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Bioinformatics Analysis of 5HT2C Gene Cys23ser Polymorphism and Epigenetics Insights into the HTR2C Receptor Gene Regulation: Implications for Physiological Roles in the Brain

Kiran Kumar Halagur Bhogegowda [a*], Sajeeda Niketh [b++], Sowmyashree BS [c#] and Tenkanidiyooru Ramamoorthy Prashith Kekuda [d#]

[a] Former Post-doc NCBS, Bangalore. Wilson Garden, Bangalore-560030, affiliated to Nrupathunga University, Bengaluru, 560001, India.
[b] Department of Biochemistry, Nrupathunga University, Bengaluru, 560001, India.
[c] Department of Biotechnology, Nrupathunga University, Bengaluru, 560001, India.
[d] Department of Microbiology, S.R.N.M College of Applied Science. N.E.S Campus. Balraj urs Road, Shimoga-577201, India.

Authors' contributions

This work was carried out in collaboration among all authors. Design, reviewing of literature, conduction of in silico experiments, collation, drafting of manuscript Author KKHB and SN design, collation, drafting of manuscript. Author SBS literature collation, collation of data from databases help in drafting the manuscript. Author TRPK literature collection, retrieving data from databases, manuscript preparation. All authors read and approved the final manuscript.

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[++] Associate Professor;
[#] Assistant Professor;
**Corresponding author: E-mail: kirankumarhb@gmail.com;*

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Review Article

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ABSTRACT

The expression profile, signaling and neuronal functions of the 5-HT_{2C} receptor makes it a candidate of interest for the treatment of several neuropsychiatric diseases. The encoded ligand-gated G-protein coupled receptor (GPCR) protein responds to cell signaling through several neurotransmitters. Signal transduction through G-proteins is a prominent feature of several eukaryotes. In this review and analysis paper, we provide background literature on the unique biochemical, structural, and pharmacological properties of the 5-HT_{2c} receptor and gene architecture, regulation, RNA editing and association studies of polymorphisms.Also on the evolutionary, and phylogenetic paths. of the 5*HT2C* gene. We conduct *in silico* predictions using bioinformatics tools SIFT and POLYPHEN to assess the effect of Cys23ser substitution in the N-terminal extracellular loop. Further, epigenetic analysis of the promoter and regions flanking the exon such as (DNase I Hypersensitivity, enhancer with TF binding site); methylation analysis of promoter (CpG methylation and CpG island); and histone marks with FAIRE. The results suggested that the cys23ser substitution can affect the 3D-protein structure. The additional cysteine amino acids (position-23,235,341) in human receptor could enable additional structural stability to the protein and may have evolved from previous ancestors (various mammals and primates) to aid the modulation of behavioral traits under evolutionary pressure. The alterations in DNA methylation and associated regulatory elements in promoter and upstream could impact gene expression, inactivation, genome stabilization, and inheritance which could have relevance in pathological states in the Central nervous system (CNS).

Keywords: GPCR protein; RNA editing; Cys23ser variant; methylation; FAIRE.

1. INTRODUCTION

The *5HT2C* gene is X-linked gene with 6 exons and 5 introns located on the human with higher proportions in higher organisms [1]. The receptor is expressed in various human brain regions such as the midbrain, the lateral septal complex, the hypothalamus, the olfactory bulb, the pons, the choroid plexus, the nucleus pallidus, striatum and amygdala, the nucleus accumbens and the anterior cingulated gyrus. In these regions receptor demonstrates high-affinity interactions with a wide variety of psychiatric medications [2]. The receptor is involved in the regulation of serotonin, dopaminergic, GABAergic, and glutamatergic neurotransmitter-related physiological activities respectively. The 5-HT neurons are grouped in 9 nuclei, located in the medial part of the brainstem called the raphe nuclei. It dimerizes as a homodimer with another ($5HT_{2C}$) and as heterodimer with ($5HT_{2A}$) receptor. The receptor couples to G protein-dependent signaling and through other G proteins such as G12/13 and Gi/o. Also, through non-G protein downstream proteins like the PDZ containing scaffolding proteins. The protein is a GPCR with characteristic seven transmembrane domain-containing protein (TM I – VII), three extracellular (ECL 1–3) and three intracellular loops (ICL 1–3), an intracellular carboxyl (C)-terminus, and an extracellular amino (N)-terminus (reviewed in [3]. Another particular characteristic of the GPCRs is the high conservation of the DRY sequence of the intracellular end of transmembrane domains. It also harbors serine/threonine residues for phosphorylation and post-translational modification in the form of glycosylation sites. In mammals, glycosylation sites are different with one in rats and four in mice and humans [4]. These findings illustrate the nature and complexity of cellular and region-specific roles of this receptor in neurobiology.

1.1 5-HT_{2c} Receptor and Physiological Roles

The 5-HT_{2c} receptor is also involved in various physiological roles such as endocrine, appetite, anxiogenic stimuli, and stress and circadian responses [5]. Further, it functions as a receptor for various drugs and psychoactive substances, including ergot alkaloid derivatives, 1-2,5,-dimethoxy-4-iodophenyl-2-aminopropane (DOI)

[6], and lysergic acid diethylamide (LSD) [7]; antipsychotic and antidepressant drugs such as imipramine and fluoxetine [8]. CNS role of 5-HT2C neurons contributes to the regulation of energy homeostasis and glucose homeostasis. Knock-out studies demonstrate increased food intake, insulin resistance, and obesity while pharmacological activation inhibits food intake. Moreover, 5-HT in the gut has paracrine signal effects on β-islet cells. Several genetics association studies also show positive association with drug response and glucose metabolism. Alterations in receptor editing, splicing and density is found in pathological conditions such as Prader-Willi Syndrome (PWS) (multigene disorder characterized by hyperphagia and obesity) underscoring the role of the receptor in glucose homeostasis [9]. Also, the 5-HT signaling pathway is closely related to individual energy storage and expenditure [10]. Sodium ions are important minerals for maintaining extracellular fluid and blood volume. 5-HT_{2C} receptors enable sodium balance since sodium appetite is a powerful form of motivation that can drive ingestion of high, yet aversive concentrations of sodium [11]. The receptor interacts with signal molecules like leptin, ghrelin, and cholecystokinin and regulate body weight [12] .These observations are supported through animal and genetic studies. Finally, the receptor in brain affects psychosis, reward, substance abuse, anxiety, and other physiological measures such as sleep, exercise, and body temperature [13].

1.2 5-HT_{2c} Receptor as a GPCR and Target for Various Ligands

GPCRs mediate the effects of numerous endogenous and exogenous ligands such as neurotransmitters, hormones, cytokines, therapeutic drugs, and drugs of abuse [14]. They transduce downstream signaling and Class A/1 (Rhodopsin-like receptors) corresponding to 30% of all identified drug targets and are a major target for new drug development [15]. The 5-HT_{2C} receptor detects extracellular effector molecules leading to protein conformation change ending in activation of intracellular responses through the β-arrestin pathway. The canonical G protein-dependent signaling leads to coupling to Gαq/11 to activate the enzyme phospholipase Cβ (PLCβ) mediated hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to generate the intracellular second messenger inositol-1,4,5-trisphosphate (IP3), accumulation of the metabolite inositol monophosphate (IP1), and diacylglycerol (DAG) (Reviewed in [16]); subsequently mobilizes intracellular calcium (Ca^{2+}) [17]. Agonist-dependent desensitization is associated with phosphorylation involving G protein receptor kinase2 (GRK2), binding of β-arrestin and uncoupling of the receptor from the G protein. This results in receptor internalization into endosomes; and recycling to the plasma membrane through de-phosphorylation and glycosylation. There is approximately 80% sequence homology in the TM region (orthosteric binding site) between members of the 5-HT2R family, while the ECL and ICL sequences are known to vary across receptor subtypes. Opportunities to regulate the biological function (s) of the receptor at various levels continue to fuel drug discovery. Impaired ligand concentration, GPCR protein expression, or mutation and signaling are implicated in many pathophysiological conditions such as cardiovascular and metabolic diseases [18], cancer and immune diseases [19], and musculoskeletal pathologies. Also, in several central nervous system (CNS) disorders [20].

1.2.1 CNS drug targets

The repertoire of ligand molecules that bind to the receptor makes it a frequent target of CNS drugs and biomedical research involving *in vivo* biochemical, mutagenic studies, enzymatic, drug-protein (structure-based design). Also,through *in silico* methods such as docking. Intense pharmacological research has led to therapeutically active 5-HT_{2C} receptor ligands, both agonists and antagonists (or inverse agonists) [21]. Several antidepressants and antipsychotics are 5-HT_{2C}R antagonists/inverse agonists such as Agomelatine an antagonist used for the treatment of major depression; Lorcaserin used in the treatment of obesity and addiction (nicotine/ smoking) [22], antagonists/inverse agonists such as cyproheptadine or SB206553 is used in spinal cord injury- and Vabicaserin [23]. Unique feature of 5-HT2C-GPCR signaling involves signaling bias or functional selectivity depending on the ligand/agonist. The receptor displays divergent levels of activation through multiple signaling pathways such as activation of PLCβ over PLA2, and vice versa. To counter these biases lead series of potent benzodiazepine agonists with high selectivity are in use [24]. Allosteric modulation has enabled specificity in target receptors to this end several lead compounds such as 4-Phenylpiperidine-2-Carboxamide [25]; Oleamide analogues [26] and several others are

currently is use. These results have propelled drug design efforts for various neuropsychiatric diseases such as schizophrenia (SCZ), depression, anxiety, Parkinson's, and epilepsy. However, several advantages such as selective agonists suppressed cocaine intake and the resurgence of drug-seeking, smoking cessation rates and disadvantages as weight gain, greater relative risk of metabolic dysfunction. Also, diabetes and suppressed food intake and relapses coupled. with genetic variation (pharmacogenetics) have limited their use.

1.2.2 Structure-based drug design

Using computational (Docking, SAR) and chemical pharmacophore methods researchers have elucidated various structure-function relationships, pharmacological properties, and coupling and interaction(s) with signaling ligands and drugs of the receptor [27]. Drugs targeting the 5-HT_{2C} receptor are useful for treating obesity, drug abuse, and SCZ referred to as polypharmacy which take advantage of the conserved orthosteric binding pockets in the TM. Cumulatively, these studies implicate the role of TM, intra and extra loops of the transmembrane domain (ECL) and (ICL) sequences which are known to vary in the organization and protein sequence across receptor subtypes. Drug interaction is mediated through non-covalent binding of the drug with its target (catalytic or non-catalytic sites) exploiting the hydrophobic and/or hydrophilic interactions [28]. Approximately, 50% of cysteine residues play crucial roles in cellular processes a feature which impacts the interaction with xenobiotics and drugs. Cysteine residues are targets of drug discovery due to the combination of the high reactivity of thiol/thiolate groups and the low abundance in proteins conferring specificity in target and limiting the number of potential off-target reactions [29].

1.3 5-HT2C Gene Alternate Splicing and RNA Editing

A unique characteristic of the 5-HT_{2C} receptor, which differentiates it from other GPCRs, is RNA editing where the mRNA is subject to post-transcriptional modifications [30,31]. RNA splicing and editing alter levels of 5-HT_{2C} receptor function with implications in multiple human diseases [32] and in brain diseases such as anxiety and aggression [33]. The receptor is encoded by a complex transcription unit spanning at least 326 Kilo-base (Kb) pairs. Its pre-mRNA undergoes extensive processing that includes alternative splicing as well as editing of exon 5b, and skipping generating a truncated protein isoform (Fig. 1a. b). Studies in transfected cells show that the truncated isoform forms a heterodimer with the full-length receptor, causing an entrapment in the endoplasmic reticulum and a decrease in active cell surface receptors [34]. Several physiological changes are observed in the brain due to these alterations such as hyper-excitability, over-eating, and epileptic convulsions. Knockout mice corroborate these observations. Moreover, several factors affect this mechanism, in C57BL/6 mice the hypothalamic-pituitary-adrenal axis and mood are altered in a neuronal cell type-specific way [35], antidepressant treatments change receptor mRNA expression in rat brains [36] and epigenetic processes affects have a site-specific effect. A unique feature of these mechanisms is the transgenerational transmission "parent to offspring" effect. To this end, research shows that female rats exposed to chronic stress and fluoxetine before reproduction affect editing in brain of newborn offspring at birth [37].

Adenosine to inosine (A-to-I) RNA editing on double-stranded RNA changes the transcript sequence and structure. The human editing sites identified to date reside in non-coding repetitive transcripts such as Alu elements [38] mediated by Adenosine Deaminase Acting on RNA (ADAR) family of proteins [39]. Multiple RNA editing events and SNORD115 action alter the structure of the second intracellular loop (important region for proteins).This generates alternate protein forms with a decreased ability to interact/coupling with G-protein which subsequently affects downstream signaling cascades [40]. Sites on the receptors A, B, C, D, and E are sites of editing in exon 5 (Fig. 2). The editing at each of the sites results in changes in three amino acid sequences at positions- 156 (isoleucine-I); 158 (asparagine-N) and 160 (isoleucine-I) respectively. Editing at A and B sites the causes isoleucine to valine (V) or methionine (M) substitution. At C and E site asparagine could change to aspartic acid (D), serine (S), or glycine (G). Finally, at the D site isoleucine could be substituted for valine (V) and editing at all sites, a VGV-type isoform is generated. Differences in editing both between species and in different brain regions in various neuropsychiatric diseases such as suicide (sites- D, E,) no difference in depression, and normal brain (site – A) suggesting variations [41].

1.4 Phylogeny of GPCR and 5-HT$_{2c}$ Receptor

Evolutionary analysis suggests that the gene families involved in the GPCR signaling system were already present in the last common ancestor of eukaryotes (Metazoan) as shown in model ciliate, *Tetrahymena thermophile*. Conservation of the signaling transduction machinery and a burst of receptor diversification through gene/genome duplication events enabled the transition to multicellularity [42]. Interplay of conserved transmembrane core and the invariant set of intracellular signaling mechanisms properties are proposed to be mechanism. The rhodopsin family is the largest and forms four main groups with 13 sub-branches. Further, synteny of chromosomal paralogs regions (Xq24; 96.1 to 99.6% homology) implicates tetra-ploidizations or local gene duplication events [43]. The contemporary classification based on the phylogeny of 5-HT receptor proposes a time frame of 700-800 million years ago (MYA), further radio-ligand and physiological studies have enabled identification of 13 subtypes [44]. Baring 5HT3 a ligand ion-gated receptor all other 5HT 1, 5HT 2, 5HT 4, 5HT 5, 5HT 6, and 7 are GPCRs indicative of the repertoire of biological and behavioral functions they transduce. Comparative genome analysis using primate sequences and studies on evolution (Ka/Ks ratios) have helped gain insights into the various evolutionary mechanisms operating on these receptors. Studies show that the 5HT receptors are clustered into 6 clusters with the cluster2 consisting of 5-HT2a, 5-HT2c, 5-HT2b, and 5-HT6 with 92 to 98% homology (Fig. 3). A total of 32 amino acids are identical which are distributed across the protein implicating conservation between human and non-human primates [45]. Several genes involved in diverse nervous system functions showed accelerated evolution in primates when compared to rodents, and pronounced in the primate lineage leading to humans. Estimation of synonymous and non-synonymous (Ka) nucleotide substitution to test selection pressures acting on the coding sequences suggests negative purifying selection acting on the receptors (Ka/Ks <1). However, few residues under positive and negative pressure were identified in the *5HT2C* gene. The amino acid residues mapped to the hypervariable

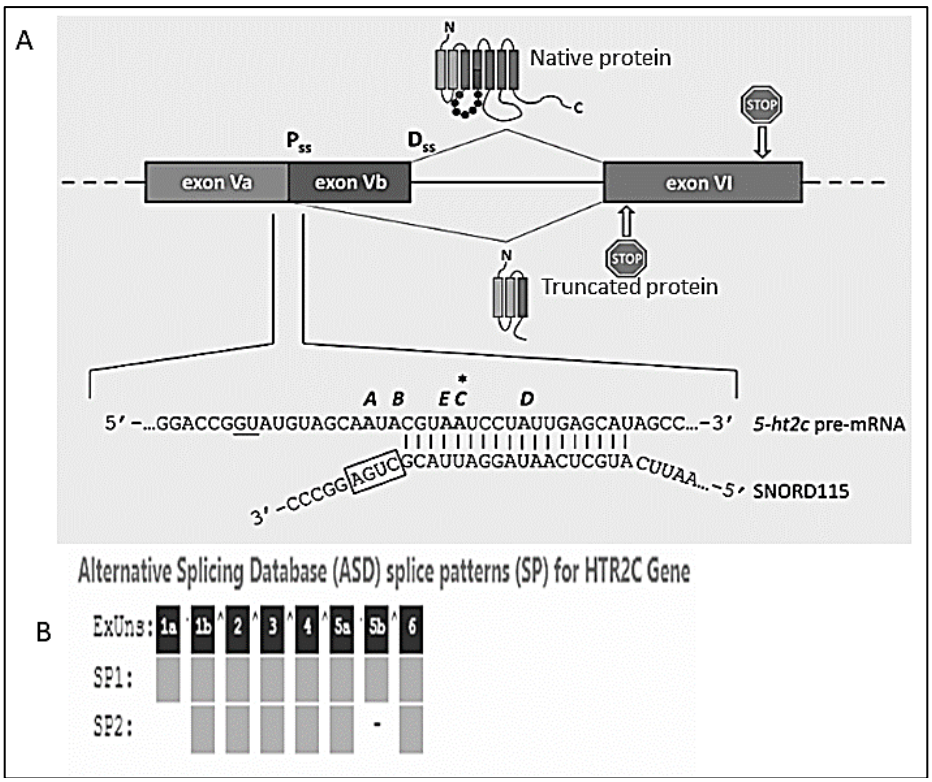


Fig. 1. a. Depicting HT2C gene transcript with exon splicing at exon (V-VI), and b. Spice variants oth the gene. Adapted from (a. Tomaz bratkovic et al., Sci rep. 2018;b. Genecards)

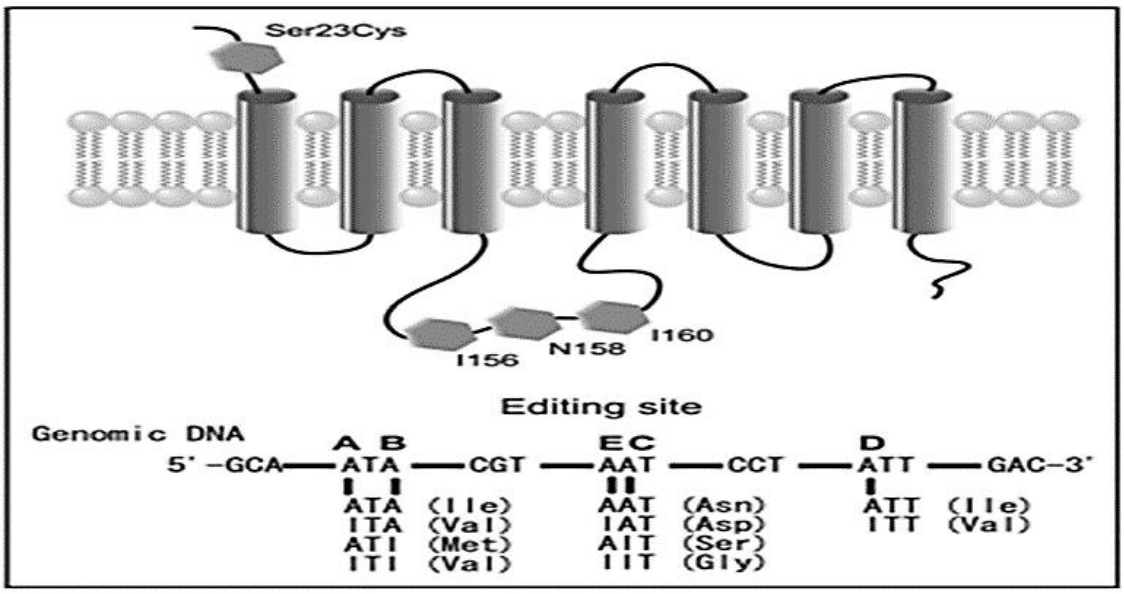


Fig. 2. Depicting RNA editing sites, relative amino acid positions on the 5HT2C protein (Figure adapted from Masaki Tanaka and Yoshihisa Watanabe Front. Neurosci., 14 January 2020)

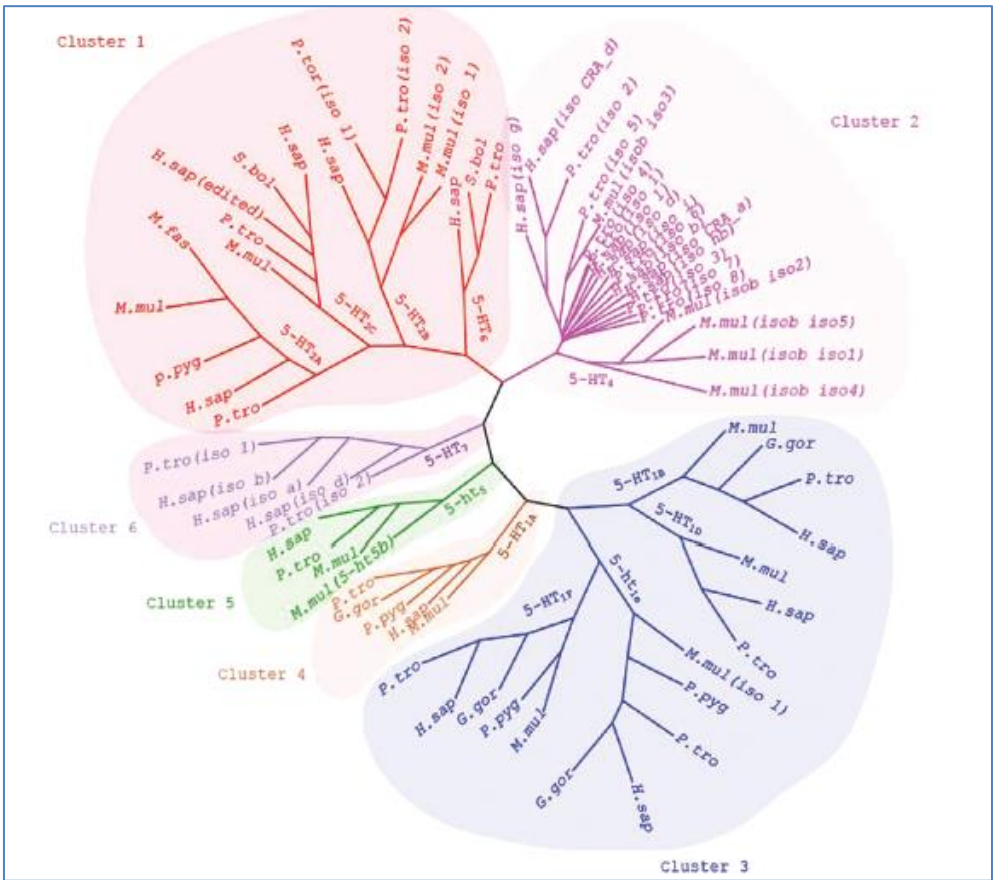


Fig. 3. Unrooted phylogentic tree of 5HT receptor clustered into 6 groups(arrow indicates cluster-1 with 5HT2C). Figure adapted from (A.padmanabhan et al., 2010)

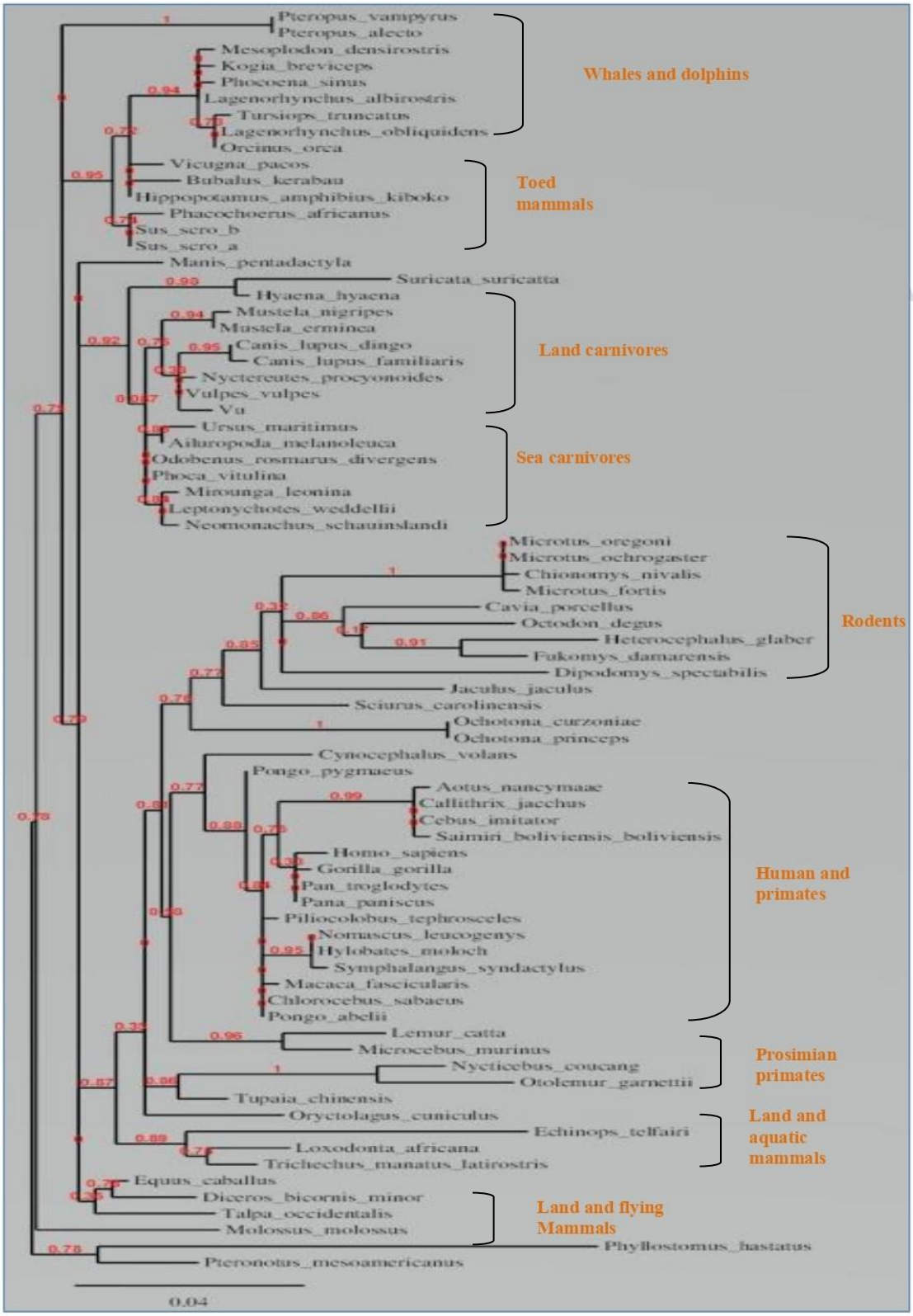


Fig. 4. Phylogenetic tree of HTR2C protein in mammals and primates generated using http://www.phylogeny.fr/index.cgi

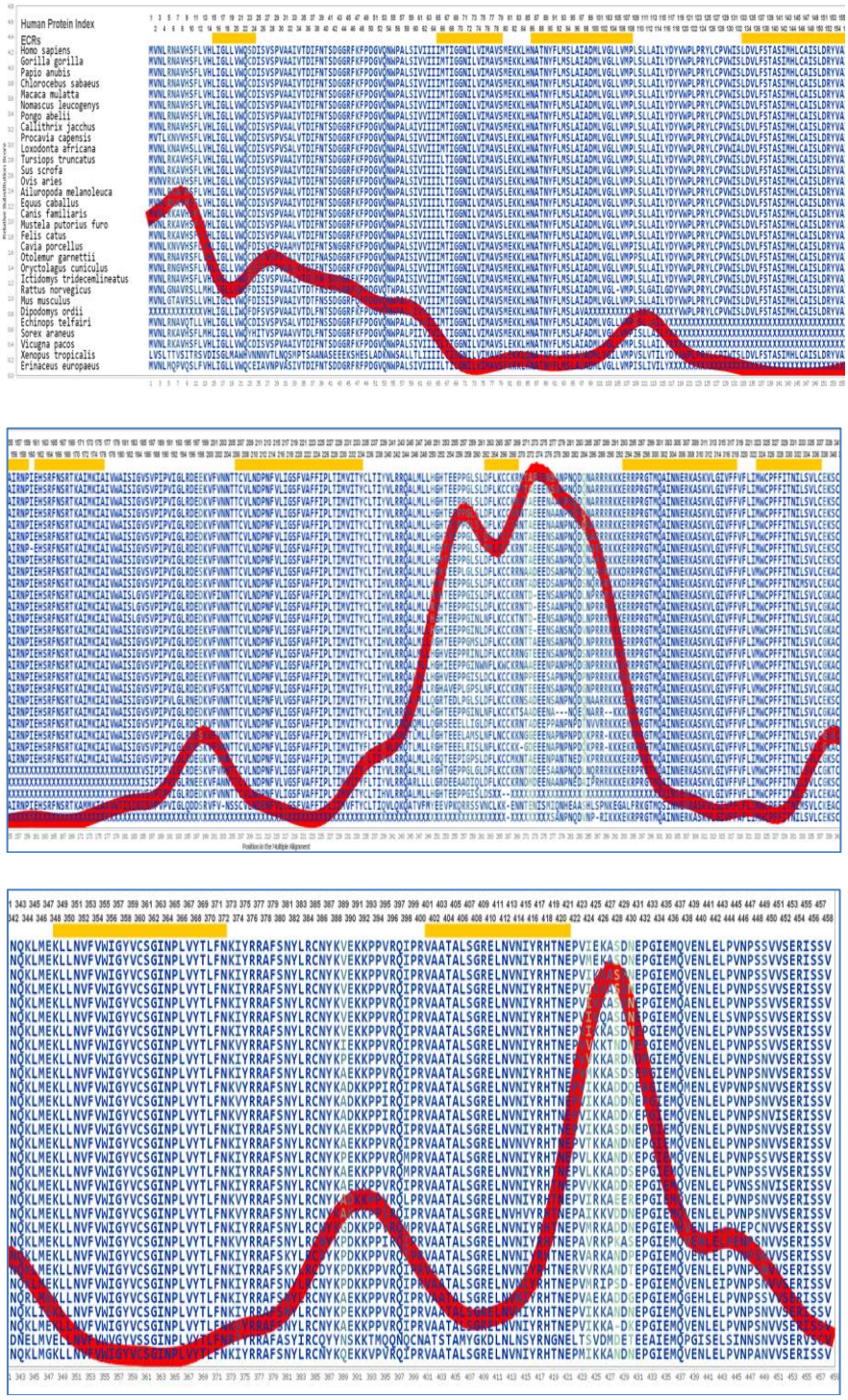


Fig. 5. Evolutionary conserved regions (ECR)analysis of HTR2C protein. (Red line) represents the relative rate of amino acid substitution calculated at each protein position. Constrained regions with relatively low substitution rates (Local minima-highlighted by Yellow bars) and regions with relatively high substitution rates (Local maxima indicate by Red peaks). The amino acid sequences for each ortholog are shown in shades of blue (high conserved) to green to (less conserved). Cysteine at position23 in h5HT$_{2C}$ and conserved at 127,207,337,341

regions on N and C terminal, intracellular and extracellular loops (for details refer [45]). Finally, the Haplotter base test suggests a moderate trend toward selection at the *5-HT2C* locus in the African population (p=0.06) suggesting positive selection and founder effects. In summary, these studies highlight the unique features of the receptor with respect to organization, evolution, and phylogeny.

1.5 5-HT2C Gene Variants / Polymorphisms and Association

Several variants in the gene are reported in *the MASTERMIND* database (33 missense; 15 non-synonymous; 1 truncating; 1 in-frame; 27 non-coding) [46]. Further, 5 structural variants (CNV) and loss-of-function variants associated with obesity and maladaptive behavior are also reported [47]. Several polymorphisms in the gene are reported and have been tested for association with various neuropsychiatric phenotypes (Table 1). Frequently studied the Cys23Ser (rs6318) is proposed to influence suicidal behavior [48], and cortisol stress reactivity in homozygous females and hemizygous males [49]. Further, it is associated with CSF monoamine metabolite concentration [50]. Other reports of association of the polymorphism are in bipolar disorder [51], lithium prophylaxis in mood disorders [52], Major affective disorder [53], Puerperal psychosis [54], and clinical course of SCZ [55]. Another polymorphism -759C/T is associated with antipsychotic-induced weight gain [56] and higher risk for hypertension in pharmacogenetics studies of antipsychotics [57]. Polymorphism rs1414334(C/G) is associated with metabolic syndrome in atypical antipsychotic treatment [58], and in metabolic syndrome in users of clozapine or risperidone [59], and cigarette consumption [60]. [61] Confirmed these previous findings. Finally, several researchers have also reported a lack of association of variants with various phenotypes.

1.5.1 Cys23ser variant and its effect on the receptor

The Cys23ser polymorphism (rs6318 ; p.C23S) in the N-terminal of protein leads to amino acid change at position 23 (Cys-hydrophobic and ser-hydrophilic). Further, this substitution is in the hydrophobic domain, and modification may affect the receptor function, and since it is the only cysteine in the N-terminal the Ser-23 substitution can disrupt di-sulphide bridges within and between 5-HT_{2C} receptors [62]. The unique property of cysteine is its relevance as both a free amino acid and a targetable protein residue with functional group thiol (–SH) as a side chain enabling the formation of disulfide bonds (S–S) leading to intra- and intermolecular covalent interactions affecting the three-dimensional structure of proteins. This assigns the protein several advantages such as extreme, physiological conditions of temperature and pH [63]. Several *in vivo* studies suggest differential effects to desensitization and inverse agonists of the ser-23 variant in cos-7 lines [64], variation of human wild-type and C23S variant in response to inverse agonist-induced re-sensitization [65] and lower function and shift in the subcellular localization profile in response to cocaine[66]. Also, biochemical analyses demonstrate lower Ser23 plasma membrane localization versus the Cys23 and subcellular localization studies demonstrate O-linked glycosylation of the Ser23 variant, but not the wild-type Cys23. Further, both the Cys23 and Ser23 variants are present in the recycling pathway with the Ser23 variant showing decreased co-localization with the early endosome [67]. Finally, Sf-9 studies provide evidence for higher binding of the Ser23 variant to m-cop and 5-HT [68]. In summary, several lines of evidences suggest that Ser23 influences inter-individual variations in behavior, susceptibility to behavioral diseases, and drug response.

1.6 Drug(s) and DNA/RNA Interaction Implications for Receptor Regulation

Several synthetic and natural compounds are shown to bind the receptor and alter its physiology in the brain differentially. Carbamazepine and *B. moniker* treatments reversed the alterations of receptor binding, gene expression, and inositol triphosphate content in the hippocampus of pilocarpine-treated epileptic rats [69]. Inactivation of the receptors reduced hypoplasia and motor response to MDMA (3, 4-Methylenedioxy-N-methamphetamine (MDMA or 'ecstasy') an appetite suppressant drug [70]. Serotonin satiety systems are altered in transgenic rats in response to angiotensin as indexed by higher expression of mRNAs in the hypothalamus [71]. Many developmental neurobehavioral effects are associated with ethanol exposure on the basal ganglia [72] and brain [73]. [74] Reported acute exposure to cocaine results in increased histone acetylation in the nucleus accumbens implicating epigenetic effects. [75] Suggested cocaine and opioid exposure results in deficits in hippocampal

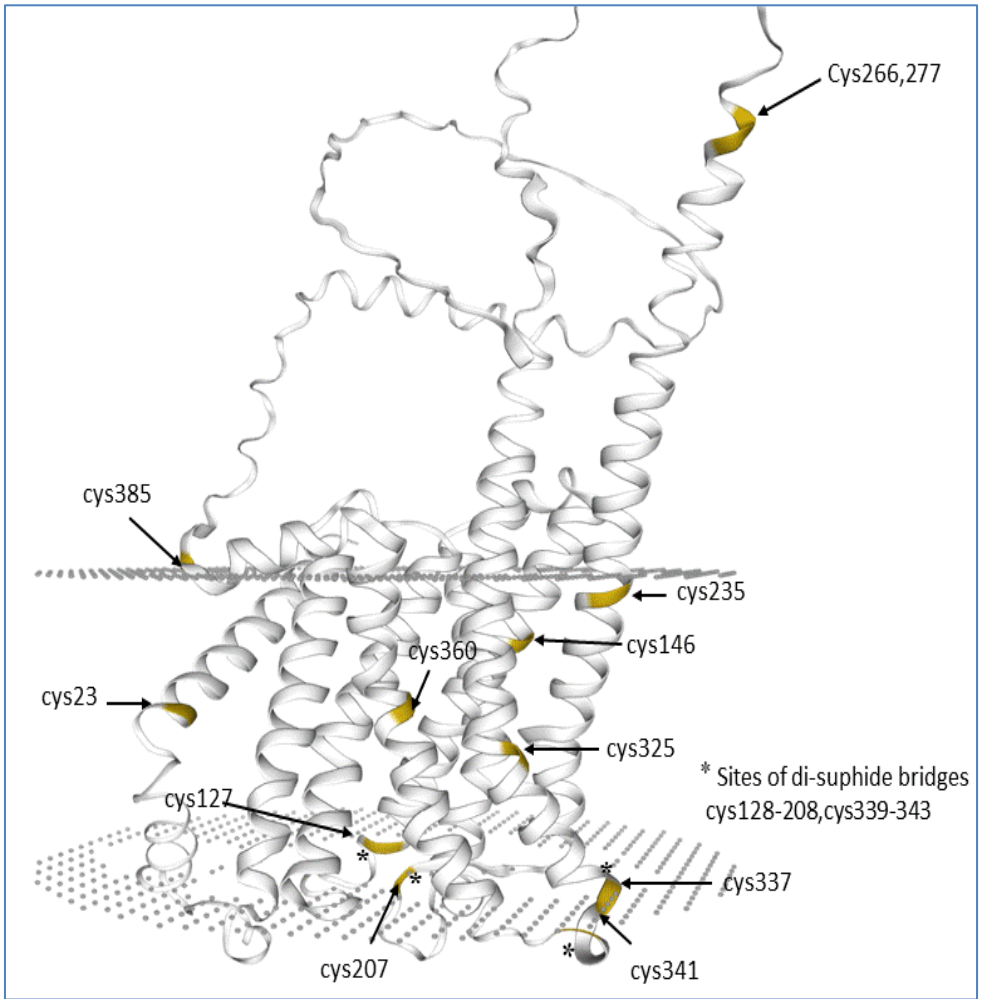


Fig. 6. AlphaFold DB model(ribbon) of 5HT2C_human receptor generated using SwissProt depicting cysteine residues(brown colour/arrow) and* highlighting disulphide bridges

plasticity and corpus callosum. Drugs are shown to affect editing [76] and ADAR2 activity [77]. A unique feature of these effects is inter and transgenerational transmission after drug exposure [78], psychoactive drugs [79] and epigenetic effects. These effects are proposed as mechanisms through which the dynamic genome interacts with the environment.

1.7 Homology Modelling of the 5-HT$_{2c}$ Receptor

Molecular docking studies assign important functions to the intracellular and extracellular N-C-terminal amino acid loops emerging from the transmembrane domains such as a binding interface for agonists and antagonists in the GPCR signal transduction [80]. Three-dimensional structures of the receptor are generated through homology modeling to study its interactions with diverse ligands. Of these, a. bovine rhodopsin with agomelatine [81], highlights two binding sites: a hydrophilic site involving Asp134, which plays a role in activation, and a more hydrophobic site with Ser138, Asp134, and Asn204, which enable interaction with aromatic rings, b. azepines with the β2-adrenergic receptor (β2AR) [82] highlights the roles of electrostatic interactions through hydrogen bonds aiding interactions with TM. Extracellular domains are distinct from the conserved transmembrane domains in terms of non-conservation of sequence, diversity in length, and conformational heterogeneity [83]. In site-directed mutagenesis experiments, hydrophobic cysteine cannot be unambiguously substituted with a single amino acid residue, in some cases the hydrophilic serine; or with hydrophobic alanine. These studies highlight the unique features of transmembrane domains and loops.

1.8 Analysis of the Variants using Bioinformatics Methods

Advances in computational biology and bioinformatics, have made available many *in silico* tools to researchers to predict the effects and potential significance of missense coding variants in the human genome. Several known disease mutations in GPCR genes cause close to 66 monogenic diseases [84]. Hence it becomes important to catalogue variants based on their impact on the protein. SIFT (Sorting Intolerant From Tolerant) and Polyp hen (polymorphism phenotyping) methods enable the prediction of variants based on protein sequence homology, alignment, and conversation among species and structural features characterizing the amino acid substitution.

1.9 Epigenetics Analysis of the *5HT2C* Gene

In the last decade, genetic studies repeatedly implicate the involvement of non-genetic factors/environmental factors in the causation of a range of human disorders [85]. Diet, chemicals (including drugs), and metals are known to affect DNA methylation and other epigenetic processes [86]. Methylation is the common epigenetic modification involved in the regulation of transcription (through transcriptional repression, formation of closed heterochromatin), imprinting, establishment of X-inactivation, and the formation of a chromatin structure [87]. Cytosine residue methylation is the most common DNA modification in mammalian cells accounting for 70–80% and 25–50% in stem cells and neurons [88]. Recent findings highlight methylation alterations in several genes caused by various drugs such as cocaine, opioids, cannabinoids (drugs of abuse), amphetamine, phenobarbital, and alcohol (addiction substances) in various human and animal model studies [89]. Methylation mediates long-lasting changes in gene promoters in response to environmental factors and acts as an intermediate process imprinting dynamic environmental experiences on the 'fixed' genome, resulting in stable altered phenotypes. Alterations of epigenetic pathways are shown to be associated with several neuropsychiatric disorders through several candidate genes with SCZ, such as *HTR1A, HTR2A*, glutamic acid decarboxylase 1 (*GAD1*), *REELIN*, *COMT* [90] in BPAD [91], and MDD [92]. Also, through epigenome-wide profiling [93] and network/pathway of proteins in reward and addiction such as deltaFosB [94].

Recent technological advances and resources in bioinformatics, genomics, and epigenetics provide new promising opportunities to explore the role of protein(s) structure, function and gene regulation mechanisms in neurotransmitter receptor genes during normal and disease processes. We test the hypothesis of gene-environmental interaction (drugs, stress) and their roles in transcription, and splicing using *in silico* methods.

2. MATERIALS AND METHODS

a. Protein BLAST- blastp of the receptor protein was carried out using the NCBI database and sequences retrieved. The phylogenetic tree was constructed using [95] to gain insights into the protein domains, conservation, and phylogenetic evolution.
b. Bioinformatics methods-SIFT and POLYPHEN were analyzed using servers [96] and [97].
c. Protein 3D model was generated using [98]
d. Epigenetics analysis was carried out using web-based resources available at ENCODE [99] accessed on May 2024. The genomic sequence of *5HT2C* gene 2 kb 5' flanks to transcription start sites (TSSs) and regions around exon 1 were analyzed (around 1kb (-) upstream and (+) downstream (defines the ROI). Specific tracks representing various epigenetic modifications were activated and images were acquired.

3. RESULTS AND DISCUSSION

Protein BLAST (blastp) search of the $5HT_{2C}$ protein retrieved homologous sequences from diverse species. The majority of these were mammals and primates (including prosimian), carnivores (land and sea), rodents, land and aquatic mammals, sea mammals (seals and whales) land and flying mammals, and toed mammals (Fig. 4). The presence of the receptor in diverse mammals suggests its evolutionary conservation across various taxa from sea mammals to land mammals with carnivores and primate intermediates hinting at the nervous system and physiological roles of GPCR in these species. Several animal behaviors such as aggression, feeding, defense and hierarchy, and executive functions including attention, have neurochemical correlates involving several neurotransmitters pathways, supported through

aberrant behavioral traits in Knockout mice (2CKO) [100]. The Phylogenetic analysis suggested moderate conservation at several residues in the protein. The Cys residue is moderately conserved with Cys residue at positions 127, 207, 337, and 341.

The human 5-HT_{2C} (human 5-HT_{2C}) has an additional Cys at position 23. Evolutionary and proteomic analysis suggests that cysteine residues appeared later in evolution, together with glycine, proline, and tryptophan and Cys residues constitute 2.3% of the human proteome with higher proportions in higher organisms. Further, in higher mammalian behavior traits such as motor activity, anxiety, learning and memory, sleep arousal, and circadian functions 5HT acts as a species-specific adaptation modulator enabling adaptation.

The seven transmembrane receptors are central and versatile components of the evolution of the GPCR signaling mechanism after introduction into early eukaryotic blueprint. Phylogenetic studies demonstrate rhodopsin and glutamate receptor families, known to be involved in neurotransmission in higher animals are also widely found in pre-bilaterian metazoans. Study of conservation of editing in shark and bowhead whales, pigs, and humans with higher expression of *5HT2C* and *ADAR2* mRNAs supports this rationale [101]. 5-HT receptors in the brain have a unique distribution pattern with varied functions and are implicated in neuro-psychiatric diseases. This could be explained through the diversity and distribution pattern of each 5-HT receptor enabling better cognitive and physiological functions. RNA modifications, gene-environment interactions and gene expression patterns are possible mechanisms with brain region specificity. Neuropsychiatric disorders tend to occur at the interface of normal social interactions associated with several social and cognitive skills and commonality exists in behaviors in primates and human societies at several social and cognitive traits. Distribution of SERT, 5HT1B, and 2C receptors between rats, primates and humans in major brain areas is observed. DNA variation(s) at several candidate gene/s are shown to affect these traits [102] suggesting conservation.

The rationale of SIFT is based on the premise that amino acid substitutions in conserved protein families will be intolerant towards substitutions and deletions. The Cys23ser polymorphism resulted in TI score (tolerant and intolerant) of 0.00, implicating its plausible effect on protein structure. POLYPHEN is based on the impact of amino acids on substitution structure and function the analysis showed the substitution was probably damaging (PRB), with consequences for an altered 3D structure. The effect of non-synonymous variants in a protein includes gain-of-function or loss–of–functions. Gain-of-function variants confer features such as selectivity, biased signaling, kinetics, and trafficking to the protein. Further, the domain of occurrence of variant also affects the "disease propensity" [103]. The unique protein motifs that have evolved through the recombination and duplication of discrete evolutionary units are referred to as evolutionary-constrained regions (ECRs). These regions are under functional constraint owing to their roles in protein stability, post-translational modifications, subcellular localization, interactions, and functions. Pathogenic variants like Cys23ser are enriched in ECRs ($P<10^{-4}$). Because constraints can vary widely along a given protein sequence, profiling the rates of evolutionary changes can provide useful information to identify the key residues [104]. ECRs analysis of $5HTR_{2C}$ is depicted in Fig. 5. The analysis demonstrated that Cys amino acid is conserved at several regions (moderate to high) and two regions with high substitution which could enable adaptation in the protein structure. Amino acid composition affects sequence-structure relationships and a large contribution to protein stability originates from the sequestration solvent properties of hydrophobic residues in the protein core [105]. Further, the physical nature of hydrophobic and electrostatic interactions, either hydrophobic-hydrophobic or opposite-type interactions such as negatively-positively is dependent on the protein residues in the core [106]. The 5-HT_{2C} receptor-mediated signaling network topology consists of several hub and node proteins thus hydrophobic cysteine residues allow the network flexibility and evolve in response to various ligands.

Alphafold model of h5-HT_{2c} receptor based on the rat-5-HT_{2c} 3D structure suggests di-sulfide bridges between Cys-127 and Cys-207; between Cys-337 and 341(Fig. 6). It is suggested that the disulfide bridge in the N-terminus provides rigidity, thus providing evidence for the role of Cys23 in ligand recognition/or interactions. Cys residues of rat-5-HT_{2c} are at positions 128,147,208,268,269,270,327,339,362,387, whereas in predicted h5-HT_{2c} are at positions 23,127,146,207,266,267,235,325,337,341,360,385. Suggesting that the Cys at 23,235, 341, are

unique and recent additions to human receptors. Further, it could be hypothesized that Cys23 residue in the second extracellular domain participates in the formation of disulfide bridges either through Cys337 or Cys341. A hypothetical structure of the receptor depicting alternative cysteine di-sulfide bridges due to substitution is shown in Fig. 7. Such changes due to different 3D structures of the resulting variant protein could alter the receptor structure, alter binding profiles, and downstream signaling. Unique clustering of cysteines in the three-dimensional structure of proteins correlates well with the fast and reversible SH/S–S exchange among vicinal residues. Several chemical processes take place at the level of thiol group such as redox potential regulation, the coordination of metals and metalloid cofactors, and reactions with gaseous signaling molecules such as NO and H2S [107]. The additional cysteine in the human receptor confers evolutionary advantages to the receptor at both at the structure and functional level to adapt to diverse ligands and signaling mechanisms in response to a changing environment. Future high-resolution computational research and cellular models will provide additional credence to the observations.

Various reasons have been ascribed to the conflicting reports about the association of variants in the gene and phenotypes such as population and founder effects [108], linkage disequilibrium (LD) patterns [109], complex promoter architecture, DNA secondary structure [110], use of post-mortem brain, buccal or leucocyte DNA which is now proposed to vary according to age, storage, drug treatment and limitations of bioinformatics methods which often predict based on sequence or structure. Also, several modifying genes may influence clinical variables adding noise to the genetic model. Lack of association of promoter haplotypes, and neuroleptic treatment implicates the role of regulatory variants or trans-acting factors [111]. Hence, a need for detailed case review to account for these variables before inclusion for genetics studies is suggested.

Gene expression is orchestrated by numerous control elements that may be located anywhere in the gene/genome through the cis/trans effect and can regulate distal genes by physically interacting with them. Identification of active gene regulatory elements is key to understanding transcriptional control of neural processes in different cells of CNS during differentiation and development, and in responses to the environment also environment. Aberrant DNA methylation is a hallmark of disease [112]. DNA methylation status within a gene's promoter upstream-downstream of the start codon and regions around exon 1 and intron 2 is associated with gene regulation. The Reduced Representation Bisulfite Sequencing (RRBS) of HeLa-S3 cells promoter methylation suggests a high to moderate methylation signal, however, no methylation was visible in H1-hESC and Brain cells (Fig. 8a). DNase I Hypersensitivity results from the loss or remodeling of nucleosomes and enables occupancy of regulatory factors *in vivo* at nucleotide resolution. The signal intensity at the DNAse I cluster and Master DNaseI HS was moderate, and the Txn factor ChIPE3 signal was high indicating a probable site of peak cluster of transcription factors (Fig. 8a).

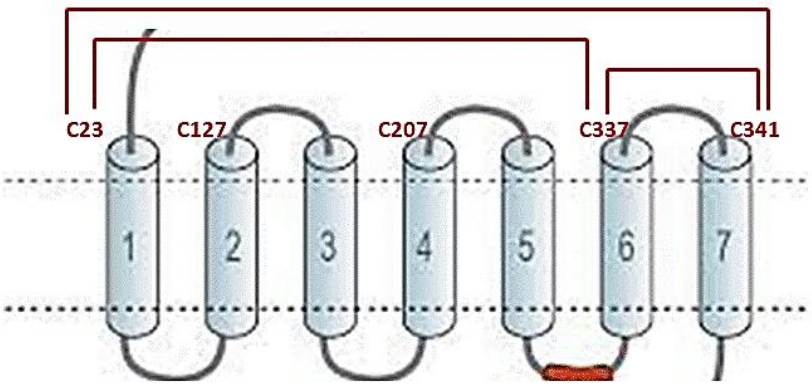
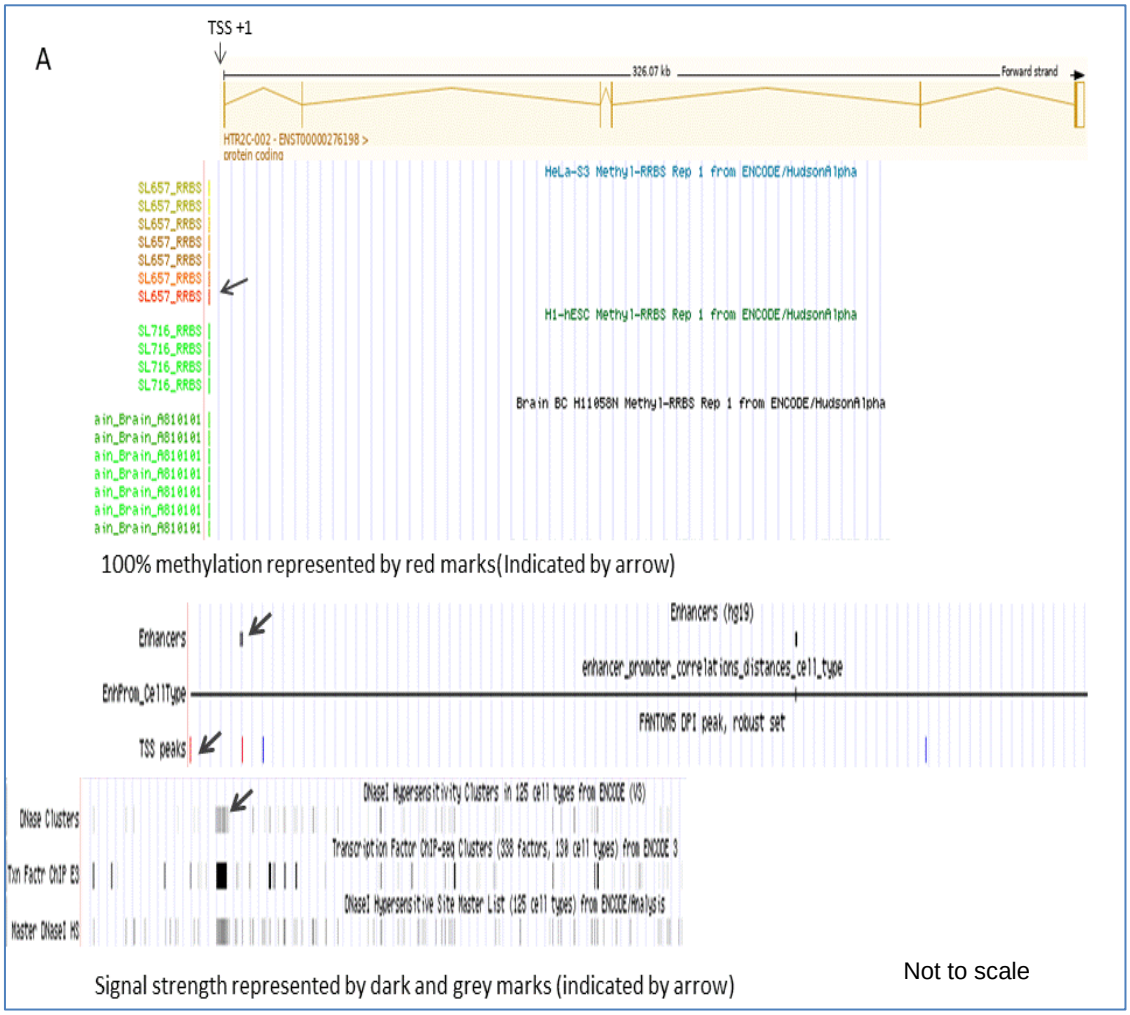
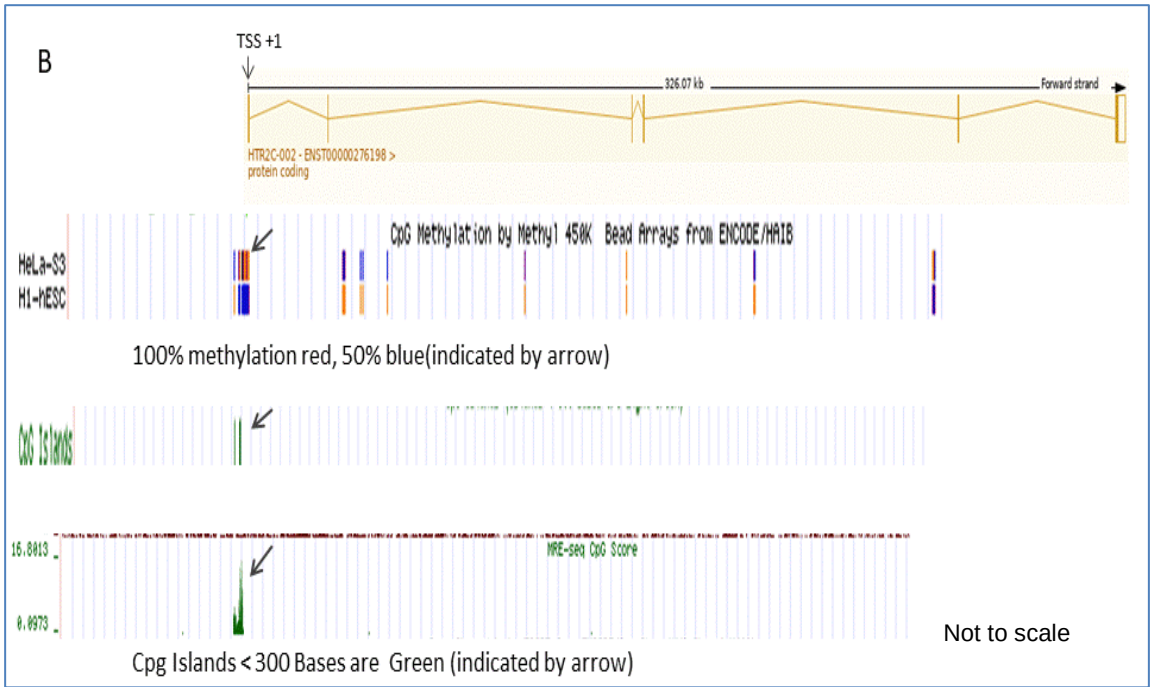


Fig. 7. Putative HTR2C receptor structure with cysteine residue (C23,127,207,340,344), connecting lines indicate probable cys-disulphide bridges


A
TSS +1
326.07 kb
Forward strand
HTR2C-002 - ENST00000276198 >
protein coding
HeLa-S3 Methyl-RRBS Rep 1 from ENCODE/HudsonAlpha
H1-hESC Methyl-RRBS Rep 1 from ENCODE/HudsonAlpha
Brain BC H11058N Methyl-RRBS Rep 1 from ENCODE/HudsonAlpha
100% methylation represented by red marks(Indicated by arrow)
Enhancers (hg19)
Enhancers
enhancer_promoter_correlations_distances_cell_type
EnhProm_CellType
FANTOM5 DP1 peak, robust set
TSS peaks
DNase Clusters
Transcription Factor ChIP-seq Clusters (338 factors, 130 cell types) from ENCODE 3
Txn Factr ChIP E3
DNaseI Hypersensitive Site Master List (125 cell types) from ENCODE/Analysis
Master DNaseI HS
Signal strength represented by dark and grey marks (indicated by arrow)
Not to scale


B
TSS +1
326.07 kb
Forward strand
HTR2C-002 - ENST00000276198 >
protein coding
CpG Methylation by Methyl 450K Bead Arrays from ENCODE/HAIB
HeLa-S3
H1-hESC
100% methylation red, 50% blue(indicated by arrow)
CpG Islands
MRE-seq CpG Score
Cpg Islands < 300 Bases are Green (indicated by arrow)
Not to scale

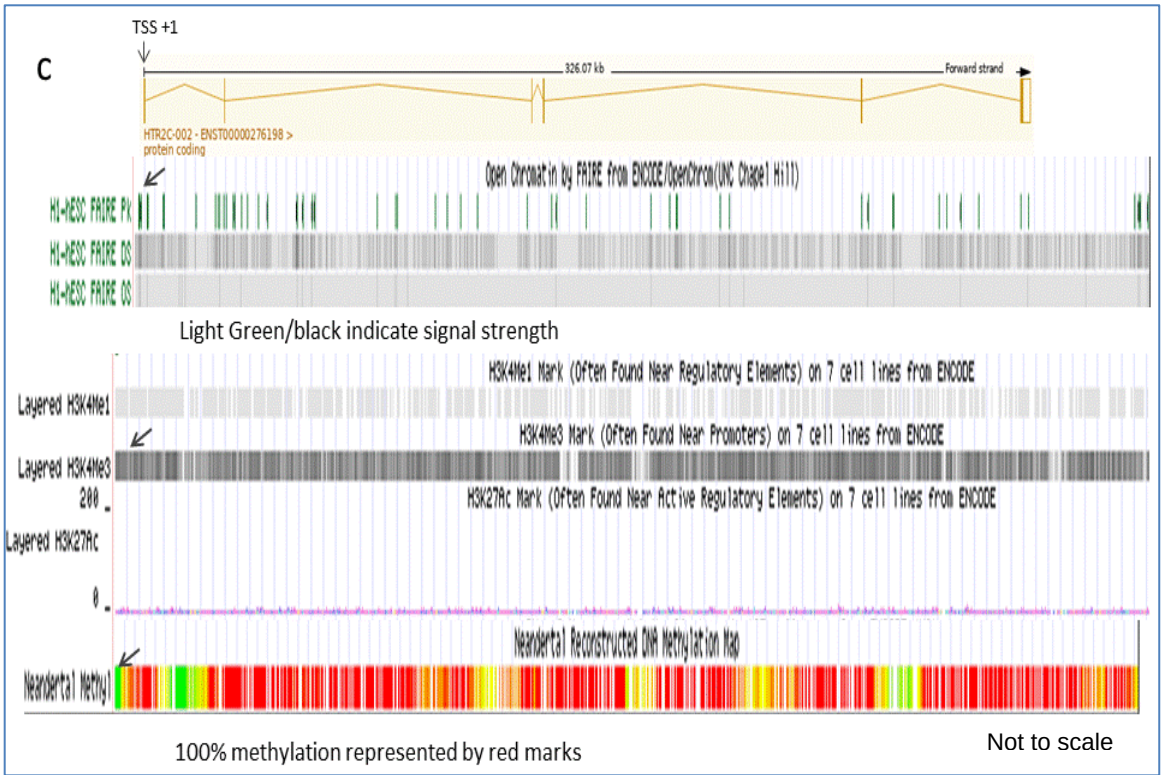


Fig. 8. Depicting the organisation of HTR2C gene with exons and transcription start site (TSS). A,B,C-UCSC-ENCODE analysis

A-RRBS methylation, Enhancer and TF binding and DNAse I hypersensitivity.
B-Promoter methylation ,CpG methlation, and CpG island.
C-FAIRE, Histone methylation and Neanderthal genome analysis.

Active transcription foci contain clusters—hubs—of enhancer-promoter interactions, transcriptional activators, and the stepwise assembly of RNA Polymerase II (Pol II). Complexes of promoters and enhancers networks at specific sets of genes in the transcription start site (TSS+/-) along with Transcription factors (TF) interact with RNA polymerases to regulate gene expression dynamically [113]. An enhancer in the promoter was observed and three TF binding sites were observed one in the promoter and two around the exon1 (Fig. 8b). Database search using [114] suggested three TF, a. *EZH2*-Enhancer Of Zeste (Drosophila) Homolog 2- belongs to the Polycomb-group (PcG) family of protein which enables transcription (-200 bp), b. *POLR2A*-DNA-Directed RNA Polymerase II Subunit protein responsible for synthesizing messenger RNA (+800bp) and c. *ZBTB40*-Zinc Finger and BTB Domain Containing 40 which enables DNA-binding of transcription factors (+740 bp). These results suggest the region is a hub for transcription related activities.MeDIP-seq enables investigation of the role of promoter-specific, intragenic, tissue-specific CpG and island methylation controlling gene expression. As depicted in Fig. B, the HeLa-s3 cells analysis showed differential methylation (orange, blue, and purple peaks) whereas weak signal intensity was seen in H1-hESC indicating no methylation. Two CpG 34 and 83 were observed near the promoter, also the CpG islands overlapped most of the ENCODE marks suggesting a probable role of methylation in gene expression. The unique cell type specific methylation signatures have relevance in gene regulation. Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) is a method to isolate and identify nucleosome-depleted regions of the genome it identifies functional regulatory elements that include promoters, enhancers, silencers, insulators, and locus control regions [115]. FAIRE peaks in H1-hESC were seen near the promoter and upstream of the promoter and exon 2 suggesting open nucleosome regions Fig. 8c. These results along with previous observations of DNaseI hypersensitivity, TF, and enhancer regions suggest a unique nucleosome region with affinity for differential regulation based on environmental cues.

Table 1. Summary of Polymorphisms in the *5HT2C* gene associated with neuropsychiatric diseases

Sl.no	SNP/Variant	Description of variant	Associated conditions	References
1.	rs1414334	intron_variant/ benign	Metabolic syndrome in Schizophrenia Treatment-resistant Schizophrenia Risperidone-induced weight gain in children with autism spectrum disorders	Mulder H et al.,2007 Fernandez-Egea E et al.,2024 Hoekstra PJ et al.,2010
2.	rs2192372	intron_variant	Suicidal personality traits in suicide attempters and controls	Molina-Guzman G et al.,2017 Serretti A et al.,2007, 2009
3.	rs2428707	intron_variant	Suicidal personality traits in suicide attempters and controls BPAD	Tovilla-Zarate CA 2014 Sadkowski M 2009,2013 Mazza M
4.	rs3813928	2KB_upstream_variant/ likely-benign	Nutritional status in children Metabolic Syndrome in Patients with Schizophrenia Antipsychotic-induced weight Obesity in psychiatric patients using antipsychotics	Miranda RC et al.,2015 Kang SH et al. ,2011 Opgen-Rhein C et al., 2010;Mulder H et al.,2007
5.	rs3813929	2KB_upstream_variant/ likely-benign	Circadian prolactin secretion related to pharmacogenetics Antipsychotic-induced weight gain Risperidone-Induced Insulin Resistance Syndrome	Sonkurt MD et al.,2022 Koller D et al.,2020 Chen Y et al.,2020;Das S et al.,2018
6.	rs4272555	intron_ variant	Suicidal behavior Personality traits in suicide attempters Suicide attempters and completers	Molina-Guzman G et al.,2017 Serretti A et al.,2009 Serretti A et al., 2007
7.	rs498207	2KB_upstream_variant / benign	Diabetes Mellitus and Obesity Antipsychotic-induced weight gain	Oh CM et al., 2016 Opgen-Rhein C et al.,2010;Wallace TJ et al.,2011.
8.	rs518147	5_prime_UTR_variant	Tardive Dyskinesia Antipsychotic-Induced Weight Gain Risperidone- or clozapine-induced hyperglycemia Risperidone-Induced Insulin Resistance	Sonkurt MD et al.,2022 Tsermpini EE et al.,2021 Luo C et al.,2019 Puangpetch A et al.,2019;Das S et al.,2018
9.	rs521018		Antipsychotic-Induced Metabolic Dysfunction in Schizophrenia Response to Treatment with Antidepressant Drugs Metabolic syndrome in patients with schizophrenia with atypical antipsychotics.	Paderina DZ et al.,2021 Xu Z et al., 2016 Bai YM et al., 2011

Sl.no	SNP/Variant	Description of variant	Associated conditions	References
10.	rs6318	Intron variant ,missense_ variant/benign	Cocaine use disorder Genetic Factors Associated With Tardive Dyskinesia Suicidal ideation and aggression in childhood, Adult depression depression in temporal lobe epilepsy Psychopathological symptoms in children and adolescents.	Ma L et al.,2022 Tsermpini EE et al.,2021 Hill SY et al.,2020 Vincentiis S et al.,2018 Paes LA et al.,2018 Bordoni L et al.,2018

Specific modification of histone proteins is referred to as histone mark, methylation and acylation to the histone proteins which influence gene expression by altering accessibility to the chromatin. The H3K4me1 histone mark is the mono-methylation of lysine 1 of the H3 histone protein associated with enhancers and regions downstream of transcription start [116]. The H3K4Me3 is the tri-methylation of lysine 4 of the H3 histone protein, associated with promoters that are active or yet to be activated. Enrichment of the H3K4Me3 marks was observed, however, no significant enrichment of H3K4me1 or H3K27Ac was observed, implicating its role in enhancer-promoter contacts (Fig. 8c). Finally, archaic DNA analysis enables the detection of potential regulatory elements as key drivers of phenotypic divergence. Two archaic humans the Neanderthal and the Denisovan, based on the natural deamination of cytosine in ancient DNA are presently used by researchers [117]. Moreover, methylated region differences between the genomes may explain phenotypic differences. In the present study, no significant signature methylation was observed implicating that the locus is conserved (8c).

4. CONCLUSION

Several questions in the context of Neuropsychiatric disorders remain unanswered such as a) why action of pharmacological drugs persists long after drug cessation and how and why gene expression profiles change? b) How do allelic variations alter these effects? Studies in model organisms propose that repeated exposure to drugs/chemicals can lead to cumulative effects on the chromatin and the effect may last for months or years and could be reversible/irreversible [118]. Pharmacological studies demonstrate the dominant effect of promoter on 5-HT_{2C} receptor hetero-dimerization and ligand binding, demonstrating the importance of promoter [119]. Further, the *5HT2C* gene shows a complex profile of transcriptional regulation, exon splicing, and RNA editing patterns with different isoforms. Moreover, several lines of research each of these processes could be altered. [120] showed psychotropic drugs/drugs of abuse can impact alternate splicing of *Dclk1* gene isoforms in a brain region-specific manner; [121] demonstrated epigenetic alterations impact splicing in the *ELOVL7* gene and finally, trans mechanism regulation of the promoter as shown in methyl-CpG binding protein 1(Mbd1) [122]. It is tempting to propose similar mechanisms could operate on the *5HT2C* gene, as discussed previously environmental effects through drug(s), dosage, interactions affect the gene regulation through several chromatin and methylation mechanisms and at transcription or translation. Specific drug, dose, or stimuli or a combinatorial combination could alter the signatures with specificity at cell, signature and at regulation levels. Future, high-resolution computational methods and *in vivo* chromatin level validation will enable a detailed picture.

Wide array of cellular pathways are aided by cysteine thereby impacting several neuro physiological processes [123]. Several events in GPCR signaling such as receptor G protein activation, coupling, and oligomerization processes are facilitated through Cysteine residues. Several observations and experimental data highlight the importance of cysteine in the protein structure-function relationship assigned to the physicochemical properties with a range of hydrophobicity scales that enable drug/ligand targeting. The residues are predicted to be crucial in molecular interactions between ADAR and targeting with small molecules represents a therapeutic strategy for modulating RNA editing [124]. The location in the protein structure of conserved residues (primates studies) implicate their roles in signaling, ligand selectivity and SNP variations in the N and C terminal loop can affect these processes. The Cys23ser is suggested to be part of the N-terminal signal peptide which acts as a cleavable peptide to direct the receptor to the plasma membrane. Hence, it could be inferred that the gain of function Cys23ser polymorphism could have conferred an advantage to the protein in the course of evolution to the human and closely related primates.

Several recent studies implicate epigenetic regulation of genome functions in several brain processes including neurogenesis, maturation, and differentiation and underlying behavioral plasticity such as learning, memory, and aging. Disruptions in these processes lead to the pathogenesis of many brain disorders [125]. Neurons are dynamic cells that respond and adapt to various stimuli throughout their long post-mitotic lives and regulation of transcription and its controls is a prerequisite. Dynamic and ordered 5mC and 5hmC changes are components of effective neurotransmission. Several unique features in chromatin states in the promoter such as DNaseI hypersensitive region, enhancer, TF binding, and H3K4me1

methylation were observed in the study**.** Evidence for similar observations is accruing, in cell lines of neural progenitor cells where KMT2B promotes catalysis of H3K4me1 at enhancers during differentiation. Also, a cell-type–specific enrichment of FAIRE coincident with the location of DNaseI hypersensitive sites, transcriptional start sites, and active promoters. This adds another layer of gene-specific unique complexity in the secondary and tertiary organization of the genome around the promoter and flanking regions.

Changes in the genetic sequence are often regarded as sources of observed heritability. Recent studies assessing mouse and human germ cell reprogramming have reported certain variations in methylation are erased in metabolic and neurological disorders [126] and erasure could occur during transgenerational epigenetic inheritance [127]. This observation is appealing since the dynamic genome could respond to environmental challenges given the instability over time, and the phenotypic effects disappear in a few generations. It is possible that the epigenetics marks observed in the study could also undergo such changes in response to various drug(s), and environmental stimuli (stress, anxiety) differentially and impact genetic stability.

Finally, the evolutionary trajectory of many DMR differentially methylated sites) in archaic and modern humans have an impact on human evolution and adaption may hint at their impact on fitness and influence species-specific inheritance. The lack of conservation of methylation at the promoter region suggests that the region is conserved.

In summary, the study highlights the role of protein residue conservation/flexibility at unique amino acids in the evolution of GPCRs. Also, the roles of promoter chromatin signatures and methylation-related differential regulation and expression of the *5HT2C* gene. Recent, studies implicate miRNAs could influence gene expression and regulation, database also suggest several miRNA in the promoter and flanking regions, hence exploring these factors and prudently designed cellular models will provide credence to the *in silico* observations. Therefore, future studies investigating genetics should carefully consider the variables of drugs, dosage, interactions at the DNA/RNA levels in the design and interpretation of results.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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Dedications-To the sacred feet of Bhaghavan and Gurus of Sringeri peetams.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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