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► **To cite this version:**

Luc Maroteaux. Gene Structure, Expression, and 5-HT2B Receptor Signaling. 5-HT2B Receptors. The Receptors, vol 35., pp.1-32, 2021, 10.1007/978-3-030-55920-5_1 . hal-03217218

HAL Id: hal-03217218

<https://hal.science/hal-03217218>

Submitted on 5 May 2021

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Chapter 1

Gene structure, expression, and 5-HT_{2B} receptor signaling.

Luc Maroteaux¹

Abstract: Although the contractile effects of serotonin on stomach has been described since long time, the identification of the receptor mediating such a response was only obtained by molecular cloning in 1992. The pharmacological characterization of the 5-HT_{2B} receptor subtype in various species identified proximity but differences in its pharmacology, as compared to 5-HT_{2A} or 5-HT_{2C} receptors. Initially thought to be restricted in periphery, 5-HT_{2B} receptor expression was also detected in various cell types in the central nervous system. Although quite low at adult stage, a strong expression of 5-HT_{2B} receptors has been found early in embryos in migrating neural crest cells, neural tube, hematopoietic tissue, and heart primordia. In this chapter, we will develop and discuss the complexity of its signaling in relation with its expression, including in various types of cancer cells.

Keywords: Signaling pathways, embryogenesis, pharmacology, tumors, gene regulation.

Abbreviations: Adenine nucleotide translocator (ANT-1); Brain natriuretic peptide (BNP); Bioluminescence resonance energy transfer (BRET); Cyclooxygenase (COX); cAMP response element-binding protein (CREB); Dihydro-ergotamine (DHE); Ergotamine (ERG); Extracellular loop 2 (ECL2); Extracellular signal-regulated kinase 2 and 2 (ERK2/ERK1); G-protein coupled receptor (GPCR); G-protein coupled receptor kinase (GRK); Heparin-binding epidermal growth factor (HB-EGF); Hepatic stellate cell (HSC); Hepatocellular carcinoma (HCC); Histone deacetylase (HDAC); Ligand of Numb protein X (LNx); Lysergic acid diethylamide (LSD); Mammalian target of rapamycin (mTOR); Mitogen-activated protein kinase (MAPK); microphthalmia-associated transcription factor (MITF); NADPH oxidase (NOX); Negative allosteric modulator (NAM); Neural crest cell (NCC); Nitric oxide (NO); Nuclear factor-κB (NF-κB); Phosphatidylinositol-3 kinase (PI3K); Phospholipase A2 (PLA2); Phospholipase C (PLC); Platelet derived growth factor receptor (PDGFR); Positive allosteric modulator (PAM); Postsynaptic density protein of 95 kDa, disc large, zonula occludens-1 (PDZ); Prostaglandin (PGE2); Prostacyclin (PGI2); Protein kinase A (PKA); Pulmonary arterial hypertension (PAH); Retinoic acid (RA); Serotonin transporter (SERT); Tissue-nonspecific alkaline phosphatase (TNAP); Toll-like receptor (TLR); Transforming growth factor (TGF); Tryptophan hydroxylase (TPH).

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1 5-HT_{2B} receptor gene expression and regulation

Because of its exquisite sensitivity to serotonin, the rat stomach fundus was used as a bioassay for serotonin concentration before the development of analytical assays for this biogenic amine (1). Although the potency for the contractile effects of serotonin has been known since 1957, the receptors mediating such a response eluded definitive characterization. Pharmacological attempt to characterize the contractile serotonergic receptor in the rat stomach fundus initially documented its similarity to the 5-HT_{2C} receptor. In the absence of detectable 5-HT_{2C} receptor mRNA in the rat stomach fundus, only molecular cloning allowed the identification of a new receptor in 1992 in rat and mouse (2-6) and in 1994 in humans (7-10), now called 5-HT_{2B} receptor. That's only in 1994 that the group of T. Blackburn concluded about "the close pharmacological identity of 5-HT receptors in rat stomach fundus and the recently cloned 5-HT_{2B} receptor" (11). Subsequent pharmacological characterization of this receptor subtype in various species identified differences in its pharmacology, and confirmed the close identity of this receptor to 5-HT_{2C} receptors. Its developmental, physiological, and pathophysiological functions include many differentiation steps both in periphery and central nervous system that were not previously identified or attributed to other receptor subtypes.

1.1 5-HT_{2B} receptor expression

By Northern blot and RT-PCR, the 5-HT_{2B} receptor mRNA expression was detected in the stomach, in rats (2, 4) but also in liver, kidney, pancreas, spleen and lung, as well as in the brain of mice (5, 12) and later in several species including rats (13), and humans (4, 7-9, 12, 14). In the human brain, 5-HT_{2B} receptor expression has been reported in cerebral cortex, cerebellar nuclei and their projection areas, lateral septum, dorsal hypothalamus and medial amygdala. Expression of serotonin 5-HT_{2B} receptor mRNA was also confirmed in several brain nuclei including the dorsal raphe nuclei by gene expression profiling in the rat brain, and by *in-situ* hybridization (15). Human brain expression was confirmed by RT-qPCR in frontal, temporal, parietal, and occipital lobe, olfactory region, cerebellum, diencephalon, hippocampus, thalamus, pituitary gland, pons, medulla oblongata and nucleus accumbens (16).

The mRNA and protein expression of 5-HT_{2B} receptors has been found in mouse embryos in migrating neural crest cells (NCC), neural tube, hematopoietic tissue, and heart primordia by immunohistochemistry and *in situ* hybridization (17), for review see (18). The 5-HT_{2B} receptor mRNA was detected in rat embryos since 8.0 days post-coitum and confirmed by pharmacological assays (17, 19). In *Xenopus*, 5-HT_{2B} receptors have also been shown to modulate, in a cell-autonomous manner, postmigratory NCC without altering early steps of cranial NCC development and migration (20). In the zebrafish, the 5-HT_{2B} receptor was also

found expressed in the pharyngeal arches and in the development of NCC-derived tissues (21) (see also chapter 4).

1.2 Genomic organization and control of the 5-HT_{2B} receptor gene *HTR2B*.

In humans, the *HTR2B* gene is located on the reverse strand at the tip of the second chromosome at 2q37.1 (22). The 5-HT_{2B} receptor's cDNA, which is 2,246 base pairs (bp) long, is encoded by four exons, three of which are coding, and produces a pre-mRNA transcript of approximately 51 Kbp before splicing (7-10). Interestingly, the *PSMD1* gene, which encodes the non-ATP regulatory subunit 1 (RPN2) of the 26S proteasome, is located at the same locus on the forward strand and overlaps *HTR2B* gene, which is entirely encoded within intron 16 as revealed in the Ensembl database (23) (**Fig. 1.1**). Similar organization with the 5-HT_{2B} receptor's cDNA encoded in the reverse strand of a large intron of the proteasome *PSMD1* gene has been found not only in humans, rats, and mice but also in all mammals and vertebrates, for review see (18). The 26S proteasome is a highly conserved multicatalytic protease from yeast to mammals, which functions to degrade proteins following ubiquitination *via* the ubiquitin-proteasome system. The 26S proteasome contains many distinct subunits and is predominantly composed of two large structures (24); the 19S complex (regulatory particle), within which RPN2 is found, functions to recognize ubiquitinated proteins (25); it then processes and transports them to the 20S complex (core particle), where proteolysis occurs.

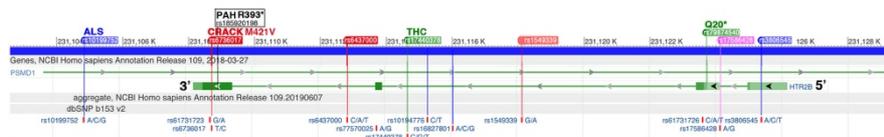


Figure 1.1. *HTR2B* gene organization on the reverse strand of *PSMD1* intron 16 with SNPs position. This is a schematic representation of the *HTR2B* gene on the reverse strand of *PSMD1* locus with the location of different SNPs, including SNPs previously associated with disorders: THC: cannabis-induced aggression (26); Q20*: stop codon in exon 2, impulsivity (16); ALS: amyotrophic lateral sclerosis (27), Crack addicts (M421V) (28), and pulmonary arterial hypertension (PAH) R393* (29), adapted from (28).

Transcription of the *HTR2B* gene is under the regulatory influences of many transcription factors. Recent publication identified transcription factor binding sites in *HTR2B* gene promoter (30), including GATA protein 1 and 2 (GATA-1 and GATA-2), forkhead box A2 (HNF-3B), SRY-box 5 (SOX-5), runt related transcription factor 2 (RUNX2), MYB proto-oncogene transcription factor (c-Myb), RUNX1, nuclear factor IA (NFIA), some of the CCAAT/enhancer binding protein (C/EBP), signal transducer and activator of transcription (STAT) family members,

and the activator protein-1 (AP-1). Both the transcription factors nuclear factor 1 (NF1) and Runt-related transcription factor I (RUNX1) interact with regulatory elements from the *HTR2B* gene to either activate (NF1) or repress (RUNX1) *HTR2B* expression in uveal melanoma cells (30).

Similarities between the phenotypes of 5-HT₂ antagonists- and retinoic acid (RA)-treated embryos, as well as AP-2 knockout mice, have led to speculation about a possible reciprocal relationship between RA and 5-HT_{2B} receptor signaling during embryogenesis (19). However, it was found that there was no negative regulation of 5-HT_{2B} receptor by RA, as previously considered (31). Inhibitory effects of RA on chondrogenic differentiation in hindlimb cultures appeared not mediated by negative transcriptional regulation of the 5-HT_{2B} receptor, but by increased expression of RAR β , and decreased activation of p38 mitogen-activated protein kinase (MAPK). On the contrary, stimulatory effects of 5-HT_{2B} receptor activation on chondrogenic differentiation appeared to be mediated by activation of the p42/44 MAPK pathway. Therefore, RA and 5-HT may exert opposing effects on chondrogenesis in the developing hindlimb by using different MAPK pathways (31). Liu *et al.*, (32) described putative interactions between peroxisome proliferator-activated receptor (PPAR γ) and 5-HT_{2B} receptor expression in pulmonary arterial hypertension (PAH). However, the relation between PPAR γ and 5-HT_{2B} receptors regulated expression remains controversial and further research to determine if the *HTR2B* is a direct target of PPAR γ action on the vascular contraction and remodeling during PAH (33).

In conclusion, both the particular *HTR2B* gene organization that may have evolutionary consequences, and the complex transcriptional control needs further studies to fully understand its regulated expression.

2 Structure and pharmacological impact

2.1 5-HT_{2B} receptor structure

The 5-HT_{2B} receptor is a G-protein coupled receptor (GPCR), with a N-terminus of about 55 amino-acids. In this region, a weak consensus site of N-glycosylation can be found, which is missing in the rat 5-HT_{2B} receptor, questioning the N-glycosylation of the N-Terminus of this receptor. The receptor consists in 481 amino acids in human and 479 amino acids in rat or mouse, with 79% homology for human *vs.* rat and 82% homology for human *vs.* mouse. A new and unanticipated role of the 5-HT_{2B} receptor N-terminus as a negative modulator, affecting both constitutive and agonist-stimulated activity of the receptor has been evidenced (34). The available 5-HT_{2B} receptor bound to ergotamine (ERG) crystal structure showed that it exhibits conformational characteristics of both the active and inactive states: an active-like state in the helix VII conformation of the 5-HT_{2B} receptor, but only partial changes in helix VI, mirrored the strong β -arrestin bias of ERG at 5-HT_{2B} receptors observed in pharmacological assays.

The differential signaling patterns were also mirrored in the crystal structures, which showed features of a β -arrestin-biased activation state for the 5-HT_{2B} receptor (35, 36). A likely structural explanation for the distinct conformational features and biased pharmacology of ERG for 5-HT_{2B} receptors can be found in the region of the extracellular loop 2 (ECL2) junction with helix V, E212-R213-F214 forming an additional helical turn stabilized by a structured water molecule at the extracellular tip of helix V. The segment of ECL2 connecting helices III and V *via* the conserved disulfide bond is, therefore, shortened in the 5-HT_{2B} receptor, and creates a conformational constraint on the position of the extracellular tip of helix V (37). However, this structured water molecule involved in ECL2 junction with helix V has been challenged since differential interactions of ERG with the top of helices V and VI could determine the rotational freedom of helix VI (38) (See also chapter 2).

2.2 Selective agonists

There is virtually no highly selective agonist for a particular 5-HT₂ receptor: BW723C86: 1-methyl-2- [5-(2-thienylmethoxy)-1H-indole-3-yl] ethylamine hydrochloride, has been reported to have 10-fold selectivity over the human 5-HT_{2C} and 100-fold selectivity over the 5-HT_{2A} receptors (39-42). Nor-dexfenfluramine (metabolite of dexfenfluramine), methylergonovine (metabolite of methysergide), and Ro 60-0175: 2(S)-1-(6-chloro-5-fluoro-1H-indol-1-yl)-2-propanamine fumarate are all preferential 5-HT_{2B} agonists with about 10-fold selectivity over other 5-HT₂ receptor (43). 2,5-dimethoxy-4-iodoamphetamine (DOI), MDA (3,4-methylene dioxyamphetamine-MDA, metabolite of 3,4-Methylenedioxy methamphetamine-MDMA) (44), tryptamine, lysergic acid diethylamide (LSD), and alpha-methyl-5-HT are non-selective nearly full agonists at 5-HT₂ receptors with similar affinity to 5-HT_{2A} 5-HT_{2B} and 5-HT_{2C} receptors (39-42). Many substances from the class of "new" drugs known as "legal highs" were also found to display notable affinity for 5-HT_{2B} receptors, including 5-APB (K_i = 14 nM) and 6-APB (K_i = 3.7 nM), and 5-iodo-aminoindane (K_i = 70 nM). Functional assays of 5-APB and 6-APB confirmed that these compounds acted as potent (*i.e.*, nanomolar EC₅₀ values) full agonists at 5-HT_{2B} receptors (45, 46). 5-APB, commonly marketed as 'benzofury' a new psychoactive substance was shown to cause contraction of rat stomach fundus, which was reversed by the 5-HT_{2B} receptor antagonist RS127445 (47). This finding is potentially important because previous studies have shown that there was a correlation in a series of phenylisopropylamines between hallucinogenic activity and affinity for the 5-HT_{2B} receptor (48) (see also chapter 3 and Table 1).

		h5-HT2A	h5-HT2B	m5-HT2B	h5-HT2C	m5-HT2C
		pKi	pKi	pKi	pKi	pKi
BW723C86	(49)		7.89±0.01	8.04±0.15	6.90±0.01	6.78±0.05
	(41) h2CINI	7.2±0.08	7.33±0.03		7.11±0.21	
	(39) h2CVSV	6.63±0.06	7.85±0.11		7.11±0.01	
RO600175	(49) h2CINI		9.01±0.13	8.64±0.14	7.72±0.22	7.35±0.29
	(41) h2CINI	7.44±0.04	8.27±0.06		8.22±0.29	
	(39) h2CVSV	6.80±0.08	8.66±0.13		7.67±0.07	
WAY161503	(49) h2CINI		7.28±0.19	7.84±0.12	7.46±0.05	6.92±0.11
	(50) 2006	7.74±0.11	7.22±0.03		8.48±0.14	
CP809101	(51)	8.22±0.15	7.19±0.25		8.80±0.11	
	(49) h2CINI		7.86±0.18	8.41±0.18	8.35±0.02	7.72±0.15
DOI	(49) h2CINI		8.29±0.18	7.87±0.06	7.60±0.02	7.41±0.24
	(41) h2CINI	9.02±0.11	7.55±0.05		8.08±0.11	
	(39) h2CVSV	8.04±0.05	7.78±0.09		7.73±0.04	
Norfenfluramine	(49) h2CINI		8.02±0.19	6.76±0.23	7.09±0.62	6.21±0.07
	(41) h2CINI	6.82±0.29	7.00±0.06		7.29±0.04	
D-LSD	(41) h2CINI	9.12±0.06	9.01±0.09		8.96±0.06	
	(39) h2CVSV	9.49±0.03	9.22±0.02		8.52±0.06	
Lorcaserine	(52)	6.95±0.03	6.76±0.09		7.82±0.03	
clozapine	(49) h2CINI			7.97±0.09		
	(41) h2CINI	7.60±0.08	7.99±0.09		7.87±0.05	
	(53)	8.39±0.03	8.79±0.09		8.56±0.06	
aripiprazole	(49) h2CINI			7.21±0.09		
	(54) h2CINI	8.06±0.10	9.44±0.16		7.12±0.09	
	(53)	8.02±0.16	9.59±0.17			
RS1022221	(49) h2CINI		6.47±0.02	6.52±0.08	8.01±0.30	7.72±0.22
	(41) h2CINI	5.54±0.03	5.95±0.06		8.30±0.05	
	(43) h2CVSV		6.63±0.05		8.83±0.04	
SB215505	(49) h2CINI		8.12±0.01	7.61±0.21	7.40±0.02	7.24±0.26
	(41) h2CINI					
	(43) h2CVSV		8.83±0.09		7.95±0.06	
SB206553	(49) h2CINI		8.29±0.04	7.06±0.41	8.24±0.01	8.21±0.24
	(41) h2CINI	5.64±0.09	7.65±0.07		7.79±0.07	
	(43) h2CVSV		8.26±0.17		8.50±0.13	
SB242084	(49) h2CINI		6.36±0.02	6.07±0.01	8.19±0.22	5.93±0.27
	(41) h2CINI	6.07±0.18	6.84±0.28		8.15±0.10	
	(43) h2CVSV		7.34±0.07		9.32±0.06	
Mesulergine	(49) h2CINI		8.39±0.2	7.81±0.15	9.01±0.01	8.53±0.21
	(41) h2CINI	7.34±0.03	8.46±0.05		8.74±0.03	
	(43) h2CVSV		8.71±0.02		8.95±0.06	
RS127445	(49) h2CINI		8.51±0.07	8.22±0.24	5.63±0.05	5.33±0.45
	(41) h2CINI	6.03±0.13	8.97±0.09		6.33±0.10	
MDL100907	(49) h2CINI		5.79±0.60	5.03±0.23	6.79±0.51	6.64±0.17
	(41) h2CINI	8.73±0.20	5.99±0.06		7.52±0.13	
SB204741	(41) h2CINI	<5.00	6.90±0.27		5.56±0.07	
	(43) h2CVSV		7.29±0.04		5.67±0.11	
Sarpogrelate	(55)	8.52±0.12	6.57±0.12		7.43±0.03	
Ketanserin	(55)	9.67±0.12	6.55±0.09		7.39±0.11	

Table 1 : Pharmacological properties of human and mouse 5-HT_{2B} receptors Reprinted from (56).

2.3 Selective antagonists

A few selective antagonists are available for the 5-HT_{2B} receptor subtype. The first highly selective 5-HT_{2B} receptor antagonist reported was LY266097: 1-(2-chloro-3,4-dimethoxybenzyl)-6-methyl-1,2,3,4-tetrahydro-9Hpyrido [3,4-b]indole hydrochloride with a pK_i of 9.7 for the human cloned 5-HT_{2B} receptor and a 100-fold greater selectivity over human 5-HT_{2C} and 5-HT_{2A} sites (57). SB204741: N-(1-methyl-5-indolyl)-N'-(3-methyl-5-isothiazolyl)urea has been reported as a selective 5-HT_{2B} receptor antagonist with approximately 100-fold selectivity over the 5-HT_{2C} and 5-HT_{2A} receptors but with a lower potency (K_i around 100 nM) (14). The tetrahydro-β-carboline, LY272015 [6-chloro-5-methyl-N-(5-quinolinyl)-2,3-dihydro-1H-indole-1-carboxamide] is also a fairly selective and potent antagonist (58). RS127445 [2-amino-4-(4-fluoronaphth-1-yl)-6-isopropyl pyrimidine] was found to have sub-nanomolar affinity for the 5-HT_{2B} receptor (pK_i = 9.5) and 1,000 fold selectivity for this receptor as compared to numerous other receptor and ion channel binding sites and appears as the most selective, high affinity 5-HT_{2B} receptor antagonist available (59). SB215505 [6-chloro-5-methyl-N-(5-quinolinyl)-2,3-dihydro-1H-indole-1-carboxamide] behaves as a high affinity and preferential inverse agonist at 5-HT_{2B} receptors (60). SB206553 [5-methyl-N-(3-pyridyl)-1,2,3,5-tetrahydrobenzo[1,2-b:4,5-b']dipyrrole-1-carboxamide] is a mixed 5-HT_{2C}/5-HT_{2B} receptor antagonist. It has been reported as a selective 5-HT_{2C}/5-HT_{2B} receptor inverse agonist with 50- to 100-fold lower affinity for 5-HT_{2A} and other sites (41, 61).

Non-selective 5-HT₂ receptor antagonists such as ritanserin and mesulergine block 5-HT₂ receptor-mediated effects. Atypical antipsychotics including clozapine, asenapine, or cariprazine also have fairly high antagonistic affinity for all 5-HT₂ receptors (10, 54, 62, 63). Aripiprazole (OPC-14597) is a novel atypical antipsychotic drug, which has higher antagonist affinity (EC₅₀ = 11 nM) for the human 5-HT_{2B} receptor than for 5-HT_{2A} or 5-HT_{2C} receptors (53), (see Table 1).

Hertz and coworkers (64) proposed the possibility that fluoxetine and other SSRIs act as direct 5-HT_{2B} receptor agonists independently of the serotonin transporter (SERT), based on their work on astrocytes. However, the absence of antidepressant effects of fluoxetine in mice lacking the 5-HT_{2B} receptor (*Htr2b*^{-/-}), knockout for the SERT (*Sert*^{-/-}), or lacking most of differentiated serotonin neurons knockout for *Pet1* (*Pet1*^{-/-}) (65) (i) rules out that the antidepressant effects of fluoxetine could be independent of SERT; (ii) indicates that serotonin neurons expressing SERT (and 5-HT_{2B} receptors) are necessary for the 5-HT_{2B} receptor effects independently of other cell types; (iii) rules out the possibility that SSRIs mediate antidepressant effects only by stimulating directly putative astrocytic 5-HT_{2B} receptors, which should be intact in these mutant mice (*Sert*^{-/-} and *Pet1*^{-/-}). It is clear from experiments in mice that acute or chronic effects of SSRIs cannot be due to direct 5-HT_{2B} receptor stimulation independently of SERT and serotonergic neurons (65). Furthermore, pharmacological determination in mice is in accordance with affinity of SSRIs for human 5-HT_{2B} receptors with K_i values over 5 μM (65)

and with no agonist activity (**Fig. 1.2**), while SSRI K_i values for SERT are in nanomolar range (66).

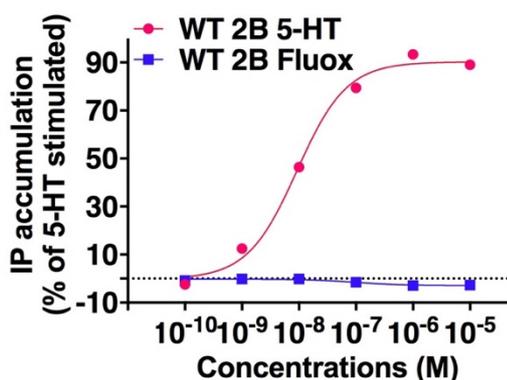


Figure 1.2. Inositol phosphate production by 5-HT_{2B} receptors transiently expressed in Cos-7 cells. The quantification of inositol phosphate (IP) signaling pathway activity was performed in COS-7 cells transfected with 5-HT_{2B} receptor and exposed to increasing concentrations of a full agonist of the receptor (5-HT) or of fluoxetine. If serotonin fully stimulated the inositol phosphate production by these cells with an EC₅₀ of 9 nM, fluoxetine reduced basal levels of inositol phosphate, behaving as inverse agonist.

2.4 Allosteric modulators

Positive allosteric modulators (PAMs) represent alternative approaches to orthosteric agonists (i.e., compounds that interact with the native ligand-binding site). PAMs can increase the affinity and/or efficacy of the orthosteric agonist for its target receptor by acting at a site other than the native ligand-binding site (allosteric). Importantly, so-called pure GPCR PAMs, which lack intrinsic agonist activity within a specific signaling pathway, have been described. These compounds modulate the basal tone of the endogenous ligand in a manner that conserves spatial and temporal elements of native neurotransmission (67). Indeed, multiple PAMs have been identified for GPCRs and may circumvent the challenges of orthosteric agonists: (1) PAMs would amplify endogenous signaling through the 5-HT₂ receptors, likely resulting in a more physiologically relevant enhancement of function compared to a direct orthosteric agonist; (2) because of a generally higher sequence divergence in allosteric sites relative to the conserved orthosteric domain, PAMs could potentially achieve higher receptor selectivity than orthosteric agonists. ERG has been shown to occupy two distinct sites in 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. The allosteric site in the muscarinic M₂ receptor is the same extracellular region as that interacting with the tripeptide portion of ERG. These similarities 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 5-HT_{2B} receptor. It is now thought that a sodium ion allosterically alters the binding pocket to dampen G-protein signaling, leaving β -arrestin recruitment intact. Recent structural consideration support that this sodium pocket is collapsed in the 5-HT_{2B} receptor

structure (68). Recently, imidazole linked phenyl cyclopropyl methanones were shown to display PAM activity on both 5-HT_{2C} and 5-HT_{2B} receptors. Furthermore, piperazine linked phenyl cyclopropyl methanones were active as PAM at 5-HT_{2C} (increased the E_{max} of serotonin), and as negative allosteric modulator (NAM) at 5-HT_{2B} receptors (decreases EC₅₀ of serotonin 10 times without affecting E_{max}) (69). The identification of specific PAMs at 5-HT_{2B} receptors may conceivably lead to improved therapeutics.

2.5 Biased agonists

Another area for 5-HT₂ receptors agonist development might emerge from compounds so-called biased agonists sharing a functional selectivity for specific intracellular signaling pathways (70). 5-HT₂ receptors couple to multiple intracellular pathways. LSD and ERG displayed bias for β -arrestin signaling at 5-HT_{2B} receptors, as well as other ergolines such as dihydro-ergotamine (DHE), methylergonovine, pergolide, and cabergoline. ERG and dihydro-ergotamine displayed more extreme signaling bias at the 5-HT_{2B} receptor compared to LSD (35). Furthermore, structural studies of the human 5-HT_{2B} receptor in complex with methysergide, methylergonovine, lisuride or LY266097 illuminated key structural determinants essential for activation and revealed binding pocket residues that are essential for agonist-mediated biased signaling and β -arrestin2 translocation. LSD presents a slow binding kinetics may be due to a "lid" formed by ECL2 at the entrance to the binding pocket. Furthermore, the structure of 5-HT_{2B} receptors captured in an active-like state revealed the mechanism of selectivity in extracellular recognition of GPCRs by monoclonal antibodies (71-74) (See also chapter 2).

Currently, it is unknown whether this functional selectivity could be translated into any therapeutic gain, although this does open up an interesting opportunity for future drug discovery. Further work on the structure function relationships is required to fully understand the 5-HT_{2B} receptor pharmacological complexity.

3 5-HT_{2B} receptor heteromeric receptor associations

3.1 Heterodimers of Gq-coupled protomers: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors

Many members of the GPCR family have the capacity to form homo- or hetero-oligomers with biochemical and functional characteristics, including receptor pharmacology, signaling, and regulation, which are unique to these oligomeric conformations (75). In the 5-HT₂ receptor subfamily, 5-HT_{2A} and 5-HT_{2C} receptors

have been shown to be able to form homodimers (76-79), whereas 5-HT_{2B} receptors overexpressed in COS-7 cells are not (79). However, when co-expressed in heterologous expression systems, saturating bioluminescence resonance energy transfer (BRET) experiments indicated that the formation of heterodimers is favored over homodimerization (79). Signaling from these heterodimers is exclusively driven by the 5-HT_{2C} protomer. Indeed, in 5-HT_{2C}-containing 5-HT_{2A}-5-HT_{2C} and 5-HT_{2B}-5-HT_{2C} heterodimers, the binding of ligands selective for the 5-HT_{2A} or 5-HT_{2B} protomers is eliminated despite normal surface expression of these receptor subtypes. Concomitantly, 5-HT_{2A} or 5-HT_{2B} selective antagonists are unable to block signaling in the presence of the 5-HT_{2C} protomer, whereas antagonists of the 5-HT_{2C} protomer are totally inhibiting signaling in 5-HT_{2A}-5-HT_{2C} and 5-HT_{2B}-5-HT_{2C} heterodimers (79). By contrast, signaling in 5-HT_{2A}-5-HT_{2B} heterodimers could be blocked either by 5-HT_{2A} or 5-HT_{2B} selective antagonists.

To further investigate this issue, the 5-HT_{2C} receptor was deleted for its C-terminal tail (5-HT_{2C}ΔCter), still able to bind serotonin but unable to activate Gq and to generate inositol phosphate production. Co-expression of 5-HT_{2C}ΔCter with 5-HT_{2A} or 5-HT_{2B} protomers abolished serotonin-dependent inositol phosphate accumulation by 5-HT_{2A}-5-HT_{2C}ΔCter and 5-HT_{2B}-5-HT_{2C}ΔCter heterodimers, despite their retained dimerization ability (79). Conversely, co-expression of 5-HT_{2C} with 5-HT_{2B}ΔCter, a 5-HT_{2B} receptor impaired for Gq activation, had no impact on 5-HT_{2C} signaling in 5-HT_{2B}ΔCter-5-HT_{2C} dimers since inositol phosphate production in response to serotonin could still be detected and abolished by a 5-HT_{2C} receptor-selective antagonist (79, 80). This coupling seems related to a dominant negative effect of the 5-HT_{2C} protomer on ligand binding and coupling ability of the other partner. A dominant negative effect of the 5-HT_{2C} protomer over the 5-HT_{2A} protomer (and potentially 5-HT_{2B}) was also observed *in-vivo* that pinpointed the physiological relevance of a putative switch in the pharmacological profile of 5-HT_{2A} receptor expressing neurons, depending on the 5-HT_{2C} receptor co-expression levels (79).

The study of association between 5-HT_{2A}-5-HT_{2C} or 5-HT_{2B}-5-HT_{2C} receptors revealed the asymmetry in Gq-protein coupling, and signaling from 5-HT_{2A} and 5-HT_{2B} protomers.

3.2 Heterodimers among Gq-activating protomers: AT1-5-HT_{2B} heterodimers

In *ex-vivo* primary cultures of cardiac fibroblasts, endogenously expressed AT1 receptors for angiotensin II and 5-HT_{2B} receptors shared common Gq-protein-dependent signaling pathways leading to release of cytokines, which triggers cardiac hypertrophy (81). Through metalloproteinases activation, responsible for Heparin-binding EGF-like growth factor (HB-EGF) shedding, a subsequent EGF-receptor transactivation is induced by either angiotensin II or serotonin. These findings support that AT-1 and 5-HT_{2B} receptors share common EGF-receptor-dependent

signaling pathways leading to cytokine release. Blockade of one of the two receptors prevents cytokine release induced by stimulation of the other receptor at a dose that is inactive to the other receptor in endogeneously expressing cardiac fibroblasts or in COS7 transfected cells, supporting transinhibition between 5-HT_{2B} and AT-1 receptors (81). Confocal microscopy to assess colocalization and a pull-down assay in cotransfected COS7 cells demonstrated the interaction of 5-HT_{2B} and AT-1 receptors and their organization in heterodimeric complexes (81). Signaling of each protomer is not modified by the heterodimerization but inhibiting one protomer is sufficient to block the Gq activation by the second protomer, supporting the presence of a single active G-protein per heterodimer. A symmetrical Gq coupling between Angiotensin II and serotonin signal has thus been found in respect to coupling to hypertrophic cytokine release in adult cardiac fibroblasts, with transinhibition and transactivation properties (see also chapter 17).

The propensity of 5-HT_{2B} receptors to form heterodimers may be a critical property that needs to be taken into account in further physiological studies. The dominance of a protomer over the others may have strong consequences in interpreting pharmacological approaches.

4 5-HT_{2B} receptor internalization

In transfected cells, 5-HT_{2B} receptors were found to exhibit high degree of desensitization, with prior exposure to serotonin reducing subsequent response to serotonin with an extremely rapid time-course ($t_{1/2} = 5$ min) (82). Internalization of 5-HT_{2B} receptors was found caveolin1-independent and clathrin- G-protein coupled receptor kinase (GRK)2,3- and β -arrestin2-dependent, while that of 5-HT_{1B} receptors was clathrin-independent and caveolin1-dependent (83). Upon co-expression of these two receptors, serotonin-induced 5-HT_{2B} receptor internalization became partially caveolin1-dependent, and serotonin-induced 5-HT_{1B} receptor internalization became caveolin1-independent in a protein kinase C ϵ -dependent fashion. Serotonin-induced internalization of 5-HT_{2B} receptors was accelerated five-fold, and insensitive to a 5-HT_{2B} receptor antagonist. In this context, 5-HT_{2B} receptors did internalize in response to a 5-HT_{1B} receptor agonist. In contrast, co-expression did not render 5-HT_{1B} receptor internalization sensitive to a 5-HT_{2B} receptor agonist. The altered internalization kinetics of both receptors upon co-expression was not due to direct receptor interaction as no co-localization could be detected (83). The crystal structure of the serotonin 5-HT_{2B} receptor in complex with ERG, which was identified as a highly β -arrestin biased ligand for the 5-HT_{2B} receptor (84), provides clues to the molecular determinants of functionally selective biased ligands. The 5-HT_{2B} receptor crystal structure reveals an intermediate state of activation stabilized by the extracellular-facing tripeptide portion of ERG, which likely drives β -arrestin bias and is not present on unbiased ligands such as serotonin itself. The lack of C-terminal tail containing the

palmitoylation site in the R393X mutant 5-HT_{2B} receptor (85) was associated with a patient having developed PAH (**Figure 1.1**). This receptor displayed a loss of rapid internalization and a striking increase in proliferative capacity resulting from a switch from a dual G_{αq}/G_{α13} coupling in wildtype receptor to a nearly exclusive coupling to G_{α13} in the R393X 5-HT_{2B} receptor (29).

Again, more work on the structure function relationships is required to fully understand the 5-HT_{2B} receptor coupling and desensitization pathways.

5 5-HT_{2B} receptor interacting proteins

Proteins known to interact with 5-HT_{2B} receptors include constitutive and inducible nitric oxide (NO) synthase, Gα_q, Gα₁₁, and Gα₁₃, involved in signaling of the receptor. MUPP1 a multivalent postsynaptic density protein of 95 kDa, disc large, zonula occludens-1 (PDZ) scaffolding protein was shown to interact with the C-terminus of the 5-HT_{2C} receptor -SSV sequence. Moreover, 5-HT_{2A} and 5-HT_{2B} receptors sharing the C-terminal -E-X-V/I-S-X-V sequence with 5-HT_{2C} receptors also bind MUPP1-PDZ domains *in-vitro* (86). The PDZ motif at the C-terminus of the 5-HT_{2B} receptor was also found necessary for the recruitment of the constitutive NO synthase (87).

Ubiquitin ligases (E3s) confer specificity to ubiquitination by recognizing target substrates. The Ligand of Numb protein X (LNX) family of E3 ubiquitin ligases, is a group of PDZ domain-containing RING-type E3 ubiquitin ligases. The substrate recognition mechanism of LNX E3 ubiquitin ligases involves the recognition of substrates *via* their specific PDZ domains by binding to the C-termini of the target proteins. Guo *et al.* (88) showed that the C-terminal LNX1 PDZ3-binding motifs of the 5-HT_{2B} receptor promoted ubiquitination by LNX1ΔPDZ4. Another study on uveal melanoma cell lines (89), in which one of the most reliable predictive markers at risk of metastasis is an abnormally elevated level of expression of 5-HT_{2B} receptors, revealed important alterations in the expression of some of its transcripts and of those encoding E3 ubiquitin ligases and various subunits of the proteasome. These alterations also correlated with significant changes in the enzymatic activity of the proteasome and 5-HT_{2B} receptor turnover (89).

It appears, therefore, necessary to further investigate putative 5-HT_{2B} receptor interacting proteins and their impact on the receptor turnover *via* proteasome regulation.

6 5-HT_{2B} receptor transduction system(s)

6.1 In transfected cells

The 5-HT_{2B} receptor, when stably transfected in mouse fibroblast L-cells, has been shown to activate GTPase activity and inositol 1,4,5- triphosphate production upon agonist stimulation, which could be blocked by antibodies against Gα_{q/11}, but not by pertussis or cholera toxins or by anti-Gα_i or anti-Gα_s antibodies. This GTPase activation was thus mediated by the G-protein Gα_{q/11}, but not by Gα_s or Gα_i. The GTPase activation was also blocked by anti-β1-4, or -γ2 subunit antibodies. Agonist stimulation of the 5-HT_{2B} receptor caused a rapid and transient activation of the proto-oncogene product p21^{ras} in response to serotonin, as measured by an increase in GTP bound-Ras (90). Furthermore, 5-HT_{2B} receptor stimulation activated the MAPKs, extracellular signal-regulated kinase 2 and 2 (ERK2/ERK1). In addition to a mitogenic action, a transforming activity of serotonin was mediated by the 5-HT_{2B} receptor as it led to the formation of foci and to the formation of tumors from these foci in nude mice (90). Moreover, the 5-HT_{2B} receptor-dependent cell cycle progression occurred through retinoblastoma protein hyperphosphorylation and the activation of both cyclin D1/cdk4 and cyclin E/cdk2 kinases. The induction of cyclin D1 expression, but not that of cyclin E, was under MAPK control, indicating an independent regulation of these two cyclins in 5-HT_{2B} receptor mitogenesis. Platelet derived growth factor receptor (PDGFR) kinase activity was essential for 5-HT_{2B}-triggered MAPK/cyclin D1, but not cyclin E, signaling pathways. The 5-HT_{2B} receptor activation also increases activity of the SRC family kinases SRC, FYN and YES. Strikingly, SRC, but not FYN or YES, was the crucial molecule between the Gq protein-coupled 5-HT_{2B} receptor and the cell cycle regulators (91). Inhibition of SRC activity was sufficient to abolish the serotonin-induced: (i) PDGFR tyrosine kinase phosphorylation and MAPK activation; (ii) cyclin D1 and cyclin E expression levels; and (iii) thymidine incorporation. Thus, SRC activation by the 5-HT_{2B} receptor controlled cyclin E induction, and in concert with the receptor tyrosine kinase PDGFR, induced cyclin D1 expression *via* the MAPK/ERK pathway (91). The 5-HT_{2B} receptor also coupled to the phospholipase A2 (PLA2)-mediated release of arachidonic acid (92). In addition, stimulation of the 5-HT_{2B} receptor triggered intracellular cGMP production through dual activation of constitutive nitric-oxide synthase (NOS) and inducible NOS. The group I PDZ motif at the carboxy terminus of the 5-HT_{2B} receptor was shown to be required for recruitment of the constitutive NOS transduction pathways, and inducible NOS stimulation was under control of the Gα13 pathways (87). Only the NOS pathway seems to be 5-HT_{2B} receptor-specific over other 5-HT₂ receptors (**Figure 1.3**).

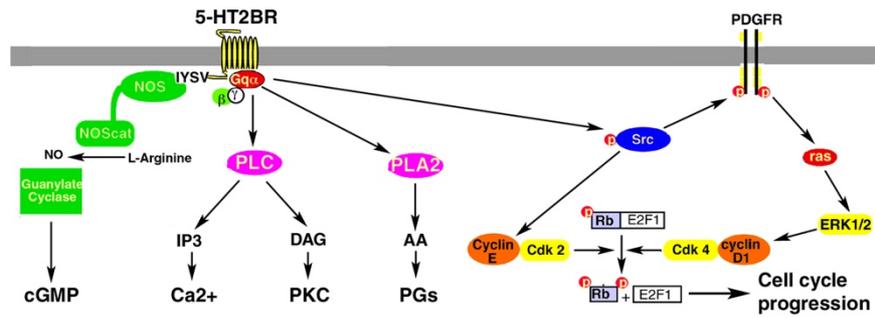


Fig. 1.3. (A) Signal transduction pathways of the transfected 5-HT_{2B} receptor in fibroblasts. The 5-HT_{2B} receptor stimulation triggers intracellular cGMP production through activation of nitric-oxide synthase (NOS) via the type I PDZ motif (VSYI) at its C-terminus; inositolphosphate (IP₃) that releases intracellular calcium (Ca²⁺), and diacylglycerol (DAG), which activates protein kinase C (PKC) via phospholipase C (PLC); phospholipase A₂ (PLA₂)/arachidonic acid (AA)-dependent cyclooxygenase (COX) pathway. Activation of the 5-HT_{2B} receptor also stimulates a ras-mitogen-activated protein kinase (ERK/MAPK) cascade via c-Src and PDGFR that regulate cell-cycle by controlling cyclin E and cyclin D1 expression via retinoblastoma (Rb) phosphorylation, adapted from (93).

6.2 Heart

Inactivation of *Htr_{2B}* gene by homologous recombination in mice leads to partial embryonic lethality due to defects in heart development, for review see (94). Neonates exhibit a second wave of partial lethality due to cardiac dilation resulting from contractility deficits and structural deficits at the intercellular junctions between cardiomyocytes. Echocardiography and electrocardiography studies in animals that live past the first week and survive until adulthood, confirm the presence of left ventricular dilation and decreased systolic function. Serotonin, *via* the 5-HT_{2B} receptor, regulates heart differentiation and proliferation during embryonic development as well as cardiac structure and function in adults (95). The 5-HT_{2B} receptor has been shown to be functionally coupled to reactive oxygen species synthesis through NADPH oxidase (NOX) stimulation in 1C11 cells (96) and in angiotensin II and isoproterenol-induced cardiac hypertrophy (97). In human atrial myocytes, serotonin reduced the amplitude of L-type calcium currents and influenced the strength of gap junctional intercellular communication, which was markedly reduced when 5-HT_{2B} receptors were inhibited, showing that activation of these receptors antagonistically regulated gap junctional intercellular communication (98). Upon pulmonary artery banding, the 5-HT_{2B} receptor antagonist SB204741 was shown to reduce right ventricular fibrosis and to improve heart function in mice (99).

A compound that modulates calcineurin signaling *via* the 5-HT_{2B} receptor (100), was shown to blocks the hypertrophic effects of α -adrenergic receptor agonists. A

model, in which 5-HT_{2B} signaling promoted cardiac hypertrophy by stimulating calcineurin/NFAT signaling, has been proposed with consequent recruitment of histone acetyl transferases to regulatory regions of NFAT target genes. A selective agonist for 5-HT_{2B} receptors induced hypertrophy of cardiac muscle cells through a signaling pathway involving calcineurin and a kinase-dependent mechanism that inactivates class II histone deacetylases (HDAC), which act as repressors of cardiac growth (100). Since it also stimulated nuclear export of class II HDACs, MEF2 may play a role in the mechanism by which 5-HT_{2B} receptor signaling triggers cardiac remodeling (101). A cDNA encoding the 5-HT_{2B} receptor was found in a screen for genes encoding HDAC5 modulators and the ability of 5-HT_{2B} receptors to promote HDAC5 phosphorylation and cardiomyocyte hypertrophy was confirmed (101). The 5-HT_{2B} receptor triggered intracellular calcium release and PKC activation, which likely accounted, at least in part, for the ability of the overexpressed receptor to induce HDAC5 phosphorylation (102).

The 5-HT_{2B} receptor was shown to protect newborn post-mitotic cardiomyocytes against serum deprivation-induced apoptosis as manifested by DNA fragmentation, nuclear chromatin condensation, and TUNEL labeling. Serotonin prevented cytochrome c release and caspase-9 and -3 activation after serum deprivation *via* cross-talks between phosphatidylinositol-3 kinase (PI3K)/Akt and ERK1/2 signaling pathways. Serotonin binding to 5-HT_{2B} receptor activated ERK kinases that inhibited Bax expression induced by serum deprivation. Serotonin *via* PI3K/Akt activated nuclear factor- κ B (NF- κ B) that was required for the regulation of the mitochondrial adenine nucleotide translocator (ANT-1) and mitochondrial permeability. These findings identified serotonin as a novel cardiomyocyte survival factor targeting mitochondria (103). Interestingly, NF- κ B regulation by 5-HT_{2B} receptors was confirmed in a large screen for genes regulating NF- κ B and the MAPK pathways (104). Furthermore, in C-reactive peptide (CRP)-stimulated pulmonary artery endothelial cells, the 5-HT_{2B} receptor was found downregulated by 25%, inhibitor of NF- κ B kinase subunit epsilon (I κ BK ϵ) by 30%, and toll-like receptor (TLR)-4 and -6 by 18 and 39%, respectively. CRP induced RelA/NF- κ Bp65 phosphorylation that represses expression of 5-HT_{2B} receptor, TLR-4, and TLR-6, and I κ BK ϵ gene (105). Following mechanical stretch of cardiomyocytes and incubation with serotonin, the level of 5-HT_{2B} receptor and brain natriuretic peptide (BNP) protein increased time-dependently. Therefore, 5-HT_{2B} receptor expression is involved in pressure-induced cardiomyopathy and its downstream signaling involves NF- κ B to modulate BNP expression in cardiomyocyte (106). These data revealed a dual role of 5-HT_{2B} receptors on both cardiomyocytes and cardiac fibroblasts in regulating cardiac hypertrophy *in-vivo*. Collectively, these results revealed that convergent action of NE, AngII and serotonin *via* interactions between AT1 and 5-HT_{2B} receptors coexpressed by non-cardiomyocytes are limiting key events in cardiac hypertrophy (See also chapter 17).

6.3 Liver

Serotonin is a potent growth factor for the liver development and regeneration. The expression of 5-HT_{2A} and 5-HT_{2B} receptor subtypes in the liver has been reported by several authors to increase after hepatectomy. 5-HT₂ agonists significantly enhanced hepatocyte proliferation after liver transplantation in mice. Evidence for a contribution of 5-HT_{2B} receptors resulted from the observation of the loss of the protective effects of agonists in animals exposed to SB206553, an antagonist of the 5-HT_{2B/2C} receptor subtype (107). In hepatocyte parenchymal cells grown in serum-free defined medium, the proliferative mechanism of serotonin is mediated mainly through 5-HT_{2B} receptor-stimulated Gq/phospholipase C (PLC) and epidermal growth factor (EGF)/transforming growth factor (TGF)- α -receptor/PI3K/ERK1/2/mammalian Target of Rapamycin (mTOR) signaling pathways in primary cultured hepatocytes (108). On the other hand, serotonin-induced phosphorylation of p70S6K, which was blocked by a selective 5-HT_{2B} receptor antagonist LY272015, a specific PLC inhibitor U-73122, a membrane-permeable Ca²⁺ chelator BAPTA/AM, an L-type Ca²⁺ channel blocker verapamil, somatostatin, or a specific p70S6K inhibitor LY2584702 (109).

Hepatocytes are known to express also 5-HT_{2A} receptors that may interact with and 5-HT_{2B} receptors (110). Hepatic stellate cells (HSCs) are key cellular components of hepatic wound healing and fibrosis. However in a pathophysiological setting, the regenerative influence of serotonin acting through 5-HT_{2A} receptors on hepatocytes may be subjected to opposite anti-regenerative effects arising from serotonin acting through 5-HT_{2B} receptors in HSCs (111). After HSC activation, expression of 5-HT_{2A} and 5-HT_{2B} receptors was found 100 and 50-fold over-expressed, respectively. Treatment of HSCs with 5-HT₂ receptor antagonists suppressed proliferation and elevated their rate of apoptosis. Serotonin synergized with PDGF to stimulate increased HSC proliferation (112). Distinct from quiescent cells, activated HSCs exhibited [Ca²⁺]_i transients following treatment with serotonin. Pretreatment with 5-HT₂ antagonist inhibited [Ca²⁺]_i changes upon application of serotonin. Ca²⁺ binding proteins, including calreticulin, calnexin and calquestrin, were up-regulated following activation of HSCs (113).

The 5-HT_{2B} receptor expression was strongly associated with fibrotic tissue in diseased liver. Stimulation of 5-HT_{2B} receptors on HSC by serotonin was shown to activate expression of TGF- β 1 (a powerful suppressor of hepatocyte proliferation) *via* ERK/JunD signaling. Selective antagonism of 5-HT_{2B} receptors enhanced hepatocyte growth in models of acute and chronic liver injury. Similarly, antagonists of 5-HT_{2B} receptor have been shown to decrease mRNA levels of TGF- β 1, connective growth factor, plasminogen activator inhibitor-1, Smad-3 and JunD in lung and skin fibroblasts (114). Activation of the 5-HT_{2B} receptor leads to sustained phosphorylation of two downstream targets of mTOR, p70S6K and 4E-BP1, thereby facilitating survival and inhibiting autophagy of hepatocellular carcinomas (HCC) (115). Similar effects were observed in mice lacking 5-HT_{2B} receptor or JunD and when HSCs have been selectively depleted. Antagonism of 5-HT_{2B} receptor attenuated fibrogenesis and improved liver function in disease models, in which

fibrosis is pre-established and progressive (111). Therefore, 5-HT_{2B} receptor appears to have a dual role on liver, promoting regeneration physiological conditions and fibrosis in pathological conditions (See also chapter 21).

6.4 Lung

In human pulmonary artery endothelial cells, 5-HT_{2B} receptors stimulate calcium release from intracellular stores (116). Ellis *et al.*, (117) showed that antagonizing 5-HT_{2B} receptors caused endothelium-dependent relaxation of rat jugular vein. Another study showed that serotonin induced relaxation of pig pulmonary artery was mediated by endothelial 5-HT_{2B} receptors (118). Ishida *et al.*, (119) reported that activation of 5-HT_{2B}/5-HT_{1B} receptors stimulated NO production in human coronary artery endothelial cells. Other work identified a cardioprotective function of the 5-HT_{2B} receptors in an integrated model of heart failure with preserved ejection fraction that could be explained by a contribution of the endothelial 5-HT_{2B} receptors to coronary vasodilatation (120).

PAH is a progressive and often fatal disorder in humans that results from an increase in pulmonary blood pressure associated with abnormal vascular proliferation. Serotonin is associated with the pathogenesis of PAH (121). Therapeutic drugs with PAH as a side effect, like the amphetamine derivative and anorexigen dexfenfluramine, are potent serotonin releasers acting at SERT and (or their metabolite) agonists at 5-HT_{2B} receptors (122). Serotonergic anorexigen-dependent PAH is clinically indistinguishable from the heritable form of disease, associated with *BMPR2* mutations. Both *BMPR2* mutation and agonists to the serotonin receptor *HTR2B* have been shown to cause activation of SRC tyrosine kinase; conversely, antagonists to *HTR2B* inhibit SRC trafficking and downstream function. In *Bmpr2R899X* knock-in mice, which spontaneously develop pulmonary hypertension, the 5-HT_{2B} receptor antagonist, SB204741, blocks the SRC activation caused by *Bmpr2R899X* mutation. SB204741 prevented the development of pulmonary hypertension, reduced recruitment of inflammatory cells to their lungs, reduced muscularization of their blood vessels, reduced SRC phosphorylation and downstream activity in *Bmpr2R899X* mice (123). Using bone-marrow transplantation, the restricted expression of 5-HT_{2B} receptors to bone-marrow cells was shown as necessary and sufficient for pulmonary hypertension to develop *via* an action at hematopoietic stem cell differentiation (124). Bone-marrow cells play thus a key role in genetic pulmonary hypertension pathogenesis that was further validated by transplanted *Bmpr2R899X* bone-marrow cells, which were able to drive the lung phenotype (125). Together, these findings reveal the limiting role of serotonin *via* 5-HT_{2B} receptors in PAH development and shift the contribution of serotonin to PAH to an extrapulmonary, hematopoietic event (see also chapter 18 and 20).

Serotonin was shown to increase proliferation and collagen synthesis by lung fibroblasts. Serotonin concentrations in lung homogenates increased significantly

over the time course of bleomycin-induced fibrosis, with a maximum at day seven, together with the expression of serotonin receptors 5-HT_{2A} and 5-HT_{2B} (126). Blockade of 5-HT_{2B} receptors by SB215505 reduced bleomycin-induced lung fibrosis, as demonstrated by reduced lung collagen content and reduced procollagen 1 and procollagen 3 mRNA expression. 5-HT_{2B} receptor antagonists promoted an antifibrotic environment by decreasing the lung mRNA levels of TGF- β 1, connective growth factor and plasminogen activator inhibitor-1 and JunD mRNA. Interestingly, the 5-HT_{2B} receptor was strongly overexpressed by fibroblasts in the fibroblastic foci of human idiopathic pulmonary fibrosis samples (127). Serotonin contribution to lung fibrosis is thus controlled by 5-HT_{2B} receptors regulating TGF- β 1 levels.

6.5 Skin

Dermal fibrosis was independently shown to be reduced in *Htr2b*^{-/-} mice using both inducible and genetic models of fibrosis. Pharmacologic inactivation of 5-HT_{2B} receptor also effectively prevented the onset of experimental fibrosis and ameliorated established fibrosis by decreasing mRNA levels of TGF- β 1, connective growth factor, plasminogen activator inhibitor-1 and Smad-3 (114). Moreover, inhibition of platelet activation prevented fibrosis in different models of skin fibrosis. Consistently, mice deficient for tryptophan hydroxylase (TPH)-1, the rate-limiting enzyme for serotonin production outside the central nervous system, showed reduced experimental skin fibrosis (114). Serotonin contribution to skin fibrosis is thus controlled by 5-HT_{2B} receptors *via* regulation of TGF- β 1 levels. Stimulation of dermal fibroblasts with serotonin led to increased expression of pro-fibrotic genes which was significantly reduced by antagonists, and decreased type I collagen and α -SMA, ERK1/2 and STAT3 phosphorylation independently of Smad3 phosphorylation (128). 5-HT_{2B} receptor antagonists can thus suppress TGF- β 1-mediated non-canonical pathways, ERK1/2 and STAT3, which have been implicated in the regulation of pro-fibrotic genes and in the development of fibrosis (See also chapter 22).

6.6 Bones

The *Htr2B* mRNA expression, which was undetectable in anaplastic osteoblasts, appears in differentiated and matured osteoblasts (129, 130), (131). The differentiation and maturation of osteoblasts is thus regulated by the activation of the 5-HT_{2B} receptor (132). Optimal bone matrix mineralization involves both NO and PLA2 signaling pathways and the 5-HT_{2B} receptor promotes prostaglandin (PGE2) production through cyclooxygenase (COX) activation. The 5-HT_{2B} receptor

contributed in an autocrine manner to osteogenic differentiation (133). A functional link between the 5-HT_{2B} receptor and the activity of the tissue-nonspecific alkaline phosphatase (TNAP) was established during the initial mineralization phase. Previous observations indicated that the 5-HT_{2B} receptor coupled to PLA2 pathway and prostaglandin production at the beginning of mineral deposition. The 5-HT_{2B} receptor also controlled leukotriene synthesis *via* PLA2 at the terminal stages of differentiation. These two 5-HT_{2B} receptor-dependent eicosanoid productions delineate distinct time-windows of TNAP regulation during the osteogenic program. Finally, prostaglandins or leukotrienes were shown to relay the post-translational activation of TNAP *via* stimulation of the phosphatidylinositol-specific PLC. In agreement with the above findings, primary calvarial osteoblasts from *Htr2b*^{-/-} mice were shown to exhibit defects in TNAP activity (134). Brain serotonin was proposed to favor indirectly bone mass accrual following activation of 5-HT_{2C} receptors on ventromedial hypothalamic neurons and 5-HT_{2B} receptors on arcuate neurons (135). Compared to control osteoblasts, the lack of 5-HT_{2B} receptors was associated with a 10-fold over-production of prostacyclin (PGI₂). Also, a specific prostacyclin synthase inhibitor (U51605) rescued totally osteoblast aggregation and matrix mineralization in *Htr2b*^{-/-} osteoblasts. Prostacyclin is the endogenous ligand of PPAR-β/δ, and its inhibition in *Htr2b*^{-/-} cells rescued totally the TNAP and osteopontin mRNA levels, cell-cell adhesion, and matrix mineralization. The absence of 5-HT_{2B} receptors leads to the overproduction of prostacyclin, inducing reduced osteoblast differentiation due to PPAR-β/δ - dependent target regulation and defective cell-cell adhesion and matrix mineralization (136). The 5-HT_{2B} receptor contributes thus in an autocrine manner to osteogenic differentiation, *via* a physiological negative control of prostacyclin by 5-HT_{2B} receptors (See also chapter 5 and 16).

As in transfected cells, endogenously expressed 5-HT_{2B} receptors can stimulate various transduction pathways according to the cell subtype including SRC, NO, metalloproteinases, PLA2 activities. The complex signal transduction pathways highlighted by the study of 5-HT_{2B} receptors makes very likely that biased agonists or antagonists will appear as valuable therapeutics.

7 5-HT_{2B} receptor in cancer cells

A recent screen of a large tumour set using functional genomic mRNA, high *HTR2B* mRNA overexpression was found on all melanoma, in gastro-intestinal stromal tumour cells, and endothelial cells of colon, ovarian, breast, renal and pancreatic tumours (137).

7.1 Carcinoid tumors

Strong expression of 5-HT_{2B} receptors was observed in spontaneous human carcinoid tumors, along with coupling to p21^{ras} activation (90). The tumor proliferative activity of small intestinal neuroendocrine tumors (including cell growth and the development of desmoplasia) is associated with particular microenvironment in peritoneum that is controlled by tumor cells through the secretion of profibrotic/ angiogenetic factors (138).

7.2 Melanoma

Activation of 5-HT_{2B} receptors reduced melanin synthesis and intracellular tyrosinase activity in human melanocytes. The expression of melanogenesis-related proteins (tyrosinase, TRP-1 and TRP-2) and microphthalmia-associated transcription factor (MITF) decreased after agonist treatment. The reduced level of MITF was associated with inhibition of protein kinase A (PKA) and cAMP response element-binding protein (CREB) activation (139). Independently, *HTR2B* is among the genes, which show the highest overexpression in class 2 uveal melanoma (140). A PCR-based 15-gene assay comprising 12 discriminating genes including *HTR2B* are now part of a prognostic assay for managing patients with uveal melanoma (141), by providing candidates for distinguishing whether uveal melanomas contain liver metastases. This set of genes thus aid in the diagnosis and prevention of uveal melanoma liver metastases, based on their different features (142). Metastatic uveal melanomas primary tumors show important alterations in the expression of 5-HT_{2B} receptor, the E3 ubiquitin ligases, and various subunits of the proteasome. This finding suggested that the inability of the proteasome to degrade 5-HT_{2B} receptor in metastatic uveal melanomas cells might rely on an increased stability of the ubiquitinated receptor in these cells (89). The selective 5-HT_{2B} receptor antagonist PRX-08066 has impact on the proliferation and migration of uveal melanoma cells, through activation of many signaling pathways such as WNT, Focal adhesion kinase and Janus kinase/STAT (143). The upstream regulatory region of the *HTR2B* gene contains a combination of alternative positive and negative regulatory elements functional in uveal melanomas cells (30).

7.3 Adrenocortical carcinoma

Gene expression profiles of adrenocortical tumors identified underexpression of *HTR2B* mRNA as a marker of malignant adrenocortical carcinoma (144). Analysis of biomarkers of malignancy of adrenocortical cancers in the meta-analysis has revealed that the combination of overexpressed anillin (*ANLN*) and underexpressed

HTR2B mRNA appeared to be the best predictor of malignancy (145). However, chronic adrenal stimulation by glucose-dependent insulinotropic peptide in adrenal hyperplasia was shown to lead to the significant induction of the *GPR54*, *HTR2B*, *GPR4*, and endothelial differentiation sphingolipid receptor *EDG8* (146).

7.4 Hepatocellular carcinoma

Serotonin has been reported to promote proliferation of serum-deprived HCCs. Among 64 genes for which mRNA expression levels differed between non-hepatitis B, non-hepatitis C compared to hepatitis C-type HCC, the most affected was *HTR2B* (147). The function of serotonin as a survival factor of HCC cells was demonstrated: activation of the 5-HT_{2B} receptor leads to sustained phosphorylation of two downstream targets of mTOR, p70S6K and 4E-BP1, thereby facilitating survival and inhibiting autophagy. Inhibiting the 5-HT_{2B} receptor reduced cancer cell growth *in-vitro* and *in-vivo*. The presence of 5-HT_{2B} receptors in HCC and the activation of autophagy-related mechanisms demonstrated new insights of serotonin in cancer biology (115). The 5-HT_{1B} and 5-HT_{2B} receptors were found expressed in about one third of the patients with HCC. Both receptors were associated with an increased proliferation index (148). The 5-HT_{2B} receptor mediates serotonin-induced proliferation in the serum-deprived HCC Huh7 cells. Additionally, inhibition of 5-HT_{2B} receptor in Huh7 cells using SB204741 significantly decreased the expression of FOXO3a, a member of class O of the fork head box family of transcription factors (149). *In-vitro* data suggest also that serotonin increased total β -catenin, active β -catenin and decreased phosphorylated β -catenin protein levels in serum deprived HuH-7 and HepG2 cells. Activation of WNT/ β -catenin signaling was evidenced by increased expression of β -catenin downstream target genes, Axin2, cyclin D1, dickkopf-1 (DKK1) and glutamine synthetase (GS) by qPCR in serum-deprived HCC cell lines treated with serotonin. Additionally, serotonin disrupted Axin1/ β -catenin interaction, a critical step in β -catenin phosphorylation (150).

7.5 Pancreatic ductal adenocarcinomas

Under metabolic stress, autocrine serotonin exhibits pro-survival and anti-apoptotic roles in pancreatic ductal adenocarcinomas cells. Intriguingly, peripheral serotonin is critically implicated in the regulation of energy homeostasis. Agonists of 5-HT_{2B} receptor, but not other serotonin receptors can promote proliferation and prevent apoptosis of pancreatic ductal adenocarcinomas cells. Knockdown of *HTR2B* in pancreatic ductal adenocarcinomas cells, or incubation of cells with 5-HT_{2B} receptor antagonists, reduced their growth as xenograft tumors in mice. Levels of metabolic enzymes involved in glycolysis, the phosphate pentose pathway, and hexosamine biosynthesis pathway increased significantly in pancreatic ductal adenocarcinomas

cells following serotonin stimulation. The mTOR signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation, and survival. Serotonin stimulation led to formation of the 5-HT_{2B} receptor-LYN-p85 complex, which increases PI3K-Akt-mTOR signaling and the Warburg effect by increasing protein levels of MYC and HIF1 α . Administration of 5-HT_{2B} receptor antagonists slowed growth and metabolism of established pancreatic tumors and prolonged survival of the mice (151). Furthermore, preincubation (6 h) of MIN6 cells with serotonin or 5-HT_{2B} receptor agonist BW723C86 reduces glucose stimulated insulin secretion and the effect of serotonin could be prevented by 5-HT_{2B} receptor antagonist SB204741. Preincubation with BW723C86 increases PPAR γ co-activator 1 α (PGC1 α) and PPAR γ mRNA and protein levels and decreases mitochondrial respiration and ATP content in MIN6 cells (152). Prolonged 5-HT_{2B} receptor activation in murine β -cells decreases glucose-stimulated insulin secretion and mitochondrial activity by mechanisms likely dependent on enhanced PPAR γ expression (See also chapter 23).

7.6 T-cell Leukemia

A proteasome inhibitor, bortezomib, could be a potential therapeutic agent in treating adult T-cell leukemia (ATL) patients. *HTR2B* was identified in a network that converges to secreted protein acidic and rich in cysteine (*SPARC*) gene. *SPARC* is a tumor-invasiveness related gene, which may act as a possible modulator of bortezomib-induced cell death in adult T-cell leukemia cells (153).

7.7 Tumor angiogenesis

In tumor-infiltrating macrophages, serotonin does not enhance cancer tumor cell proliferation but may act as a regulator of angiogenesis by reducing the expression of metalloproteinase-12, entailing lower levels of angiostatin, an endogenous inhibitor of angiogenesis (154). Serotonin can stimulate the phosphorylation of ERK1/2 in bovine endothelial cells, and the 5-HT_{2B} receptor was reported to play a role in the activation of endothelial NOS in human endothelial cells. In SB204741-treated mice, the selective blockade of 5-HT_{2B} receptor resulted in the reduction of tumor angiogenesis and growth through the inhibition effect of ERK1/2 and endothelial NOS (155). Therefore, the possibility that 5-HT_{2B} receptors participate in tumor angiogenesis is a likely possibility that remains to be validated.

The 5-HT_{2B} receptor appears thus associated with various types of cancer cells. In addition, its contribution varies according to each tumor and may contribute to many processes in tumor differentiation, proliferation, survival, or angiogenesis. The relation between its strong embryonic expression in particular in NCC and in

certain tumors cells needs to be investigated. Nevertheless, it appears that, at least in particular situations, blocking its activation may have therapeutic potential.

8 Outlook and Prospects

The characterization of the 5-HT_{2B} receptor subtype in various species identified similarities, but also but differences in its pharmacology, as compared to 5-HT_{2A} or 5-HT_{2C} receptors. Its physiological functions include many differentiation steps both in periphery and central nervous system. Early expression of 5-HT_{2B} receptors has been found in embryos in post-migrating NCCs, neural tube, hematopoietic tissue, and heart primordia. Although quite low at adult stage, the expression of 5-HT_{2B} receptor was confirmed in several brain nuclei of all investigated vertebrate species. The particular *HTR2B* gene organization and its complex transcriptional regulation are not yet fully understood. Similarly, more work is needed on the structure-function relationships of the 5-HT_{2B} receptor, in particular the preferential heteromeric association between 5-HT₂ receptors, the asymmetry in Gq-protein coupling, and signaling. How its structure impacts on 5-HT_{2B} receptor coupling, desensitization pathways, or possible proteasome regulation remains poorly understood but needs to be taken into account in further physiological studies. As in transfected cells, endogenously expressed 5-HT_{2B} receptors can stimulate various transduction pathways including SRC, PI3K-Akt-mTOR, MAPK, NO, metalloproteinases, PLC or PLA2 activities. Finally, the 5-HT_{2B} receptor appears associated with various types of cancer cells, but its contribution varies according to each tumor and may be involved in tumor differentiation, proliferation, survival, and/or angiogenesis.

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