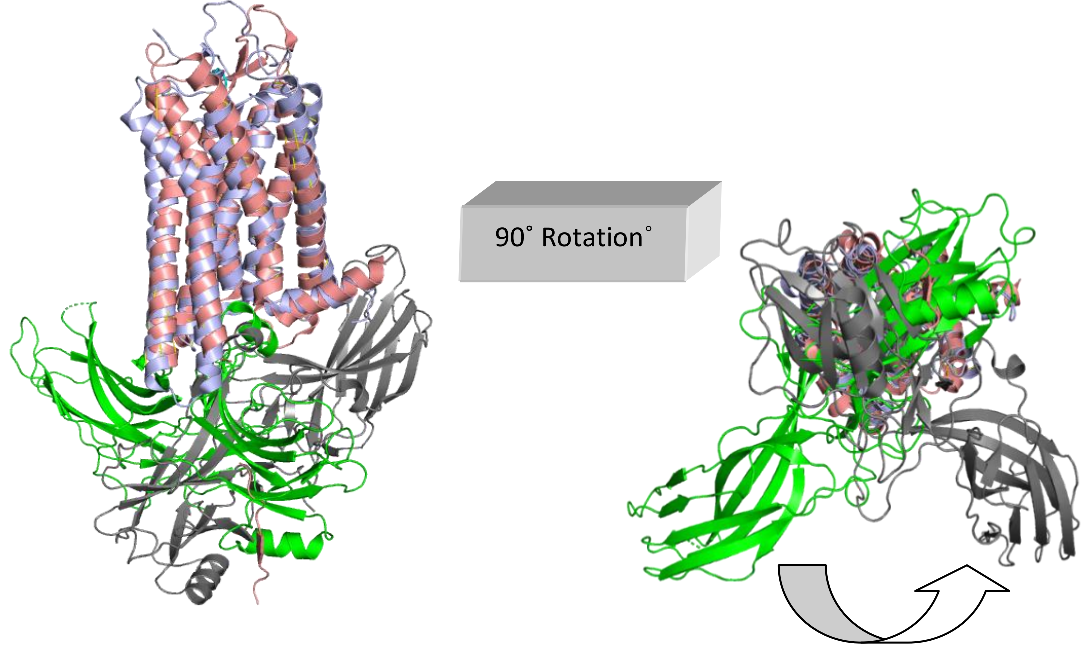
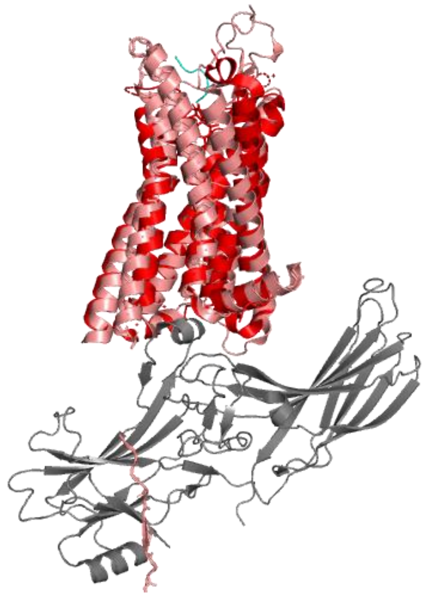


Figure 3 (a) Structural alignment of Rhodopsin (Blue) v-arrestin (green) with NTSR-1(Salmon), β-arrestin1 (Grey), (b) Cytoplasmic view of v arrestin vs. β-arrestin1 position after 90 ° rotation



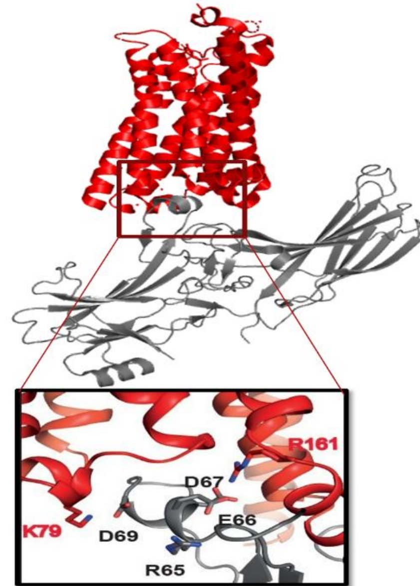


Figure 4 (a) Alignment and identification of residues participating in the $5HTR_{1B}$- B-Arrestin 1 complex. $5HTR_{1B}$ (Red), NTSR-1(Salmon), β-Arrestin1 (Grey); (b) A closed look at the participating residues in the $5HTR_{1B}$- β-Arrestin1 complex

(Figure 4a and b). It has shown striking results, identifying specific and unique residues that are gravely engaged in the ligand-receptor interaction. Notably, such residues are not found in the NTSR-1 receptor but only in $5HTR_{1B}$, confirming the stringent specificity of such interactions (Yin et al. 2019).

2 Materials and methods

2.1 Construct preparation

The complex formed by the Biological Resource Imaging Laboratory (BRIL), NTSR1-Arr2-Fab30, has been taken as a structural reference for our experimental studies**.** The structures referred for studies conducted for this paper are the human Neurotensin receptor 1 (NTSR1) (UniProtKB ID: P30989), the human Arrestin-2 with 3A mutations (residues 1-3334 I386A, V387A, F388A, UniProtKB ID: P49407), and the human Serotonin receptor ($5HTR_{1B}$) with UniProtKB ID: P28222. The Arr2 mutants were cloned into pcDNA6 vector, modified to encode 3× Flag tags at the C-terminal. The 5-HTR_{1B} receptor was PCR amplified and cloned into pcDNA6, previously modified to encode Hemagglutinin (3× HA) tags at the C-termini. The information from PDB structures (4IAQ, 4ZWJ and 6PWC) and their sequence alignments were used to design the mutations to probe the receptor/arrestin interface (Kang et al. 2015; Yin et al. 2019).

2.2 Site-directed Mutagenesis

The finger loop mutations of β-Arrestin1 were designed by Site-directed mutagenesis. The mutation constructs were created by the quick-change method (Agilent). The protein sequencing was performed to confirm all plasmid constructs before protein expression and protein interaction studies by Tango-Assay (Kang et al., 2015; Yin et al., 2019).

2.3 Tango Assay

The Tango Assay was performed with pcDNA6.1 vector carrying $5HTR_{1B}$ wild-type receptor (1-418). cDNA in the fusion of tobacco etch virus protease (TEV protease) cut site followed by transcriptional inducer tTa at the C-termini of vector in the expression cassette (pcDNA6-$5HTR1_B$-TEV site-tTA). Similarly, the Arr2(1-393, wild type) construct was prepared by fusing it with cDNA of TEV protease at C-termini (pcDNA-Arr2-TEV protease), which will be responsible for cleaving the reporter gene (Figure 5a,b). The possible interacting residues between $5HTR_{1B}$ and Arr2, if in proximity, will result in TA's separation by cutting at the TEV site, which activates the expression of reporter gene TA-dependent luciferase. Before conducting the assay in HTLA cells, a 24-hour culture of cells in a 24-well plate was done to a population of 5 $\times 10^4$ cells/well. This was followed by cotransfection with Arr2-TEV protease (10 ng) plasmids, $5HTR1_B$-TEVsite-tTA (10 ng) and phRG-tkRenilla luciferase-expressing plasmid (5 ng) utilizing X-treme GENE 9 DNA Transfection Reagent (Roche). The cells were transfected for 24 hours and kept in 24 well plate overnight with PBS (vehicle), 50 μM Ergotamine, and 5 μM ML314, respectively. Then, luciferase assay was used to evaluate the interactions following the protocol provided by Dual-Luciferase Kit (Promega) (Kang et al. 2015; Yin et al. 2019).

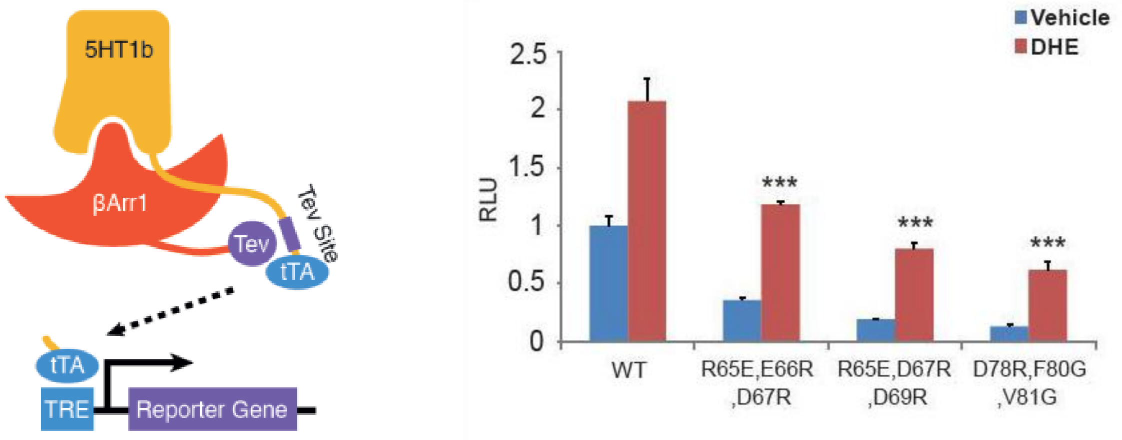


Figure 5 (a) The amino acid-mediated interaction between 5HTR$_{1B}$- β-arrestin1 by Tango Assay; (b) A detailed graphical depiction of the Ergotamine-bound serotonin receptor-β-arrestin1 interaction in the wild type and the mutant arrestin residues showing a gradual decrease in the interaction, confirming a specific interaction between them.

3 Results and Discussion

The amino acid-mediated interaction interface between the serotonin receptor and β--the Tango Assay analyzed Arrestin1. A remarkable GPCR analytical assay binds the receptor to arrestin, leading to GPCR desensitization. Our study was conducted with the Serotonin receptor and β-arrestin1. A successful receptor-ligand binding could be confirmed by Tango Assay, where β-arrestin1has an attached Tev protease cassette, and the serotonin receptor has a Tev cleavage site. On successful interaction between the receptor binding cleft of serotonin receptor and the β-arrestin1 finger loop, cleavage of the protease site results, confirming the interactions leading to further cues for studying and identifying the residues participating in the 5HTR$_{1B}$- β-Arrestin1 complex formation. A detailed investigation of β-arrestin1 and 5-HTR$_{1B}$ (Serotonin receptor) provided astounding findings. It was observed that some intricate ionic and hydrophobic interactions mediated complex formation between receptor binding cleft and finger loop of β-arrestin1. Some specific mutations were generated, R65, D67and D69, in the finger loop of β-arrestin1 by Tango assay, confirming arrestin's possible interaction with the electropositive pocket of K79 and R161 in 5-HTR$_{1B}$. The interaction between the Ergotamine-bound serotonin receptor- β-arrestin1 complex showed well-ordered interaction.

In contrast, the various mutants generated in the finger loop of β-arrestin1showed a decreased degree of interaction because serotonin possibly interacts specifically with β-arrestin1. The interaction seems to be mediated by the ionic and hydrophobic interactions. It is also important to understand the molecular conformation of long ICL3 of 5HTR$_{1B}$ while in complex with arrestin. The exchanges are highly GPCR sequence-specific. Similarly, the role of phosphorylation sites in ICL3 was evaluated by biochemical studies (Gupta et al. 2023)

Conclusions

The mutations generated in the β-arrestin1 finger loop display a unique possibility of interaction between β-arrestin1and 5HTR$_{1B}$ receptors. This implication can be exploited for new research avenues and has been found to add another bright feather to the insurmountable progress in GPCR research. This development has rendered the serotonin receptor a vital drug target with options in the future direction. This paper contributes to the significance of the specificity of the interaction of the ligand-receptor complex. This study can also contribute to drug design, assist with virtual screening, and develop vaccines for treating cancer and other deadly diseases in the near future, which will be a boon to humanity.

Acknowledgement

Nil

Conflict of Interest

The authors declare that there is no conflict of interest.

Appendices

The protein structures have been prepared in PyMOL. (Reference: https// pymol.org/2). The notion of experimental design and sample preparation techniques were taken from the corresponding author's paper published in 2015 (Reference 1 and 12).

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