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Structure and Function of Serotonin G protein Coupled Receptors

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Abstract

Serotonin receptors are prevalent throughout the nervous system and the periphery, and remain one of the most lucrative and promising drug discovery targets for disorders ranging from migraine headaches to neuropsychiatric disorders such as schizophrenia and depression. There are 14 distinct serotonin receptors, of which 13 are G protein coupled receptors (GPCRs), which are targets for approximately 40% of the approved medicines. Recent crystallographic and biochemical evidence has provided a converging understanding of the basic structure and functional mechanics of GPCR activation. Currently, two GPCR crystal structures exist for the serotonin family, the 5-HT_{1B} and 5-HT_{2B} receptor, with the antimigraine and valvulopathic drug ergotamine bound. The first serotonin crystal structures not only provide the first evidence of serotonin receptor topography but also provide mechanistic explanations into functional selectivity or biased agonism. This review will detail the findings of these crystal structures from a molecular and mutagenesis perspective for driving rational drug design for novel therapeutics incorporating biased signaling.

Keywords

serotonin; 5-HT; GPCR; 7TM; 5-HT_{1B}; 5-HT_{2B}; functional selectivity; β -arrestin; ligand bias

1. Introduction

Serotonin or 5-hydroxytryptamine (5-HT) remains one of the most widely studied chemical messengers. Serotonin produces a myriad of physiological effects in humans, mediated through 14 distinct receptor subtypes, of which 13 are G protein coupled receptors (GPCRs), and one ligand-gated cation channel (Berger et al., 2009; Hoyer et al., 1994). 5-HT receptors have evolved over the course of 700–800 million years (Kroeze and Roth, 1998; Peroutka and Howell, 1994). In the human central nervous system (CNS) alone, all the serotonin

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receptor subtypes, with the exception of 5-HT_{5b}, are expressed, and they are involved in the modulation of sleep-wake cycles, emesis, appetite, mood, memory, breathing (Ray et al., 2011), cognition, and many other functions (Berger et al., 2009; Meltzer and Roth, 2013). Much of the serotonin in the body, however, is not found in the CNS, rather in the gastrointestinal (GI) tract, where it causes peristalsis through either smooth muscle contraction or enteric nerve depolarization (Gershon et al., 1990). Lesser known, but historically important, serotonin is found in the blood, isolated from platelets in the serum, where it is involved in blood coagulation and vasoconstriction, a function which led to its name “sero” (from serum) and “tonin” (to induce contraction)(Rapport et al., 1948). Not surprisingly, more than 125,000 published articles as of 2014 (pubmed search) have been published on serotonin and its receptors.

Much research regarding serotonin has been in the area of neuropsychiatric drug discovery in treatment of affective disorders, where there continues to be an extreme interest in the design of more efficacious pharmaceuticals. Drugs either targeting serotonin receptors or serotonin itself represent a large share of the top selling pharmaceuticals in the past decade, with more being approved for future use. This is most evident with antidepressants such as SSRIs (serotonin-selective reuptake inhibitors) dominating the drug market, with over \$11 billion in sales in 2008 alone, and Cymbalta (duloxetine), a dual serotonin-norepinephrine reuptake inhibitor (SNRI), being in the top 10 drugs sold in the US in 2012 (Lindsley, 2013). Additionally, newer antipsychotics, such as aripiprazole (Abilify) and quetiapine (Seroquel), which have partial agonist activity and/or antagonist activity at serotonin receptors, were also in the top 10 drugs sold in the US. Recently, newer SNRIs are being approved by the FDA with an indication to treat depression, including the approved levomilnacipran (Fetzima), and SSRIs with direct agonist activity at the 5-HT_{1A} receptor have been approved by the FDA including vilazodone (Viibryd) and vortioxetine (Brintellix) for treatment of major depressive disorder (Celada et al., 2013). There is, however, a need for the discovery of more efficacious CNS pharmaceuticals with reduced side effects associated with off-target activity. For example, agents for Parkinson's disease, such as pergolide and bromocriptine, were discontinued in the US because of their ability to induce cardiac valve hypertrophy, which has been linked to off-target agonist action at the 5-HT_{2B} receptor (Horvath et al., 2004; Roth, 2007; Rothman et al., 2000; Setola et al., 2003).

In addition to drug selectivity, the concept of functional selectivity (Urban et al., 2007) in pharmacology has revolutionized the drug discovery process with recent findings highlighting that functionally selective β -arrestin biased drugs can show efficacy in preclinical models of schizophrenia (Allen et al., 2011), and β -arrestin biased angiotensin II type 1 compounds are currently in Phase 2 clinical trials for treatment of congestive heart failure (Soergel et al., 2013). Thus, rational drug design of newer serotonin receptor drugs incorporating ligand bias appears to be a fruitful area of drug development that potentially could be facilitated by insights into the structure and function of their respective targets.

Recently, there has been a GPCR structural “renaissance” with crystal structures from several representative receptor classes being solved, including rhodopsin (Palczewski et al. 2000), β_2 adrenergic (Cherezov et al., 2007), β_1 adrenergic (Warne et al., 2008), A_{2A} adenosine (Jaakola et al., 2008), H₁ histamine (Shimamura et al., 2011), D₃ dopamine

(Chien et al., 2010), smoothened (Wang et al., 2013b), CXCR4 (Wu et al., 2010), sphingosine 1-phosphate (Hanson et al., 2012), protease-activated receptor 1 (Zhang et al., 2012), M2 muscarinic (Haga et al., 2012), M3 muscarinic (Kruse et al., 2012), mu opiate (Manglik et al., 2012), delta opiate (Granier et al., 2012), kappa opiate (Wu et al., 2012), nociceptin/orphanin (Thompson et al., 2012), and recently the 5-HT_{1B} and 5-HT_{2B} serotonin receptors (Wacker et al., 2013; Wang et al., 2013a). These receptor crystal structures provide a wealth of structural information for the design of more receptor subtype selective agents, and also provide insights into the chemo-mechanical processes involved in GPCR activation (Mustafi and Palczewski, 2009). Thus GPCR structures likely will serve medicinal chemists and pharmacologists to design new functionally selective drugs as potential therapies or pharmacological tools (Kenakin and Christopoulos, 2013).

The recent publications of two serotonin receptor crystal structures in complex with ergotamine (ERG), a former antimigraine agent, has opened up two major discussions relevant to the serotonin field, which concern the molecular basis for 1) serotonin receptor subtype recognition and 2) functional selectivity. The 5-HT_{1B} crystal structure illustrates the molecular basis for the selectivity of antimigraine triptan drugs that are devoid of 5-HT_{2B} receptor off-target activity, and the 5-HT_{2B} crystal structure provides clues into the functional mechanics of a receptor in a β -arrestin biased state. These crystal structures not only further our understanding into serotonin's therapeutic and off-target effects in the current drug discovery process, but also serve to refine our understanding of GPCRs in general.

This review will be divided into two major sections. The first part will provide a background and relevance of serotonin as it relates to our current understanding of its actions in the body and in medicine, with a particular emphasis on the 5-HT_{1B} and 5-HT_{2B} receptors. The second part will focus on the structural importance of the recently published serotonin receptor crystal structures, especially as it relates to our current understanding of GPCR structure and function. The final section will suggest future areas for the development of our understanding of functional selectivity and biased agonism.

2. Historic aspects and significance

2.1 Discovery of 5-HT and 5-HT receptors

Serotonin was actually discovered independently by two laboratories, Vittorio Erspamer's lab in Rome, Italy (Erspamer and Boretti, 1950) and Irvine Page's lab at the Cleveland Clinic (Rapport et al., 1948). Although the action of serotonin is usually associated with CNS function, the majority of serotonin actually resides in the gastrointestinal (GI) tract, produced by enterochromaffin cells lining the lumen of the gut, where it causes increased peristaltic activity (Beleslin and Varagic, 1958). It was from the GI tract that Vittorio Erspamer first isolated serotonin in 1938, a substance that he deduced was an indole amine, which he named enteramine (Erspamer and Asero, 1952). Years later in 1948, a substance in blood serum that produced vasoconstriction was isolated by Maurice Rapport, Arda Green, and Irvine Page of the Cleveland Clinic (Rapport et al., 1948; Rapport et al., 1947), which was later named serotonin ("sero" serum, "tonin" constrict). It wasn't until 1953, however, when Betty Twarog in Irvine Page's lab analyzed brain extracts using a serotonin-sensitive

mollusk bioassay (Twarog and Page, 1953), an assay initially discovered by Erspamer (Erspamer and Ghiretti, 1951), that the presence of serotonin in brain extract was recognized. Interestingly, Erspamer had initially posited an indole nucleus being contained in enteramine's structure, and later Rapport determined the structure of serotonin to be 5-hydroxytryptamine (Rapport, 1949) shown in Figure 1.

Prior to the discovery of serotonin in the 1930's, Sandoz pharmaceuticals had been marketing ergotamine, a drug that produced uterine contractions and was used to induce labor (Hofmann, 1978). Ergotamine is part of a larger class of compounds called ergolines, which includes ergopeptines like ergotamine, lysergamides like lysergic acid diethylamide (LSD), and ergoclavines, all of which retain the indole nucleus in their structures (Figure 1). By 1938, research into other ergolines at Sandoz by the Swiss chemist, Albert Hofmann, who under Arthur Stoll, synthesized several hundred analogs of ergotamine. Albert Hofmann is often famously noted for the discovery of LSD, which serendipitously had been discovered by him via synthesizing analogs of ergotamine and changing the amide substituent into simpler alkyl chains, a subclass of ergolines usually termed lysergamides. LSD, however, did not satisfy the pharmacological profile using rat uterus contraction assays, and interest in it as an obstetric drug waned. Hofmann, however, was compelled to re-synthesize LSD in 1943 based on the diethyl substitution found in a known analeptic drug coramine (nicotinic acid diethylamide, forming the D-ring of LSD, Figure 1), which was known to induce CNS stimulation and increased respiration. On April 16th 1943, Hofmann experienced what he thought was a "mystical" experience around his newly synthesized LSD. Thinking he may have accidentally ingested a small amount, three days later on April 19th, he ingested what he thought was a small test dose, 0.25 mg or 250 µg, which today is known to be a substantial dose of LSD, and the unexpected effects were far beyond any respiratory or CNS stimulant as seen with coramine. Instead, they were "kaleidoscopic, fantastic images" and "alterations that I perceived in myself, in my inner being" (Hofmann, 1980). From that time forward, the unique and powerful psychoactive properties that LSD can evoke became widely known.

Later in the 1940s, LSD was promoted as a research tool in the psychiatry, especially in understanding schizophrenia. Until then, the roots of schizophrenia's etiology had been argued to be more nurture-based rather than nature-based, and attributed to improper parenting. LSD's ability to induce transient thought disorders, perceptual and visual hallucinations, and delusions in certain individuals, however, began to spur more research into LSD's pharmacological actions. After the structure of serotonin was published, a hypothesis was put forth that linked serotonin to several mental disorders (Woolley and Shaw, 1954). Woolley, who was almost completely blind from diabetes, conceived the importance of serotonin in mental disorders simply by realizing that the tryptamine scaffold of 5-HT was embedded within LSD's structure (Figure 1). It wasn't until much later, however, that the importance of serotonin receptors in schizophrenia and depression was fully appreciated.

LSD became a powerful tool at the disposal of pharmacologists to clarify the physiological actions of serotonin in whole tissue assays. John Gaddum pioneered the serotonin receptor pharmacology field by first discovering that LSD antagonized the action of 5-HT in these

tissues (Gaddum, 1953), which ultimately led to the classification of two types of serotonin receptors, then referred to as tryptamine receptors (Gaddum and Picarelli, 1957). In serotonin-induced contractions of guinea pig ileum, the D-type was designated as being sensitive to blockade by the irreversible antagonist, dibenzyline, also known as phenoxybenzamine, and the M-type was sensitive to blockade by morphine (Gaddum and Picarelli, 1957). In that study, Gaddum and Picarelli hypothesized that serotonin-induced contractions of the ileum by the D-type receptors acted in smooth muscle and the M-type acted in nerve ganglia. With only a delineation of serotonin receptors based on available pharmacological agents in animal tissues, much of serotonin receptor research waned for more than two decades.

In the 1970's, the development of radioligands specifically to label receptors in animal tissue were developed, leading to the use of [^3H]-LSD and [^3H]-5-HT. Peroutka and Snyder (1979) used [^3H]-LSD to label two distinct receptor populations, one that had higher affinity for [^3H]-5-HT, which they named 5-HT₁, and another that had higher affinity for [^3H]-spiperone, which they named 5-HT₂. The affinity for 5-HT at these receptor types was sensitive to the presence of guanine nucleotides (Peroutka et al., 1979), indicating that these receptors were likely G protein coupled. Later, it was found that 5-HT₂ receptors were correlated with the actions of the previously named D-type receptor (Bradley et al., 1986). The M-type was then found to be a new serotonin receptor type, the 5-HT₃ receptor, which was revealed to be a ligand-gated ion channel permeable to cations (Derkach et al., 1989), in contrast to the 5-HT₁ and 5-HT₂ receptors.

On the basis of selective agonist/antagonist ligand affinities, cloned sequence homology, and intracellular transduction mechanisms, serotonin receptors were reclassified into seven types, comprised of 14 subtypes (Hoyer et al. 1994). With this new classification system, much serotonin receptor research has flourished in the past 20 years, linking receptor distribution to physiological actions (Berger et al., 2009), and the design of subtype selective agents, which will be discussed in the next section.

2.2 5-HT receptors and canonical G protein signaling

According to the current classification system, there are seven types of 5-HT receptors, 5-HT₁₋₇. All 5-HT receptors are GPCRs with the exception of 5-HT₃, which is a ligand-gated cation channel. Serotonin GPCRs couple to all three canonical signaling pathways through G α_i , G $\alpha_{q/11}$, and G α_s , allowing this receptor family to modulate several biochemical signaling pathways, and leading to several physiological consequences, a discussion which is beyond the scope of this review, (but see Nichols and Nichols (2008) for a more comprehensive review).

The G α_i -coupled serotonin receptors encompass the 5-HT₁ and 5-HT₅ types. The 5-HT₁ subfamily, which is comprised of 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} subtypes, usually couple to G $\alpha_{i(2)}$ leading to inhibition of adenylyl cyclase and a decrease of intracellular cAMP (Bockaert et al., 1987; Lin et al., 2002). There is no 5-HT_{1C} receptor because this subtype was re-designated the 5-HT_{2C} receptor based on its functional G α_q coupling. In this subfamily, the 5-HT_{1A} receptor is expressed as both a pre-synaptic receptor in the raphe nuclei, where it serves to inhibit further release of CNS serotonin, and as a post-

synaptic receptor in medial prefrontal cortex and other cortical areas to modulate dopamine release (Altieri et al., 2013; Bockaert et al., 1987). The 5-HT_{1A} receptor has been implicated in anxiety, and as mentioned, novel anti-depressants and antipsychotics have begun to incorporate partial 5-HT_{1A} agonist activity to enhance their efficacy in such diseases (Celada et al., 2013). Historically, the other 5-HT₁ subtypes have been a focus on for the treatment of migraine and will be discussed in more detail in the following sections.

Little is known about the other G_q-coupled serotonin receptor family, the 5-HT₅ subfamily, which includes subtypes 5-HT_{5A} and 5-HT_{5B}. Only 5-HT_{5A} has only been identified in human cortical layers and cerebellum (Pasqualetti et al., 1998). 5-HT_{5A} has been shown to couple to G_q in HEK cells (Hurley et al., 1998), and the selective 5-HT_{5A} antagonist, SB-6995516, impairs memory (Gonzalez et al., 2013) and reduces acoustic startle (Curtin et al., 2013) in animal models. It has been suggested that the 5-HT₅ receptor may be a therapeutic target in psychiatric disorders (Thomas, 2006), but to date, no selective agonists for this subtype exist to clarify its role.

The G_{q/11}-coupled serotonin receptors include the 5-HT₂ receptor subtypes, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}. These receptors traditionally have been linked to G_q-related pathways, with activation of phospholipase C producing inositol triphosphate (IP₃) and diacylglycerol (DAG), ultimately leading to an increase in intracellular calcium (Roth et al., 1984; Roth et al., 1998). There is evidence, however, that 5-HT₂ receptors may exhibit G protein promiscuity, at least in cultured cell systems. For instance, inhibition of cAMP possibly through G_q activation has been found to occur for 5-HT_{2A} (Garnovskaya et al., 1995), and in the case of 5-HT_{2C} with high expression levels (Lucaites et al., 1996). The 5-HT_{2A} receptor is, by far, one of the most studied types of serotonin receptors owing to its widespread expression in the cortex (Willins et al., 1997), its involvement in the mechanism of hallucinogen action (see Nichols, 2004 for a review), and its implications and effectiveness as a receptor target in a handful of mental illnesses including schizophrenia, depression, and Tourette's syndrome to name a few (Meltzer and Roth, 2013). The 5-HT_{2C} is also widely distributed in the CNS (Mengod et al., 1996; Molineaux et al., 1989), and is also found in the ventral tegmental area negatively regulating dopamine release into nucleus accumbens (Di Giovanni et al., 2000). In fact, 5-HT_{2C}-induced control of dopamine release has been proposed as a mechanism for some antidepressants (Millan et al., 2005), and 5-HT_{2C} antagonism may be responsible for the rewarding effects associated with these antidepressants (McCorvy et al., 2011). In addition, selective 5-HT_{2C} agonists such as lorcaserin have shown efficacy as appetite suppressants (Thomsen et al., 2008) and are currently FDA approved (Hess and Cross, 2013), albeit with slight off-target effects at the 5-HT_{2B} receptor. Less well-known is the ability for 5-HT₂ receptors to induce cell proliferation (Banasr et al., 2004), especially during tissue development, which will be further discussed as it pertains to off-target effects stemming from chronic 5-HT_{2B} receptor activation.

The G_s-coupled receptors include 5-HT₄, 5-HT₆, and 5-HT₇. The 5-HT₄ receptor has been shown to couple positively to adenylyl cyclase leading to increased levels of cAMP in guinea pig hippocampus (Bockaert et al., 1990), but may also result in increased calcium current, at least in human atrial myocytes (Ouadid et al., 1992) through a possible G_{q/11}-

coupled mechanism, which has been demonstrated in reconstituted systems (Ponimaskin et al., 2002). 5-HT₄ receptor distribution varies widely in humans (Bonaventure et al., 2000), and select 5-HT₄ activation can range from peristalsis in the gut (Kadowaki et al., 2002), to long-term potentiation (LTP) in hippocampus (Kulla and Manahan-Vaughan, 2002). Selective 5-HT₄ knock-out mice exhibit stress-induced feeding disorders (Compan et al., 2004). Selective 5-HT₄ antagonists have been proposed to treat atrial fibrillation, irritable bowel syndrome, and urinary incontinence (Brudeli et al., 2013).

The 5-HT₆ and 5-HT₇ receptors, also G α_s -coupled, lead to an increase in cAMP, and are located in the CNS in the thalamus, hypothalamus, hippocampus, as well as the peripheral tissues. Many typical and atypical antipsychotics function as antagonists at cloned human 5-HT₆ and 5-HT₇ receptors (Roth et al., 1994). There is evidence that selective 5-HT₆ receptor antagonists, such as SB 271046, may also have cognitive enhancing properties, and may potentially be used for “add-on” therapy in conjunction with antipsychotics with low affinity for the 5-HT₆ receptor (Roth et al., 2004). In addition, the 5-HT₇ receptor is a potential drug target for depression and 5-HT₇ receptor antagonism has been posited as necessary for amisulpride’s antidepressant (Abbas et al., 2009).

2.3 Non-canonical signaling and functional selectivity

In addition to canonical G protein signaling, GPCRs have also been found to be involved in non-canonical signaling via arrestin recruitment. Arrestin recruitment is a now appreciated as a mechanism in GPCR desensitization (Freedman and Lefkowitz, 1996) with clinical importance, especially regarding the tolerance and therapeutic utility of drugs (Violin et al., 2014). GPCR desensitization occurs through GPCR kinase (GRK) recruitment and subsequent GPCR phosphorylation, which recruit β -arrestin1 and β -arrestin2 (also known as arrestin 2 and arrestin3, respectively) from which the binding of β -arrestin sterically prevents further G protein coupling, ultimately leading to desensitization (Krupnick and Benovic, 1998). The GPCR- β -arrestin complex can also target the GPCR to clathrin-coated pits (Krupnick et al., 1997), ultimately leading to internalization (Ferguson et al., 1996). GPCR internalization serves to control both the duration of the response and subsequent downstream signaling of the receptor-ligand complex. In addition to β -arrestins acting to attenuate G protein-dependent signaling, they can simultaneously initiate parallel, G protein-independent signals (Lefkowitz and Shenoy, 2005). In view of the recent findings with non-canonical arrestin-dependent signaling, it was previously thought that all agonist-receptor complexes stimulated all available signaling pathways to an equal extent. However, over the last decade, mounting evidence questions such a simplistic view.

The introduction and use of recombinant cell systems to express and measure specific receptor functional responses ushered in a new concept in pharmacology. Functional selectivity, sometimes called “agonist-directed trafficking” or more recently “ligand bias” was proposed to account for differences between two or more functional responses resulting from receptor activation by an agonist (Urban et al., 2007). The earliest conceptual framework for functional selectivity was proposed by Roth and Chuang (1987) to account for the phenomenon whereby one or more distinct biochemical transduction mechanisms may be activated by a single GPCR. Roth and Chuang (1987) also predicted that this

phenomenon would lead to the development of selective agonists for a particular receptor-linked effector. Although functional selectivity exhibited by a given ligand may be system-dependent in select cell lines, it has been well-established that functional selectivity reflects a ligand's ability to select different conformations of the receptor, leading to a specific functional response over another (Kenakin, 2011), thus exhibiting conformational ligand bias. Selecting different conformations of the receptor may be key to the design of novel functionally selective agonists or antagonists with improved therapeutic potential over currently available medications; however our understanding of the molecular determinants that lead to biased agonism is not well-understood.

2.4 Serotonin 5-HT_{1B/1D} receptors as a target for antimigraine medication

The first serotonin receptor to be cloned was the 5-HT_{1A} receptor subtype (Kobilka et al., 1987; Fargin et al., 1988). Interestingly, the 5-HT₁ subtype exhibits a high degree of sequence and pharmacological similarity to the β -adrenergic receptors, and initially, it was thought to be a β -adrenergic receptor. Later, the 5-HT_{1B} subtype was designated as a distinct receptor subtype from 5-HT_{1A} on the basis that it did have high affinity for β -adrenergic receptor antagonists, but also had low affinity for spiperone (Pedigo et al., 1981; Nelson et al., 1981), whereas the 5-HT_{1A} receptor binds spiperone with high affinity. Confusion still arose, however, over the existence of a human ortholog of 5-HT_{1B}, because it exhibited responses in rodents (Hoyer et al., 1992) that were not similar in humans (Peroutka, 1994). Therefore, the 5-HT_{1D α} and 5-HT_{1D β} receptor subtypes were classified to account for the human orthologs (Hoyer and Middlemiss, 1989). These two receptor subtypes were often confused as being pharmacologically distinct based on their ability to be activated by β -adrenergic antagonists, such as propranolol and pindolol, where a single amino acid residue distinguishes this reverse pharmacology (Hamblin et al., 1992; Metcalf et al., 1992). This species difference will be discussed more in detail as it pertains to ligand selectivity in later sections.

As mentioned, the 5-HT_{1B/1D} receptors have been shown to couple negatively to adenylyl cyclase resulting in lower intracellular cAMP, which has been demonstrated in vivo in substantia nigra (Bouhelal et al., 1988; Schoeffter et al., 1988), rabbit mesenteric artery (Hinton et al., 1999), and in transfected heterologous cell lines (Levy et al., 1992; Adham et al., 1992; Weinshank et al., 1992). Expression of 5-HT_{1B} receptors is most prevalent in human cerebral arteries (Nilsson et al., 1999), where vasospasm has been implicated in the pathogenesis of migraine. In fact, activation of 5-HT_{1B/1D} receptors in cerebral arteries by agonists causes vasoconstriction and has been posited as a therapeutic explanation for the antimigraine drugs.

Ergotamine, as discussed previously, was developed by Sandoz in the early 1930's as a drug to induce pronounced uterine contraction, but ergotamine also shows vasoconstriction in the periphery as well as the cerebral arteries, suggesting therapeutic efficacy for migraine. The ergolines as a class of drugs, in general, are recognized as having several receptor targets including but not limited to 5-HT_{1A-1F}, 5-HT_{2A-C}, D₁₋₅, as well as α 1 and α 2 adrenergic receptor types (Silberstein, 1997). The antimigraine effects, however, are likely mediated through agonist activity at the 5-HT_{1B}, 5-HT_{1D} and possibly 5-HT_{1F} receptors present on

trigeminal nerve terminals (Ramirez Rosas et al., 2013). However, ergotamine as well as other antimigraine ergolines, such as dihydroergotamine and methysergide, suffer from acute side-effects due to vasoconstriction in the periphery leading to hypertension and coronary vasoconstriction. Ultimately with continued use, severe chronic side-effects can occur such as retroperitoneal fibrosis, pleuropulmonary fibrosis, and cardiac valvulopathy due to off-target activity at the 5-HT_{2B} receptor (Roth, 2007; Rothman et al., 2000), which will be discussed in the next section.

In light of the serious side-effects and off-target activity of the ergolines, newer generation antimigraines incorporate an extended substitution on the tryptamine scaffold giving rise to the triptan class of drugs (Humphrey, 2008) (Figure 1). Triptans, such as sumatriptan, are extremely effective as antimigraine drugs potentially alleviating symptoms by vasoconstriction at cerebral arteries via 5-HT_{1B} receptors and inhibition of proinflammatory neuropeptides in trigeminal fibers via 5-HT_{1D} receptors. This class of medications avoids the cardiac valvulopathy and coronary vasoconstriction by having lower affinity at 5-HT₂ receptors, yet retaining selectivity and agonist activity at 5-HT_{1B/1D/1F} receptors.

2.5 Serotonin 5-HT_{2B} receptors as an off-target for cardiac valvulopathy

The rat 5-HT_{2B} receptor, formerly known as the 5-HT_{2F} receptor, was first cloned and characterized from the rat stomach fundus (Kursar et al., 1992; Foguet et al., 1992), which was previously discovered to cause contraction upon application of several tryptamine agonists (Vane, 1959). The human 5-HT_{2B} receptor was cloned from a placental genomic library (Kursar et al., 1994; Schmuck et al., 1994), and shares considerable homology with the previously cloned human 5-HT_{2A} and 5-HT_{2C} (formerly 5-HT_{1C}) receptors. Functional activation of 5-HT_{2B} was linked to inositol triphosphate (IP₃) production in cultured cells (Wainscott et al., 1993) and calcium influx (Cox and Cohen, 1996), which leads to contraction of the stomach fundus. The *ex vivo* stomach fundus contraction assay was commonly used for detection of early serotonergic agents including hallucinogenic drugs like LSD, but this assay likely reflects 5-HT_{2B} receptor activity rather than 5-HT_{2A} activity, which is more predictive for hallucinogenic agents (Nelson et al., 1999).

Expression patterns of 5-HT_{2B} receptor using mRNA detection in mouse (Kursar et al., 1994) showed high levels in the liver and kidney, with lower amounts in the heart and brain (Loric et al., 1992). Other mRNA expression studies confirmed the presence of 5-HT_{2B} receptors in brain, specifically in the septal nuclei, dorsal hypothalamus and medial amygdala, with comparable levels detected in the stomach fundus (Duxon et al., 1997). Later, it was shown that the cardiovascular (Choi et al., 1994) and cardiac physiological functions of 5-HT_{2B} were more prominent compared to its role in the CNS compared to 5-HT_{2A} and 5-HT_{2C} receptor CNS functions (Bonhaus et al., 1995).

The hypothesis that 5-HT_{2B} activation leading to cardiac hypertrophy gained prominence with the observation that the 5-HT_{2B} knockout mouse demonstrated a lethal phenotype as a result of hypotrophy of the trabeculae cardiac muscles (Nebigil et al., 2000), which help control backflow of blood through the mitral (bicuspid) and tricuspid valves. Studies linked the involvement of the 5-HT_{2B} receptor in cardiac hypertrophy through G_q activation and

concomitant phosphatidylinositol-3 kinase/Akt and extracellular signal-regulated kinase (ERK) 1/2 signaling pathways (Nebigil et al., 2003).

Coincidentally, the fenfluramine and phenteramine weight loss combination (fen-phen) marketed by Wyeth in the early 1990s resulted in patients exhibiting abnormal echocardiograms (Connolly et al., 1997), pulmonary hypertension (Brenot et al., 1993), among other cardiac valve deficiencies (Smith et al., 2009). Indeed, the major metabolite of fenfluramine, norfenfluramine (Figure 1), was found to have potent 5-HT_{2B} agonist activity in Gq calcium release assays, whereas fenfluramine exhibits little activation (Fitzgerald et al., 2000; Roth, 2007; Rothman et al., 2000), indicating that the off-target effects of the fenfluramine metabolite, norfenfluramine, can lead to serious side-effects. This fact coupled with the finding that ergotamine also exhibits 5-HT_{2B} agonist activity clearly indicates that the 5-HT_{2B} receptor is an important off-target receptor to avoid in drug design. Finally, recent evidence regarding ergotamine's pharmacological actions may be explained by its differential signaling profile at the 5-HT_{2B} receptor (Huang et al., 2009), for which the major biochemical signaling pathway leading to cardiac valvulopathy remains unknown.

3. Structural features of serotonin 5-HT receptors

Since the cloning and annotation of the human genome, over 5% of the genome encodes for receptors, which more than 800 are GPCRs (Kroeze et al., 2003; Stevens et al., 2013). GPCRs are usually divided up into five classes and are named according to a representative prototypical receptor: rhodopsin (Class A), secretin (Class B), metabotropic glutamate (Class C), adhesion and smoothened/frizzled/taste (Fredriksson et al., 2003). Within these families, they share considerable sequence homology, and usually pharmacological similarities, but without evidence for an endogenous ligand, or for their physiological role, many are left orphaned and un-mined for their therapeutic potential (Armbruster and Roth, 2005), or their side-effect profile (Allen and Roth, 2011). Lately, there has been an initiative to crystallize representative GPCRs from each class with the goal of understanding their structure-function relationships (Stevens et al., 2013), which will serve to expand drug development of novel therapeutic agents.

In the early 1900's, when the terms "receptive substance" or "receptor" were introduced by John Langley and Paul Erlich, respectively (Langley, 1905; Ehrlich, 1913), to account for agonist versus antagonist drug action, pharmacologists believed that receptor activation was binary, that receptors contained an active state associated with agonist activity versus an inactive state, which was thought to be stabilized by antagonists. The phenomenon of partial agonism, however, began to question this notion, when it was found that ligands possess an "intrinsic" activity, which is to be contrasted with ligand affinity or the ability of a given ligand to bind to the receptor. Intrinsic activity or efficacy can reflect a ligand's ability to maximally activate a receptor in a given pathway and is still poorly understood. Although receptor crystal structures provide an incredible amount of structural information about receptor conformations, they still do not provide a converging concept of intrinsic activity, especially with respect to different functional outcomes exhibited with biased ligands. However, in the case of the recent 5-HT_{1B} and 5-HT_{2B} crystal structures in complex with the same ligand, ergotamine, which exhibits a differential functional selectivity profile at

each of the receptors, it may now be possible to begin to uncover the molecular basis of functional selectivity.

This section of the review will offer a “tour” of the serotonin receptor crystal structures detailing findings from the conserved and non-conserved residues in the binding pocket to microdomain switches involved in the activation process. In addition, this review will also outline possible residues involved in the activation process that possibly lead to functional selectivity or biased signaling.

3.1 General GPCR features

Most of the well-studied GPCRs are in the rhodopsin-like family (Class A), which includes amine, peptide (e.g. opiate, vasopressin, angiotensin), and more than 200 types of olfactory receptors. The first Class A GPCR to be crystallized was bovine rhodopsin in an inactivated state covalently bound to cis-retinal (Palczewski et al., 2000), followed by a lower resolution opsin crystal structure, rhodopsin without retinal, in a photoactivated state (Salom et al., 2006). These structures led to insights into the overall GPCR activation process; however, these structures without the G protein present in complex with the receptor lack the necessary information to provide a present mechanistic explanation needed for full receptor activation.

It wasn't until the Kobilka and Stevens groups published the first amine GPCR crystal structure, the β_2 adrenergic receptor (Rasmussen et al., 2007) followed by the structure of the β_2 adrenergic receptor in complex with a G protein by Kobilka's group (Rasmussen et al., 2011) that insights into the ligand-induced GPCR G protein activation were available. Using the β_2 adrenergic receptor as a model for the GPCR activation process, crystal structures are now available with a variety of ligands including antagonist, inverse agonist, agonist, and full agonists to understand further the complex underpinnings of G protein agonism.

Together, these well-studied receptor systems revealed that Class A GPCRs contain a conserved architecture of seven transmembrane (TM) α -helical domains, and an intramembrane helix 8 (H8), which are connected by three intracellular loops (IL) and three extracellular loops (EL). Although the TM domains are relatively similar, except for slight differences in tilt and twist, there is considerable variability with the loops, especially EL2. In all Class A GPCRs, EL2 is tethered to TM3 by a conserved disulfide bridge between Cys3.25 and Cys^{EL2}. Joined by Cys^{EL2}, EL2 is essentially divided in two parts, the N-terminus and C-terminus region, termed herein as EL2a and EL2b, respectively. With respect to the conserved disulfide bridge, the length of EL2b differs considerably between receptor types and even between subtypes, whereas EL2a is much longer and can adopt secondary structures such as the α -helix seen in the β_2 adrenergic receptor crystal structure (Rasmussen et al., 2007). In addition, there are also conserved residues within each TM, but most of the differences between GPCRs of this class are in the binding pocket, which is lined by inward facing residues located in TM3, 5, 6, and 7, EL2 and EL3, and to a lesser extent, TM2. Differences in the binding pocket allow the selection of the ligand and hence, allow their homology to be associated with particular receptor types.

For Class A GPCRs, Ballesteros and Weinstein conceived of a numbering system based on the most conserved residue within each TM designated as X.50, where X is the number of the TM and subsequent residues toward the C-terminus increase in number (X.51, X.52, etc.), and residues toward the N-terminus decrease (Ballesteros and Weinstein, 1995). Although there are other numbering systems that can be used (Baldwin, 1993; Schwartz, 1994), the Ballesteros-Weinstein numbering system establishes a nomenclature that is better adapted to usage with visualization of crystal structures, and its use is indicated in superscripts when the relative receptor residue number is present.

Not surprisingly, each of the conserved residues in Class A GPCRs designated as X.50 have functional significance, to be discussed in detail in the following sections. Briefly, Asn1.50 and Asp2.50 are important residues in the allosteric sodium site (Fenalti et al., 2014), Arg3.50 is part of the conserved DRY motif important in the activation process, Trp4.50 arguably serves to stabilize the packing of TM2, 3, 4, and Pro5.50, Pro6.50, and Pro7.50 introduce kinks into these helices and likely allow for dynamic movement toward the intracellular side. These residues are astounding in their conserved nature, which likely allows the formation of 7TM receptors scaffold to bind and function in a prototypical fashion.

3.2 Binding pocket

The binding pockets of the 5-HT_{1B} and 5-HT_{2B} receptors are strikingly similar in their overall architecture and exhibit little divergence in their residues. It is proposed that the orthosteric site, or the site of 5-HT binding, is occupied by the indole nucleus present in ergotamine (ERG), whereas the extended binding site is occupied by the peptide portion of ERG (Figure 2).

3.2.1 Orthosteric site—Class A GPCR orthosteric sites are relatively conserved in their overall architecture, where the majority of the orthosteric site occupies the extracellular portion of TM3, TM5, and TM6, and to a lesser extent TM2, TM7, and the C-terminal part of EL2. The orthosteric site, however, can differ depending on the crevice or depth, where histamine and muscarinic GPCR orthosteric sites occupy a deeper portion compared to serotonin and dopamine receptors. In TM3, the conserved aspartate (Asp3.32) serves to anchor the charged amine portion of the ligand, forming a stable salt bridge (Kristiansen et al., 2000) as shown in Figure 3A. Insights into the strength of this bond with mutagenesis reveal that D3.32A mutations severely reduce the affinity for amine-containing agonists and antagonists, but can be insensitive to certain ligands, especially in the case of 5-HT₄ ligands (Claeysen et al., 2003). Residue Asp3.32 could be an advantageous starting point to prevent endogenous neurotransmitter binding for the design of novel engineered GPCRs including RASSLs (receptors activated solely by synthetic ligands) and DREADDs (designer receptors exclusively activated by designer drugs). DREADDs have the potential to illuminate the biochemical roles that GPCRs play in disease states and ultimately could translate into use as novel human therapeutics (Conklin et al., 2008; Urban and Roth, 2014).

One turn below the critical conserved Asp3.32, residue 3.36 also faces the binding pocket, where this residue is a Ser in 5-HT₂ receptors, a Thr in 5-HT₄ receptors, and a Cys in all

other 5-HT receptors, including the 5-HT_{1B} receptor (Figure 3A). At least among 5-HT₂ receptors, this residue has been hypothesized to recognize the amine by possible coordination of the conserved aspartate (Almaula et al., 1996b). In this report, these authors clearly showed that alanine mutations of Ser3.36 in the 5-HT_{2A} receptor detrimentally affected the affinity of primary amine ligands such as 5-HT and tryptamine, but for secondary or tertiary amine serotonin ligands, such as N,N-dimethyl tryptamine (DMT) and 5-methoxy-DMT, the affinity was spared for the most part. This suggests that this residue likely is involved in recognition of the amine portion of the ligand and that the N-alkyl substitution of ligands can differentially be recognized dependent on the type of residue present.

Also, of importance in TM3 is a conserved Thr3.37, which is shown to engage the indole N-H of ERG in both structures (Figure 3B). In both 5-HT_{1B} and 5-HT_{2B} structures, Thr3.37 likely acts as a hydrogen bond acceptor, whereas the adjacent residue, Ala5.46, may be forming an additional van der Waals interaction with the N-H of the indole nucleus (Figure 3B). This finding is particularly interesting in light of the fact that methysergide is an ergoline that contains a methyl at the N1 position and yet exhibits antagonist activity at the 5-HT_{2B} receptor. Moreover, residue 5.46 has been heavily investigated in mutagenesis studies at the β -adrenergic receptor as also serving to confer agonist activity with catechol-containing ligands (Strader et al., 1989), where it forms part of a conserved serine motif in TM5. It remains to be seen whether residue 5.46 confers agonist activity at serotonin receptors similar to β -adrenergic receptors. In fact, at all 5-HT receptors residue 5.46 is an alanine, except at the 5-HT_{2A} and 5-HT₆ receptors, where 5.46 is a serine and threonine, respectively. Interestingly, an alanine at 5.46 is a known rodent species difference at the 5-HT_{2A} receptor that likely explains 5-HT₂ receptor drug selectivity (Almaula et al., 1996a; Johnson et al., 1994).

In TM6, Phe6.51 and Phe6.52 are interacting with the A and B aromatic ring systems of ERG in an edge-to-face π - π interaction (Figure 3C). Residue Phe6.52 was first posited to be involved in the high affinity binding conformation in the 5-HT_{2A} receptor, because mutation of this residue abolishes agonist affinity yet retains antagonist affinity (Choudhary et al., 1993; Choudhary et al., 1995; Roth et al., 1997). These aromatic residues are located just above the conserved Pro6.50 kink of TM6, and are likely involved in the activation process, especially as it pertains to the movement of helix 6, which has been demonstrated in the β 2 receptor as important for overall activation.

Lesser understood are polar residues located in TM5, which are conserved at positions 5.42 and 5.43 for all 5-HT receptors, with notable exceptions at 5-HT₂, 5-HT₄, and 5-HT₆ receptors. Unique to the 5-HT₂ receptors is a glycine present at 5.42, whereas 5.43 is a serine. Ser5.43 has been investigated for ligand selectivity at the 5-HT_{2A} receptor, where it shows a definitive role for hydrogen bond formation with the 5-methoxy substituent of phenethylamines like DOI (Braden and Nichols, 2007), which are selective 5-HT₂ ligands, and also with possibly 5-HT itself (Johnson et al., 1997). In both the 5-HT_{1B} and 5-HT_{2B} crystal structures, ERG lacks a substituent capable of forming a hydrogen bond in this region, and in fact, residue 5.43 is interacting with an adjacent polar residue at position 6.55 in TM6 (Figure 3D). This hydrogen bond between Ser5.43 and Asn6.55 in the

5-HT_{2B} receptor likely serves as an anchor for the tight distance between TM5 and 6, which has been posited as a possible explanation for ERG's β -arrestin bias by possible dampening of G protein activation (Wacker et al., 2013; Figure 4).

3.2.2 Extended binding site—Compared to the orthosteric site, the extended binding sites of the 5-HT_{1B} and 5-HT_{2B} receptors are less similar in their interactions with ERG. Overall, the extended binding site encompasses EL2b and also the extracellular portions of TM5 and TM7, as well as select residues in TM6. The tripeptide of ERG occupies the site between hydrophobic residues located in EL2b and residue 7.35 forming a hydrophobic cleft between these two regions of the receptor (Figure 4A). In fact, EL2b and residue 7.35 in TM7 can be thought of as a cap or lid forming over the tripeptide of ERG.

Arguably, one of the most important differences between the 5-HT_{1B} and 5-HT_{2B} structures with respect to the extended binding site is M5.39 in the 5-HT_{2B} receptor and M6.58 in the 5-HT_{1B} receptor, which interact with the phenylalanine peptide portion of ERG (Figure 4B), yet from opposite sides of the receptor. In fact, residue M5.39 in the 5-HT_{2B} receptor has been investigated as determining ligand selectivity, where valine mutation of M5.39 results in loss of affinity for norfenfluramine (Setola et al., 2005), the active metabolite of fenfluramine. In the 5-HT_{1B} receptor, however, this residue is a smaller, polar Thr, which likely allows space for extended bulky 5-substituted tryptamines like the triptan class of antimigraine drugs (see Figure 1). It appears therefore, that residue M5.39 would prevent binding of larger triptans in the 5-HT_{2B} receptor, thus explaining the lack of 5-HT_{2B} affinity and overall safety for the triptan class of antimigraine drugs.

Related to divergence of residue 5.39, residue 6.58 in TM6 is also strikingly different between the 5-HT_{1B} and 5-HT_{2B} receptors. In the 5-HT_{1B} receptor, M6.58 interacts with the phenyl of the phenylalanine of the tripeptide of ERG causing a shift of this phenyl group closer to TM5 (Figure 4B). Comparison of the TM5 and TM6 distances between the two receptor structures reveals that in the 5-HT_{2B} receptor these helices are closer than observed in the 5-HT_{1B} receptor (Figure 4C), likely a result of these divergent residues 5.39 and 6.58, as well as previously discussed residues 5.43 and 6.55.

3.3 Conserved motifs and microswitches

3.3.1 DRY motif—In TM3, the most conserved residue is Arg3.50, which is part of the conserved DRY motif (Asp-Arg-Tyr). In the inactive state of rhodopsin, Arg3.50 forms a salt bridge to neighboring Asp3.49 (Palczewski et al., 2000), and the DRY motif was originally thought to stabilize the ground state of the receptor (Rovati et al., 2007), because in photoactivated rhodopsin, the salt bridge between Asp3.49 and Arg3.50 is broken (Salom et al., 2006). Mutagenesis studies further support the role of the DRY motif to stabilize the ground state of the receptor because mutations of this motif in the 5-HT_{2A} receptor usually result in constitutive activity (Shapiro et al., 2002), and mutations of Arg3.50 completely abolish measurable G protein activation, at least in muscarinic M1 receptors (Jones et al., 1995). The crystal structure of the β_2 adrenergic receptor-Gs complex also revealed that Arg3.50 likely forms a hydrogen bond with Tyr5.58 or with the G protein itself, where the Arg3.50 is sandwiched between Tyr5.58 and a Tyr located in the Gs-protein C-terminal tail

(Rasmussen et al., 2011) in a cation- π electrostatic interaction. However, the role of the DRY motif is still not entirely clear because several GPCRs lack the D(E)RY motif, and examination of inactive (antagonist-bound) crystal structures reveal that the Asp3.49 and Arg3.50 is indeed broken, as is the case for the β_1 and β_2 adrenergic receptor, A2A, and CXCR4 receptors (Cherezov et al., 2007; Jaakola et al., 2008; Warne et al., 2008; Wu et al., 2010). In fact in the H1 histamine receptor, Arg3.50 forms a novel contact with a Gln residue in TM6 (Shimamura et al., 2011). In summary, D(E)RY motif is not entirely conserved, but does point to a general mechanism of G protein activation with respect to GPCRs.

In the 5-HT_{1B} receptor, this ionic lock between Arg3.50 and Asp3.49 is broken, and surprisingly in the 5-HT_{2B} crystal structure, this ionic lock is preserved (Figure 5A). The lack of the intact salt bridge suggests that ERG in the 5-HT_{1B} receptor is in a fully active state of the receptor, whereas 5-HT_{2B} is not, partially explaining that an intact salt bridge in the DRY motif may be indicative of a β -arrestin biased state in the 5-HT_{2B} receptor.

3.3.2 NPxxY motif—The highly conserved NPxxY motif located toward the cytoplasmic end of TM7 is thought to be involved in GPCR activation (Nygaard et al., 2009), where the conserved Tyr7.53 can move and rotate toward the helical bundle just below the binding site. In the β_2 adrenergic receptor, ¹³C-methionine labeling reveals conformational states not detected in crystal structures, which argue the existence of a multitude of receptor activation states (Nygaard et al., 2013). In this study carvedilol, a β_2 adrenergic β -arrestin biased ligand, shows a large shift in movement of the cytoplasmic domain of TM7, an area containing the NPxxY motif. The NPxxY motif has been implicated as crucial in the recruitment of β -arrestin by its movement toward intramembrane helix 8, which contains a known phosphorylation site responsible for the binding of arrestin (Gehret et al., 2010). The NPxxY motif is thought to form an opening for a water channel to flow through upon receptor activation (Yuan et al., 2014) and residue Tyr7.53 is thought of as “gate keeper.”

In both the 5-HT_{1B} and 5-HT_{2B} receptor structures, Tyr7.53 rotates toward the helical core, suggestive of a classical active state, but there appears to be more pronounced inward rotation of Tyr7.53 in the 5-HT_{2B} structure (Figure 5B). As mentioned, the movement of Tyr7.53 and TM7 in general is thought to be critical for the arrestin recruitment event moving the C-terminus, possibly allowing for phosphorylation and for subsequent arrestin recruitment to occur (Shukla et al., 2011). This suggests perturbations in the NPxxY motif may be predictive of β -arrestin bias. The finding that the inward rotation and overall movement of TM7 toward the core of the 5-HT_{2B} receptor is evidence that the 5-HT_{2B}-ERG receptor complex is indeed in a β -arrestin biased state.

3.3.3 P-I-F motif—The most conserved residue in TM5 P^{5.50} forms the P-I-F motif together with I^{3.40} and F^{6.44}. This motif is only now gaining attention as a critical trigger motif for receptor activation, especially in light of the 5-HT_{1B} and 5-HT_{2B} crystal structures. These three residues form an interface at the base of the binding pocket and show striking differences related to the active versus inactive crystal structures of aminergic GPCRs. For example, comparison of active and inactive crystal structures of the β_2 adrenergic receptor (Rasmussen et al., 2007), an inward shift of TM5 P^{5.50} leads to a rotamer switch of I^{3.40}

resulting in an inward shift of F^{6.44}, overall leading to a rotation of TM6 (Figure 5C). Rotation and overall movement of TM6 has been implicated in GPCR activation (Ghanouni et al., 2001), especially because the intracellular portion of TM6 forms the interface with the G protein (Rasmussen et al., 2011) and shifts 14 Å outward toward the cytoplasmic end upon activation.

Residues I^{3.40} and F^{6.44} were first proposed to account for active versus inactive states of the β₂-receptor through in silico simulations (Dror et al., 2011). For the 5-HT_{1B} and 5-HT_{2B} crystal structures, the P-I-F motif shows two different configurations, where the 5-HT_{1B} resembles the active state of the β₂ adrenergic receptor, and the 5-HT_{2B} is posited to be in an intermediate state. The 5-HT_{1B} receptor shows an almost identical display of the P-I-F motif compared with both active crystal structures of the β₂ adrenergic receptor with I^{3.40} rotated up toward the extracellular side and F^{6.44} shifting inward. The 5-HT_{2B} receptor, however, shows a similar rotation of I^{3.40}, but does not show the same degree of inward shift of F^{6.44}, which is distinct from the inactive crystal structure of the β₂ adrenergic receptor, suggestive of an intermediate state of activation.

As discussed previously related to the binding pocket, critical residues for manifesting agonist activity reside in the TM3, TM5, and TM6 interface, most notably residues 3.37, 5.46, and 6.52, which allow for high agonist binding affinity. It is conceivable that the P-I-F motif serves as an initial relay from the binding pocket to the other trigger motifs, D(E)RY and NPxxY. The P-I-F motif is located in close proximity to the binding pocket, and therefore can be argued to be a primary trigger motif to recognize ligand-induced conformation changes from the binding pocket, thus transducing this shift down to the other trigger motifs. This is especially relevant given that ergotamine is a β-arrestin biased agonist with very low G protein activation and that the intermediate state of the P-I-F motif observed in the 5-HT_{2B} receptor may be indicative or possibly predictive of a biased state of the receptor.

3.3.4 Allosteric sodium site—The most conserved residues in TM1 and TM2 are Asn1.50 and Asp2.50, which participate in an extensive hydrogen bond network with structured waters in rhodopsin (Pardo et al., 2007). Recent evidence indicates that Asp2.50 also forms part of the sodium ion binding site in the A_{2A} receptor (Liu et al., 2012), and can also serve as an efficacy switch for β-arrestin versus G protein signaling in the delta opioid receptor (Fenalti et al., 2014). These findings are particularly interesting because the presence of sodium in binding buffer was known to inhibit high affinity agonist binding in opioid receptors, leaving antagonist affinity intact (Pert et al., 1973). The sodium site as a possible allosteric level of control of functional selectivity is now becoming appreciated, and it is thought that the sodium ion allosterically alters the binding pocket to dampen G protein signaling, leaving β-arrestin recruitment intact. Therefore, the sodium ion may serve as a general mechanism for Class A GPCRs to keep the receptor in a low affinity state, and upon activation by agonists, the sodium pocket collapses leading to robust G protein signaling (Katritch et al., 2014).

In the 5-HT_{1B} and 5-HT_{2B} structures, the resolution is too low to observe the sodium ion, but alignment between the delta opioid receptor (DOR) containing the sodium ion and the

serotonin receptor structures reveals that the sodium pocket is structurally similar. The conserved aspartate in TM2 (D2.50) is present in a similar orientation (Figure 5D), as well as W6.48, located across the sodium site in TM6. Some striking differences include the conserved serine, S3.39, which is oriented closer to the location of the putative sodium ion in the serotonin structures, suggesting the sodium pocket may be contracted or possibly collapsed in both 5-HT structures, further supporting the notion that these structures are in an active state. Residues 3.35 (F138^{3.35} in the 5-HT_{2B} and C132^{3.35} in the 5-HT_{1B} receptors) are oriented away from the putative sodium ion and likely provide very little coordination. In contrast, the analogous residue N131^{3.35} in the DOR was found to mediate high β -arrestin constitutive activity (Fenalti et al., 2014), which suggests the electronic identity and orientation of this residue may impart a bottom to the sodium site preventing the escape of sodium, thus balancing the basal state of G protein versus arrestin activity. In addition, the 5-HT_{2B} receptor residue S373^{7.45} located one turn above the NPxxY motif in TM7 is oriented much closer to the position of the putative sodium ion, which is in contrast to N360^{7.45} in the 5-HT_{1B} receptor and N310^{7.45} in the DOR. The close orientation of S373^{7.45} in the 5-HT_{2B} structure into the sodium site suggests that the sodium pocket is indeed collapsed. Mutation of this residue imparted an efficacy switch to β -arrestin signaling in the DOR (Fenalti et al., 2014), and the close orientation of S373^{7.45} to the sodium pocket further supports the finding that the 5-HT_{2B} structure is in a β -arrestin biased state. However, more studies are needed to elucidate fully the effects of sodium on serotonin receptor signaling.

3.3.5 Comparison to the muscarinic M2 allosteric site—Recent crystallographic evidence for the allosteric site in GPCRs has indicated that the extracellular portions of the muscarinic M2 receptor form the binding site for positive allosteric modulator, LY2119620 (Kruse et al., 2013). In the M2 crystal structure (PDB 4MQS), the orthosteric agonist, iperovo, roughly occupies the same site as the indole nucleus of ERG in the 5-HT_{2B} receptor. Most strikingly, however, the allosteric site that LY2119620 occupies is the same extracellular region as the tripeptide portion of ERG (Figure 6). These similar poses in both the M2 and 5-HT_{2B} receptors suggest that the location of the extracellular allosteric site for Class A GPCRs is quite similar, and in fact, argue that ERG likely functions as a bitopic ligand; that is it occupies both the orthosteric and putative extracellular allosteric site in the serotonin receptor. These similar binding poses open a potential therapeutic avenue for drug development of novel serotonin receptor allosteric modulators.

4. Conclusions and future directions

Crystal structures provide a rather static template for the design of novel drugs aimed at selectivity among the serotonin receptors. Currently, the 5-HT_{1B} and 5-HT_{2B} crystal structures have guided the development of novel compounds selective for the 5-HT_{1B} receptor over the 5-HT_{2B} receptor (Rodriguez et al., 2014). In fact, with respect to the design of functionally selective ligands, efforts are already underway to understand and exploit the extended binding site with molecular modeling, in particular, the dopamine D₃ receptor (Lane et al., 2013).

One of the greatest challenges facing the design of functionally selective drugs is the lack of structural information available for each receptor target. With the recent renaissance in GPCR crystallography, however, the molecular mechanisms of GPCR activation are now becoming clearer. Examination of GPCR crystal structures in complex with a variety of ligands (from full/partial agonists, inverse/silent antagonists) and effectors (e.g. G proteins, arrestins, etc.) provide medicinal chemists and molecular pharmacologists with an opportunity to design more efficacious drug treatments at their respective receptor targets. More importantly, this review focuses on the serotonin 5-HT_{2B} and 5-HT_{1B} receptor crystal structures in complex with ergotamine (Wacker et al., 2013), as offering a more focused approach to design functionally selective compounds with ligand bias.

The process by which GPCRs activate one pathway over another, however, is still rather complex. Using the β 2 adrenergic receptor as a prototypical system, progress into overall receptor mechanics is still being investigated in regard to ligand sampling of various conformational states observed upon agonist versus inverse agonist activated receptors (Nygaard et al., 2013). However, our insights into the molecular mechanics of receptor activation are still rather limited, because crystal structures provide only a “snapshot” of the receptor’s lowest energy state (Katritch et al., 2013). Currently, crystal structures of Class A GPCRs reveal that several conformations exist that correlate with in vitro pharmacology profile of the complexed ligand. These additional receptor states or conformations are now being equated with biased states of the receptor as detected by NMR in the β 2 adrenergic receptor (Nygaard et al., 2013). Therefore, the plethora of such “active” receptor states seems immense and possibly exploitable by understanding the structure-activity-relationships (SAR) of druggable ligands that give rise to such states. In the future, advanced structural biology methods including NMR (Ding et al., 2013) and X-ray free electron laser (XFEL) diffraction (Deupi, 2013) will likely lead to a greater understanding into the dynamics of receptor activation. In fact, the structure of the 5-HT_{2B} receptor has been recently solved using serial femtosecond XFEL diffraction, and shows remarkable similarity to the crystal structure obtained by X-ray diffraction, with the notable exceptions of the intracellular and extracellular loops (Liu et al. 2013). Hopefully, future structures using XFEL diffraction will resolve conformational states of the receptor in a biased state, and possibly identify new conformations not yet observed with X-ray crystallography.

In summary, the crystal structure of the serotonin 5-HT_{2B} receptor in complex with ergotamine, a β -arrestin biased ligand, provides SAR clues to the molecular determinants of functionally selective biased ligands (Wacker et al., 2013). The 5-HT_{2B} crystal structure reveals an intermediate state of activation stabilized by the extracellular-facing tripeptide portion of ergotamine, which likely drives β -arrestin bias and is not present on unbiased ligands such as serotonin itself. This tripeptide portion interacts with the extracellular end of TM6 and the C-terminal portion of EL2 (Figure 4), forming the extended binding site of the receptor, as previously discussed. Also previously discussed is that the extended binding site overlaps with the allosteric site of the M2 muscarinic receptor (Figure 6), implicating ergotamine is a bitopic ligand. That suggests that the tripeptide portion of the ergotamine can serve as starting point for the design of not only functionally selective ligands, but also functionally selective allosteric ligands, which are potentially capable of superior therapeutic

utility. Overall, these crystal structures provide much needed insight into novel therapeutics incorporating biased signaling for a number of serotonin-related diseases.

Methods used for generation of GPCR alignments

Figures utilized the 5-HT_{1B} and 5-HT_{2B} crystal structures with ERG bound (Wang et al. 2013; Wacker et al. 2013), and also the muscarinic M2 receptor crystal structure with iperovo and LY2119620 bound (Kruse et al. 2013). All structures were downloaded from the Protein Data Bank (5-HT_{1B} PDB code 4IAR; 5-HT_{2B} PDB code 4IB4; M2 PDB code 4MQT). Using only the GPCR and complexed ligand, structures were aligned using the PyMOL Molecular Graphics System, Version 1.1 Schrödinger, LLC.

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
GPCR	G protein coupled receptor
CNS	central nervous system
GI	gastrointestinal
IP₃	inositol triphosphate
DAG	diacylglycerol
GRK	G protein receptor kinase
LSD	lysergic acid diethylamide
ERG	ergotamine
DHE	dihydroergotamine
TM	transmembrane
EL	extracellular loop
IL	intracellular loop

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Figure 1. 5-HT receptor ligands

Shown are the structures of 5-hydroxytryptamine (5-HT or serotonin) and ergolines, lysergic acid diethylamide (LSD), ergotamine (ERG), and dihydroergotamine (DHE), which are ligands at all serotonin receptors. Also shown is an example triptan ligand, sumatriptan, which possesses 5-HT_{1B} receptor selectivity and also norfenfluramine, the active metabolite of fenfluramine, which possesses 5-HT_{2B} agonist activity.

Figure 2. ERG occupies both the orthosteric and extended binding sites in 5-HT_{1B} and 5-HT_{2B} receptors

Ergotamine (ERG) occupies two distinct sites in both the 5-HT_{1B} and 5-HT_{2B} receptors, the orthosteric site, where the indole nucleus of ERG resides, and the extended binding site, where the tripeptide portion is engaged.

Figure 3. Orthosteric sites of the 5-HT_{1B} and 5-HT_{2B} receptor crystal structures

Alignment of the 5-HT_{1B} (purple) and 5-HT_{2B} (blue) crystal structures reveals similar binding poses for ERG in the orthosteric binding pocket. A) Conserved aspartate (D3.32) forms a salt bridge with protonated N⁶ of ERG and is also coordinated with residue 3.36. D3.32 also forms a hydrogen bond with Y7.43. B) Conserved residues, T3.37 and A5.46, engage the indole N-H portion of ERG. C) Aromatic residues F6.51, F6.52 and W6.48 involved in an edge-to-face π - π stacking with the ring system of ERG. D) Polar residues 5.43 and 6.55 involved in an interhelical hydrogen bond between TM5 and TM6, which are

the putative residues for 5-hydroxy recognition of 5-HT. E) Summary of residue interactions with ERG in the orthosteric site.

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Figure 4. Extended binding sites of the 5-HT_{1B} and 5-HT_{2B} receptor crystal structures
 Alignment of the 5-HT_{1B} (purple) and 5-HT_{2B} (blue) crystal structures reveals similar yet strikingly different poses for ERG in the extended binding site of the binding pocket. A) Tripeptide portion of ERG forms similar interactions with nonpolar contacts in EL2 and residue 7.35 B) Rotamer state of tripeptide phenyl differs depending on presence of methionine at either 5.39 (5-HT_{2B}) or 6.58 (5-HT_{1B}) C) TM5 is pushed toward TM6 in 5-HT_{2B} receptor compared to the 5-HT_{1B} receptor, likely a result of the tripeptide phenyl rotamer state D) Summary of residue interactions with ERG in the extended binding site.

Figure 5. Microswitch trigger motifs of the 5-HT_{1B} and 5-HT_{2B} receptor crystal structures
 The 5-HT_{1B} (purple) and 5-HT_{2B} (blue) crystal structures show select differences in their trigger motifs. A) Ionic lock between Asp3.49 and Arg3.50 in the DRY motif is broken in the 5-HT_{1B} structure but intact in the 5-HT_{2B} structure, suggestive of an inactive state. B) In the NPxxY motif, movement of Tyr7.53 toward the helical bundle is more pronounced in the 5-HT_{2B} structure compared to the 5-HT_{1B} structure. C) Inward movement of Phe6.44 in the P-I-F trigger motif is only present in the 5-HT_{1B} structure. D) Sodium (purple) in the sodium site in the DOR (orange) aligned with 5-HT structures show a condensed or collapsed sodium pocket in both 5-HT structures.

Figure 6. Tripeptide portion of ERG occupies a site overlapping with the allosteric modulator, LY2119620, in the muscarinic M2 receptor

Alignment of the 5-HT_{2B} (blue) and muscarinic M2 (green; PDB 4MQS) receptor crystal structures show that M2 orthosteric agonist iperoxo (IPX) and the ergoline nucleus of ERG occupy the same site deep in the helical bundle. Similarly, the tripeptide portion of ERG and M2 allosteric modulator, LY2119620 (LY), also occupy the same site encompassing the extracellular portion of the receptor.