MOLECULAR MECHANISM OF AGONIST AND ANTAGONIST WITH 5-HT RECEPTORS, STRUCTURAL CONSEQUENCES OF SNP's AND EVOLUTIONARY TRACE ANALYSIS OF 5-HT RECEPTORS

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter in the mammalian central nervous system (CNS) involved in numerous physiological and behavioral. Serotonin belongs to the evolutionarily oldest biogenic amines acting as neurotransmitters in the central nervous system. It was isolated from mammals in 1946 as a substance in the serum with tonic

actions on the vasculature, explaining its name. Seven years later it was also found in the brain and subsequently characterized as neurotransmitter. 5-HT plays a significant role in the regulation of many vital functions of the organism such as sleep, circadian rhythm, mood, cognition, reproductive behaviors, thermoregulation, nociceptive transmission, motor, endocrine, cardiovascular and respiratory functions, and intestinal peristalsis, and in the etiology of the related pathological states such as depression, anxiety, mania, schizophrenia, autism, obesity, drug addiction, migraine and hypertension (Alenina et al., 2009; Filip et al., 2005; Greek 2006).

Serotonin receptors have been implicated in modulating complex social behaviors (anxiety, depression, aggression, fear etc) in many mammals. In particular, 5-HT_{1A}, 5-HT_{1B}, and 5-HT2 receptors have been thought to be involved in such complex social behaviors. Evolutionary data from both sequenced genomes and targeted retrieved orthologs are increasingly used as a source of structural information. Mining of evolutionary data provides an additional source for understanding the functional relevance of individual receptor proteins, for interpreting naturally occurring receptor mutations in patients and for guiding structural modeling and mutagenesis studies of the target protein. Furthermore, the shared evolutionary ancestry of many proteins has enabled the development of comparative approaches designed to identify conserved sequence motifs that are responsible for certain functions. The presence of 5-HT receptors in diverse organisms from *Caenorhabiditis elegans* (worm) to humans and the larger multiplicity of the 5-HT receptor subtypes are suggestive of varied biological and behavioral functions further underlines its importance. The availability of large scale genome sequencing data from diverse species will contribute to the better understanding of human diseases. Moreover, sequence information from non-human primate genomes, which are thought to be closely related to the human genome sequences may aid in understanding the correlates of specific genes across primates and further help us to understand their impact on biology in health and disease (Varki and Altheide 2005).

The serotonin (5-hydroxytryptamine, 5-HT) receptors are G protein coupled receptors (GPCRs) belongs to the largest class of rhodopsin superfamily. The receptors belonging to the Rhodopsin family have several structural characteristics in common, such as the NSxxNPxxY motif in TMHVII or the DRY motif (also defined as D(E)-R-Y(F)) at the border between TMHIII and IL2. The diverse effects of serotonin are mediated via 5-HT receptors (5-HTR). The serotonin receptor family is larger than any other family of G-protein

coupled (GPCR) neurotransmitter receptors. They are located in the cell membrane of neurons and other cell types, including smooth muscle cells, in animals. In the brain the function of many 5-HTR is associated with specific physiological responses, ranging from modulation of the neuronal activity and transmitter release to behavioral changes. Based on the phylogenetic and molecular evolution studies of G protein-coupled receptors it has been proposed that 5-HT receptors have evolved more than 700-800 million years ago (Peroutka 1992; Peroutka and Howell, 1994). Being the ancient neurotransmitter receptor, comparative phylogenetic analysis with closely related species (Anbazhagan et al 2010) and in other mammalian and vertebrate species can aid in better understanding the molecular evolution of these 5-HT receptors (*manuscript in preparation*). 5-HT receptors are also pharmacologically important drug targets, especially the 5-HT2 sub-type receptors known to interact with large number of compounds. However, due to unavailability of the exact 3-dimensional structure of these receptors it becomes crucial in understanding the binding nature of the ligands. Homology based molecular modeling in combination with Evolutionary Trace (ET) analysis could provide opportunities to combine the sequence analysis results together with comparative docking studies in identifying functionally important residues (*manuscript in preparation*).

In this thesis I have carried out detailed sequence analysis of 5-HT receptors in human and non-human primate species (Anbazhagan et al 2010) which was further extended to look for the diversity and evolutionary relationships across species. As part of the structural insights, molecular modeling was mainly focused on the 5-HT2 receptor family viz., 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors and docking was carried out with available ligands. Further comparative analysis was carried out to look for the variation in binding affinities of the ligands among these receptors.

The thesis has been portioned as: 1) Introduction 2) Review of Literature 3) Comparative analysis of 5-HT receptors in human and non-human primates 4) Genome-wide survey of 5-HT receptors 5) Molecular modeling of 5-HT_{2A} and 5-HT_{2B} receptors and comparative docking 6) Focus on 5-HT_{2C} receptors: molecular modeling, Evolutionary trace analysis and preliminary analysis of single nucleotide polymorphism (SNP) 7) Conclusions 8) Appendices and 9) References.

Chapter 1: Introduction

This chapter gives the historical overview of serotonin and its isolation has been discussed. Further, the synthesis and metabolism of 5-HT was described briefly. The presence of 5-HT in the brain was reported by Gaddum in 1954 and it was Gaddum who also demonstrated that the action of 5-HT (in the gut) was antagonised by the potent hallucinogen lysergic acid diethylamide. This provoked the notion that 5-HT played a pivotal role in the control of mood and subsequent investigations have generally confirmed this hypothesis. A detailed overview of G protein-coupled receptors (GPCRs) with respect to the structure and its classification was discussed. The general overview of 5-HT receptor classification and its structural features were presented. To date, seven serotonin receptor families have been identified which are further sub-divided into at least 14 distinct receptor subtypes based on pharmacological and structural characteristics, and transductional mechanisms (Hoyer et al., 2002). Of the 5-HT receptors group, the 5-HT3 receptors is a ligand gated ion channel receptor; while the other 5-HT receptors viz., 5-HT_1, 5-HT_2, 5-HT_4, 5-ht_5, 5-HT_6 and 5-HT_7 belong to the G-protein coupled-receptors (GPCRs) superfamily. A detailed overview of location of serotonin receptors in the brain in context to the behavioral effect mediated by these receptors has been discussed. The significance of comparative sequence analysis focusing on specific genes (Varki and Altheide) using non-human primates in understanding the function and disease processes is discussed. Finally, a short description about the available crystal structures along with homology modeling and docking has been presented. Further, various tools, softwares databases has been described briefly which has been extensively used in this thesis work. Finally a short review on Evolutionary Trace analysis along with the significance of opting neurotransmitter receptors for evolutionary studies is described.

Chapter 2: Review of literature

In this chapter, I have briefly elaborated the evolutionary aspects of 5-HT receptors. A brief description for each single receptor which describes the length of the protein, expression, chromosomal mapping and function of each receptor were presented. Along with the description for the 5-HT2 receptors, a detailed scientific review in relation to the structure-function aspects is discussed. Recently, the first human GPCR, the β2 Adrenergic receptor (β2AR) which belongs to the class A of the GPCR superfamily has been made available (Cherezov et al, 2007; Rasmussen et al, 2007); a real breakthrough in GPCR structure modeling. Other GPCR structures, squid rhodopsin (Murakami and Kouyama, 2008) and human adenosine A2 receptor structure have also been completed (Jaakola et al, 2008). A

review of previous modeling studies along with mutagenesis is briefly described. A comprehensive description of various ligands used in this thesis work is also presented.

OBJECTIVES AND SCOPE OF THE STUDY

The present study was about the sequence and structural aspects of the 5-HT receptors. The overall aim of this thesis is to understand the 5-HT receptors at the sequence level between humans and non-human primate sequences and with other species as well. The other objective was to study the 5-HT2 receptors at the structural level. .

Thus, the specific aims of our study are:

1. Comparative and phylogenetic analysis of 5-HT receptors between human and non-human primates to study clustering pattern and sequences diversity.

2. Molecular modeling of 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors and docking of ligands to identify the functionally important residues.

3. Genome-wide survey and phylogenetic analysis of 5-HT receptor family to identify sub-class, sub-type and species specific residues within the 5-HT receptor family.

Chapter 3: Phylogenetic analysis and selection pressures of 5-HT receptors in human and non-human primates: receptor of an ancient neurotransmitter.

In this chapter, I have compared and analysed the human 5-HT receptors sequences with non-human primate sequences which has provided insights about the level of conservation as well as the diversity. The non-human primate 5-HT orthologous sequences were obtained from NCBI protein database through BLAST (Altschul *et al.,* 1990) search. Multiple sequence alignment (MSA) of 5-HT receptor sequences was carried out with ClustalW 1.83 (Thompson et al 1994) To understand the evolutionary relationships among the human and non-human primates 5-HT receptors, phylogenetic analysis with Maximum likelihood (ML) method tree construction was performed using PHYLIP package V 3.65 (Felsenstein, 1989). Sequences showing a bootstrap value of more than 50 were identified and considered for clustering. Upon clustering, it was observed that compared to the other 5-HT subgroups,

larger multiplicity and expansion was seen within the 5-HT_4 receptor subtype in both human and non-human primates.

Human and non-human primate 5-HT receptor gene coding sequences were subjected to non-synonymous (Ka) and synonymous (Ks) substitutions per nucleotide (*Ka/Ks)* ratio study using the Nei-Gojobori method as implemented in Molecular Evolutionary Genetics Analysis (MEGA 4.0 version) software (Nei and Gojobori, 1986; Tamura et al 2007). Analysis of signature gene sequences reveals negative (purifying) selection of 5-HT receptors over the course of evolution in human, chimpanzee and rhesus monkey. However, site-wise analysis using Selecton server version 2.4 (Stern et al 2007; Doron-Faigenboim 2005) revealed few residues that are under positive selection pressure and most of these residues were found to be in the highly variable region of the GPCR superfamily. Further, when we looked for the ongoing and recent selective sweeps in the human 5-HT receptors based on the population genetic data using Haplotter (Voight et al 2006, (http://hg-wen.uchicago.edu/selection/haplotter.htm), we found that 5-HT_{1D} and 5-HT_7 are under positive selection in the Asian and African population respectively. Similarly, we also observed that 5-ht_{1e} and 5-HT_{2C} show a trend towards positive selection in the African population.

Chapter 4: Genome-wide survey and phylogenetic analysis of 5-HT receptors

In continuation to the previous chapter, in this chapter I have analyzed the 5-HT receptors in the other genomes to study the clustering pattern, identification of motifs specific to the 5-HT receptors and also to understand the diversity of 5-HT receptors in other species among the sub clusters. Human 5-HT receptor protein sequences (5-HT1 to 7; excluding 5-HT3) were obtained from NCBI protein database (http://www.ncbi.nlm.nih.gov/). BLAST (Altschul *et al.,* 1990) search against non-redundant (nr) database for every human 5-HT receptor sequence including the subtypes was carried out to identify the orthologous sequences in the other genomes. The final data set contained 202 5-HT receptor sequences. All these receptor sequences were grouped based on their subtypes. We used the best bidirectional hit method to determine the orthologous pairs. Transmembrane helical domains were predicted using TMHMM (Krogh *et al.,* 2001) and HMMTOP (Tusnady and Simon, 2001) for the sequences

obtained and were considered for the analysis. Neighbor-joining (NJ) method tree construction was carried out using PHYLIP V 3.65 (Felsenstein, 1989). Also, maximum likelihood (ML) tree construction has been carried out for each sub-class individually. Our previous comparison study of 5-HT receptors between human and non-human primate have identified conserved subtype specific residues (Please see chapter 3, Figure 3.2 and Table 3.1). In this present study, it is interesting to note that the sub-type specific residues which are conserved in human and non-human primate are also found to be highly conserved in other species as well.

Tree construction by NJ method of the 5-HT receptors has been grouped into eight different clades. The first three clades comprise of the 5-HT1 receptor sub-class, which includes 5-HT1B and 5-HT1D in clade 1, clade 2 consists of 5-HT1E and 5-HT1F and the clade 3 consist of 5-HT_{1A}. Clade 4 encompasses the 5-HT2 receptor sub-class, the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. Clade 5 includes the 5-HT_4 receptor sequences and clade 6 contains 5-HT_6 sub-class. The last two clades, clade 7 and clade 8 comprise the 5-HT5 (5-ht_{5a} and 5-ht_{5b}) and 5-ht_7 receptors respectively. Interestingly, our sequence analysis of 5-HT receptors across several species revealed motifs specific to 5-HT receptor family as well as specific to sub-classes of 5-HT receptors. Study shows that the primordial 5-HT receptor should have evolved more than 700-800 million years ago and later on the further differentiation occurred since the 3 major classes of G protein-coupled 5-HT receptors (i.e. 5-HT1, 5-HT2 and 5-HT6 receptors) are less than 25% homologous (Peroutka and Howell 1994). This is consistent with our study and based on the larger sample set of orthologous sequence identity, the three major receptor classes clearly show lower sequence identity. Based on our study, it can be hypothesized that among the three major classes the 5-HT6 receptor might have evolved first, then the 5-HT_{2B} and 5-HT_{1A} receptor. In conclusion, this is the first detailed sequence analysis of the 5-HT receptors which led us to identify sub-class specific, sub-type specific and species specific residues.

Chapter 2: Molecular modeling of 5-HT_2 receptors: A comparative study of binding properties of various ligands.

The 5-HT2 family of receptors consists of three subtypes, namely, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, which have been grouped together on the basis of molecular sequence, secondary-messenger system, and operational profile. Sequence analysis indicates approximately 80% amino acid identity in the transmembrane regions of these three subtypes, and it is not

surprising that many compounds once thought to be selective for the 5-HT_{2A} receptor also bind with high affinity to the 5-HT_{2B} and 5-HT_{2C} sites and vice versa. Given that the variety of ligands bind to the 5-HT2 receptors with similar and with varying affinity, it might be interesting to look for the nature of the ligand binding in all the three receptors which could provide useful insights regarding the variation in affinities based on the type of interactions at the residue level. The human 5-HT2 receptor sub-types amino sequences were retrieved from the NCBI protein database, 5-HT2A (NP_000612.1), 5-HT2B (NP_000858.3) and 5-HT2C (NP_000859.1). The amino acid sequence length of the 5-HT2A receptor is 471 residues and for the 5-HT2B and 5-HT2C its 481 and 458 residues in length respectively.

In this chapter, we have modeled all the full length 5-HT2 receptors suing MODELLER9v1 software package (Sali A and Blundell T.L 1993). First, the7 TM domains of human 5-HT2 receptor were constructed using the recently available crystal structure of human β2 Adrenergic receptor (β2AR) as a template. The human 5-HT2 receptor and the β2AR belongs to the aminergic receptor class of the the α-Group of Rhodopsin receptors and are found to be phylogenetically closely related (Fredriksson et al 2003). Twenty different models were generated for each 5-HT2 receptors and the one with the lowest possible energy and least restraints defilement was picked up for further analysis. Second, the N-terminal region, Intracellular loop3 and C-terminal were constructed to build the full length model of the 5-HT2 receptor by searching for Protein Loops against the inbuilt PRODAT Database in SYBYL software package (SYBYL 7.1, Tripos Inc., MO, USA). The final full length models was then subjected to Powell and conjugate gradient minimization using the standard Tripos force field until the RMS gradient was lower than 0.05 kcal/mol with the distant-dependent dielectric constant of 1.0 and non-bonding interaction cut off as 15. All hydrogen atoms were included during the minimization process. All these calculations were performed using the SYBYL software package (SYBYL 7.1, Tripos Inc., MO, USA) running on the high end Linux work station. The final model was validated for stereochemical properties of the coordinates using PROCHECK (Laskowski R. A et al 1993) available through RCSB ADIT server while the 3D profiles of the model was checked using the Verify 3D (Luthy et al 1992).

A series of ligands belonging to different chemical classes viz., tryptamine derivatives, ergolines, piperazine class of drugs, phenothiazines, diazepines, Benzisoxazole derivatives , butyrophenone and indole derivatives were docked into the modeled 5-HT2 receptors. Docking of all these ligands to the modeled 5-HT2 receptors was carried out using AutoDock 4.0 (Morris G.M et al 1998). Analysis of the docked complexes revealed ligands with similar

chemical group prefer to be oriented in similar fashion inside the binding cavity. The conformational position of the tryptamines and other synthetic agonists such as DOI (4-iodo-2,5-dimethoxyphenylisopropylamine) inside the binding pocket of the 5-HT2 receptors prefer to orient inbetween TM3, TM5, TM6 and TM7. However, it was interesting to note that Leu209 and Lys211 which is present in the extra cellular loop 2 (ECL2) of the 5-HT2B receptor siginificantly contributed in ligand binding. Lys211 participates in hydrogen bond formation with 5-HT, 5-CT (3-(2-aminoethyl)-1H-indole-5-carboxamide) and with α-Methyl-5-HT; 3-(2-aminopropyl)-1H-indol-5-ol). Evolutionary Trace analysis (chapter 6) and sequence comparison of (chapter 6) of human 5-HT2 receptors has revealed Lys211 as class-specific residue. In the corresponding position Aspartic acid (D) residue is present in both 5-HT2A and 5-HT2C receptors. Analysis of ergolines found to orient in between TM2, extracellular loop 1 (ECL1), TM3, ECL2, TM5, TM6 and TM7 except for the metergoline for which there was no residue found to interact in TM2 and ECL1. The priperazine class of drugs majority of the residues from TM3, TM5 and TM6 were found to interact with the ligand except for trazodone for which significant contribution of residues from TM7 was found to participate in the ligand binding. The phenoththiazines group of compounds such as Chlorpromazine and Trifluoperazine found to prefer different orientations in the binding pocket of 5-HT2A and 5-HT2C receptor respectively. Chlorpromazine was found to accommodate in between TM3, TM5 and TM6. However, the Trifluoperazine compound was found to be oriented in between TM3, ECL2, TM6 and TM7. ECL2 and TM7 have significant contribution in ligand binding for Trifluoperazine. Whereas, no contribution of residues from ECL2 and TM7 for the drug Chlorpromazine except for the Val354 residue in TM7 of the 5-HT2C receptor. The diazepine class of drugs found be oriented inbetween TM3, ECL2, TM5, TM6 and TM7. The butyrophenone and indole derivatives aslo found to be preferred in the same orientation as observed for diazepine class. Apart from the previously reported residues that contribute for binding, our study in combination with ET and sequence analysis had identified newer residues which could have a functional importance to these receptors.

Chapter 6: 5-HT_{2C} Receptor and Its Role in Neuropsychiatric Disorders: Evolutionary Trace Analysis and Molecular Docking Study

This chapter describes similar kind of work as the previous one. In this chapter detailed

analysis of the 5-HT2C receptor has been carried out. Evolutionary trace (ET) analysis was carried out using a set of homologous sequences to map the functionally important residues onto the modeled 5-HT_{2C} receptor structure. Using this method, we find regions that are highly conserved and likely play a role in the function of the receptor. In addition, we find a number of class-specific residues that distinguish the 5-HT_{2C} receptor from the other subtypes in the 5-HT2 family, and may involve differing in their substrate specificities. Finally, docking was performed using a series of ligands such as the endogenous ligand (5-HT, serotonin), synthetic agonist (DOI), partial agonists (Lisuride, m-CPP), antagonists (Asenapine, Chlorpromazine and Ketanserin) and inverse agonists (Clozapine, Risperidone and Sertindole) to identify structurally and functionally important residues. The methods followed in constructing the 5-HT2C model and structure validation is as mentioned above.

Evolutionary trace (ET) analysis was carried out on a set of 47 5-HT2 receptor sequences to identify and differentiate the 5-HT_{2C} receptor residues within the 5-HT2 receptor subtypes (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}). Here, the trace for increasingly fine partitions was mapped onto the 5-HT_{2C} receptor structure. First, the phylogenetic tree was constructed and split along evolutionary time into ten evenly distributed partition: P01 to P10 in order of increasing evolutionary time cut-off (ETC). For each partition, a trace procedure was completed automatically in 3 steps: (i) Protein sequences connected by a common node with evolutionary time greater than the given ETC were clustered together; (ii) a consensus sequence was generated for each group to distinguish between conserved and non-conserved position and; (iii) a trace was generated by comparing the consensus sequences of receptors. Residues were classified into three types: invariant, class specific, or neutral. A total of 91 residues that are invariant in the transmembrane domain of the 5-HT2 receptor family had been identified. We focused mainly on the class-specific residues which are present in the TM regions and nearby invariant residues believing that these residues could be important in distinguishing ligand specificity. Analysis of partition P08 identified a set of class-specific residues and most of these residues were located in between the invariant residues or in the close proximity of the invariant residues. We have also identified 5-HT_{2C} receptor "strictly" specific residues from the alignment which are replaced by a common residue in the human 5-HT_{2A} and 5-HT_{2B} receptor sequences respectively. Analysis of the docking results had showed some of the class-specific residues, Ser110, Ala113, Ile114 (TM2), Ile131 (TM3), Val215 (TM5) and Asn351 (TM7) identified through ET analysis have shown to contribute to the ligand binding. Apart from the residues present in the TM regions, two residues Val208

and Asn210 present at the end of the ECL2 also participated in the ligand binding suggesting that residues present in the ECL regions might have a role to play in the ligand binding.

Molecular modeling of the human 5-HT_{2C} receptor showed two cysteine residues, Cys337, Cys341 in the ECL3 which are in the close proximity to Cys23 present in the N-terminal region. Since, these residues were present in the flexible loop regions; we propose that a disulphide bridge may form either between Cys23 and Cys337 or Cys23 and Cys341.

Modification due to the Cys23Ser substitution of the 5-HT_{2C} receptor N-terminal region was analysed using **SIFT** (Sorting Intolerant From Tolerant) (Ng et al. 2003, http://blocks.fhcrc.org/sift/SIFT.html) and **PolyPhen** (*Poly*morphism *Phen*otyping, (Ramensky 2002, http://www.bork.embl-heidelberg.de/PolyPhen). The SIFT analysis showed the substitution was predicted to be affecting the protein function (Tolerant and Intolerant Score = 0.00). Polyphen analysis also showed that the substitution is probably damaging. Point mutation study of Cys23 at the structural level will be of further interest as the Cys23Ser polymorphism has been shown to be associated with many psychiatric illnesses. Comparative molecular dynamics study of the 5-HT_{2C} receptor between wild type (Cys23) and mutated (Ser23) in agonist or antagonist bound form may shed light in better understanding the structural consequences of this polymorphism.

Conclusions

Serotonergic signaling participates in several essential biological processes. They are also critical for complex phenomena such as aggression, behavior, and various aspects of cognition. The 5-HT receptors have thus been associated with psychiatric disorders including depression, anxiety, social phobia, schizophrenia, obsessive-compulsive and panic disorders, and other disease states like migraine, hypertension, eating disorders, vomiting and irritable bowel syndrome (IBS). Sequence studies such as Comparative genome analysis is a useful method to understand the diversity and complexity observed in biology. In this thesis I have attempted detailed sequence analysis of the 5-HT receptors and attempted to correlate the information obtained to the 5-HT2 receptors at structural level.

The present study was aimed at understanding the evolution of 5-HT receptors, given the involvement of 5-HT receptors in various psychiatric disorders, both as susceptibility loci as

well as drug targets. Analysis on selection pressure revealed primate 5-HT receptors are under functional constraint (purifying or negative selection), which could be due to the high level of sequence conservation. Such comparative and detailed analysis of specific genes associated with these disorders, as well correlation of behaviors in relation to genetic variations, in closely related non-human primates, may aid in better understanding of the nature of these disorders, and the complex interactions between genes responsible for them. Similarly, a genome-wide survey of 5-HT receptors has identified sub-class specific, sub-type specific and species specific residues. Further, our detailed sequence analysis revealed motifs specific to the 5-HT receptor class. Taken together, these residues could have a role to play in the functional aspects of the receptor.

The structural studies of the 5-HT2 receptors in combination with ET analysis led us to identify invariant and class specific residues. Further, molecular docking experiment was performed with different sets of agonists and antagonists, which identified residues that might play a crucial role in binding of the ligands. Preliminary analysis of the Cys23Ser polymorphism at the N-terminal region is predicted to have an impact on the structure and function of the protein. Further, detailed analysis may aid in better understanding of the receptor in terms of ligand interaction, conformational changes, downstream signaling and trafficking.