

Chapter 11

Structure, Function and Physiology of 5-Hydroxytryptamine Receptors Subtype 3



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Abstract 5-hydroxytryptamine receptor subtype 3 (5-HT₃R) is a pentameric ligand-gated ion channel (pLGIC) involved in neuronal signaling. It is best known for its prominent role in gut-CNS signaling though there is growing interest in its other functions, particularly in modulating non-serotonergic synaptic activity. Recent advances in structural biology have provided mechanistic understanding of 5-HT₃R function and present new opportunities for the field. This chapter gives a broad overview of 5-HT₃R from a physiological and structural perspective and then discusses the specific details of ion permeation, ligand binding and allosteric coupling between these two events. Biochemical evidence is summarized and placed within a physiological context. This perspective underscores the progress that has been made as well as outstanding challenges and opportunities for future 5-HT₃R research.

Keywords 5-HT · Serotonin · 5-HT₃R · Pentameric ligand-gated ion channel · Neurotransmission · Membrane biophysics

Introduction

5-hydroxytryptamine (5-HT) or serotonin is a versatile neurotransmitter whose functions range from visceral reactions to higher-order neuronal processes. 5-HT affects cellular processes by binding to a large family of membrane-bound 5-HT receptors (5-HTR). 5-HTRs are mostly G-protein coupled receptors (GPCR), and act through

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relatively slow metabotropic processes. The sole exception is 5-HT₃R, which is a pLGIC that participates in fast neurotransmission (Lummis 2012).

5-HT₃Rs are expressed throughout the body and are especially known for their specialized roles in brain-gut circuitry. In this context, the receptors regulate gut motility, secretion, emetic reflex and visceral perception and are important therapeutic targets for chemotherapy-induced vomiting and irritable bowel syndrome (IBS). Elsewhere in the brain, 5-HT₃Rs play a mostly modulatory role in synaptic signaling and are potential targets of several psychiatric disorders (Rojas and Slusher 2012). 5-HT₃Rs share a quaternary structure with three other vertebrate pLGICs, nicotinic acetylcholine receptors (nAChR), γ -amino butyric acid A receptors (GABA_AR) and glycine receptors (GlyR). Together, these ion channels are integral components of neurotransmission with significant interplay. Therefore, the underlying mechanisms of 5-HT₃R function and modulation are not only relevant to 5-HT signaling but general neuronal communication.

5-HT₃Rs were first recognized as ion channels in 1989 (Derkach et al. 1989). In 1991, the first of five 5-HT₃R genes, 5-HT_{3A}, was discovered and the amino acid sequence revealed that 5-HT₃Rs are members of the pLGIC family (Maricq et al. 1991). pLGICs assemble as a complex of five homologous subunits that are often heterogeneous. To date, there are five 5-HT₃R genes termed A-E (Niesler et al. 2003; Davies et al. 1999). Only 5-HT_{3A} can assemble as a functional homopentamer, 5-HT_{3A}R. (5-HT_{3A} refers to a single subunit and 5-HT_{3A}R refers to an assembled pentamer). 5-HT_{3A} is an obligate participant in all other 5-HT₃R complexes and the most well-studied complexes are 5-HT_{3A}R and heteromeric 5-HT_{3A/B}R.

The first section of this chapter is a general overview of the physiological role of 5-HT₃R and its general structure. The physiological role of 5-HT₃R requires three fundamental properties. (1) 5-HT₃R selectively conducts and gates ionic flux across the membrane, (2) 5-HT₃R strongly and specifically binds 5-HT and (3) 5-HT₃R allosterically connects channel activity to ligand binding. Each of these properties is presented in its own section that discusses its physiological implications, underlying mechanisms and regulation by endogenous and exogenous ligands. The chapter concludes with a perspective on outstanding questions and future directions of 5-HT₃R research.

General Overview

Expression and Physiological Role

5-HT₃Rs in the PNS

5-HT₃Rs participate most prominently in CNS-GI signaling, consistent with the fact that more than 90% of 5-HT is found in the gut. Specifically, 5-HT₃Rs are found on nodose ganglion afferent neurons that innervate the GI tract (Leal-Cardoso et al.

1993). The receptors are found at multiple locations on nodal afferents including, dendrites in the gut, cell bodies at the ganglion (located at the base of the skull) and presynaptic terminals in the brain stem (Powley et al. 2011). Dendritic 5-HT₃Rs directly respond to 5-HT released by enterochromaffin cells during digestion. (Hillsley et al. 1998; Hillsley and Grundy 1998). Receptors at the ganglion and brainstem can be activated by 5-HT circulating in the bloodstream because the barrier between them and the bloodstream is leaky (Lacolley et al. 2006a,2006b; Baptista et al. 2007). Once activated, 5-HT signals are transmitted to the brainstem via glutamatergic synapses in the nucleus tractus solitarius (NTS). There, 5-HT₃Rs are found presynaptically where they modulate glutamate release, and there is at least one report of postsynaptic 5-HT₃Rs that shape the NTS response (Glaum et al. 1992; Jeggo et al. 2005). Nodal afferent stimulation is summarized in Fig. 11.1.

CNS Regions with High 5-HT₃R Expression

- Basal Ganglia
- Hippocampus
- Brain Stem

Physiological Responses

- Gut motility
- Secretion
- Visceral perception
- Emetic reflex

Intestinal EC Cells

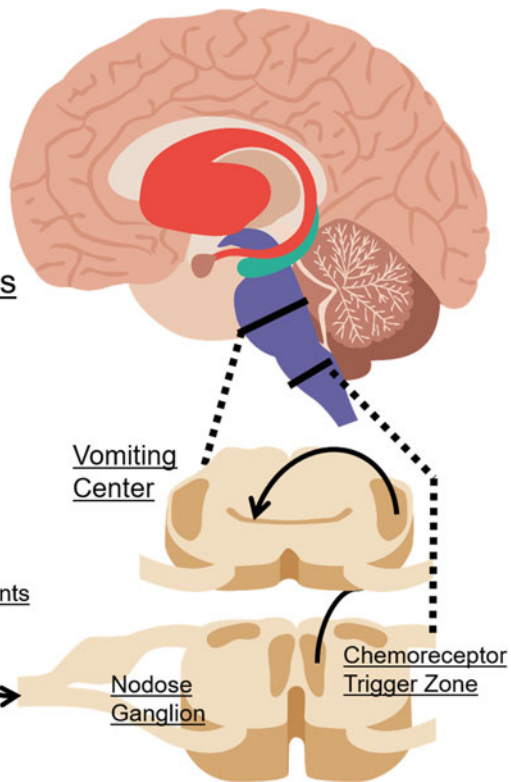
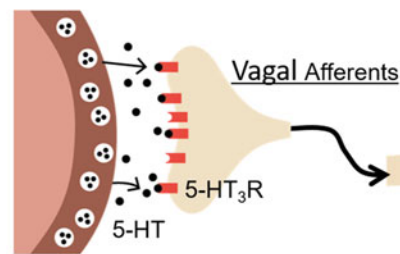


Fig. 11.1 Localization and physiological signaling of 5-HT₃R. 5-HT released from enterochromaffin cells in the intestine stimulates 5-HT₃Rs on the nerve terminals of vagal afferents. In addition, circulating 5-HT can stimulate 5-HT₃Rs in the nodose ganglion or within the brain stem. These initiate signals within the brain that contribute to the listed physiological responses. 5-HT₃Rs are also present in other CNS regions where they play a mostly modulatory role at glutamatergic, GABAergic and dopaminergic synapses

5-HT₃Rs on vagal nerves are not required for enteric motility but are important in modulating motility according to environmental factors (Keating and Spencer 2010; Blackshaw and Grundy 1993). For example, in the gut, glucose stimulates enterochromaffin cells which in turn release 5-HT. 5-HT then activates 5-HT₃Rs that inhibit gastric motility, allowing time for digestion (Raybould et al. 2003). Glucose in the bloodstream can further impact nodal afferents by directly increasing excitability and by increasing surface expression of 5-HT₃Rs throughout the cell membrane and presynaptic terminals (Grabauskas et al. 2010; Babic et al. 2012; Wan and Browning 2008). The influence of glucose on 5-HT₃R signaling under both normal and pathophysiological conditions requires further investigation. In addition to nutrient modulation, antigens also indirectly activate 5-HT₃Rs via enterochromaffin cells. Antigen-induced 5-HT₃R stimulation leads to surface expression of immunogenic NK1 receptors on afferent vagal neurons, implicating 5-HT₃Rs in long-term nausea, antigen-induced pain and inflammation (Moore et al. 2002). 5-HT₃R antagonists have been used effectively as anti-emetics during cancer treatment and more recently for treatment of IBS (Zheng et al. 2017; Rojas and Slusher 2012). This is discussed further in the section on ligand binding.

Though peripheral 5-HT₃R input is generally associated with vagal afferents, 5-HT₃Rs are also found in other neurons though there are significant variations between species. In some species, 5-HT₃Rs have been found in the enteric nervous system, spinal nociceptive neurons and in the trigeminal nucleus, which innervates the jaw and face (Parker et al. 1996; Bertrand et al. 2000; Glaum et al. 1990). 5-HT₃Rs are also found in non-excitabile cells within the GI tract including interstitial cells of Cajal, which act as pacemaker cells for smooth muscle, and enteroendocrine cells, though not enterochromaffin cells (Glatzle et al. 2002).

5-HT₃Rs in the CNS

Radioligand binding and mRNA expression show that 5-HT₃Rs are found in several brain structures. In the brainstem, 5-HT₃Rs are found on structures that integrate inputs from other sensory neuronal circuits (Parker et al. 1996; Miyake et al. 1995). 5-HT₃R expression is particularly high in the NTS and area postrema. The receptor is also present in the adjacent dorsal motor nucleus of the vagus (DMV), implicating 5-HT₃R activity in physiological responses such as vomiting and blood pressure regulation (Babic and Browning 2014; Sevoz et al. 1997; Mussa et al. 2008).

In humans, 5-HT₃Rs are also detected at relatively high levels in the hippocampus and basal ganglia and at lesser levels in the cortex (Bufton et al. 1993; Parker et al. 1996). In these regions, there are some serotonergic synapses with postsynaptic 5-HT₃Rs, but 5-HT₃Rs are especially found presynaptically where they modulate GABAergic and dopaminergic synapses (Sugita et al. 1992; Mylecharane 1995; Puig et al. 2004). In particular, 5-HT_{3A}R homomers have conductance, ligand binding and kinetic properties tailored to this role, as will be discussed throughout the chapter. Understanding the effects of this modulation is difficult as there are significant variations in 5-HT₃R expression and function between species (Miyake et al. 1995).

5-HT₃R modulation is implicated in complicated neurobehaviors such as depression, anxiety, bipolar disorder, pain perception and appetite regulation (Fakhfour et al. 2019; Rajkumar and Mahesh 2010; Wu et al. 2012; Jones et al. 1988; Liang et al. 2011; Thompson et al. 2006b). 5-HT₃Rs are attractive drug targets as they exhibit less adverse side effects and use dependence than traditional drugs that directly target synaptic activity. 5-HT₃R antagonists have been used successfully to treat schizophrenia and alcohol abuse and several studies suggest potential in other psychiatric disorders (Dorostkar and Boehm 2007).

Differential Expression of 5-HT₃ Subunits and Isoforms

Expression patterns of 5-HT₃R are further complicated when considering different isoforms and subunit types. As will be discussed through the chapter, the properties of 5-HT_{3A}Rs are different to 5-HT_{3A/B}Rs and expression of one over the other has physiological implications. In humans, 5-HT_{3A} and 5-HT_{3B} are expressed in near equal ratios in the small intestine but 5-HT_{3B} is expressed at much higher levels in the brain (Tzvetkov et al. 2007). Relative expression also varies between different brain structures. For example, 5-HT_{3B} transcription is six times higher than 5-HT_{3A} in the amygdala while the ratio is roughly equal in the hippocampus (Brady et al. 2007; Tzvetkov et al. 2007). Surface expression of 5-HT_{3A}R homomers and 5-HT_{3A/B}R heteromers is also dependent on chaperone proteins and glycosylation (Boyd et al. 2002). In particular, RIC-3 specifically promotes 5-HT_{3A}R surface expression over 5-HT_{3A/B}R (Cheng et al. 2007).

Similarly, the 5-HT_{3C}, 5-HT_{3D} and 5-HT_{3E} subunits all form functional channels with the 5-HT_{3A} subunit (Niesler et al. 2003). These subunits are expressed in peripheral tissues, particularly in the colon, and may be expressed in the brain as well (Niesler et al. 2007; Kapeller et al. 2011). The study of these subunits is limited by the lack of subunit-specific antibodies and their physiological function is largely unknown. For simplicity subunits C-E will not be discussed in further detail.

5-HT₃ subunit isoforms also display tissue-specific expression. In humans, 5-HT_{3A} isoforms have different localization patterns and functional properties (Brüss et al. 2000). 5-HT_{3A} isoform expression also shifts during development in mouse cell lines (Emerit et al. 1995). 5-HT_{3B} isoforms transcribed in the brain are not found in peripheral tissue, and *visa-versa* (Tzvetkov et al. 2007; Jensen et al. 2008). The major 5-HT_{3B} isoform in either tissue encodes the same protein but the isoforms differ in peptide signaling sequences. A minor yet significant portion of brain isoforms are truncated up to and including the β 1- β 2 loop. Overall, isoform expression appears tissue and development specific and thus physiologically important.

Basic Structural Topology

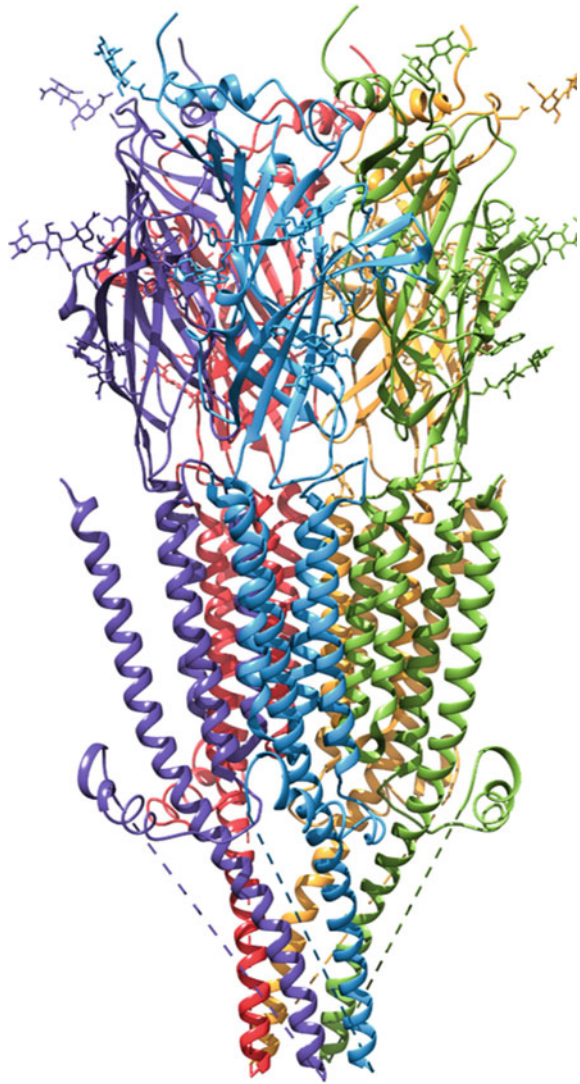
Early pLGIC studies identified amino acids important for specific functions and clues about the underlying structure. However, until recently, direct structural information had been elusive as eukaryotic pLGICs are not easily crystalized. Prior to the last five years, pLGIC structural studies were mostly limited to bacterial homologues, the naturally enriched torpedo nAChR or acetylcholine binding protein (AChBP) (Unwin 2005; Brejc et al. 2002; Kesters et al. 2013; Hilf and Dutzler 2008). In 2014, mouse 5-HT_{3A}R was finally able to be crystallized in the presence of stabilizing nanobodies (Hassaine et al. 2014). There has been a recent explosion of pLGIC structural data with major technological advancements in cryo-electron microscopy (cryo-EM). These include several 5-HT_{3A}R structures bound to different ligands and in multiple conformational states (Basak et al. 2018a, b; 2019; Polovinkin et al. 2018; Basak et al. 2020; Zarkadas et al. 2020). Figure 11.2 shows the fully-assembled structure of 5-HT_{3A}R and Fig. 11.3 highlights the historical nomenclature of different regions. Throughout the chapter, residue numbering is consistent with that of the apo mouse 5-HT_{3A}R structure published by Basak and colleagues (PDB 6BE1).

pLGICs assemble as five homologous subunits about a pseudo-symmetric five-fold axis as shown in Fig. 11.2. The subunit matching is not fully understood, but important factors include complementary shape and charge at subunit interfaces, subunit-specific glycosylation patterns, specific chaperone proteins and spatial constraints in closing the five-fold ring structure (Walsh et al. 2018; Boyd et al. 2002; Phulera et al. 2018). It is generally believed that unmatched subunits are retained in the ER and eventually degraded (Davies et al. 1999). Specific to 5-HT_{3R}, functional expression requires at least one 5-HT_{3A} subunit, but the stoichiometry of heteromeric receptors is unknown. The stoichiometry has implications for ligand binding and is discussed further in that section.

Individual subunits of pLGICs can be divided into three domains, the extracellular domain (ECD), transmembrane domain (TMD) and intracellular domain (ICD). The structure of the ECD is remarkably well-conserved across pLGICs (Brejc et al. 2002). It consists of an N-terminal alpha helix followed by ten beta strands forming a beta-barrel structure. Clusters of residues important for ligand binding are named loops 'A-F' though it was later determined that not all of these clusters are technically loops (Brejc et al. 2002). Another set of loops at the ECD-TMD interface are important for coupling ligand binding to channel gating. These include the β 1- β 2 loop, the β 6- β 7 loop (also known as the cys-loop), the β 8- β 9 loop and the covalent link between β 10 and the TMD.

The TMD has consists of 4 bundled membrane-spanning alpha helices named M1-M4. The M2 helix forms the channel pore and is flanked by M3 counterclockwise and M1 clockwise when viewed from the ECD. M4 is most exposed to the membrane environment and acts as a lipid sensor (Hénault et al. 2015). The loops between the TM helices are also important for channel function. The extracellular M2-M3 linker transduces movements at the ECD-TMD interface to the channel pore. The ICD is comprised of the intracellular loops between the M1 and M2 helices and between

Fig. 11.2 Full pentameric structure of mouse 5-HT_{3A}R (PDB 6NP0) (Basak et al. 2019)



the M3 and M4 helices. The M1-M2 linker is relatively short but plays a role in ionic charge selectivity and desensitization. The M3-M4 linker is 70–120 amino acids long, has the most heterogeneity among pLGICs and includes a large stretch of structurally disordered residues (Basak et al. 2018b). It has an important role in membrane trafficking, regulating channel conductance and interactions with other signaling proteins. The two structured elements of the ICD are the MX helix, which lies parallel to the membrane shortly following the M3 helix, and the MA helix, which directly precedes the M4 helix.

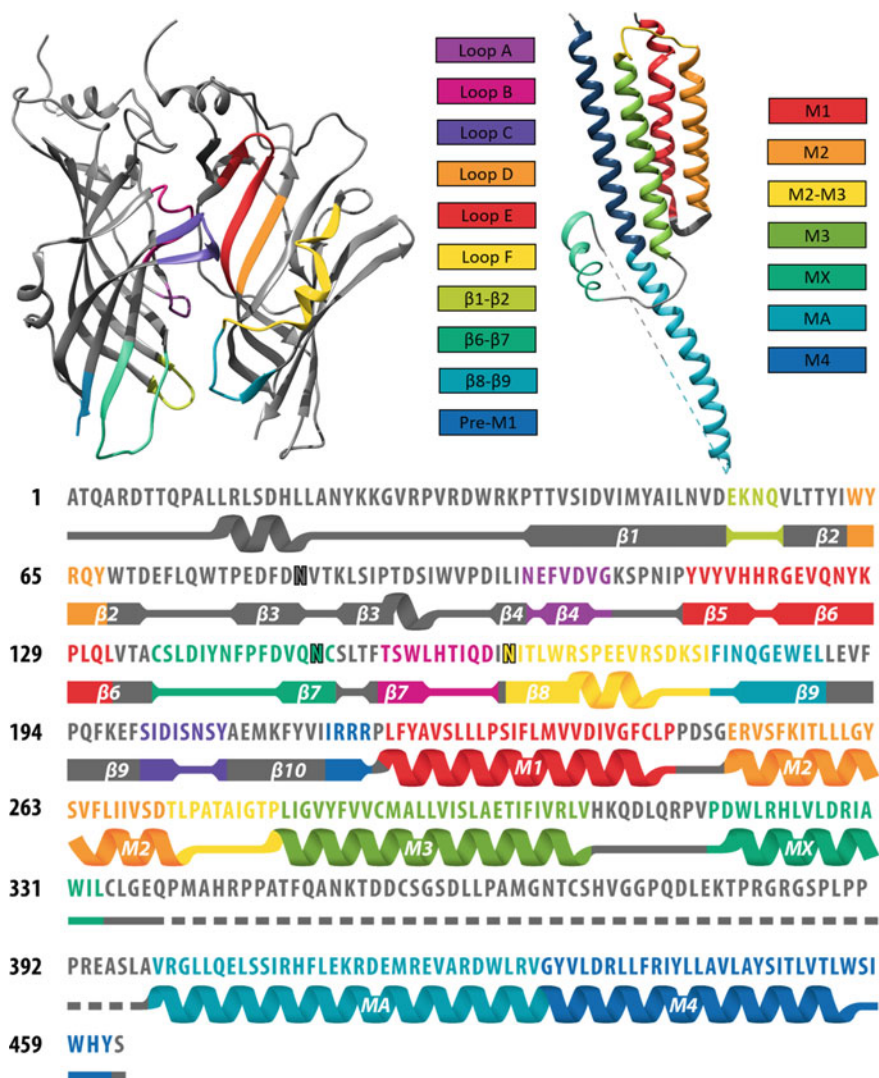


Fig. 11.3 Labeled sequence and 3D-structure of 5-HT_{3A} subunit components (PDB 6NP0) (Basak et al. 2019). Top left, two neighboring subunit ECDs are shown. Binding site Loops A-F and ECD-TMD interface regions are color-coded by historical nomenclature. Top right, the TMD and ICD of a single subunit are similarly colored. Note that the colors are repeated from the ECD image. Below, the amino-acid sequence of 5-HT_{3A} color coded to match the above images. The break between the ECD and TMD-ICD image is between β 10 and M1. Glycosylated asparagines are outlined

Ion Permeation and Channel Gating

5-HT₃R was first recognized as an ion channel from electrophysiology studies and characterized as a pLGIC from its amino acid sequence (Maricq et al. 1991; Derkach et al. 1989). As with all pLGICs, the M2 helix forms the channel pore, but elements of the ECD and ICD also affect ion permeation (Schofield et al. 1987). This section describes observations of 5-HT₃R ion permeation and their physiological implications. It then addresses the underlying physical mechanisms of these observations. The section closes with a brief summary of antagonists that act at the channel pore.

Observed Properties of 5-HT₃R Ion Conductance

Early electrophysiology studies agreed that 5-HT₃Rs are cationic with a similar relative conductance for Na⁺/K⁺ with a reversal potential near 0 mV, but other properties diverged significantly (Gill et al. 1995; Derkach et al. 1989; Yang 1990). Some studies reported 5-HT₃Rs had sub-picosiemen single channel conductance, were calcium permeable and displayed strong voltage rectification (Gill et al. 1995; Yang et al. 1992; Brown et al. 1998). Other studies reported a channel with much a higher single channel conductance (9–15 pS), reduced divalent cation permeability and no intrinsic rectification (Hussy et al. 1994; Derkach et al. 1989; Jones and Surprenant 1994). Discovery of the 5-HT_{3B} subunit confirmed that most of these differences could be attributed to channel subunit composition (Davies et al. 1999; Dubin et al. 1999; Hapfelmeier et al. 2003). Specifically, 5-HT_{3A}R homomers fit the first description and 5-HT_{3A/B}R heteromers fit the latter. Interestingly, the M2 portions of these channels are quite similar suggesting that elements outside the pore helix regulate conductance properties.

Differences in channel conductance and permeability have important implications in neuronal communication. For example, presynaptic 5-HT₃Rs modulate inhibitory GABA release in a calcium-dependent manner (Koyama et al. 2000; Rondé and Nichols 1998; Choi et al. 2007). This suggests that these presynaptic receptors are 5-HT_{3A}R homomers as only 5-HT_{3A}Rs are calcium permeable. Another example, the high conductance of 5-HT_{3A/B}R will lead to larger changes in membrane potential upon activation. A common theme of 5-HT₃R is that 5-HT_{3A}Rs seem better suited for presynaptic modulation and 5-HT_{3A/B}Rs seem better for postsynaptic transmission. However, mRNA expression and western analysis suggest that the roles of 5-HT_{3A}R and 5-HT_{3A/B}R in vivo are more subtle and vary across neuronal populations (Férézou et al. 2002; Brady et al. 2007).

In addition to fast synaptic signaling, 5-HT_{3A}R calcium activity has long-term effects. 5-HT_{3A}R calcium flux is prolonged by 5-HT open-pore block at higher 5-HT concentrations, which does not occur in 5-HT_{3A/B}Rs (Hapfelmeier et al. 2003; Corradi et al. 2009). Prolonged calcium signaling is associated with synaptic plasticity, suggesting 5-HT_{3A}R may be important in memory and development. Indeed,

5-HT_{3A} expression is relatively high in the hippocampus compared to other regions of the brain and 5-HT_{3R} expression is important in the development and pathology of rat pyramidal and Purkinje cells (Brady et al. 2007; Oostland et al. 2013; Rosenberg et al. 1997; Roerig et al. 1997; Zhang et al. 2011; Choi et al. 2007). Both 5-HT_{3AR} and 5-HT_{3A/B}R are blocked by elevated extracellular calcium. Elevated extracellular calcium occurs near dying cells and calcium block can protect further damage by overstimulation following injury.

The distinct properties of 5-HT_{3AR} and 5-HT_{3A/B}R are relatively well known, but their physiological roles are not yet understood. This is partially due to the complex interplay between channel subtypes and other neurotransmitter signaling, but also due to the lack of quality antibodies to distinguish between the two populations. This technical challenge is further exasperated in the C, D and E 5-HT₃ subunits. Future research will hopefully reveal how subunit-specific channel properties influence the diverse physiological roles of 5-HT_{3R}.

Mechanisms Underlying Ion Permeation

Physical Description of the Permeation Pathway

Unlike many selective ion channels, pLGICs do not have a unique well-defined ion selectivity filter. Rather, channel properties are influenced by spatially dispersed elements in the ECD, TMD and ICD as shown in Fig. 11.4. The extracellular conductance pathway consists of a bowl-shaped vestibule with an approximate average inner-diameter of 20 Ångstroms. It has been proposed that ions can enter this vestibule either through the top and/or from gaps between the subunits and that glycosylation sites help define the conduction pathway for some pLGICs (Miller and Aricescu 2014; Phulera et al. 2018; Zhu et al. 2018). 5-HT_{3AR} structures show several charges along the inner vestibule wall, including D91 and D105 (Basak et al. 2018b; Hassaine et al. 2014). On the background of a high conductance 5-HT_{3AR} mutant (with mutated intracellular arginines as discussed below), further mutation of D91 or especially D105 reduced channel conductance, calcium permeability and inhibition by elevated extracellular calcium (Livesey et al. 2011). Molecular dynamic simulations of nAChR show that these residues coordinate positively charged cations and there is an elevated cation concentration throughout the extracellular vestibule (Wang et al. 2008). Residues corresponding to D105 are conserved in other cationic pLGICs while anionic pLGICs have positively charged residues at this position, suggesting extracellular charge selection is a conserved mechanism across the family (Hansen et al. 2008).

D271 or D20' (The prime notation is the position relative to the cytoplasmic end of M2), is a pore facing negatively charged residue at the ECD-TMD interface. This is the most extracellular of three 'rings of charge' that were identified as early determinants of cation conductance and selectivity in nAChRs (Miyake et al. 1995; Imoto et al. 1988). D20' in 5-HT_{3A} is conserved across species and in some 5-HT_{3B}

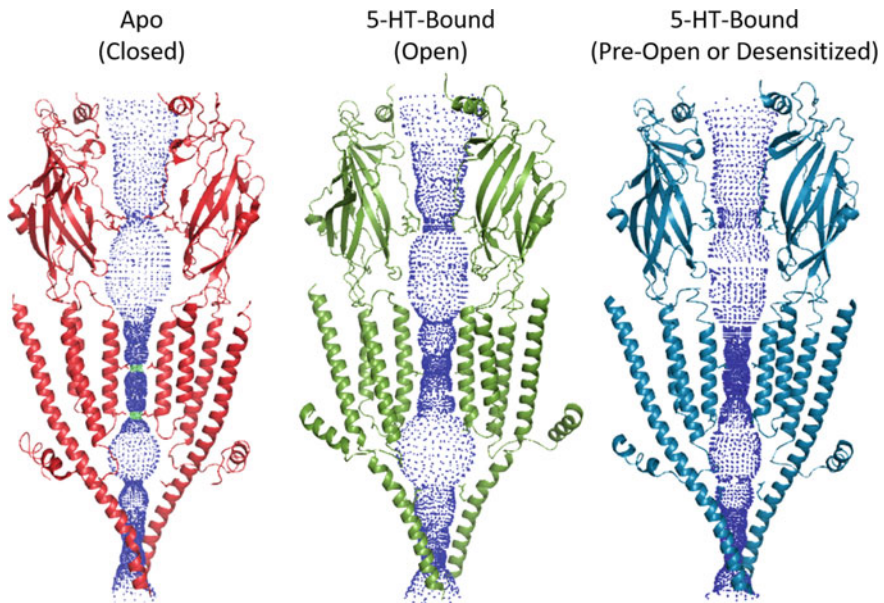


Fig. 11.4 Channel permeation pathway in different conformations (PDBs 6BE1, 6DG7 and 6DG8) (Basak et al. 2018a; Basak et al. 2018b). The L9' and E-1' gates are shown most clearly in the apo structure near the constriction points shown in green. The residues above L9' form a hydrophobic barrier that requires ion solvation for passage. The M2 helices splay outwards and rotate in the 5-HT-bound (open) state compared to the apo (closed) state, widening the channel pore. The 5-HT-bound (pre-open or desensitized) state show an intermediate M2 helix position where the E-1' gate is the most constricted point. MD simulations suggest that this state is mostly impermeable

subunits as well, though in human 5-HT_{3B} it is an asparagine. Homomeric receptors with a 5-HT_{3A} mutant D20'A do not differ in monovalent cation selectivity but have a lower channel conductance and altered kinetics (Livesey et al. 2008; Hu and Lovinger 2005). This could occur because anions are already disfavored within the extracellular vestibule but mutations to D20' disrupt cation flow. The D20'A mutant also shows reduced calcium permeability, possibly due to weakened electrostatic interactions at the pore entry. N20' in the 5-HT_{3B} subunit may contribute to the calcium impermeability of 5-HT_{3A/B}Rs.

E250 or E-1' forms the second ring of negative charge in 5-HT_{3A}R at the cytosolic end of the M2 helix. This glutamate is conserved across several nAChR subunits. A non-polar mutation of E-1'A results in a channel that shows no monovalent charge selectivity and charge selection can be reversed when combined with other mutations (Thompson and Lummis 2003; Gunthorpe and Lummis 2001). Recent structures depicting 5-HT_{3A}R in an open conformation show that the channel pore is wider at the extracellular end than at the cytoplasmic end as seen in Fig. 11.4. This supports evidence that E-1' is a stronger determinant of charge and size selectivity than D20' (Basak et al. 2018a; McKinnon et al. 2011; Polovinkin et al. 2018). However, 5-HT_{3B} subunits and some nAChR subunits do not contain a negatively charged residue in

the E-1' position and monovalent cation selectivity is not dramatically altered (Peters et al. 2005). Thus negative charge is not required in every subunit within the pentamer for cation selection. The overall charge reduction may however contribute to calcium impermeability or lack of voltage rectification in 5-HT_{3A/B}R (Gunthorpe and Lummis 2001).

The pore lining residues of M2 lie between E-1' and D20'. L260 or L9' is at the center of the pore and the narrowest part of the pore in 5-HT_{3A}R apo and antagonist-bound structures. The position of L9' can be seen in the Apo (Closed) pore profile in Fig. 11.4. L9', V13' and I17' form a hydrophobic barrier within the channel pore that is generally conserved across pLGICs (Corry 2006; Revah et al. 1991; Filatov and White 1995). Mutations to L9' and V13' affect open channel stability (Dang et al. 2000). Cysteine labelling studies suggest that these residues form a vestibule large enough to accommodate solvated ions and MD simulations show ions must be solvated to cross this barrier (Reeves et al. 2001). In 5-HT_{3A}Rs, a pair of polar residues, S2' and T6' are intracellular to the hydrophobic barrier. However, polar residues in these positions are not conserved across pLGICs, including the 5-HT_{3B} subunit, which has an alanine at the 2' position (Davies et al. 1999). The uncharged alanine may however, once again, contribute to specific properties of 5-HT_{3A/B}Rs (Das and Dillon 2003).

The 5-HT₃ structure shows that the 5 MA helices coil and form an inverted pyramid that occludes intracellular ion permeation along the pore axis but ions can pass through the pore through intracellular portals that line the MA helix (Kelley et al. 2003; Basak et al. 2018a; Deeb et al. 2007). Structural and mutational studies show this is a common feature of pLGICs, though the MA helix is unstructured in anionic pLGIC structures (Carland et al. 2009; Zhu et al. 2018; Phulera et al. 2018; Du et al. 2015; Everitt et al. 2009). One key piece of evidence for ion permeation via intracellular portals is the remarkably low single channel conductance of 5-HT_{3A}R relative to 5-HT_{3A/B}R and other pLGICs. This is particularly surprising as 5-HT_{3A/B}R contains hydrophobic residues within the channel pore. It was found that three arginines within the MA helix, R416, R420 and R424, were responsible for the low conductance of 5-HT_{3A}R. Mutation of these residues to their 5-HT_{3B} counterparts, R416Q, R420D and R424A gave 5-HT_{3A}R a single channel conductance comparable to 5-HT_{3A/B}R (Kelley et al. 2003; Hales et al. 2006). Cysteine mutant labelling, MD simulations and 5-HT_{3A}R structures show that these arginines line the intracellular portals (Basak et al. 2018a; Deeb et al. 2007). These structures also suggest that the MX helix may be positioned to act as another conformational gate in different functional states. Mutation of these arginines also eliminates phosphate-dependent inhibition at hyperpolarized membrane potentials and it is likely that negatively charged phosphates that block the channel are coordinated by arginine residues (Noam et al. 2008).

Conformational Changes to the Channel Pore with Different Functional States

In the extracellular permeation pathway, ligand binding leads to a counter-clockwise rotation of individual 5-HT₃ subunits and loop rearrangement (Basak et al. 2018a). This rotation exposes more negative charges to the vestibule particularly near the entrance to the TMD pore. There, the mobile β 1- β 2 loop, β 6- β 7 loop and M2-M3 linker form a strong electronegative pocket. The mobile loops are important for allosteric coupling, making it difficult to clearly define their effects on channel conductance, though the anionic glycine receptor has more neutrally charged residues at this interface (Du et al. 2015).

Within the membrane pore, the M2 helix undergoes significant changes between different functional states, which can be visualized in Fig. 11.4. Cysteine labelling and, more recently, ligand-bound structures of 5-HT_{3A}R have captured these different states (Reeves et al. 2001; Basak et al. 2018a; 2019; Panicker et al. 2002; Polovinkin et al. 2018). Assigning structures to specific functional states is challenging, but in general, the positioning of the M2 helix in apo and ligand-bound pLGIC structures is consistent with the expected functional state (Corrie and Baenziger 2013).

Apo 5-HT_{3A}R structures show the M2 helix mostly perpendicular to the membrane with two constriction points, the narrowest being L9'. MD simulations show that the L9'/V13' hydrophobic barrier is impermeable to ions and water in this state. Apo 5-HT_{3A}R is also constricted at E-1'. Unlike the hydrophobic gate, this constriction remains closed in the desensitized state and E-1' is referred to as the desensitization gate. Antagonist-bound structures show the M2 helices in a similar conformation to the apo state and it is reasonable to assign these structures to a closed channel state (Basak et al. 2019; Polovinkin et al. 2018).

Different M2 arrangements have been observed in serotonin-bound 5-HT_{3A}R structures (Basak et al. 2018a; Polovinkin et al. 2018). In one observed serotonin-bound state, the M2 helix is slightly kinked at the L9' position and rotated clockwise relative to the apo state. Both extracellular and intracellular entrances to the TMD pore are splayed radially outward (Basak et al. 2018a). L9' is also shifted away from the pore and slightly rotated. This tilt and rotation widen the pore, similar to a camera iris. Due to the kink, the desensitization gate is also slightly wider and the net effect is a pore wide enough to allow permeation of solvated ions. These structural observations are supported by open-state dependent labeling of mutated cysteines near the L9' position and MD simulations. Thus one can assign these structures to open states (Panicker et al. 2002).

In another serotonin-bound state, the M2 helix is no longer kinked but is still tilted relative to the closed position (Basak et al. 2018a). The extracellular end of M2 is splayed outward, though not as much as the open state structure, and the intracellular end is now positioned radially inward. The L9' barrier is still wide enough to allow ion passage like the open state, but the desensitization gate at E-1' is even further constricted than the closed state. MD simulations show that permeation events are much rarer and the simulations do not include residues from the M3-M4 linker, which

may form an additional barrier to ion permeation in this state. It is unclear however whether this state is a pre-open or desensitized state (Corradi et al. 2009; Solt et al. 2007).

Pore Blocking Antagonists

Several 5-HT₃R antagonists bind within the pore region and block ion conduction including picrotoxin, bilobalide and ginkgolide B. In human 5-HT₃Rs, pore block by these compounds is eliminated by T6'S or S12'A mutations suggesting these residues may hydrogen bond with the antagonist (Thompson et al. 2011b). Interestingly, S2'A mutations enhance channel block and even cause ginkgolide B to become trapped in the pore upon channel closure, likely by hydrophobic contacts that further stabilize the blocker deeper in the pore. 5-HT_{3A/B}R and mouse 5-HT_{3A}R are 4–6 times less sensitive than human 5-HT_{3A}R to these antagonists, consistent with differences in pore lining residues (Das and Dillon 2005, 2003; Thompson et al. 2011b). In addition to natural compounds, tricyclic antidepressants such as fluoxetine and antipsychotic medications can act as pore blockers and this may contribute to their therapeutic effects (Gumilar and Bouzat 2008; Rammes et al. 2004).

Other compounds bind at both the orthosteric binding site as well as the channel pore, including the primary agonist 5-HT, the blood pressure medication diltiazem and morphine (Hapfelmeier et al. 2003; Thompson et al. 2011b; Baptista-Hon et al. 2012). The anti-malarial compound mefloquine also acts as a channel blocker while structurally similar anti-malarial compounds act at the orthosteric binding site (Thompson and Lummis 2008). The binding site for these compounds overlaps with that of picrotoxin but is slightly higher as diltiazem binding is affected by 7' and 12' but not 2' or 6' mutations. 5-HT₃R pore block may contribute to the side-effects of certain drugs and as previously mentioned prolongs 5-HT_{3A}R signaling. Improved understanding of pore block mechanisms could improve drugs by increasing the efficacy of anti-depressants or limiting off-target effects of other drugs.

Ligand Binding

Ligand binding is the initial signal that governs channel activity. This section first presents common 5-HT₃R agonists, antagonists and partial agonists that bind at the orthosteric binding site and their role in physiology or medicine. Then we look at specific interactions that mediate ligand binding and subsequent rearrangements within the binding pocket.

Description of the Ligand Binding Site and Orthosteric Ligands

The neurotransmitter binding site is located at subunit interfaces as shown in Fig. 11.5. 5-HT_{3A}R presents five identical binding sites, but not all of the subunit interfaces are equivalent in heteromeric channels. Considering only the 5-HT_{3A} and 5-HT_{3B} subunits, there are four possible interfaces, A(+)/A(-), A(+)/B(-), B(+)/A(-) and B(+)/B(-), where + and - refer to the principal and complementary subunits. There is strong pharmacological evidence that only the A(+)/A(-)

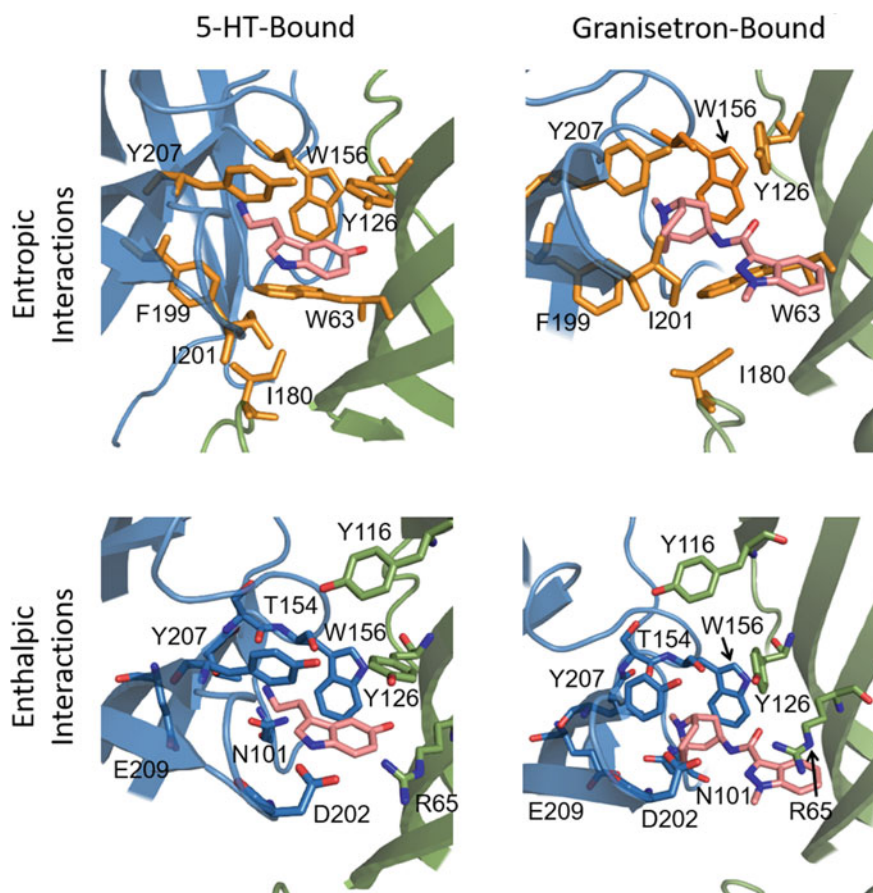


Fig. 11.5 Interactions within the binding pocket for both 5-HT and the competitive antagonist granisetron (PDBs 6DG7, 6NP0) (Basak et al. 2018a; Basak et al. 2019). Residues discussed within the text are shown and labelled. Top panels show a selection of residues involved in entropic interactions, shown in orange, and bottom panels show enthalpic interactions, colored by heteroatom. Note the tropane moiety of granisetron more completely fills the binding pocket, and how the elongated structure widens the gap between subunit interfaces

interface binds both agonists and competitive antagonists. Mutations at the binding interface that swap 5-HT_{3A} residues for those found in 5-HT_{3B} dramatically reduce the receptor's EC₅₀ while 5-HT_{3B} to 5-HT_{3A} swaps have little effect (Lochner and Lummis 2010). Additionally, cysteine mutant labels and double cysteine mutations across the A(+)/A(-) interface ablate agonist activity but can be rescued with DTT, while this treatment at other possible binding interfaces has no effect (Thompson et al. 2011a). Similar results show granisetron, a competitive antagonist, only binds to the A(+)/A(-) interface, though other antagonists may bind multiple interfaces. This suggests 5-HT_{3A/B}Rs have at least one A(+)/A(-) interface. However, atomic force microscopy experiments showed exogenously expressed receptors do not contain an A(+)/A(-) interface, but these experiments may not be physiologically representative (Barrera et al. 2005). 5-HT_{3A/B}Rs have a Hill coefficient close to one, which may suggest there is only one A(+)/A(-) interface. It is also possible that multiple stoichiometries are expressed similar to nAChR $\alpha 2\beta 4$ receptors.

5-HT₃Rs are less sensitive to 5-HT than 5-HTRs in the GPCR subfamily. This allows for a differential physiological response where short-term spikes in 5-HT produce rapid transient signaling by 5-HT₃R and baseline 5-HT levels regulate slower metabotropic processes via other 5-HTRs. 5-HT₃Rs do have a lower threshold for 5-HT than other pLGICs have for their primary agonist. This is consistent with a modulatory role of 5-HT₃R as activation can occur at concentrations lower than those encountered during synaptic events. 5-HT_{3A}R also have a lower 5-HT activation threshold than 5-HT_{3A/B}R, consistent with 5-HT_{3A}R being more suited for presynaptic modulation than 5-HT_{3A/B}R (Dubin et al. 1999). 5-HT is the only endogenous full agonist of 5-HT₃R but there are other natural and synthetic ligands that act as agonists or partial agonists.

5-HT₃R antagonists generally bind with higher affinity than 5-HT. Setrons are an important class of 5-HT₃R antagonists. They are used clinically to treat chemotherapy or post-operative induced nausea and vomiting. This nausea and vomiting occurs in an acute and delayed phase. First generation setrons only treat acute nausea. These include alosetron, granisetron, ondansetron and tropisetron. The only second generation setron, palonosetron, treats both acute and delayed nausea (Rojas and Slusher 2012). Palonosetron is a competitive inhibitor like other setrons but is more effective because it remains bound to 5-HT₃R for up to several days, which has lasting effects on NK1 signaling, not entirely understood (Hothersall et al. 2013; Lummis and Thompson 2013; Wong et al. 1995; Hesketh et al. 2003). Setrons have also been investigated for the treatment of IBS though side-effects include severe constipation (Zheng et al. 2017). Alosetron is the only FDA-approved setron for IBS treatment. Other orthosteric antagonists act non-specifically on 5-HT₃R in addition to their primary targets. For example, the constipating effects of drugs such as morphine, methadone and anti-malarial drugs are at least partially mediated by 5-HT₃R (Baptista-Hon et al. 2012; Deeb et al. 2009; Thompson and Lummis 2008).

There are also several 5-HT₃R partial agonists that compete with 5-HT for binding, but elicit smaller maximal currents than 5-HT. Dopamine is an endogenous partial agonist of 5-HT_{3A}R that produces substantially less current than serotonin and favors

receptor deactivation to desensitization (Solt et al. 2007). This could lead to dampened but prolonged 5-HT₃R signaling at dopaminergic synapses. Several therapeutics also act as partial agonists. The smoking cessation agent Varenicline is a strong partial agonist of 5-HT₃R, which is thought to cause nausea (Lummiss et al. 2011). While this is undesirable, weaker partial agonists are being investigated as useful therapeutics because they can reduce 5-HT₃R signaling without completely eliminating it (Roberts et al. 2020). This may prevent side effects sometimes caused by 5-HT₃R antagonists, such as severe constipation and ischemic colitis.

Mechanisms Underlying Ligand Binding

The key functional properties of 5-HT that allow it bind 5-HT₃R are the basic ethylamine group and the aromatic indole group. 5-HT₃R orthosteric antagonists have a similar structure to 5-HT, but generally have a bulkier ring-embedded basic nitrogen, sometimes a larger hydrophobic moiety, and extended linker between the two containing a hydrogen bond acceptor (Thompson 2013). The increased size and bonding partners give 5-HT₃R antagonists low nanomolar affinities compared to the micromolar affinity of 5-HT.

The free energy associated with ligand binding arises from both entropic and enthalpic contributions. Enthalpic contributions come from local interactions, such as hydrogen bonding, charge delocalization like pi-pi or pi-cation interactions, or electrostatic interactions. Entropic contributions come from increasing the conformational variability of the protein and surroundings. Though it is easy to understand how ligand binding decreases enthalpy, it may seem that ligand binding would also decrease entropy by restricting spatial flexibility and conformational variability. However, these entropy losses can be more than offset by solvent entropy, an essential consideration in many protein–ligand interactions. Changes in solvent-accessible surface area are a useful surrogate for changes in solvent entropy (Hilser et al. 2006). In particular, burying hydrophobic residues increases solvent entropy. Though empirical analysis of thermodynamic properties is beyond the scope of this chapter, it is useful to consider these principles in analyzing 5-HT₃R binding interactions. Note that all structures to date are from mouse 5-HT_{3A}R, though many of the interactions discussed are conserved in human 5-HT_{3A}R.

Entropic Contributions to Ligand Binding

The 5-HT₃R binding pocket is formed by the solvent-accessible gap between the protruding Loop C, Loop B and the (–) interface as shown in Fig. 11.5 (Basak et al. 2018b). This gap is lined with hydrophobic residues that give an entropic penalty to the apo conformation. Several of these residues are bulky aromatics that form the so-called aromatic box common to all pLGICs. These include F199 and Y207 from Loop C, W156 from Loop B, W63 from Loop D and Y126 from Loop E. The

shape of the aromatic box plays a role in neurotransmitter selectivity. For example, in GlyR and GABA_AR, the residues corresponding to W63 and W156 are either phenylalanine or tyrosine. This results in a smaller binding pocket more adapt to binding glycine or GABA (Du et al. 2015; Miller and Aricescu 2014). However, tryptophans are conserved in nAChR, consistent with acetylcholine's larger size (Morales-Perez et al. 2016).

In addition to the aromatic box, other hydrophobic residues also make entropic contributions to binding. They do not directly contact the ligand but their solvent-accessible area does change upon ligand binding. For example, conformational changes in Loop F and Loop C bury I180 against I201. Though neither residue directly contacts the ligand, their effect on binding is apparent in mutational studies (Zhang et al. 2007).

Comparing 5-HT-bound 5-HT_{3A}R structures with the granisetron-bound 5-HT_{3A}R structure further demonstrates the importance of solvent-accessible surface area (Basak et al. 2019). The 5-HT-bound structure only partially fills the binding pocket, allowing for conformational flexibility as shown in Fig. 11.5. The nitrogen-containing tropane moiety of granisetron more fully occupies this space. Additionally, the aromatic indazole group, similar in shape to serotonin, makes more contacts with the (–) interface due to the linker between the tropane and aromatic moieties. The large surface area of 5-HT_{3R} antagonists bury large portions of otherwise solvent-accessible surface area, which largely accounts for increased affinity of granisetron compared to 5-HT.

Enthalpic Contributions to Ligand Binding

5-HT_{3R} mutagenesis studies have produced a long list of charged or hydrogen-bonding residues implicated in ligand binding. However, many of these residues are too spatially dispersed to simultaneously interact with the ligand. One possible explanation is that these spatially dispersed residues directly interact with bound ligand at different times in different conformations (Corrie and Baenziger 2013; Solt et al. 2007; Corradi et al. 2009). Another non-exclusive possibility is that water molecules coordinate indirect ligand–protein interactions within the binding pocket. Here we describe residues that structural or mutagenesis studies suggest make enthalpic contributions to the 5-HT_{3R} ligand binding, though the exact nature of these interactions is as yet undetermined. The position of these residues in 5-HT-bound or granisetron-bound structures are shown in Fig. 11.5.

Most enthalpic 5-HT interactions occur near the negatively charged pocket between Loop B and Loop C, which is conserved across pLGICs (Du et al. 2015; Laverty et al. 2019; Morales-Perez et al. 2016). In mouse 5-HT_{3A}R, this pocket is formed by side chain oxygen atoms from N101, T154, Y207 and E209 and the main chain carbonyl oxygen from S155. Multiple 5-HT-bound structures show the ethylamine of 5-HT in a position near the side chain of T154 and the carbonyl oxygen of S155 (Basak et al. 2018a; Polovinkin et al. 2018).

Directly below this pocket are three Loop A residues, N101, E102 and F103 (Price et al. 2008; Sullivan et al. 2006; Boess et al. 1997; Steward et al. 2000; Nyce et al. 2010). In published structures, N101 faces towards the 5-HT ethylamine, but is too far away to directly interact. However, mutations to N101 significantly affect the EC_{50} of 5-HT (Nyce et al. 2010; Sullivan et al. 2006). It is possible that N101 interacts with 5-HT's ethylamine in a different conformation than that observed in present 5-HT-bound 5-HT₃R structures. This is observed in the 5-HT-bound structure of the soluble ECD surrogate of 5-HT₃R, 5-HTBP (Kesters et al. 2013). 5-HTBP is AChBP with an engineered agonist-binding pocket to mimic 5-HT_{3A}R. 5-HTBP has a tyrosine in the position of N101, but the tyrosine hydroxyl is positioned near the ethylamine of bound 5-HT. However, a 5-HT_{3A} N101V mutation improves the EC_{50} of 5-HT_{3A}R, inconsistent with possible hydrogen bonding (Price et al. 2008). E102 and F103 are positioned further away from the binding pocket than N101, but also have significant effects on 5-HT₃R binding (Sullivan et al. 2006; Boess et al. 1997). One intriguing possibility is that these form a less stable but more accessible 5-HT binding site that precedes a more stable binding conformation (Solt et al. 2007; Corradi et al. 2009). It is also possible that these residues play a role in coupling binding to channel gating.

Loop B lies above Loop A and forms part of the interior wall of the binding pocket. 5-HT induced currents are extremely sensitive to mutations from S150 through N164, but 5-HT₃R structures suggest only T154, S155 and W156 are exposed near the binding pocket (Basak et al. 2018a; Polovinkin et al. 2018). The other residues are likely involved with allosteric coupling or shaping the binding pocket. As previously mentioned, the side chain of T154 and carbonyl oxygen of S155 are positioned to form hydrogen bonds with the ethylamine of 5-HT in published 5-HT-bound 5-HT_{3A}R structures. Mutagenesis has also shown that W156 is critical for 5-HT binding (Spier and Lummis 2000; Beene et al. 2002; Thompson et al. 2008). This aromatic residue helps form the binding pocket as part of the aromatic box, but unnatural amino-acid mutagenesis shows that W156 also participates in pi-cation interactions with the 5-HT ethylamine. This suggests that the ethylamine can be in yet another conformation different from the 5-HT-bound 5-HT₃R structures, this one placing the ethylamine further out of the binding pocket from T154 and S155 towards the (–) interface.

The indole group of 5-HT is mostly involved in hydrophobic interactions in the aromatic box as previously discussed. However, the aromatic residues may also form pi-pi interactions with the indole rings. Additionally, the 5' hydroxyl is positioned near the carbonyl oxygen of K127 and computational docking studies suggest several other 5' hydroxyl positions that could contribute to ligand binding (Reeves et al. 2003).

Similar to entropic contributions, changes in protein conformation upon ligand binding also introduce enthalpic protein–protein interactions that make binding more favorable. Many of these interactions occur in residues across the binding interface. Y114 on Loop E is positioned to interact with H158 on Loop B upon ligand binding. Similarly, Y116 and Y126 on Loop E are positioned to hydrogen bond with Y207 on loop C in 5-HT-bound structures but not in apo or granisetron-bound structures. Unnatural mutagenesis shows that hydrogen bonding from both Y116 and Y126 is

important for channel function. It is likely that Loop C movements are coupled to Loop E via these hydrogen bonds (Beene et al. 2004; Venkataraman et al. 2002; Price and Lummis 2004). 5-HT_{3A}R structures show another possible cross-interface interaction between R65 on Loop D and D202 on Loop C. R65 is also positioned to bond with D42 on the β 1 strand and R169 on Loop F. These residues are generally conserved across pLGICs and their mutation affects ligand binding (Yan et al. 1999; Thompson et al. 2006a).

The structurally observed enthalpic interactions between granisetron and 5-HT₃R are similar (Basak et al. 2019). The tropane nitrogen is in the ethylamine pocket. The carbonyl oxygen on the linker between tropane and the indazole moieties occupies almost the exact same position as the 5' hydroxyl in the 5-HT-bound structure. This reinforces the indazole protruding towards the (–) interface more than the indole group of 5-HT as previously discussed. The protrusion changes interactions across the subunit interface compared to 5-HT binding and this may account for different effects on channel gating.

Conformational Changes Due to Ligand Binding

The binding of 5-HT or other ligands induces changes important for allosteric coupling, which is discussed in the next section. Here we address changes in the immediate vicinity of the binding pocket. The most obvious changes in protein structure come from Loop C movements. It has been shown that agonist binding in pLGICs induces an inward motion of Loop C relative to the apo state and most antagonists induce outward motion. However, it is unclear if these movements are necessary for channel gating, as discussed further in the section on allosteric coupling. The inward motion closes the gap between the two subunits and results in a smaller pocket accommodating the hydrophobic and hydrogen bond interactions previously discussed. Dissimilar to most pLGIC antagonists, the granisetron structure shows Loop C in-between the 5-HT-bound and apo positions (Basak et al. 2019). Perhaps setron inhibition of 5-HT₃R occurs via a mechanism distinct from that of other pLGIC antagonists.

Rearrangements in loop B are more subtle, but channel activity is very sensitive to mutations in this region. One source of protein rearrangements is the rotameric flexibility of W156. Both the 5-HT-bound 5-HTBP structure and the nanobody-stabilized 5-HT₃R crystal structure show W156 in a different conformation than later cryo-EM 5-HT₃R structures (Hassaine et al. 2014; Kesters et al. 2013). This may be an experimental artifact, but molecular dynamic simulations have been used to propose that this rotameric flexibility is important for coupling ligand binding and channel opening (Yuan et al. 2016).

Ligand binding also results in rearrangements in the relative position of neighboring subunits. When TMD helices are aligned, beta sheets 1, 2 (Loop D) and 6 (Loop E) are twisted counter-clockwise in granisetron-bound structures and more so in 5-HT-bound structures relative to the apo structure. This closes the gap between the interfaces around the bound ligand. The previously discussed interactions between

Loops B and C on the (+) interface and D, E and F on the (−) interface likely help mediate this beta-sheet twist.

There have been significant efforts to understand how changes in the ligand binding pocket are connected to channel activity. This is addressed more fully in the next section but here we note that each loop is covalently linked to loops in the ECD-TMD interface known to affect allosteric coupling. It seems likely there is not a single or even dominant conformational wave that connects ligand binding to channel activity, but rather there are rather multiple conformational pathways whose combinatorial effects determine the downstream effects of a specific ligand on channel activity.

Allosteric Coupling of Ligand Binding and Channel Gating

pLGICs are able to function as signal transducers because ligand binding is allosterically coupled to channel activity. The simple framework of pLGIC coupling is that channels go from an apo (resting) state, to a 5-HT-bound (open) state to a 5-HT-bound (desensitized) state before returning to the apo (closed) state. However, single-channel kinetic studies of open and closed dwell times reveal that this is an oversimplification and there are different lifetimes within each of these conformations. The lifetimes of these conformations determine the channel's overall kinetic properties. Generally, state lifetimes are measured by single-channel recordings, but WT 5-HT_{3A}R currents are too weak for these recordings. As an alternative, 5-HT_{3R} kinetic properties have been determined from macroscopic currents, albeit with less detail than single-channel recordings (Mott et al. 2001; Solt et al. 2007). Single-channel recordings have also been done with chimeric channels containing portions of 5-HT_{3A} or a high-conductance mutant of 5-HT_{3A}R that replaces the conductance-limiting intracellular arginines with their 5-HT_{3B} counterparts (Corradi et al. 2009; Bouzat et al. 2008). Both macroscopic current measurements and mutant recordings have limitations, but these studies provide valuable insight to 5-HT_{3R} kinetics and their physiological implications.

The mechanisms underlying coupling between ligand binding and channel activity are also not fully understood, but there are residues and protein structures known to be key contributors. These processes can be significantly altered by allosteric modulators. These modulators can enhance or inhibit channel function by stabilizing particular kinetic states. Allosteric modulators of 5-HT_{3R} have significant physiological consequences and include endogenous molecules, recreational drugs and potential therapeutics.

Observed Properties of Allosteric Coupling

5-HT_{3A}R has five identical binding sites and most evidence suggests that channel activation requires binding of at least two but more likely three molecules (Rayes et al. 2009; Mott et al. 2001; Corradi et al. 2009; Solt et al. 2007). Upon binding, the channel passes through a concentration-independent pre-open state. This occurs in multiple pLGICs, but transitions from the pre-open state to an open state are longer with 5-HT₃Rs than other channels. This accounts for their unusually long activation times, about 3 ms (Solt et al. 2007).

Electrophysiology recordings showed there are multiple open-state lifetimes and it was originally proposed that different lifetimes corresponded to different binding occupancies (Mott et al. 2001). Three open-state lifetimes were observed in the high-conductance 5-HT_{3A}R mutant but their relative ratios did not change with 5-HT concentration, suggesting all three states are accessible regardless of occupancy (Corradi et al. 2009). As an exception, the duration of the longest open-state lifetime is shortened at higher 5-HT concentrations (>5 μ M), which is consistent with 5-HT pore-block.

Single channel recordings of chimeric or mutant 5-HT₃R receptors show bursts of openings separated by short channel closures, typical of most ion channels (Sakmann et al. 1980). A less common feature of 5-HT_{3A}R is that multiple bursts are separated by intermediate closures that are known as clusters. The cluster terminates with a long closure before activity resumes (Bouzat et al. 2008; Corradi et al. 2009). The intermediate closures are thought to be transitions back to the pre-open state and the long closure is a desensitized state. The effect of clustering prolongs channel activity. Indeed, macroscopic-current studies of WT 5-HT₃R show that the channel desensitizes with a time constant of 1 s, which is slower than most pLGICs. Complete desensitization recovery occurs in sigmoidal fashion over about 20 s with a Hill coefficient of about 3. This is consistent with the 5-HT_{3A}R sequentially unbinding 3 5-HT molecules before returning to a closed state that can be reactivated. The rate of desensitization also increases as a function of 5-HT and extracellular calcium, perhaps through mechanisms similar to 5-HT pore block and inhibition by extracellular calcium (Yakel et al. 1993; Solt et al. 2007). The kinetics of 5-HT_{3A}R are too slow for postsynaptic transmission, but are consistent with a mostly presynaptic modulatory role of 5-HT_{3A}Rs that persists through several synaptic events.

5-HT_{3A/B}Rs have not been examined to the same degree as 5-HT_{3A}R, but they have roughly a three times lower Hill coefficient for 5-HT activation, suggesting activation requires fewer bound 5-HT molecules. Heteromeric receptors also desensitize and recover more quickly than 5-HT_{3A}Rs, consistent with 5-HT_{3A/B}R being better suited for postsynaptic transmission (Hapfelmeier et al. 2003; Dubin et al. 1999).

Mechanisms Underlying Coupling Between Ligand Binding and Ion Permeation

For all pLGICs, loop regions in the ligand-binding domain are covalently connected to four ECD regions at the ECD-TMD interface that are critical for allosteric coupling, the $\beta 1$ - $\beta 2$ loop, the $\beta 6$ - $\beta 7$ (cys) loop, $\beta 6$ - $\beta 7$ loop and the pre-M1 helix. Movements in these regions are communicated to the TMD via covalent M1 linkage and interactions with the M2-M3 linker (Bouzat et al. 2004). Thermodynamic analysis of mutant perturbations shows that a ‘conformational wave’ proceeds from the ligand binding pocket, to ECD portions of the ECD-TMD regions, to the M2-M3 linker and eventually the pore (Chakrapani et al. 2004). Beyond this crude outline, the events communicating ligand binding to the pore domain remain unclear.

It was originally proposed that movements in the mobile Loop C displaced the pre-M1 helix which in turn moved the $\beta 1$ - $\beta 2$ and $\beta 6$ - $\beta 7$ loops that finally repositioned the M2-M3 linker (Bouzat et al. 2004; Lee et al. 2009). However, there is uncertainty over the role of Loop C in channel opening with reports differing among pLGICs (Terejko et al. 2020; Purohit and Auerbach 2013; Pless and Lynch 2009). It is possible that conformational changes in the ligand binding pocket act on multiple kinetic pathways whose combinatorial effects determine the channel state, like the strings of a puppet. To support this idea, 5-HT₃R antagonist-bound structures resemble open state structures in the peripheral beta sheets, including Loop C and the pre-M1 helix, and resemble closed state structures in the inner beta sheets, including the $\beta 1$ - $\beta 2$ loop as seen in Fig. 11.6.

Exact residues at the ECD-TMD interface are poorly conserved across pLGICs, but general principles are observed (Mukhtasimova and Sine 2013; Xiu et al. 2005). The charged residues at the ECD-TMD interface form a network of interactions with net charge varying between structures. The spatial layout of 5-HT₃R structures suggest that the pre-M1 helix contacts the $\beta 1$ - $\beta 2$, $\beta 6$ - $\beta 7$ and $\beta 8$ - $\beta 9$ loops, and that each of these loops contacts the M2-M3 linker, where movements are directly coupled to the channel pore. The pre-M1 and M1 helices are also positioned to directly interact with the M2-M3 linker.

The pre-M1 helix carries a net positive charge including three consecutive arginine residues R217-219, of which R218 is conserved across pLGICs. Structural evidence places these residues at a hub between E53 on the $\beta 1$ - $\beta 2$ loop, D145 on the $\beta 6$ - $\beta 7$ loop and E186 and W187 on the $\beta 8$ - $\beta 9$ loop (Hassaine et al. 2014; Basak et al. 2018b). Mutation of these residues affects channel gating or formation. For mutations that disrupt channel formation, similar mutations in other pLGICs affect gating, though the effects vary from one member to the next (Price et al. 2007; Bouzat et al. 2004; Hu et al. 2003).

The E53 connection between the pre-M1 helix and $\beta 1$ - $\beta 2$ loop creates a possible conformational pathway along the inner vestibule. Mutational experiments provide evidence that this pathway continues to the M2-M3 linker via K54. K54A mutants bind ligand but do not gate while K54R or K54Q mutations retain gating (Mosesso

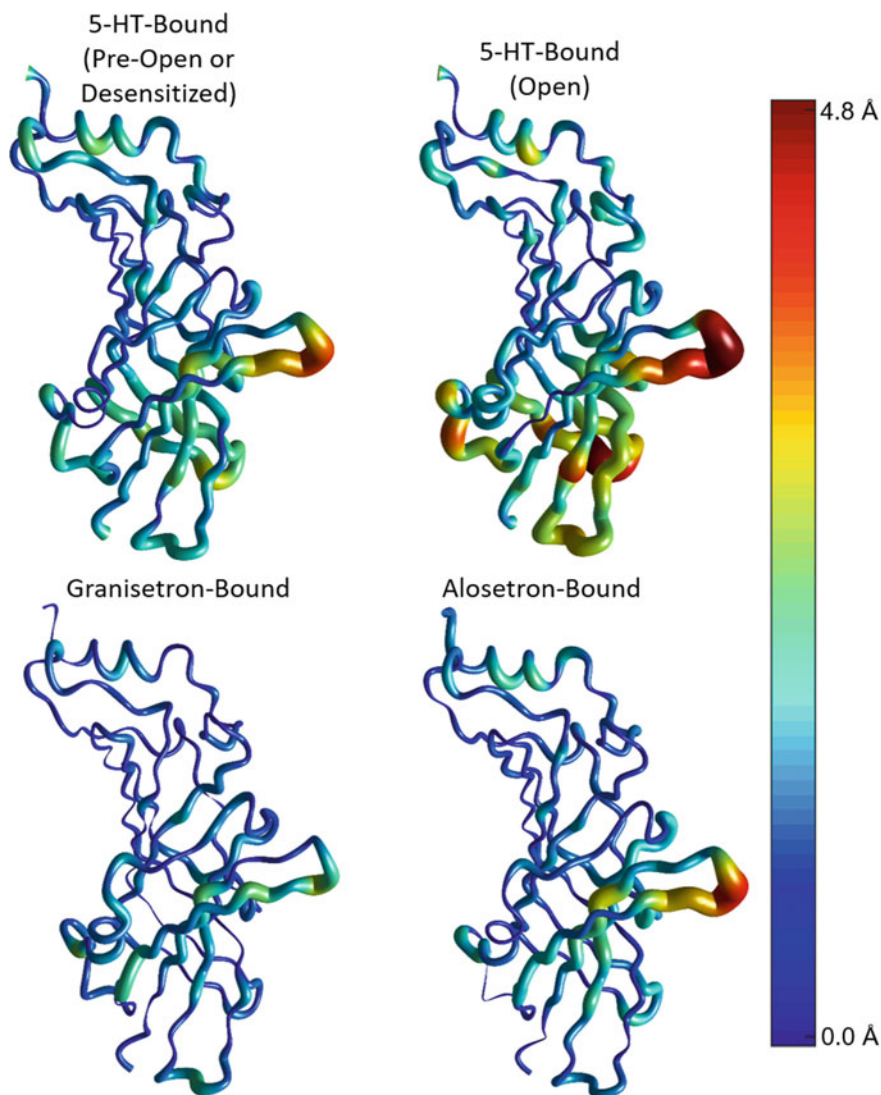


Fig. 11.6 Allosterically coupled motions of the ECD domain upon ligand binding. The ECD of each labeled structure was aligned to the ECD of apo 5-HT_{3A}R (PDBs 6BE1, 6DG7, 6DG8, 6NP0 and 6W1J) (Basak et al. 2018a, b, 2019, 2020). The rendered images show the C α chain with a thickness and color that correspond to the relative displacements from the apo structure. Note that some regions show similar displacements in 5-HT and antagonist bound structures, such as Loop C, while other motions are unique to 5-HT bound structures, such as the β 1- β 2 loop.

et al. 2019). K54C mutations have disrupted gating that can be rescued by sulfhydryl-labelling agents, regardless of charge (Reeves et al. 2005). Additionally, double mutants with A277C or I278C, near M2 on the M2-M3 linker, are completely inhibited, but currents are rescued in the presence of reducing agents. Together this suggests that K54 sterically interacts with residues near the top of the M2 helix.

The $\beta 6$ - $\beta 7$ loop provides a connection to the M2-M3 linker along the outer vestibule. This loop consists of several hydrophobic residues including the highly conserved F142, P143 and F144, or FPF motif. Structural and mutagenesis experiments suggest that these residues penetrate into the membrane interface and push L282 and I283 upon channel activation (Hassaine et al. 2014; Limapichat et al. 2010; Lee et al. 2009). These residues are located at the M3 end of the M2-M3 linker next to P281. P281 is conserved across cationic pLGICs and plays a key role in gating. Specifically, natural and unnatural mutagenesis show that propensity of the residue to be in the cis conformation correlates with gating efficiency, though isomerization is not required for channel opening (Paulsen et al. 2009; Lummis et al. 2005; Mosesso et al. 2019). The mobility of the $\beta 1$ - $\beta 2$ and $\beta 6$ - $\beta 7$ loops in 5-HT-bound states compared to antagonist-bound states can be observed in Fig. 11.6.

The M2-M3 linker is displaced radially outwards and towards the membrane in the in open 5-HT₃ structures. This is accompanied by a rotation and tilting of the M2 helix that widens the pore at the hydrophobic gate. In addition to the effects of the M2-M3 linker, there are several mutations throughout the TMD helices and ICD that affect channel gating, especially desensitization (Gunthorpe et al. 2000; Yakel et al. 1993). It has been proposed that movements in the M1 or M3 helix are communicated to M2 residues via charged residue pairs within the helix bundle. The length and composition of the M3-M4 linker also affect desensitization, perhaps through mechanisms that alter conduction through the intracellular portals (McKinnon et al. 2012; Hu et al. 2006).

Allosteric Modulators

Allosteric modulators bind to 5-HT₃Rs non-competitively and stabilize kinetic states that can either enhance or reduce channel properties. Positive allosteric modulators (PAMs) will not directly activate the channel but will potentiate the receptor so it activates at lower agonist concentrations. This has significant physiological consequences. For example, small alcohols and halogenated anesthetics are PAMs of several pLGICs including GABA_ARs and 5-HT_{3A}Rs (Jenkins et al. 1996; Lovinger 1991). At GABAergic synapses in inhibitory neurons, presynaptic activation of potentiated 5-HT_{3A}Rs results in increased GABA release, which activates potentiated postsynaptic GABA_ARs. Similarly, 5-HT_{3A}R potentiation increases dopamine release at dopaminergic synapses (Wozniak et al. 1990). These neuronal events underlie the sensations caused by these compounds. Defects in 5-HT₃R signaling are connected to various psychiatric disorders and substance abuse and 5-HT₃R

PAMs are attractive drug candidates to address these conditions (Thompson et al. 2006b; Hagan et al. 1993).

The binding cavity for alcohols and volatile anesthetics is located between M3 and M1 helices in neighboring subunits at the ECD-TMD interface. Binding at this site stabilizes the channel open state (Feinberg-Zadek and Davies 2010; Li et al. 2006). At higher concentrations, these compounds bind a distinct low-affinity site and act as negative allosteric modulators (NAMs) (Rüsch et al. 2007). This second site may act as a physiological override to excessive drug concentrations. 5-HT_{3A/B}R is also potentiated by alcohol and anesthetics but to a lesser degree, consistent with presynaptic 5-HT₃Rs being 5-HT_{3A}Rs (Solt et al. 2005).

There are many other natural 5-HT₃R NAMs. These include cannabinoids, menthol and other terpenes, ginger extracts and capsaicin. Both endogenous cannabinoids such as arachidonoyl glycerol and arachidonoyl ethanolamine (anandamide) and exogenous cannabinoids such as cannabidiol (CBD) and Δ^9 -tetrahydrocannabinol (THC) inhibit 5-HT₃R currents (Yang et al. 2010; Barann et al. 2002; Xiong et al. 2008). Notably, CBD effectively inhibits 5-HT₃R but has no psychoactive effects. Cannabinoids interact directly with 5-HT₃R and inhibit channel function by enhanced desensitization. The mechanism of inhibition is not fully understood but in GlyR, cannabinoids bind S296 within the M3 helix (Xiong et al. 2011). 5-HT₃R activity mediates analgesic and anti-emetic effects of cannabinoids in CB₁ and CB₂ KO mice (Rácz et al. 2008). This suggests that further developed 5-HT₃R modulators may offer the therapeutic benefits of cannabinoids without psychoactive effects.

The effects of alcohols and cannabinoids are applicable to many natural compounds found in traditional treatments for nausea and pain. Small terpenes and terpenoids have chemical structures similar to larger alcohols or anesthetics. Consistent with their size, molecules like menthol, boldine, citronellol and geraniol are NAMs with a range of potencies (Walstab et al. 2014; Ziemba et al. 2015). However, other similarly size molecules such as thymol and carvacrol are PAMs. Other natural NAMs include ginger and capsaicin. These lipid-embedded molecules may act through similar mechanisms as cannabinoids. These natural compounds act on many molecular targets, but 5-HT₃R modulation may contribute to their physiological effects. The range of potency and directional effects suggests that further study of these compounds will lead to a better understanding of allosteric mechanisms and perhaps useful pharmacological tools.

Conclusions

It has been about thirty years since the discovery 5-HT_{3A}R as an ion channel (Derkach et al. 1989; Maricq et al. 1991). At that time, questions were focused on the nature of 5-HT₃Rs. Where are they located? What do they look like? What are they basic properties? As covered in this chapter, substantial progress has been made on many of these questions. Still, several fundamental properties of 5-HT₃R remain unknown.

For example, most the ICD remains a black box even in recent cryo-EM structures. This domain is the most divergent region between pLGICs and is the site of many protein–protein interactions that regulate channel function, expression and localization. Future research is required to better understand this and other fundamental parts of 5-HT₃R.

Our increased understanding of what the 5-HT₃R is and does has led to further questions of how the channel operates. For example: Why is calcium permeable in 5-HT_{3A}R but not 5-HT_{3A/B}R? Why do some ligands induce channel opening while others antagonize it? What are the events that lead from ligand binding to channel opening? Answers to these questions are more than just intellectual curiosities, they help us understand channel function or dysfunction in certain conditions and provide a template for pharmacological intervention.

Ion channels seldom operate alone. The function of homomeric or heteromeric channel assembly is largely influenced by local environments that include membrane lipids, interacting protein partners related to the extracellular matrix, cytoskeletal proteins and cellular signaling molecules. While ion channels are now studied at the structural single-molecule level, future directions will increasingly be focused on the structure and function of interactome assemblies, and ultimately proteins in their native cellular environments.

A better understanding of how the channel operates will hopefully lead to an increased ability to pharmacologically manipulate its function. There is a growing list of natural and synthesized compounds that affect 5-HT₃R. In particular, there is promise in the use of weak partial agonists and NAMs as anti-nausea medications. An increased library of specific PAMs would be useful for understanding the role 5-HT₃R in synaptic signaling as present PAMs are non-specific.

Even an exact understanding of 5-HT₃R itself would be of limited practical use, since the channel is clearly tailored to produce specific events during signaling, but the downstream effects are at best crudely understood. Future research on the implications of 5-HT₃R expression and activity throughout the body can open the door to understanding complex neurobehaviors and addressing devastating mental disease. This is an immense challenge but the potential benefits touch on the ultimate aim of biomedical research.

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