The Receptors

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5-HT$_{2B}$ Receptors

From Molecular Biology to Clinical Applications

Humana Press

The Receptors

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Preface

Although quite widely expressed, but at low levels, therapeutic interest for the 5-HT_{2B} receptor has been delayed due to the lack of selective tools to study the functions of this receptor. In the past decade, selective antagonists and genetics have revealed numerous important adult physiological functions for this receptor as well as embryonic development and growth, haematopoietic and myeloid lineage control. Furthermore, its implication in cardiovascular and cardiopulmonary diseases, fibrosis and liver regeneration or cancer cells revealed the 5-HT_{2B} receptor as a new potential target for therapeutics; 5-HT_{2B} antagonists being under active investigation for peripheral pathologies including pulmonary and cardiac diseases, irritable bowel syndrome and cancer. These receptors have also been recognized as off-targets of other drugs because their stimulation plays a significant role in the pathogenesis of pulmonary hypertension and valvulopathy. Emerging evidence indicates that this serotonin receptor might regulate brain disorders including drug of abuse, psychosis and antidepressant actions as well as inflammation during neurodegenerative diseases and in turn influence the course of these diseases. This book provides a comprehensive overview of this receptor, its physiological/pathophysiological effects in periphery, its role in a number of brain functions and diseases as well as the potential therapeutic outcomes.

Paris, France — Luc Maroteaux
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Luc Maroteaux

Abbreviations

ANT-1	Adenine nucleotide translocator
BNP	Brain natriuretic peptide
BRET	Bioluminescence resonance energy transfer
COX	Cyclooxygenase
CREB	cAMP response element-binding protein
DHE	Dihydro-ergotamine
ECL2	Extracellular loop 2
ERG	Ergotamine
ERK2/ERK1	Extracellular signal-regulated kinase 2 and 2
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
HB-EGF	Heparin-binding epidermal growth factor
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HSC	Hepatic stellate cell
LNX	Ligand of Numb protein X
LSD	Lysergic acid diethylamide
MAPK	Mitogen-activated protein kinase
MITF	Microphthalmia-associated transcription factor
mTOR	Mammalian target of rapamycin
NAM	Negative allosteric modulator
NCC	Neural crest cell
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOX	NADPH oxidase

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PAH	Pulmonary arterial hypertension
PAM	Positive allosteric modulator
PDGFR	Platelet derived growth factor receptor
PDZ	Postsynaptic density protein of 95 kDa, disc large, zonulla occludens-1
PGE2	Prostaglandin
PGI2	Prostacyclin
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PLA2	Phospholipase A2
PLC	Phospholipase C
RA	Retinoic acid
SERT	Serotonin transporter
TGF	Transforming growth factor
TLR	Toll-like receptor
TNAP	Tissue-nonspecific alkaline phosphatase
TPH	Tryptophan hydroxylase

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 Chap. 2).

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 2246 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 [28]; the 19S complex (regulatory particle), within which RPN2 is found, functions to

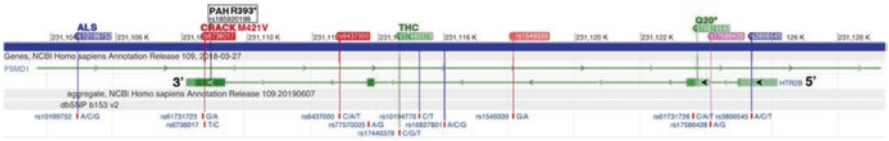

Fig. 1.1 *HTR2B* gene organization on the reverse strand of *PSMD1* intron 16 with SNPs position. This is a schematic representation of the *HTR2B* gene on the reverse strand of *PSMD1* locus with the location of different SNPs, including SNPs previously associated with disorders: THC: cannabis-induced aggression [24]; Q20*: stop codon in exon 2, implusivity [16]; ALS: amyotrophic lateral sclerosis [25], Crack addicts (M421V) [26], and pulmonary arterial hypertension (PAH) R393* [27], adapted from [26]

recognize ubiquitinated proteins [29]; it then processes and transports them to the 20S complex (core particle), where proteolysis occurs.

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-HT_2 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 RARβ, 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 (PPARγ) and 5-HT_{2B} receptor expression in pulmonary arterial hypertension (PAH). However, the relation between PPARγ and 5-HT_{2B} receptors regulated expression remains controversial and further research is needed to determine if the *HTR2B* is a direct target of PPARγ 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].

2.2 Selective Agonists

There is virtually no highly selective agonist for a particular 5-HT$_2$ receptor: BW723C86: 1-methyl-2- [5-(2-thienylmethoxy)-1H-indole-3-yl] ethylamine hydrochloride, has been reported to have ten-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 ten-fold selectivity over other 5-HT_2 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-HT_2 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 (Ki = 14 nM) and 6-APB (Ki = 3.7 nM), and 5-iodo-aminoindane (Ki = 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 phenyliso-propylamines between hallucinogenic activity and affinity for the 5-HT_{2B} receptor [48] (see also Chap. 16 and Table 1.1).

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 pKi 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 (Ki 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 (pKi = 9.5) and 1000 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-carbox amide] 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].

Table 1.1 Pharmacological properties of human and mouse 5-HT$_{2B}$ receptors. Reprinted from [49]

		h5-HT2A	h5-HT2B	m5-HT2B	h5-HT2C	m5-HT2C
		pKi	pKi	pKi	pKi	pKi
BW723C86	[50]		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	[50] h2CINI, m2CVNi		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	[50] h2CINI, m2CVNi		7.28 ± 0.19	7.84 ± 0.12	7.46 ± 0.05	6.92 ± 0.11
	[51] 2006 h2C?	7.74 ± 0.11	7.22 ± 0.03		8.48 ± 0.14	
CP809101	[52]	8.22 ± 0.15	7.19 ± 0.25		8.80 ± 0.11	
	[50] h2CINI, m2CVNi		7.86 ± 0.18	8.41 ± 0.18	8.35 ± 0.02	7.72 ± 0.15
DOI	[50] h2CINI, m2CVNi		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 ± 011	
	[39] h2CVSV	8.04 ± 0.05	7.78 ± 0.09		7.73 ± 0.04	
Norfenfluramine	[50] h2CINI, m2CVNi		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	[53]	6.95 ± 0.03	6.76 ± 0.09		7.82 ± 0.03	
Clozapine	[50] h2CINI, m2CVNi			7.97 ± 0.09		
	[41] h2CINI	7.60 ± 0.08	7.99 ± 0.09		7.87 ± 0.05	
	[54]	8.39 ± 0.03	8.79 ± 0.09		8.56 ± 0.06	
Aripiprazole	[50] h2CINI, m2CVNi			7.21 ± 0.09		
	[55] h2CINI	8.06 ± 0.10	9.44 ± 0.16		7.12 ± 0.09	
	[54]	8.02 ± 0.16	9.59 ± 0.17			
RS1022221	[50] h2CINI, m2CVNi		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	

(continued)

Table 1.1 (continued)

		h5-HT2A	h5-HT2B	m5-HT2B	h5-HT2C	m5-HT2C
		pKi	pKi	pKi	pKi	pKi
SB215505	[50] h2CINI, m2CVNi		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	[50] h2CINI, m2CVNi		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	[50] h2CINI, m2CVNi		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	[50] h2CINI, m2CVNi		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	[50] h2CINI, m2CVNi		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	[50] h2CINI, m2CVNi		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	[56]	8.52 ± 0.12	6.57 ± 0.12		7.43 ± 0.03	
Ketanserin	[56]	9.67 ± 0.12	6.55 ± 0.09		7.39 ± 0.11	

Non-selective 5-HT_2 receptor antagonists such as ritanserin and mesulergine block 5-HT_2 receptor-mediated effects. Atypical antipsychotics including clozapine, asenapine, or cariprazine also have fairly high antagonistic affinity for all 5-HT_2 receptors [10, 55, 62, 63]. Aripiprazole (OPC-14597) is a novel atypical antipsychotic drug, which has higher antagonist affinity (EC_{50} = 11 nM) for the human 5-HT_{2B} receptor than for 5-HT_{2A} or 5-HT_{2C} receptors [54], (see Table 1.1).

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

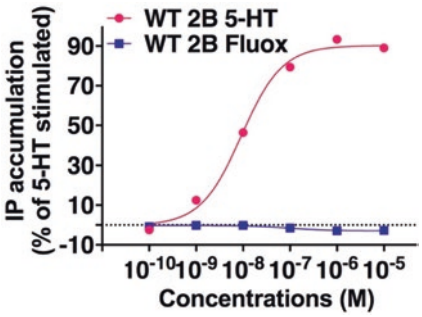

Fig. 1.2 Inositol phosphate production by 5-HT$_{2B}$ receptors transiently expressed in Cos-7 cells. The quantification of inositol phosphate (IP) signaling pathway activity was performed in COS-7 cells transfected with 5-HT$_{2B}$ receptor and exposed to increasing concentrations of a full agonist 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

knockout for Pet1 (*Pet1*$^{-/-}$) [65] (1) rules out that the antidepressant effects of fluoxetine could be independent of SERT; (2) 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; (3) 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 Ki values over 5 μM [65] and with no agonist activity (Fig. 1.2), while SSRI Ki values for SERT are in nanomolar range [66].

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-HT$_2$ 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 Emax of serotonin), and as negative allosteric modulator (NAM) at 5-HT_{2B} receptors (decreases EC50 of serotonin 10 times without affecting Emax) [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-HT_2 receptors agonist development might emerge from compounds so-called biased agonists sharing a functional selectivity for specific intracellular signaling pathways [70]. 5-HT_2 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].

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-HT$_2$ 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 Chap. 9).

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 ($t1/2$ = 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 (Fig. 1.1). This receptor displayed a loss of rapid internalization and a striking increase in proliferative capacity resulting from a switch from a dual $G_{\alpha q}/G_{\alpha 13}$ coupling in wildtype receptor to a nearly exclusive coupling to $G_{\alpha 13}$ in the R393X 5-HT$_{2B}$ receptor [27].

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, Gαq, Gα11, and Gα13, involved in signaling of the receptor. MUPP1 a multivalent postsynaptic density protein of 95 kDa, disc large, zonulla 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 Gαq/11, but not by pertussis or cholera toxins or by anti-Gαi or anti-Gαs antibodies. This GTPase activation was thus mediated by the G-protein $G\alpha_{q/11}$, but not by Gαs or Gαi. 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: (1) PDGFR tyrosine kinase phosphorylation and MAPK activation; (2) cyclin D1 and cyclin E expression levels; and (3) 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

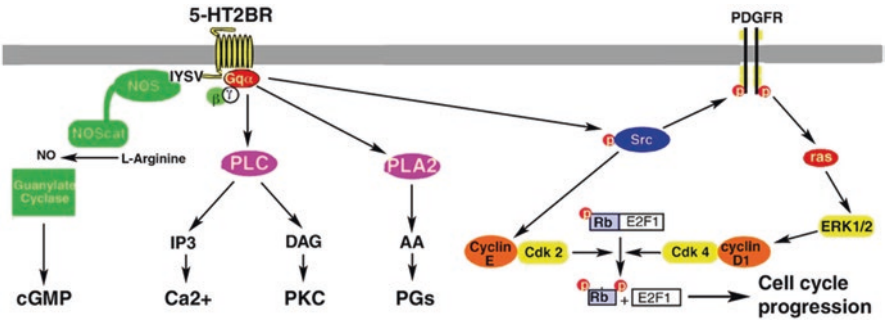

Fig. 1.3 Signal transduction pathways of the transfected 5-HT$_{2B}$ receptor in fibroblasts. The 5-HT$_{2B}$ receptor stimulation triggers intracellular cGMP production through activation of nitric-oxide synthase (NOS) via the type I PDZ motif (VSYI) at its C-terminus; inositolphosphate (IP3) that releases 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]

the Gα13 pathways [87]. Only the NOS pathway seems to be 5-HT$_{2B}$ receptor-specific over other 5-HT$_2$ receptors (Fig. 1.3).

6.2 Heart

Inactivation of *Htr2b* 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 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-κB (NF-κB) 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-κB regulation by 5-HT_{2B} receptors was confirmed in a large screen for genes regulating NF-κB 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-κB kinase subunit epsilon (IκBKε) by 30%, and toll-like receptor (TLR)-4 and -6 by 18 and 39%, respectively. CRP induced RelA/NF-κBp65 phosphorylation that represses expression of 5-HT_{2B} receptor, TLR-4, and TLR-6, and IκBKε 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-κB 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 Chap. 9).

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-HT_2 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 Ca^{2+} chelator BAPTA/AM, an L-type Ca^{2+} 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 overexpressed, respectively. Treatment of HSCs with 5-HT_2 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 $[Ca^{2+}]$i transients following treatment with serotonin. Pretreatment with 5-HT_2 antagonist inhibited $[Ca^{2+}]$i changes upon application of serotonin. Ca^{2+} binding proteins, including calreticulin, calnexin and calsequestrin, 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-β1 (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-β1, 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 Chap. 14).

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 Chaps. 10 and 12).

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-β1, 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-β1 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-β1, 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-β1 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-β1-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 Chap. 13).

6.6 Bones

The *Htr2b* mRNA expression, which was undetectable in anaplastic osteoblasts, appears in differentiated and matured osteoblasts [129–131]. 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 ten-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 Chap 7).

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, dickoppf-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 HIF1α. 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 PPARγ co-activator 1α (PGC1α) and PPARγ 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 PPARγ expression (see also Chap. 15).

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-HT_2 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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Part I
Development and Growth

Chapter 2
Serotonin Function During Embryonic Development: The 5-HT$_{2B}$ Receptor Contribution

Michela Ori and Irma Nardi

Abbreviations

5-hydroxytryptamine, 5-HT	Serotonin
AGM	Aorta-gonad-mesonephros
BM	Bone marrow
CMZ	Ciliary marginal zone
CNS	Central nervous system
ENS	Enteric nervous system
HPA/I	Hypothalamic-pituitary-adrenal/interrenal
HSPCs	Hematopoietic stem and progenitor cells
KO	Knockout
MAPK	Mitogen-activated protein kinases
MT	Melatonin
NCCs	Neural crest cells
OAVS	Oculo-auriculo-vertebral spectrum
PA	Pharyngeal arches
PAH	Pulmonary arterial hypertension
PLC	phospholipase C
POM	Periocular mesenchyme
RA	Retinoic acid
SERT	Serotonin transporter
SSRI	Serotonin reuptake inhibitor

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1 Introduction

Since the pioneer studies of Buznikov and his collaborators [1] it has becoming clear that during embryogenesis some neurotransmitters could function as morphogens before being coopted in the central nervous system. Such a role has long been suspected for serotonin (5-hydroxytryptamine, 5-HT), a biogenic monoamine that appeared very early in evolution, being present not only in vertebrates and invertebrates animals but also in plants [2]. First discovered as the secretory product of enterochromaffin cells of the gut ("enteramine") [3], it was later isolated as a constrictor factor from the blood serum and named serotonin [4]. serotonin was then identified in mammalian brain and this brought it into the field of neuroscience [5]. Sertonin is best known for its role in the nervous system since it is one of the neurotransmitters mainly involved in the etiology of many human psychiatric disorders and many substances that interfere with the serotonergic system are commonly used as therapeutic agents. However, serotonin also mediates a wide range of peripheral functions [6]. It is now well documented that serotonin also plays a crucial role during embryogenesis. Serotonin and its receptors have been detected in oocytes and early embryos of all invertebrates and vertebrates studied, from sea urchins, Drosophila, frogs, chicken to mammals, even before the appearance of neural structures suggesting a role of serotonin in cell proliferation and/or in morphogenetic movements [7–9]. In *Xenopus* embryos serotonin is present as a maternal pool in the eggs, while during mammalian embryogenesis serotonin is supplied to the embryo by the maternal blood [10, 11]. Moreover, an early transient placental source of serotonin for the fetal circulation has been detected both in mice and humans [12]. Later in the embryo development, serotonin is actively produced mainly by the raphe nuclei in the brain and in the gastrointestinal tract. In adults, most (90%) of the serotonin synthesized in the body comes from the periphery where it is produced by gut enterochromaffin cells and then taken up by the serotonin transporter (SERT) in platelets. When released by platelets, serotonin triggers biological effects through its interaction with membrane receptors**.** In addition to the sites of serotonin production, there are a number of cells that capture and store serotonin, acting as 5-HT reservoirs during development. In mice serotonin uptake sites first appear in non-neural tissues such as the heart, the liver, the cranial mesenchyme, the migrating cranial neural crest cells (NCCs), dorsal ganglia and retinal ganglion cells [13, 14]. High affinity uptake of serotonin in these structures highlights the involvement of serotonin signaling in their development and suggests that serotonin must be finely modulated. Serotonin thus plays a role as a morphogen-like signal in development before it acts as a neurotransmitter in the brain. So far evidence has been presented for serotonin signaling involvement in many important embryological events such as craniofacial and cardiac morphogenesis, neural crest migration, eye, limb, and bone development, the establishment of left-right asymmetry, the closure of the neural tube and neuronal differentiation during early neurogenesis [10, 15–17].

2 A Main Mediator of Serotonergic Signaling During Embryogenesis: The 5-HT$_{2B}$ Receptor

Most of the biological actions of serotonin are mediated by G-coupled receptors and, among these, the Gq-coupled 5-HT$_{2B}$ receptor signaling has shown to be particularly important in mediating the effects of serotonin in embryonic development. The first evidence of a role of 5-HT$_{2B}$ receptor during embryonic development, has been provided by cultured mouse embryos exposed to the 5-HT$_2$ family receptor high affinity antagonist ritanserin from 8 to 11 d.p.c. Ritanserin interferes with cranial neural crest cell migration, induces craniofacial defects including hypoplastic mandibular arches, abnormal eyes and cardiovascular defects such as reduction of trabeculation of the ventricular myocardium [15]. Similar results have been obtained in cultured Xenopus and zebrafish embryos treated with ritanserin [18, 19]. Most of these defects have been supposed to be dependent on 5-HT$_{2B}$ receptor inactivation because 5-HT$_{2B}$ receptor mRNA is expressed in the affected tissues and only other antagonists of this class with high affinity for 5-HT$_{2B}$ receptor gave similar results [15, 20]. However, the most straightforward way to understand the function of the receptors that mediate the serotonin action during development has been to investigate the effects of the abrogation of the corresponding gene function. Advances on how 5-HT$_{2B}$ receptor signaling can influence early development and its role in vertebrate morphogenesis have mainly derived from two model systems, the mouse and Xenopus, thanks to the availability of sophisticated tools for manipulating embryos and gene functions in these organisms.

3 Role of 5-HT$_{2B}$ Receptor in Cardio Vascular Development

The first genetic evidence of 5-HT$_{2B}$ receptor role in embryogenesis was obtained from its genetic ablation in mice [21]. The creation of 5-HT$_{2B}$ receptor knockout (KO) mice has allowed addressing how signaling and expression of this receptor is specifically implicated in embryonic development as well as in adult health and disease [22]. One major defect of 5-HT$_{2B}$ receptor KO embryos was a disturbed heart development leading to partial embryonic lethality and neonatal death. Histological analysis of the heart of the mutant embryos revealed a lack of trabecular cells in the ventricle, while newborn mice display severe ventricular hypoplasia, caused by impaired proliferative capacity of myocytes and cardiac dilation resulting from contractility deficits and structural defects at intercellular junctions between cardiomyocytes. In the surviving mutant adult mice echocardiography and electrocardiography both confirmed the presence of left ventricular dilation and decreased systolic function. A severe reduction in the thickness of the myocardium may induce myocardial rupture resulting in escape of blood into the pericardium followed by death. These results have shown that serotonin, via the 5-HT$_{2B}$ receptor signaling, is an important regulator of cardiac myocyte differentiation and proliferation of the

developing heart. Transduction of the 5-HT_{2B} receptor signaling is complex, including phospholipase C (PLC) and A2 stimulation, cGMP production and a mitogenic signal that integrates the tyrosine kinase -signaling pathway. Nebigil and colleagues [23] demonstrated in vitro that 5-HT_{2B} receptors activity is mitogenic. In this mitogenic signaling, c-Src is the crucial molecule that links 5-HT_{2B} receptor signaling leading to activation of the cell-cycle machinery and the cell-cycle regulators. c-Src alone controls cyclin E induction and transactivates PDGF-receptor tyrosine kinase activity to induce cyclinD1 expression through p42mapk/p44mapk (ERK2/ERK1) mitogen-activated protein kinases (MAPK) pathway.

This finding is in agreement with the observation that transgenic mice overexpressing the 5-HT_{2B} receptor, specifically in the heart, leads to ventricular hypertrophy as result of increased cell number and size. Echocardiographic analysis indicated the presence of thickened ventricular wall without alteration of the systolic function, showing that transgenic mice have compensated hypertrophy. Interestingly, the electron microscope analysis of these mutant mice revealed an abnormal mitochondrial proliferation associated to increased mitochondrial enzyme activity [24]. Parallel ultrastructural analysis of 5-HT_{2B} receptor knockout mice heart revealed pronounced mitochondrial abnormalities, such as interrupted inner membrane and swollen cristae, as well as altered mitochondrial enzyme activities (cytochrome oxidase and succinate dehydrogenase). Although damage in mitochondria is a key step leading to programmed cell death, no typical apoptotic bodies were observed in the mutated heart despite impaired myofibrillar structure, suggesting a protective role preventing apoptosis by the Gq-coupled 5-HT_{2B} receptor signaling on cardiomyocytes [24]. By using in vitro cultured cardiomyocytes and 5-HT_{2B} receptor knockout mice as an animal model of dilated cardiomyopathy, it has been shown that 5-HT_{2B} receptor signaling prevents cytochrome c release and caspase-9 and 3 activation via cross talks between phosphatidylinositol 3 kinase/Akt and ERK1/2 signaling pathways [25]. These findings are relevant since they identify serotonin via 5-HT_{2B} receptor as a novel survival factor targeting mitochondria in cardiomyocytes thus contributing to a better understanding of the pathogenesis of human congenital heart diseases [26].

4 Role of 5-HT_{2B} Receptor in Hematopoietic Lineage

Starting from the observations that serotonin may act as a growth factor for hematopoietic stem/progenitor cell and that 5-HT_{2B} receptor expression was identified in megakaryocytic cell lineage [27, 28] another aspect that has been investigated in 5-HT_{2B} receptor knockout mice is the blood composition in comparison with the wild type counterpart. Interestingly, a significant decrease in platelet number and an increase in circulating granulocyte/macrophage population have been found in adult 5-HT_{2B} receptor mutants compared to wild type mice. In accordance with these blood results, the bone marrow (BM) of 5-HT_{2B} receptor mutant mice showed alterations in cell composition such as a significant increase in granulocyte precursors

associated with a significant reduction in immature endothelial progenitor cells. Together, these observations support the idea that the lack of 5-HT$_{2B}$ receptor alters the differentiation of myeloid precursors for endothelial progenitor cells that have been proposed to participate to the development of the pulmonary arterial hypertension (PAH) [29, 30]. PAH is a very complex disease and many factors are involved in its pathophysiology characterized by progressive increase in pulmonary blood pressure associated with abnormal vascular proliferation and remodeling [31].

The role of 5-HT$_{2B}$ receptors in PAH has been shown to be restricted to bone-marrow where they contribute to the differentiation, proliferation and mobilization of endothelial progenitor cells [30]. These findings are in agreement with recent studies showing that serotonin and its receptors directly regulate hematopoietic stem and progenitor cells (HSPCs) during development [32]**.** In vitro experiments have shown that serotonin can enhance the generation of HPSCs, while in mice embryos serotonin is biosynthesized from embryonic day E10.5 in the aorta-gonad-mesonephros (AGM) region, a site of active hematopoiesis, and it is essential for the production and survival of HSPCs that then migrate to and expand in the fetal liver before colonizing the bone marrow around the birth. Although the 5-HT$_5$ receptor seems to mediate the action of serotonin in embryonic hematopoiesis via the AKT-Foxo1 signaling cascade [32], it should be reminded that by using in situ hybridization and immunocytochemical studies a region of 5-HT$_{2B}$ receptor gene and protein expression has been identified at the distal end of the mouse embryo (E10.5) corresponding to AGM, thus suggesting a contribution of this serotonin receptor to embryonic hematopoiesis as well [15]. It is interesting to note that both in mice and zebrafish serotonin is synthesized in the AGM region mainly by the tryptophan hydroxylase (TPH)2 enzyme revealing that TPH2 is expressed not only in the central nervous system (CNS), but also in the peripheral tissues [32, 33]. It has also been reported that in zebrafish embryos CNS-derived serotonin controls HSPCs production through the hypothalamic-pituitary-adrenal/interrenal (HPA/I) stress response axis via glucocorticoid receptor signaling, revealing that the CNS, as the master stress response regulator, enables the embryo to detect and react to various signals through fluctuations in HSPCs production [34].

5 Role of 5-HT$_{2B}$ Receptor in Osteogenesis

Several studies have suggested that serotonin is also involved in bone metabolism [35] and it has been shown that, among the various serotonin receptors, belonging to class 1 and 2, expressed on bone cells, the 5-HT$_{2B}$ receptor has a key role during osteogenesis. Increased 5-HT$_{2B}$ receptor expression has been reported during *in vitro* osteoblast differentiation and the 5-HT$_{2B}$ receptor knockout female mice are characterized by a reduced bone density that was significant from age 4 months and had intensified by 12 and 18 months. Cultured primary osteoblasts from the mutant mice exhibited reduced proliferation and decreased osteoblast recruitment from mesenchymal stem cells, moreover calcium incorporation was markedly reduced

after 5-HT_{2B} receptor depletion produced genetically or by pharmacological inactivation [36]. In this context, it is interesting to note that the 5-HT_{2B} receptor has been reported to regulate embryonic mouse hind limb mesenchymal cell proliferation [37] and to increase the osteogenic differentiation of a mesoblastic cell line [38]. On the whole, these studies have revealed that the 5-HT_{2B} receptor facilitates osteoprogenitors recruitment, proliferation and mineralization and that its absence leads to osteopenia that worsens with age. The 5-HT_{2B} receptor may thus be considered a main physiological mediator of serotonin in bone formation and, potentially, in the onset of osteoporosis also in aging women [39].

6 Role of 5-HT_{2B} Receptor in Neural Crest Cells (NCC)

6.1 5-HT_{2B} Receptor in Craniofacial Development

Xenopus laevis is a model system that strongly contributed to unveil new developmental roles of the 5-HT_{2B} receptor. Thanks to the availability of tools for manipulating embryos and gene functions in this organism, these studies have provided information about the role of the 5-HT_{2B} receptor signaling in two complex morphogenetic processes such as craniofacial and ocular development. Craniofacial morphogenesis is a complex developmental process requiring multiple and coordinated embryological events. In vertebrates, the viscerocranium is organized into a rostrocaudal bilateral series of segmented structures, the pharyngeal arches (PA), that are colonized by cranial NCCs, a multipotent cell population migrating in discrete streams from the mid-hindbrain segments of the dorsal neural tube. The NCC components of the pharyngeal arches give rise to skeletal cranial elements that undergo profound changes during evolution [40]. Cranial neural crest contribution in building the vertebrate head is so crucial that the acquisition of the NCC by protochordate ancestors is considered to be a turning point in the evolution of vertebrates [41]. In particular, the emergence of jawed vertebrates was accompanied by the acquisition of a buccal skeleton derived by a reshaping of the first arch into two distinct elements articulated by a jaw joint. This was one of the major novelties that shifted vertebrates from a passive filter feeding lifestyle to one of active predation [42]. A role of serotonin in craniofacial morphogenesis has been long suspected by studying the effects of in vitro exposure of mouse embryos to selective serotonin reuptake inhibitors or to receptor antagonists**.** In fact, in mouse embryos sites of serotonin reuptake and degradation are transiently expressed by epithelia of branchial arches probably protecting the underlying mesenchyme from exposure to inappropriate levels of serotonin, as indicated by patterns of cell death and cranial malformations caused by exposure of cultured embryos to selective serotonin reuptake inhibitors (SSRIs), like fluoxetine (Prozac) and sertraline (Zoloft) [14, 43, 44].

The finding of 5-HT_{2B} receptor mRNA expression in the pharyngeal arches of the mouse embryo at E9 [15, 20] as well as in the pharyngeal arches of tail bud Xenopus

embryos supported the hypothesis of a role of this receptor in mediating the serotonin action in the craniofacial developmental process [45]. A first hint for a possible role of the 5-HT$_{2B}$ receptor signaling in Xenopus craniofacial morphogenesis derived from gene gain of function experiments: 5-HT$_{2B}$ receptor overexpression resulted, in fact, in a morphological change of the craniofacial skeleton due to the formation of an ectopic cartilaginous element and by a reduction in the quadrate and subocular cartilages associated with altered muscular connectivity. Information on the origin of the ectopic cartilage derived from the analysis of the gene expression pattern of *bap*, a gene coding for a transcription factor expressed in the precursor cells of the jaw joint region [46]. In 5-HT$_{2B}$ receptor-overexpressing embryos, *bap* mRNA was ectopically expressed and resembled a mirror-image duplication of the wild-type *bap* mRNA expression site, suggesting that the ectopic cartilage derives, at least in part, from the first pharyngeal arch NCCs**.** As shown by homotypic NCC transplantation assay, the 5-HT$_{2B}$ receptor activity in NCCs was sufficient, in a cell-autonomous manner, to generate the ectopic cartilage. The overexpression of 5-HT$_{2B}$ receptor was also shown to influence the morphogenesis and/or patterning of the posterior arch NCCs by altering their dorsoventral positional information. Parallel 5-HT$_{2B}$ receptor loss of function experiments resulted in *bap* downregulation and in a fusion of a hypomorphic quadrate with the Meckel's cartilage into a single element, leading to the loss of the jaw joint. Moreover, Meckel's cartilage was altered in shape due to the lack of the cartilaginous muscular process normally located on its ventral aspect and necessary for the attachment of two muscles (hyoangularis and quadratoangularis) which failed to reach their target cartilage. The consequence of both the skeletal and the muscular abnormalities was a critical functional impairment of the mouth opening, leading to the death of the tadpole [17, 45].

These results clearly indicated that 5-HT$_{2B}$ receptor signaling is sufficient and necessary for the jaw joint formation and for shaping the mandibular arch skeletal elements by sustaining the *bap* expression. By using loss-of-function approaches it was also shown that PLC is the effector of the 5-HT$_{2B}$ receptor signaling in craniofacial development. 5-HT$_{2B}$ receptor transduction cascade via the PLC is shared with the endothelin 1 (Edn 1) pathway, the only signaling pathway known to be able to positively control *Bapx* expression [47] and possibly these two signaling pathways cooperate to sustain *bap* gene expression for a correct first arch morphogenesis. On the whole, these results show that misexpression of 5-HT$_{2B}$ receptor does not interfere with the induction or migration of NCCs but specifically influences the behavior of postmigratory pharyngeal arches NCCs. Thus the 5-HT$_{2B}$ receptor signaling can be added to the complex interactive networks of extrinsic factors that regulate mandibular arch morphogenesis contributing to one of the major vertebrate successes in evolution [48]. A role of the 5-HT$_{2B}$ receptor in pharyngeal arch morphogenesis has been confirmed in zebrafish, since its pharmacological inhibition also induced defects in visceral cranial morphogenesis similar to those observed in Xenopus [19].

Since the appearance of the jaw joint is an event at the origin of jawed vertebrates these findings support the hypothesis that this receptor is evolutionary very ancient and that it may be the common ancestor of the 5-HT$_2$ receptor subfamily as it has

been recently proposed on the basis of its genomic organization both in vertebrate and in invertebrate species [49]. Interestingly, the 5-HT_{2B} receptor gene is encoded within an intron of a large subunit of the proteasome *Psmd1* in humans, mice and rats and it has been estimated that this association appeared in pre-vertebrates and that 5-HT_2 receptor subtypes diversified approximately at the time period during which vertebrates diverged from invertebrates. In fact, while in the non-vertebrate chordate *Ciona* only one 5-HT_2-like receptor gene has been found, two whole genome duplication events have been proposed to arise in gnathostomes giving rise to a potential of four copies of the original ancestral prochordate gene. The first duplication event probably arose at the origin of the jawless vertebrates (Agnatha: lampreys and hagfish) where two 5-HT_2 receptors have been identified. The second whole genome duplication would have occurred at the divergence of the agnatans toward the jawed vertebrates (Gnathostomata). However, these duplication events were followed by loss of some redundant genes, so that in tetrapods only one copy of 5-HT_{2B}, $_{2C}$ and $_{2A}$ receptor gene is found. This loss of redundant genes appears to have not occurred in teleost such as zebrafish that displays two 5-HT_{2A} and 5-HT_{2C} receptor genes but only one copy of 5-HT_{2B} receptor having likely lost its duplicated copy. These findings indicate that a strong selective pressure seems to exist in order to keep a single copy of the 5-HT_{2B} receptor gene during evolution [49]. The results of 5-HT_{2B} receptor mRNA misexpression in Xenopus embryos strongly support this hypothesis since they clearly show that a proper 5-HT_{2B} receptor gene dosage is needed to construct a functional buccal skeleton that allows the tadpole to feed and survive.

These findings are not only relevant in terms of evolutionary biology but may have consequence in understanding of congenital defects, including human birth abnormalities. As an example, the oculo-auriculo-vertebral spectrum (OAVS) is a complex human craniofacial developmental disorder affecting the development of the structures derived from the first and the second branchial arches, with consequential maxillary, mandibular, and ear abnormalities. The phenotype in OAVS is variable and associated clinical features can involve the eye, brain, heart, kidneys and other organs and systems [50]. Although OAVS etiology is still poorly understood, a possible involvement of a dysregulation of the *BAPX1* transcription factor gene, has been suggested to occur in this syndrome [51]. It is interesting to note that the homologue of *BAPX* has been found to be regulated by the 5-HT_{2B} receptor signaling during Xenopus cranio-facial embryogenesis [45]. Understanding the role of 5-HT receptors in development could also be critical to identifying the possible effects of SSRIs on the human fetus, since these are the most commonly prescribed pharmacological treatments for depression as well as for a wide spectrum of other mood and behavioral disorders, in pregnant and lactating women [52]. SSRIs may pass to the fetus through the placenta and neonate through breast feeding, potentially exposing them to increased level of serotonin. SSRIs when used in pregnancy have been linked to cardiac and craniofacial malformations both in mice and humans [43, 52]**.** In a recent meta**-**analysis investigation, the SSRIs use in pregnant women during the first trimester has been found associated with an increased risk of cardiovascular malformations of infants including septal defects [53]. Significant

associations between fluoxetine (a member of SSRIs) use and the risk of facial dysmorphisms has also been reported [54]. Cardiovascular malformations and facial dysmorphism have been reported in two twins born to a woman who used paroxetine (SSRI) during pregnancy [55].

6.2 5-HT$_{2B}$ Receptor in Ocular Development

In Xenopus, 5-HT$_{2B}$ receptor abrogation was also found to cause ocular defects characterized by small and dorsalized eyes with a protruding lens and disorganized retinal cytoarchitecture. Evidence have been reported that a wide range of neurotransmitters and their receptors are present during early stage of vertebrate retina development and it has been suggested that neurotransmitters may be numbered among the extracellular signals contributing to the retinal development. In particular serotonin was predicted to be among the important players, as it is both produced and accumulated in the developing retina [56]. Moreover, studies carried out in mammals and in Xenopus demonstrated that the retina receives serotonergic afferents directly from serotonergic neurons located in the dorsal raphe nuclei denoted as serotonergic retinopetal projections [57]. In Xenopus, the 5-HT$_{2B}$ receptor is expressed in proliferating regions of the larval nervous system and in the ciliary marginal zone (CMZ) of the neural retina, which is the source of retinal stem cells in both larval and adult amphibian life [58]. Interestingly, 5-HT$_{2B}$ receptor mRNA appears in the retina in larval stages and this expression pattern matches the first appearance of serotonin-accumulating and -producing cells [59], suggesting that this receptor could be a candidate for mediating the serotonin action on retinal development. Loss of function experiments using morpholinos targeting the 5-HT$_{2B}$ receptor mRNA as well as ritanserin treatments resulted in the loss of the characteristic retinal laminar cytoarchitecture and in a reduction of the gene expression of the proliferation marker *Cyclin D1* in the CMZ, suggesting that the abrogation of 5-HT$_{2B}$ receptor function can influence retinal proliferation by downregulating cyclin D1. In 5-HT$_{2B}$ receptor morphants a normal expression of specific retinal cell differentiation markers was detected suggesting that the 5-HT$_{2B}$ receptor activity did not affect retinoblast differentiation while the number of apoptotic cells in the retina was strongly increased [17]. On the contrary, the upregulation of the 5-HT$_{2B}$ receptor activity did not influence apoptotic rate although a severe alteration in layering occurred in the retina [60]. These results suggest that misregulation of the 5-HT$_{2B}$ receptor activity causes alterations in the proliferation rate and survival of retinal precursors, resulting in abnormal retinal morphology, where lamination is severely compromised. It is of note that a precise coordination of retinal progenitor cell proliferation has been found to be essential for the formation of a functionally mature retina and that deregulated cell proliferation may lead to dysplasia, retinal degeneration or retinoblastoma [61]. Since the 5-HT$_{2B}$ receptor is also expressed in differentiated retinal cells, mainly in the inner nuclear layer and in the ganglionic cell layer, a post-mitotic role in differentiated cells (mainly ganglion cells) in

protecting them from cell death has been suggested for this receptor [18]. On the basis of these results it may be hypothesized that the 5-HT_{2B} receptor activity may play a role in the larval secondary neurogenesis by supporting cell proliferation and survival in the CNS as well. Further support to these findings came from *in vitro* studies showing that the 5-HT_{2B} receptor overexpression was found per se sufficient to promote cell proliferation in a neuroblastoma cell line [60]. These results are also in agreement with the observations of a role of 5-HT_{2B} receptor on survival and proliferation of cardiac myocytes during mouse development [23, 25].

Pharmacological and functional *in vivo* studies revealed other interesting aspects of the 5-HT_{2B} receptor function in Xenopus ocular morphogenesis. 5-HT_{2B} receptor morphants show an altered orientation, position and conformation of the eyes, a shorter optic nerve and a failure of the choroid fissure closure, also known as human disease named coloboma. The finding of 5-HT_{2B} receptor mRNA expression in the periocular mesenchyme (POM), that represents a key signaling center required for a correct eye morphogenesis, suggested that 5-HT_{2B} receptor signaling could be a mediator of the eye development [60]. POM is in fact a population of mesenchymal cells derived from both the cranial paraxial mesoderm and a cranial NCCs subpopulation that migrates in the first pharyngeal arch. POM cells migrate around the developing eye and then inside the optic fissure providing multiple cell lineages necessary for normal ocular development as well as essential signals for the patterning of ocular primordia including the morphogenetic extension of the optic stalk, the anterior eye segment development and the optic fissure closure [62]. The optic fissure is a ventral groove that forms during optic cup morphogenesis and once the components of the POM, that will give rise to the retinal vasculature, have entered and the retinal axons have exited, the choroid fissure fuses [63, 64]. Choroid fissure closure is a key event during eye development and failure of this process results in coloboma, a hereditary ocular malformation that can profoundly affects vision [65, 66]. Recent studies indicate that POM cells play a critical role in choroid fissure fusion [67, 68]. Experiments performed by homochronic and homotopic cranial NCCs transplantation assay in Xenopus embryos [45, 69] suggests that the 5-HT_{2B} receptor is not involved in the early migration of NCCs from the neural tube, but once these cells have migrated into the POM surrounding the eye, the autonomous action of the 5-HT_{2B} receptor would contribute to direct the final migration of NCCs into the choroid fissure [70, 71] supporting the view that the neural-crest derived POM plays a prevalent role in choroid fissure closure [68].

Retinoic acid (RA) signaling also contributes to ocular morphogenesis and choroid fissure fusion [72]. Previous works have suggested a possible functional relationship between 5-HT_{2B} receptor and RA signaling during embryogenesis [15, 20]. Bhasin and colleagues [37] established that even if 5-HT_{2B} receptor promoter contains several potential retinoids response elements, RA does not regulate 5-HT_{2B} receptor transcription but the 5-HT_{2B} receptor and RA promotes and inhibits proliferation in mouse frontonasal mass respectively, supporting the hypothesis that RA and 5-HT_{2B} receptor signals may act as opposing signals for common cellular mechanisms. In the developing eye RA is produced in the ventral retina and acts on the POM in a paracrine manner [73, 74]. In order to answer the question whether a

possible interaction occurs between 5-HT$_{2B}$ receptor and RA signaling in eye morphogenesis, the gene expression patterns of enzymes involved in RA metabolism have been analyzed in Xenopus 5-HT$_{2B}$ receptor morphants. Interestingly, the expression domain of *Raldh3,* the enzyme involved in the RA synthesis, is expanded in the ventral retina and a 6-time increase of its expression has been found by real time qPCR experiments. Accordingly, *Rdh10* and *Dhrs3*, that are target genes of negative feedback loops, change their expression levels in a manner compatible with an increase of RA [71]. Since dysregulation of the RA signaling in late phases of eye development has been implicated in ocular defects such as coloboma [74] these results suggest that the failure of optic fissure closure in 5-HT$_{2B}$ receptor Xenopus morphants could be due to an indirect action of 5-HT$_{2B}$ receptor signaling on POM NCCs by altering RA levels. Since 5-HT$_{2B}$ receptor mRNA expression has also been detected is the mouse embryonic optic vesicles [15] suggesting a conservation of this receptor signaling pathway in the eye developmental process, animal models could be useful in examining 5-HT$_{2B}$ receptor role in human ocular genetic disorders and identifying potential therapeutic targets [75].

6.3 5-HT$_{2B}$ Receptor in NCC Derivatives of the Gut

It is interesting to note that the 5-HT$_{2B}$ receptor mRNA was found to be expressed in migratory cranial neural crest cells in mice as well as in Xenopus and zebrafish and that the 5-HT$_{2B}$ receptor signaling has a crucial role in neural crest cell derivatives such as the pharyngeal arches and ocular morphogenesis. Another neural crest derivative where 5-HT$_{2B}$ receptor was found to be highly expressed and developmentally regulated are the progenitors of the enteric neurons of the gut. In vertebrates the gastrointestinal tract is the only organ whose function is controlled by its own intrinsic enteric nervous system (ENS) which is structurally and chemically very similar to CNS. During embryogenesis pluripotent migrating neural crest cells colonize the gut and the enteric microenvironment plays a role in determining enteric neuronal differentiation [76]. Since different types of enteric neurons arise in a reproducible sequential order, it has been suggested that early-developing enteric neurons, or their transmitters, might influence the fate of later-developing cells. In fact, all enteric serotonergic neurons, as well as the enterochromaffin cells (EC), which are the largest enteric source of serotonin, develop early so that serotonin could influence the fate of the late-developing *mash-1*- independent enteric neurons. mRNA encoding the 5-HT$_{2B}$ receptor is expressed in mouse fetal bowel (stomach and small and large intestine) and 5-HT$_{2B}$ immunoreactivity for the 5-HT$_{2B}$ receptor was found abundant in a subset of cells in primordial (E15-E16 embryonic stages) but not in mature myenteric ganglia. In vitro pharmacological experiments demonstrated that serotonin, by activating the 5-HT$_{2B}$ signaling pathway, affects the fate of a large subset of enteric neurons and that such action can be blocked by antagonizing 5-HT$_{2B}$ receptors [77]. The timing and the pattern of the expression of the 5-HT$_{2B}$ receptor in the mouse bowel, as well as the development of enteric

sources of serotonin, are compatible with the hypothesis that serotonin, by stimulating the 5-HT_{2B} receptor, expressed by crest-derived neuronal progenitors, acts as a growth factor to promote the development of the enteric neurons [77]. The enteric nervous system and the immune system are highly integrated in order to unite digestive function with protection from ingested environmental pathogens and the crosstalk between these two systems maintains homeostatic regulation of the gut activity. In particular, in response to microbial stimuli neuroendocrine cells can release serotonin whereas the activated immune cells can release various cytochines [6]. Interestingly, many innate immune receptors seem to affect the serotonergic system by interfering with the activity of the SERT that is a critical target for regulating extracellular serotonin levels [78]. These results have revealing for the first time a molecular mechanism involved in a putative relation between intestinal serotonergic and innate immune system. In addition to their implications in gut pathophysiology, these results are also relevant for the role of serotonin in enteric neurons development since the luminal content of the immature gut could influence the nature of the ENS thus affecting the number and composition of the neurons of the adult ENS.

6.4 5-HT_{2B} Receptor in NCC Derivatives of the Skin

5-HT_{2B} receptor transcription has also been found in melanocytes of normal and pathologic skin of both human and others animal models and it is interesting that also these cells origin from neural crests. Expression of the serotonin key biosynthetic enzyme TPH gene and protein has been detected in the human skin with predominance to normal and malignant melanocytes [79] and it has been shown that in cultured skin cells serotonin stimulates melanocytes proliferation via 5-HT_{2B} receptor [80]. A serotonin transport system that regulates serotonin availability has also been described in melanoma cells. These findings strongly support the full expression of a novel serotoninergic system in the skin that could participate not only in its normal physiological processes but also in cutaneous pathogenesis. In the skin serotonin may exert pro-edema, vasodilatory, proinflammatory and/or pruritogenic actions. In addition it has been discovered that mammalian skin, as well as melanoma cells, can transform serotonin into melatonin, as shown by the identification of the intermediate reaction products of the melatonin biosynthetic pathway [81]. Receptor gene expression for serotonin (mainly 5-HT_{2B} and 5-HT_7) and melatonin (MT1) have been detected in melanocytes, keratinocytes and fibroblasts and these receptors may represent primary targets for serotonin and melatonin signal transduction [81]. In human skin melatonin seems to have anti-proliferative effects on melanoma cells and protective effects against UV radiation induced-damage in keratinocytes, dermal fibroblasts and melanoma cells [82]. Melatonin has also been reported to exhibit tumorostatic properties in some rodent melanomas. Notably, the cutaneous melatoninergic system is organized to respond to continouos stimulation in contrast to pineal gland, which responds to discontinuous activation by the circadian clock. Overall the cutaneous serotoninergic/melatoninergic system by

modulating cell proliferation or viability could act by preserving the skin structural and functional integrity and maintaining its homeostasis [81]. The appearance of neural crest is considered a crucial event in the evolution of vertebrates and a cephalic melanocyte lineage similar to the neural crest has been identified in the tunicate *Ciona intestinalis* [83], supporting the hypothesis the 5-HT_{2B} receptor role as a regulator of neural crest cell probably appeared very early in vertebrate evolution contributing to the emergence of the main vertebrate morphological and functional innovations.

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Chapter 3
Stem Cells to Decipher the Physiological Roles of 5-HT_{2B} Receptor Signaling

Anne Baudry, Odile Kellermann, Jean-Marie Launay, Mathéa Piétri, and Benoit Schneider

Abbreviations

3′UTR	3′ untranslated region
AA	Arachidonic acid
DMP1	Dentin matrix protein
DSP	Dentin sialoprotein
dbcAMP	Dibutyryl cyclic AMP
DA	Dopamine
CCA	Cyclohexane carboxylic acid
COX	Cyclooxygenase
GPCR	G-protein coupled receptor
GPI	Glycosylphosphatidylinositol
IP3	Inositol-1,4,5-Trisphosphate
LT	Leukotriene
NO	Nitric oxide
NE	Norepinephrine
NET	NE transporter
PIPLC	Phosphatidylinositol-specific phospholipase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PrP^C	Cellular Prion protein
PKC	Protein kinase C
RAMP	Receptor activity-modifying protein

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ROS	Reactive oxygen species
5-HT	5-hydroxytryptamine
SSRIs	Selective Serotonin-reuptake inhibitors
SERT	Serotonin transporter
TACE ADAM17	TNFα converting enzyme
TNAP	Tissue-non-specific alkaline phosphatase
TNF	Tumor necrosis factor
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase

1 Introduction

In the central nervous system and at the periphery, the bioamine serotonin (5-hydroxytryptamine; 5-HT) is involved in a myriad of physiological processes including sleep, mood, memory, cognition, appetite, as well as cardiovascular, digestive and endocrine functions [1, 2]. In the brain, the source of 5-HT is the raphe nuclei and, at the periphery, 5-HT mainly originates from the intestinal enterochromaffin cells. 5-HT exerts its diverse roles through a constellation of serotonergic receptors. Since the 1970s, fifteen serotonergic receptor subtypes (5-HTR) have been evidenced and classified into seven families according to structural and pharmacological criteria as well as coupled signal transduction pathways (5-$HT_{1\text{-}7}$R) [3]. While the 5-HT_3R is a ionotropic receptor, all other 5-HT receptors are metabotropic receptors coupled to G proteins (GPCR) [4]. The intensity and duration of 5-HT receptor signaling depends on the reuptake of 5-HT exerted by the 5-HT membrane transporter, the SERT (serotonin transporter), a well-known target for selective serotonin-reuptake inhibitors (SSRIs) antidepressants such as fluoxetine (Prozac®) and paroxetine.

The 5-HT_{2B} receptor (5-HT_{2B}R) belongs to the 5-HT_2 receptor subfamily, which also includes the 5-HT_{2A}R and the 5-HT_{2C}R. The gene encoding the 5-HT_{2B}R was cloned from the mouse brain, rat stomach fundus, human liver and placenta between 1992 and 1994 [5–10]. The human 5-HT_{2B}R gene is located on chromosome 2 (2q36.3-2q37.1) [11]. During embryonic development, the 5-HT_{2B}R mRNA is detected in the mouse embryo as soon as day 8.5-9 post coitum in the heart primordia, the neural tube before its closure and in the first branchial arch at the origin of craniofacial derivatives such as maxilla, mandible and teeth [5, 12]. Of note, at this stage, the 5-HT_{2A}R and 5-HT_{2C}R mRNAs are also expressed at low levels [12]. As maternal 5-HT plays crucial trophic functions at early steps of murine development [13], this specific pattern of 5-HT_{2B}R expression just after the beginning of gastrulation indicates a key role of this receptor during the embryonic morphogenesis of the cardiovascular and craniofacial structures as well as the nervous system. This is supported by the heart and brain malformations observed in mouse embryos exposed to the 5-HT_2Rs inverse agonist ritanserin and in 5-HT_{2B}R knockout mice. Ritanserin-treated embryos exhibit an underdevelopment of the forebrain, hindbrain and

pharyngeal arches, heart defects and closure failure of the neural tube. This led to the assumption that the 5-HT_{2B}R plays a fundamental role in neural crest migration, cell proliferation and/or survival [12, 14]. Accordingly, depletion of 5-HT_{2B}R in mice leads to death of two third of the mutant population during the gestation or at birth due to severe cardiac defects [15].

In adult rodents, the 5-HT_{2B}R is mainly expressed at the periphery in the cardiovascular and gastrointestinal systems, and is also detectable at lower levels in the brain, notably in the cerebellum, hypothalamus, hippocampus, amygdala and raphe nuclei [6, 16–18]. During the past 25 years, the use of pharmacological drugs and the characterization of 5-$HT_{2B}R^{-/-}$ mice provided evidence that the 5-HT_{2B}R is involved in the control of a wide range of physiological functions, including cardiac, vascular, pulmonary, bone, gastrointestinal and cerebral functions (for reviews see [19–21] and references therein). Deregulation of 5-HT_{2B}R expression and signaling is associated with various pathological conditions such as fibrosis, pulmonary arterial hypertension and cancer [20, 22–25].

Over the last decades, the identification of the signaling pathways mobilized by the 5-HT_{2B}R and the downstream effectors led to a better understanding of the pathophysiological role of the 5-HT_{2B}R. The present review emphasizes the contribution of neuronal and mesodermal stem cell lines that endogenously express the 5-HT_{2B}R to grasp the signaling network driven by the 5-HT_{2B}R to ensure the onset of neuronal and/or bone functions and therefore homeostasis of bioaminergic neurons and mineralized tissues.

2 The 1C11 Neuronal Stem Cell Line to Dissect the Role of 5-HT_{2B}Rs in Bioaminergic Neurons

2.1 5-HT_{2B}Rs are Involved in the Neuronal Differentiation Program of 1C11 Stem Cells

Isolated in 1990 from murine multipotent cells, the 1C11 cell line behaves as a bipotential neuronal stem cell that has the intrinsic properties to differentiate upon appropriate induction into serotonergic ($1C11^{5\text{-HT}}$) and noradrenergic ($1C11^{NE}$) neuronal cells at a frequency of around 100% in a mutually exclusive manner (Fig. 3.1) [26–28].

Upon 4 days addition of dibutyryl cyclic AMP (dbcAMP) and cyclohexane carboxylic acid (CCA), there is a switch from the neuroepithelial precursor 1C11 cells to the $1C11^{5\text{-HT}}$ cells. The $1C11^{5\text{-HT}}$ neuronal cells have developed bipolar extensions, express neuron-associated markers (NCAM, synaptophysin ...) and acquired a complete serotonergic phenotype, i.e. the capacity to synthesize, store, catabolize and transport 5-HT [26, 28]. During the course of the serotonergic program, 1C11 cells acquire at definite times three 5-HT receptors (5-HT_{2B}R, 5-$HT_{1B/1D}$R and 5-HT_{2A}R) (Fig. 3.1) [29], whose expression was evidenced in vivo

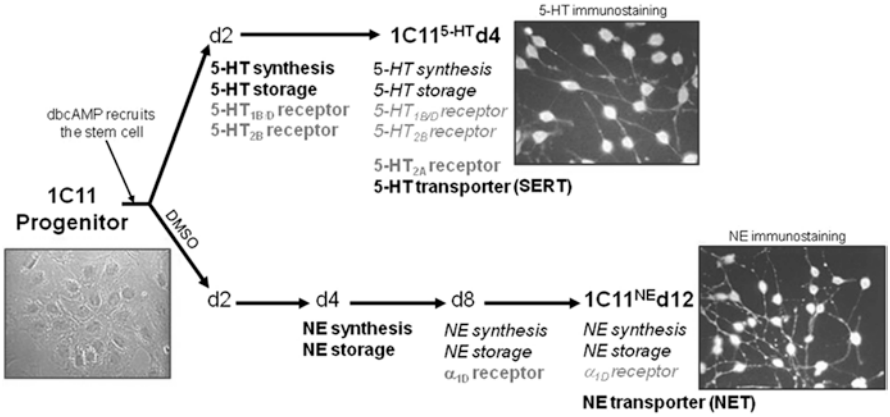

Fig. 3.1 The inducible 1C11 neuronal stem cells have the capacity to differentiate into serotonergic ($1C11^{5\text{-HT}}$) or noradrenergic ($1C11^{NE}$) neuronal cells in a mutually exclusive manner

in serotonergic neurons of the raphe nuclei [30–33]. Two days after the addition of the inducers, the 5-HT_{2B}R (Kd 21.9 nM, 2500 receptors/cells) and 5-$HT_{1B/1D}$R (Kd 0.53 nM, 1200 receptors/cell) are functionally expressed and their density remains constant along the differentiation [29]. At day 4, concomitantly with the onset of the SERT expression, a functional 5-HT_{2A}R (Kd 0.85 nM, 400 receptors/cell) is induced. Of note, at day 2, 1C11 cells start to synthesize and catabolize 5-HT. The presence of the 5-HT_{2B}R and 5-$HT_{1B/1D}$R at this stage renders 1C11 stem cells competent to respond to 5-HT during their differentiation program. Thus, 5-HT_{2B}R and/or 5-$HT_{1B/1D}$R acting as autoreceptors contribute to the onset of a complete neuronal phenotype [28]. Treatment of differentiating $1C11^{5\text{-HT}}$ cells with ritanserin from day 2 reduces the intensity of neuronal functions measured at the end of the serotonergic program.

In presence of dbcAMP, CCA and DMSO, the serotonergic differentiation is blocked. 1C11 progenitor cells then convert in 12 days into noradrenergic $1C11^{NE}$ cells, which express a complete catecholaminergic phenotype i.e. the capacity to synthesize, store, catabolize and transport norepinephrine (NE) (Fig. 3.1) [28]. Along the noradrenergic differentiation, 1C11 cells implement a unique α_{1D} adrenoreceptor at day 8 (Kd 1.1 nM, 2200 receptors/cell) and NE transporter (NET) at day 12. Of note, blocking the α_{1D} adrenoreceptor with an antagonist at day 8 avoids the onset of NET at day 12, suggesting that the α_{1D} receptor is necessary for the completion of the noradrenergic phenotype. The α_{1D} adrenoreceptor in the noradrenergic program seems to play a role similar to the 5-HT_{2B}R along the serotonergic differentiation.

The 1C11 neuronal stem cell line is thus a helpful paradigm to investigate the signaling and roles of bioaminergic receptors within an integrated serotonergic or noradrenergic context.

2.2 *The Onset of Serotonergic Functions Mainly Depends on Post-Transcriptional Controls*

In the 1C11 cell system, the transcriptional and translational control mechanisms that orchestrate the time-scheduled and effective implementation of neuronal functions during both serotonergic and noradrenergic programs remain elusive. In vivo, the differentiation of central 5-HT neurons depends on the transcription factors Nkx2-2, Lmx1b, Pet1 and Mash1 [34]. Strikingly, in the 1C11 cell line, the mRNAs encoding serotonergic and noradrenergic functions, such as neurotransmitter synthesis enzymes (tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH)), receptors (5-HTRs, α_{1D}R), transporters (SERT, NET), are expressed at the stem cell stage, but are dormant, and their levels do not vary along both differentiation programs. This indicates that post-transcriptional mechanisms are at work when 1C11 cells convert into either serotonergic or noradrenergic cells.

Concerning the SERT mRNA, variations of the poly(A) tail length at the 3′-end occur from day 1 of the serotonergic program. It increases and reaches its maximal length on day 3 corresponding to the beginning of the SERT translation [33]. The length of the poly(A) tail then decreases at day 4. As blocking the 5-HT_{2B}R by ritanserin reduces the intensity of serotonergic functions at day 4, whether 5-HT_{2B}Rs control the polyadenylation step of the SERT mRNA is an attractive hypothesis that deserves further investigations.

In 2010, we provided evidence, using fluoxetine, that a microRNA (miR-16) governs the onset of serotonergic functions. We identified miR-16 as a negative regulator of SERT translation through miR-16 interaction with the 3′ untranslated region (3′UTR) of SERT mRNA [35]. Higher levels of miR-16 were detected in $1C11^{NE}$ cells as compared to $1C11^{5\text{-}HT}$ cells. Such a disequilibrium in miR-16 levels had also been found in vivo in the noradrenergic neurons of the locus coeruleus *vs.* the serotonergic neurons of the raphe nuclei. In noradrenergic neurons, this high level of miR-16 prevents SERT expression as neutralization of miR-16 in $1C11^{NE}$ cells unlocks SERT translation and renders noradrenergic neurons competent to bind SSRI antidepressants. More surprisingly, miR-16 reduction in $1C11^{NE}$ cells also unlocks other serotonergic functions. In addition to SERT, the noradrenergic cells become capable to synthetize and catabolize 5-HT and express 5-HT_{2B}Rs. As no binding site for miR-16 was found in the 3′UTR of 5-HT_{2B}R mRNA, the molecular mechanisms by which miR-16 controls the onset of these serotonergic-specific functions are still unknown. In vivo, the injection of fluoxetine in mouse raphe provokes a rise in miR-16 levels in serotonergic neurons, thereby leading to a reduction of SERT expression [35], as observed in Prozac®-treated patients [36]. This SSRI antidepressant also induces the release of the neurotrophic factor S100β by serotonergic neurons, which mediates the action of fluoxetine on noradrenergic neurons [35]. S100β downregulates miR-16 in the locus coeruleus, which in turn, unlocks the expression of SERT as well as those of TPH and 5-HT_{2B}R. Noradrenergic neurons of the locus coeruleus thus become a new source of 5-HT in the brain. The key role of miR-16 in the antidepressant action of fluoxetine was reinforced by studies

performed in rodent models of depression. Increase of miR-16 in raphe or decrease of miR-16 in locus coeruleus improves depressive states similarly as fluoxetine [35, 37, 38]. Interestingly, 5-HT_{2B}R knockout mice display depressive-like behaviors and refractoriness to SSRI treatments [18, 39], indicating that 5-HT_{2B}R signaling contributes to antidepressant effects. Whether 5-HT_{2B}Rs implemented in noradrenergic neurons after fluoxetine/Prozac® treatment pilot the onset of 5-HT synthesis in the locus coeruleus at the origin of a new central source of 5-HT remains to be investigated.

2.3 *Identification of 5-HT_{2B}R Couplings Along the 1C11 Serotonergic Differentiation*

With the help of the 1C11 cell line, we evidenced that at day 2 of the serotonergic program, the 5-HT_{2B}R recruits the phospholipase A2 (PLA2)-arachidonic acid (AA) pathway [40] and the phospholipase C (PLC)-inositol-1,4,5-trisphosphate (IP3) pathway through the Gq proteins [27]. This latter coupling is lost at day 4 when $1C11^{5\text{-HT}}$ cells reach their terminal stage of differentiation [40]. The 5-HT_{2B}R also directly mobilizes the constitutive NO (cNOS) and the inductible NO (iNOS) synthases via the PDZ motif of its C-terminal extremity [41]. Interestingly, a special feature of the 5-HT_{2B}R among others 5-HT_2 receptors is to exhibit an intrinsic activity towards the couplings to PLC-IP3 [27], nitric oxide (NO) [41], PLA2-AA [40] and p21ras [42], which gives the receptor a major autocrine role during the serotonergic differentiation program. As described previously, the 5-$HT_{1B/1D}$R via G_i proteins is negatively coupled to adenylate cyclase. At day 4, the 5-HT_{2A}R is coupled to the PLC-IP3 and PLA2-AA cascades [29, 40]. We further demonstrated the occurrence of crosstalks between the three 5-HT receptor subtypes in $1C11^{5\text{-HT}}$ cells. The 5-HT_{2B}R exerts an inhibitory effect on the 5-$HT_{1B/D}$-mediated Gi coupling, which is relieved upon concomitant stimulation of 5-HT_{2A}R [40].

Through their couplings, all the three 5-HT autoreceptors play an essential role in modulating the intensities of 5-HT associated functions i.e. 5-HT synthesis, storage, catabolism and transport.

2.4 *The 5-HT_{2B}R Acts as a 5-HT Biosensor that Adjusts 5-HT Levels by Controlling SERT Functionality*

In vivo, plasmatic 5-HT concentration is tightly controlled to be maintained below 2 nM. When 1C11 cells are differentiated along the serotonergic pathway in a media containing high level of 5-HT (0.5–1 μM), all 5-HT functions i.e. synthesis, storage and transport are reduced compared to 1C11 cells differentiated in presence of low level of 5-HT (<1 nM) [28]. This reveals a negative feedback loop exerted by 5-HT

that tones down the intensity of neurotransmitter-associated functions. This negative feedback loop notably relies on the 5-HT_{2B}R that was shown to behave as a biosensor of external 5-HT concentration and to control 5-HT levels by acting on 5-HT transport (SERT) and 5-HT catabolism.

As previously mentioned, SERT is responsible for the reuptake of 5-HT across the plasma membrane and ensures a fine-tuned control of extracellular 5-HT concentrations. Deregulation of this precise control has been associated to diverse psychiatric diseases such as depression, anxiety and obsessive-compulsive disorders, characterized notably by reduced extracellular 5-HT levels in the brain. SSRI antidepressants used in clinics to combat depressive states block SERT function leading to a rise of central concentration of 5-HT. On the opposite, the serotonin syndrome relates to an excess of 5-HT in the central nervous system that may occur after therapeutic drug use causing adverse effects such as tremor, diarrhea, delirium, neuromuscular rigidity [43]. Exploiting the properties of the 1C11 neuronal stem cell, we firstly evidenced that the 5-HT_{2B}R governs SERT functionality (i.e. 5-HT transport and antidepressant recognition) through phosphorylation-type post-translational modifications (Fig. 3.2) [33]. At low 5-HT concentration (1–2 nM, as in in vivo conditions), the intrinsic 5-HT_{2B}R coupling to NO production governs SERT phosphorylation to basal level in $1C11^{5\text{-}HT}$ neuronal cells. All the SERT molecules are functional allowing a maximal 5-HT uptake and the binding of SSRI antidepressants. In excess of 5-HT, the agonist-dependent 5-HT_{2B}R-IP3/Protein kinase C (PKC) coupling promotes additional phosphorylation of SERT, which reduces 5-HT transport efficacy. This critical role of the 5-HT_{2B}R on SERT function was also evidenced in primary serotonergic neurons derived from the raphe. Another consequence of 5-HT_{2B}R-mediated hyperphosphorylation of neosynthesized SERT is the impairment of antidepressant recognition in $1C11^{5\text{-}HT}$ cells. In addition to SERT, the 5-HT_{2B}R via PKC signaling phosphorylates the energy source of the SERT, the Na/K ATPase electrogenic pump. This leads to a decrease of the Na/K ATPase pump activity that also contributes to the reduction of 5-HT transport. In serotonergic neurons, the autoreceptor 5-HT_{2B}R is thus a crucial regulator of 5-HT transport and sensitivity of SERT to antidepressants. As the Na/K ATPase may influence other transporters, it is likely that the 5-HT_{2B}R could contribute to the regulation of other yet to be identified neuronal and non-neuronal functions.

The 1C11 cell system has also been instrumental to identify a functional coupling between the 5-HT_{2B}R and the NADPH oxidase/TACE/Tumor necrosis factor (TNF) α that contributes to the control of 5-HT catabolism [44, 45]. By contrast to its PLC/IP3 and PLA2/AA couplings, the capacity of 5-HT_{2B}R to recruit NADPH oxidase and produce reactive oxygen species (ROS) is restricted to fully differentiated serotonergic $1C11^{5\text{-}HT}$ neuronal cells, suggesting that this bioaminergic receptor contributes to the maintenance of the redox equilibrium in mature serotonergic neurons only. This neurospecificity may relate to regulatory processes controlling partner assembly in lipid rafts during neuronal differentiation. Upon agonist stimulation of the 5-HT_{2B}R, NADPH oxidase-dependent ROS act as second message signals and govern the activation of the metalloproteinase TACE (TNFα converting enzyme, ADAM17). Activated TACE ensures the shedding of soluble TNFα, which,

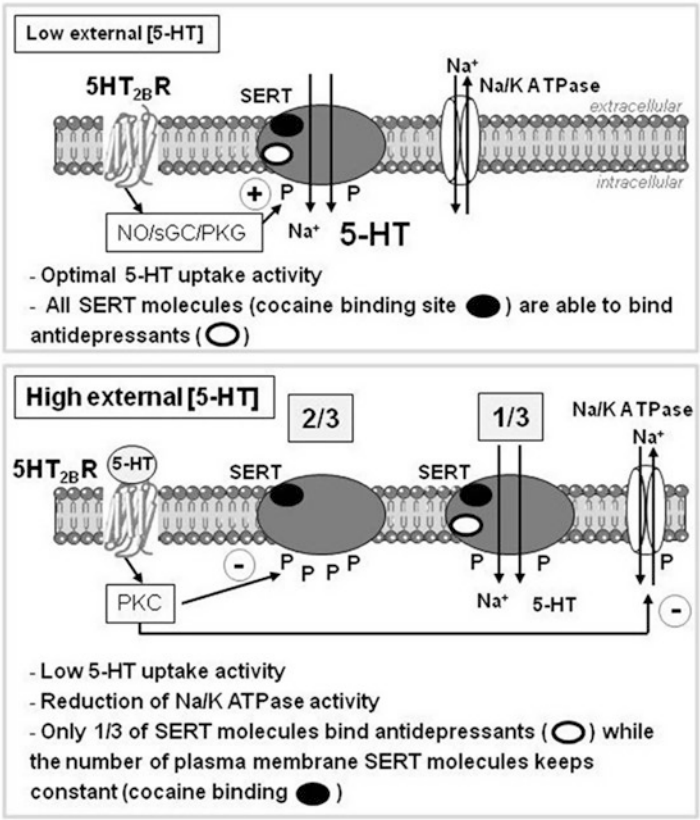

Fig. 3.2 By sensing external 5-HT levels the 5-HT_{2B}R controls SERT functionality in $1C11^{5\text{-HT}}$ neuronal cells through the phosphorylation of SERT and of its energy source, the Na/K ATPase

in turn, increases the degradation of 5-HT into 5-HIAA in $1C11^{5\text{-HT}}$ cells [44, 45]. The link between the 5-HT_{2B}R and the NADPH oxidase-TACE-TNFα pathway indicates that 5-HT_{2B} autoreceptors may play an important role in the fine-tuning of 5-HT-associated metabolism.

2.5 *5-HT_{2B}R Signaling is Modulated by the Cellular Prion Protein in Serotonergic 1C11 Cells*

As 5-HT_{2B}Rs exert a critical role as 5-HT biosensor and regulator of the intensities of 5-HT-associated functions, any modulators of 5-HT_{2B}R signaling will influence external 5-HT levels. To date, β-arrestin 2 was shown to be a negative regulator of 5-HT_{2B}R-mediated signaling by promoting its agonist-dependent internalization in LMTK-transfected cells [46]. In cardiac fibroblasts, it has been reported that the

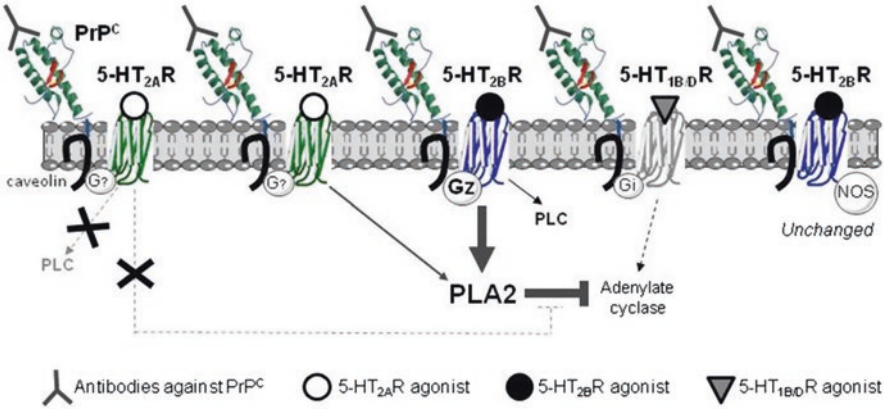

Fig. 3.3 PrP^C modulates 5-HT$_{2A,2B,1B/D}$ receptors signaling couplings in 1C11$^{5\text{-HT}}$ neuronal cells. In 1C11 serotonergic cells at day 4, co-stimulation of PrP^C with antibodies and 5-HTR with specific agonists impacts on G-dependent 5-HTR-couplings (see text for details)

5-HT$_{2B}$Rs interacts with angiotensin receptor AT1 to form heterodimeric complexes that impact cytokine release [47]. In 2005, we evidenced functional interactions between the cellular prion protein (PrP^C) and 5-HT receptors (Fig. 3.3) [48]. PrP^C, the normal isoform of the pathogenic scrapie prion protein (PrP^{Sc}) at the route of prion diseases, is a ubiquitous glycosylphosphatidylinositol (GPI)-anchored glycoprotein that exerts at the plasma membrane a role of receptor/co-receptor and is involved in signaling events [49]. Over the last decades, several signaling targets controlled by PrP^C have been identified such as PI3 kinase, PKC, NADPH oxydase, TACE, MAP kinases ERK1/2 [50]. A feature of the PrP^C-dependent neuronal response lies with the successful implementation of the signaling platform PrP^C/caveolin/Fyn kinase in cholesterol- and glycosphingolipid-rich lipid rafts of neurites [49]. Of note, bioaminergic receptors are also associated with such membrane microdomains. We demonstrated that in 1C11$^{5\text{-HT}}$ cells, the antibody-mediated-stimulation of PrP^C combined with the agonist-dependent stimulation of 5-HT receptors abolishes the 5-HT$_{2A}$R-PLC coupling, reduces the intensity of the 5-HT$_{1B/1D}$R negative coupling to adenylate cyclase, improves the efficacy of the 5-HT$_{2B}$R-PLA2 coupling and slightly restores the agonist-dependent 5-HT$_{2B}$R/PLC/IP3 coupling that is normally lost at day 4 of the serotonergic differentiation (Fig. 3.3) [48].

In these conditions, the 5-HT$_{2A}$R is no longer able to counterbalance the negative regulation of 5-HT$_{2B}$R on 5-HT$_{1B/1D}$R functions. The activation of PrP^C only disturbs receptor-couplings mobilizing G proteins. The 5-HT$_{2B}$R-NO coupling through the PDZ motif is not affected by PrP^C stimulation. Further, the impact of PrP^C on 5-HTR functionality is restricted to fully differentiated neurons, as at day 2 of the serotonergic program of 1C11 cells, PrP^C activation does not modify 5-HT$_{2B}$R and 5-HT$_{1B/1D}$R couplings. Therefore, it is unlikely that the modulation of cross-talks results from a direct interaction of PrP^C with 5-HTRs. Supporting this idea is the absence of any

effect of PrP^C stimulation on the binding affinity of agonists and antagonists for 5-HT receptors. Rather, PrP^C action on 5-HTR signaling depends on PrP^C coupling to caveolin in $1C11^{5\text{-HT}}$ cells as the immunosequestration of caveolin abrogates the modulatory effect of PrP^C on 5-HTRs. By mobilizing caveolin, PrP^C could change the dynamic of interaction between signaling partners in rafts and modify the stoechiometry of G proteins recruted in response to 5-HTR activation. PrP^C thus emerges as a physiological modulator of serotonergic functions, possibly acting as a receptor activity-modifying protein (RAMP) that interferes with the 5-HT autoreceptors-coupled signaling pathways.

It is widely recognized that the interaction of PrP^C with pathogenic prions PrP^{Sc} and its conversion into PrP^{Sc} is at the origin of prion diseases [50–52]. For 10 years, PrP^C is also known as a high affinity receptor for Aβ oligomers found in Alzheimer's disease that relays, at least in part, Aβ toxicity [53–57]. More recently, PrP^C was shown to interact with the pathological α synuclein involved in Parkinson's disease [58]. The role of PrP^C in the modulation of 5-HT receptor-mediated signaling thus raises the question of whether the corruption of PrP^C by PrP^{Sc}, Aβ or α synuclein would be at the root of deregulation of 5-HT receptor signaling pathways that could lead to a loss of homeostasis in serotonergic neurons and contribute to neurodegeneration. There are already evidences that prion infection alters 5-HT functions [59].

3 Role of the 5-HT_{2B} Receptor in Mineralized Tissues

3.1 The 5-HT_{2B}R Controls TNAP Activity During Osteogenic Differentiation of the C1 Mesodermic Stem Cells

The C1 tripotential mesoblastic cell line is endowed with the ability to recapitulate in vitro the spatio-temporal features of osteogenic, chondrogenic or adipogenic differentiations, under defined culture conditions [60]. The growth of C1 cells in three-dimensional nodules, mimicking the in vivo mesodermal condensation required for bone development and repair, is compulsory for cells to engage into osteogenic or chondrogenic programs. C1 aggregates, upon addition of β-glycerophosphate and ascorbate, start to produce an abundant extracellular matrix of type 1 collagen. The mineralization of this matrix initiates at day 7 by the deposition of hydroxyapatite crystals on collagen fibrils. At the end of the osteogenic differentiation (day 12), mature C1 osteocyte-like cells are embedded in a calcified matrix and stop to divide [61].

A functional 5-HT_{2B}R is induced at day 5 of the C1 osteogenic differentiation prior starting mineralization (Fig. 3.4) [62, 63]. From its implementation to the end stage of the osteogenic program, 5-HT_{2B}Rs are coupled to NOS/NO and PLA2/AA signaling pathways. Concerning AA metabolism, cyclooxygenase (COX) ensures the conversion of AA into prostaglandin PGE2 from day 5 to day 10. During the late phase of mineralization process, i.e. when osteoblasts are converted into

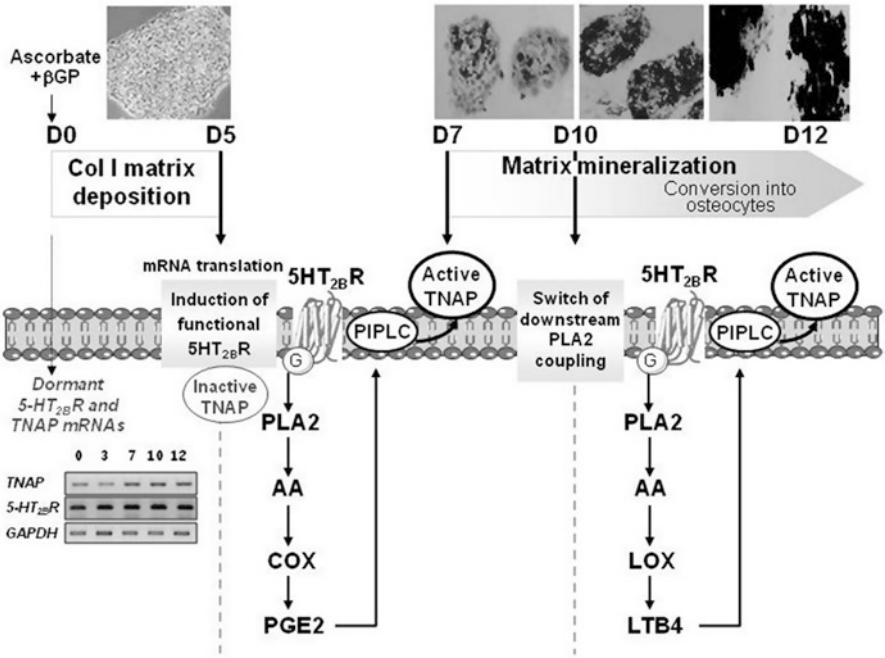

Fig. 3.4 The coupling of 5-HT$_{2B}$R to the PLA2/eicosanoids/PIPLC pathway controls TNAP activity during osteogenic differentiation of the C1 mesodermic stem cells

osteocyte-like cells, COX activity is quenched by yet unidentified molecular mechanisms. From day 10 to day 12, AA are then metabolized by lipoxygenase leading to leukotriene (LT)B4 synthesis. Both NO, PGE2 and LTB4 as intra- and/or intercellular second messengers, are well-known protagonists of bone homeostasis [64–66]. They ensure either bone-forming and/or bone-resorbing effects. The major role of the 5-HT$_{2B}$R and its downstream signalings in matrix mineralization was evidenced by a 25–40% reduction of Ca^{2+} incorporation within the bone matrix using ritanserin, an agonist inverse of 5-HT$_2$Rs, or upon inhibition of NOS and COX. The involvement of the 5-HT$_{2B}$R in osteogenesis is also supported in vivo by the skeleton damages observed in 5-HT$_{2B}$R knock-out mice. Indeed, female 5-HT$_{2B}$R$^{-/-}$ mice exhibit reduced bone mineral density with age, likely due to failure of osteoblast recruitment and/or proliferation [67].

We further demonstrated that 5-HT$_{2B}$Rs control bone mineralization by governing the activity of the GPI-anchored tissue-non-specific alkaline phosphatase (TNAP), a key player in bone formation (Fig. 3.4) [63]. As the 5-HT$_{2B}$R, TNAP is translated at day 5 of the C1 osteogenic program. Of note, both 5-HT$_{2B}$R and TNAP mRNAs are expressed as early as mesoblastic stem stage and their amounts remain unchanged throughout the 12 days of the differentiation. The signal events that switch on the translation of 5-HT$_{2B}$R and TNAP mRNAs in this specific timeframe are yet unknown. At day 5, TNAP is under an inactive form. This enzyme is activated from

the start of mineral deposition on day 7 until the end-stage of the C1 osteogenic program by phosphatidylinositol-specific phospholipase C (PIPLC)-dependent post-translational mechanisms governed by the 5-HT_{2B}R/PLA2/eicosanoids signaling. In agreement, primary calvarial osteoblasts derived from 5-$HT_{2B}R^{-/-}$ mice display defects in TNAP activity [63, 67]. Under physiological conditions, the 5-HT_{2B}R thus exerts a key role in mineralization processes. As PGE2 and LTB4 levels are altered in several bone diseases such as osteoporosis or rheumatoid arthritis [66], further investigations are needed to delineate whether deregulation of 5-HT_{2B}R signaling pathways would contribute to the development of these pathological situations.

3.2 Pulpal Stem Cell Lines to Reveal the Critical Role of Serotonergic (5-$HT_{2B,7}$R) and Dopaminergic ($D_{1,3}$R) Autoreceptors in Platelet-Mediated Tooth Repair

During mouse embryogenesis, 5-HT exerts a critical role in craniofacial and tooth development, notably through the regulation of neural crest cell proliferation and migration from rhombomeres 1 and 2 [68]. An implication of the 5-HT_{2B}R on dental tissues was considered because of its expression in day-9 mouse embryos in the neuroepithelium and the mesenchyme of the first branchial arch which is at the origin of craniofacial bones and tooth buds [12]. Of note, 5-$HT_{2B}R^{-/-}$ mice display structural alterations of teeth characterized by enhanced enamel porosities, thinner crystallites and disorganized rod structures [69, 70].

Fifteen years ago, clonal pulpal stem cell lines, such as the A4 and H8 cell lines, were derived from the first molar tooth germs of day 18 mouse embryos [71]. At the precursor state, A4 and H8 cells expressed odontogenic markers such as dentin matrix protein (DMP1), dentin sialoprotein (DSP), type 1 collagen and the LIM-domain homeobox transcription factors Lhx6 and Lhx7 which are present in the first branchial arch. In vitro, A4 cells are able to engage into odonto/osteogenic, chondrogenic and adipogenic differentiation and thus correspond to a multipotent mesoblastic stem cell. H8 cells whose potential of differentiation is limited to the odontogenic program, behaves as a monopotent precursor [72]. In vivo, both A4 and H8 pulpal stem cells favor an efficient tooth repair after implantation in injured mouse incisor or rat molar [72, 73].

Remarkably, although A4 and H8 cells are progenitors at different stages of commitment along the odontogenic lineage, both pulpal stem cells exhibit a dual serotonergic/dopaminergic phenotype (Fig. 3.5) [74]. A4 and H8 cells have the capacity to synthesize, catabolize, store and transport 5-HT and dopamine (DA). Further, they exhibit the same pattern of serotonergic and dopaminergic receptors at the plasma membrane. A4 and H8 cells expressed three serotonergic receptors, the 5-HT_{2B}R as well as the 5-HT_{1D}R and 5-HT_7R, and two dopaminergic receptors, the D_1 and D_3 subtypes. In pulpal stem cells, the 5-HT_{2B}R recruits the PLC/IP3, PLA2/

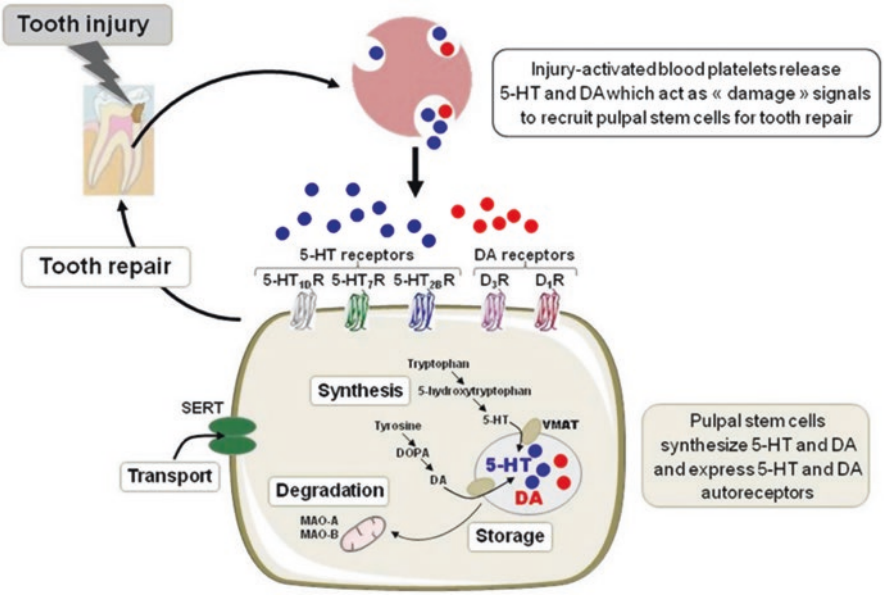

Fig. 3.5 5-HT and DA co-released by activated platelets upon tooth injury are "damage" signals that mobilize pulpal stem cells expressing 5-HT$_{2B,1D,7}$/D$_{1,3}$ receptors for tooth repair

AA as well as the NOS/NO pathways. The four other bioaminergic receptors (5-HT$_{1D,7}$Rs, D$_{1,3}$Rs) are functionally coupled to adenylate cyclase/AMPc signaling, two positively (5-HT$_7$R, D$_1$R) and two negatively (5-HT$_{1D}$R, D$_3$R). Such a specific repertoire of 5-HT/DA receptors renders odontogenic stem cells capable to respond to 5-HT and DA in an autocrine and/or paracrine manner. How 5-HT/DA receptors signaling pathways interplay physiologically to ensure pulpal stem cell homeostasis and/or the balance between proliferation/differentiation remains to be investigated.

In tooth, the dental pulp is a highly vascularized tissue [75]. Of note, peripheral systemic 5-HT and DA are predominately stored in dense granules of blood platelets [76]. We demonstrated in vivo that in pathological situations, such as tooth injury, expression of the special 5-HT/DA receptor register allows odontogenic stem cells to be mobilized by circulating 5-HT and DA released by lesion-activated platelets for tooth repair (Fig. 3.5) [74]. In wild-type rats, a natural reparative dentin is formed at the exposure site 1 month after pulp lesion of the first maxillary molar. In the opposite, in Fawn-hooded and reserpine-treated rats, two rat models exhibiting a deficit of bioamine storage in platelets, reparative dentin formation is impaired. Thus, upon tooth injury, platelet-released 5-HT/DA represent essential "damage" signals for the recruitment of pulpal stem cells expressing 5-HT/DA receptors for tooth repair. The role of 5-HT$_{2B,7}$Rs and D$_{1,3}$Rs in dentin repair is further supported by the impairment of tooth reparative processes observed in wild-type rats after addition of selective antagonists for each receptor in the damaged molar pulp. Many questions remain related to the signaling pathways and downstream targets

controlled by 5-$HT_{2B,7}$Rs and $D_{1,3}$Rs to orchestrate the mobilization /proliferation / differentiation of odontogenic stem cells for dental repair.

4 Conclusion

This review focuses on clonal neuronal and mesoblastic stem cell lines with homogeneous differentiation properties allowing the identification of several signaling pathways and key effectors governed by the 5-HT_{2B}R for the fine-tuned coordination of cell homeostasis and differentiation. These lineage progenitors used as test tubes also led to build pathophysiological scenarii implicating the 5-HT_{2B}R and provided some clues as to the events involved in disease-associated states such as depression, ectopic mineralization or tooth repair. Indeed, the 1C11 neuronal stem cell line was notably useful to reveal the key role of miR-16 as a relay of fluoxetine action both on raphe serotonergic neurons and noradrenergic neurons of the locus coeruleus. Fluoxetine, through miR-16-dependent unlocking of serotonergic functions (5-HT synthesis, 5-HT_{2B}R) in the locus coeruleus can activate a new source of 5-HT in the brain, a breakthrough in understanding the mode of action of SSRIs. In the dental field, although many challenges remain, highlighting the crucial role of the dialogue between pulp injury-dependent release of 5-HT/DA from platelets and 5-HTRs/DARs-expressing odontogenic stem cells for tooth repair could pave the road for novel therapeutic strategies.

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Chapter 4
The Serotonergic System in Hematopoiesis and Hematopoietic Disorders

Francine Côté and Tereza Coman

Abbreviations

AGM	Aorta-Gonad-Mesonephros
AML	Acute myeloid leukemia
CFU	Colony-forming unit
CMML	Chronic myelomonocytic leukemia
CMP	Common myeloid progenitor
DCs	Dendritic cells
EC	Endothelial cells
EPO	Erythropoietin
GVHD	Graft versus host disease
GVT	Graft versus tumor
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
INFγ	Interferon gamma
kyn	Kynurenine
LSCs	Leukemia stem cells
MDS	Myelodysplastic syndromes
MEP	Megakaryocyte-erythroid progenitor
NK	Natural killer
RBCs	Red blood cells

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SERT	Serotonin transporter
SSRIs	Selective serotonin reuptake inhibitors
TPH1	Tryptophan hydroxylase1
trp	Tryptophan

1 Introduction

Some notions regarding hematopoiesis will be briefly introduced before addressing the role played by components of the serotonergic system during hematopoiesis. Hematopoiesis is a continuous and complex process by which specialized mature blood cells are generated from hematopoietic stem cells (HSCs). Hematopoiesis comprises the production of (a) red blood cells (RBC) (erythropoiesis), (b) myeloid cells including lymphocytes, granulocytes, monocyte-macrophages-dendritic cells (leukopoiesis), and (c) platelets (thrombopoiesis). A definite structure, the bone marrow microenvironment, also known as the stem cell niche, is required to support differentiation, survival and proliferation of the hematopoietic system. Required as well, in a precise order and during a definite time frame, is a fine balanced of extracellular and intracellular signals namely growth factors and transcription factors activating specific genes [1].

Major advances to understand hematopoiesis were obtained from animal research, including mice, rats, guinea pigs and zebrafish [2]. Moreover, animal models that are impaired for a specific component of the serotonergic system led to the discovery of unexpected roles of serotonin in the mechanism of hematopoiesis. The study of embryonic organogenesis has shown that the hematopoietic system develops from extra medullar tissues through sequential waves [3]. In vertebrates, the primitive wave (embryonic day 7.5 -E7.5- in mouse, and day 21 in humans) occurs in the yolk sac where primitive erythrocytes, macrophages, megakaryocytes and erythroid–myeloid progenitors are generated from hemogenic angioblasts. Early yolk sac hematopoietic cells (up to E9.5) lack long-term repopulation activity [4]. During the pro-definitive and definitive stages, the hemogenic endothelium mainly situated in the Aorta-Gonad-Mesonephros (AGM) region will generate the first self-renewing HSCs and multipotent hematopoietic progenitors. Additional embryonic vascular sites, such as the vitelline/umbilical arteries, embryonic head, placenta, and yolk sac have also been shown to produce HSCs [5–8]. In mouse embryos, HSCs are detected in the AGM around 72 h after the first wave (E10.5-11.5), while in humans the definitive hematopoiesis starts at day 28. In mice, HSCs next migrate and colonize the fetal liver (E10-11) and spleen (E15), for maturation and expansion and subsequently migrate to the bone marrow (E17.5), which is the primary site of adult hematopoiesis. In humans, the HSCs colonize the bone marrow about 4 months after birth. During early childhood, hematopoiesis occurs in the red marrow of the bone and with age, it becomes restricted to the skull, sternum, ribs, vertebrae, and pelvis [9]. In the zebrafish embryo, the first wave occurs 12–24 h after fertilization and HSCs emerge at 30–72 h. Hematopoietic cells migrate through

the circulation to the caudal hematopoietic tissue, where they are matured and expanded, and subsequently migrate to the kidney, where they are maintained in adult stages [10].

Throughout history, hematopoiesis has been depicted as a hierarchically organized system, where multiple differentiated cell types are derived from a multipotent HSC, by successive branching [11, 12]. With the advent of xenotransplantation, robust in vitro clonal assays, and refined sorting strategies, significant progress toward defining the murine/human blood hierarchy has been made and extensively studied [13]. The concept of HSCs was first defined as cells in the bone marrow that could generate the complete blood cell system following bone marrow ablation. For that, HSCs have two essential properties; (a) to durably self-renew, (b) to generate daughter stem cells, and at the same time still contributing to the pool of differentiating cells. By contrast to HSCs, committed progenitors, such as the colony-forming unit (CFU) granulocyte, erythrocyte, macrophage, megakaryocyte, are unable to reconstitute the entire hematopoietic system when transplanted into an irradiated host; they have a limited self-renewal capacity and they show a restricted lineage differentiation potential.

Given the highly proliferative nature of HSCs (approximately 4–5 × 10^{11} cells arising daily in adult human), tight regulation of the bone marrow is ensured by a sophisticated interplay of numerous timely and spatially cell extrinsic signals, referred as the stem cell niche. For instance, stem cell factor and erythropoietin (EPO) are required for a myeloid progenitor cell to become an erythrocyte and thrombopoietin makes myeloid progenitor cells differentiate to megakaryocytes [14]. The initial concept of the niche has evolved from the early description to a complex network involving endothelial, osteal, neuronal, mesenchymal activity to regulate HSC localization, maintenance or differentiation [1, 15].

2 The Serotonergic System in Hematopoietic Tissues and Cells

Increasing evidences support a contribution of components of the serotonergic system to HSCs biology. Both central (CNS) and peripheral origins of serotonin (5-hydroxytryptamine or 5-HT), as well as local and distant actions have been proposed as hematopoietic regulators for the activities of self-renewal, proliferation, differentiation, and mobilization of hematopoietic stem cells. During zebrafish embryonic development, CNS-derived 5-HT has been shown to be needed for HSCs survival at the AGM site [16]. In their model, the authors proposed that Tph2 was induced by the stress sensor Hif1α and was the predominant regulator of HSCs expansion. This distant CNS-AGM axis seemed predominant, while the local activity of peripheral Tph1 and 5-HT receptors only transiently stimulated HSCs development.

In murine embryos, endothelial cells (EC) of the AGM region have been shown to express Tph2 and synthesized 5-HT with a local action on earliest HSCs survival, through a direct 5-HT_{5A} receptor signaling [17, 18]. Primary human $CD34^+$ HSC and hematopoietic progenitors express 5-HT_{1F} receptor suggesting a sensitivity to 5-HT signaling [19, 20]. In these studies, the neuronal origin of 5-HT was proposed.

With regard to a peripheral source of 5-HT, Kirouac and coll [21] have pointed out the importance of peripheral and locally secreted factors, including 5-HT, that could regulate inter- and intra-cellular interactions within the bone marrow, necessary to organize the tissue dynamics and cellular fates. These authors found that human HSC expansion was correlated with 5-HT_2 receptor signaling. The signal was delivered endogenously by hematopoietic cells and correlated with megakaryocyte development, expected by the authors to be the local source of 5-HT, rather than by niche-infiltrating neurons. Yang et al. [22] showed that 5-HT could expand early hematopoietic stem/progenitors in vitro and elicit multi-lineage progenitor commitment from human $CD34^+$ umbilical cord blood cells. These data were consistent with Kirouac findings [21] where 5-HT stimulation specifically enhanced primitive progenitors' output. In parallel, studies focusing on pulmonary hypertension identified circulating c-Kit+ progenitors that expressed the 5-HT_{2B} receptor in mice [23]. They further demonstrated ex vivo, in murine c-Kit^+ progenitors as well as in human cord blood cells ($CD34^+$) that 5-HT_{2B} receptors have a myeloid clonogenic potential and are involved in the hypoxia induced endothelial remodeling.

2.1 Megakaryocytes and Platelets

Megakaryopoiesis is a complex process during which megakaryocytes undergo a unique differentiation process to eventually produce platelets within the bone marrow [24]. Human megakaryocytes express receptors of the 5-HT_2 family (5-HT_{2A}, $_{2B}$, $_{2C}$) and 5-HT signaling has been involved in megakaryopoiesis and thrombopoiesis [25]. In vitro, the addition of 5-HT to M-07e megakaryocytic line cultures has been shown to diminish the mitochondrial membrane damage and caspase-3 expression, leading to an increased survival [25], similar to that observed with thrombopoietin, the most important hematopoietic cytokine for megakaryocytes development and platelet production. This anti-apoptotic effect was abrogated by ketanserin, a competitive antagonist of 5-HT_2 receptors [22, 26]. Consistent with this result, *Htr2b*$^{-/-}$ mice present a significant decrease in platelet number [23], suggesting the importance of 5-HT_2 receptor signaling in megakaryopoiesis. On human megakaryocytes, 5-HT_{2B} receptor activation had a dual and complementary action. On one hand, PI3K/Akt downstream signaling induced proliferation and survival of megakaryocytes and on another, induced Erk1/2 phosphorylation, F-Actin assembly and cytoskeleton reorganization leading to proplatelet release. A possible transactivation of PDGFR by 5-HT_{2B} receptor activation may further activate downstream PI3K/AKT signaling and reduce the apoptosis observed in megakaryocytes [27]. Importantly, while thrombopoietin has been showed critical for megakaryocytes growth and differentiation, 5-HT signaling through 5-HT_{2B} receptor may have a

necessary complementary action on late megakaryocytes survival and pro-platelets formation.

In the hematopoietic system, 5-HT has often been associated with platelets as the majority of peripheral 5-HT is stored in platelet dense granules (millimolar concentration) [28]. Beyond the activity of 5-HT storage, binding of 5-HT on 5-HT_{2A} receptor induces platelet reactivity, which by releasing dense granule contents, contribute to clotting, hemostasis and vascular tone [29, 30]. As such, *Tph1*$^{-/-}$ mice, deficient for peripheral 5-HT synthesis, have impaired thrombus formation [31] and the pharmacological inhibition of 5-HT_{2A} receptor attenuates recurrent coronary thrombosis in a canine model [32]. In patients with ischemic stroke, the administration of 5-HT_{2A} receptor antagonist showed a dose dependent decrease in platelet aggregation [33].

Although the 5-HT specific transporter (SERT) was shown to be expressed at different stages during megakaryopoiesis [34] its function was mostly described in platelets. 5-HT uptake in platelets is proportional to the amount of SERT expressed on the platelet membrane. The density of SERT is dynamically regulated by extracellular 5-HT concentration and vice-versa. For instance, increasing plasma 5-HT levels induce an initial rise in SERT expression, but when 5-HT levels increase, SERT expression drops below baseline [35].

2.2 Erythroid Progenitors and Red Blood Cell

RBCs production is a tightly regulated process that requires coordinated regulation of cell survival, proliferation and differentiation as well as iron availability. EPO is the main cytokine triggering erythropoiesis [14]. A key function for 5-HT in erythropoiesis and RBC survival was revealed through study of *Tph1*$^{-/-}$ mice as they present a phenotype of macrocytic anemia due to an ineffective erythropoiesis and reduced RBC survival [36, 37]. A complete 5-HT system was discovered in murine progenitor cells of the bone marrow. Tryptophan hydroxylase1 (TPH1), the key peripheral 5-HT synthesizing enzyme was selectively and highly expressed during erythropoiesis at the transition from CFU-erythroid to proerythroblast. TPH1 up-regulation, through the EPO/EPO receptor mediated STAT-5 signalling, lead to rapid 5-HT synthesis in proerythroblasts. Under EPO, 5-HT_{2A} and 5-HT_{2B} receptor genes were up-regulated, while SERT was down-regulated. Precisely, the 5-HT_{2A} receptor was required at the proerythroblast stage of erythroid differentiation while the 5-HT_{2B} receptor was significantly expressed at a later stage (basophilic erythroblasts). This allowed a local increase in 5-HT and 5-$HT_{2A/2B}$ receptor signaling necessary to cooperate with EPO signaling for the proliferation and survival of erythroid progenitors [38]. It was also shown, in vitro, that *Tph1*$^{-/-}$ progenitors' proliferation was rescued by 5-HT while 5-HT_{2A} and 5-HT_{2B} receptor antagonist led to a reduction in bone marrow erythroid precursors in wild-type mice [23]. This EPO-5-HT signaling cooperation was even more obvious during embryonic erythropoiesis. At the precise E13.5 embryonic life, where EPO signaling appears, a drastic mortality of *Tph1*$^{-/-}$ is observed, directly caused by a defect in fetal erythro-

poiesis. Serotonin loss, despite EPO signaling, induced a severe decrease in the erythroid mature compartment cellularity, while immature progenitors accumulated.

Interestingly, the 5-HT_{2B} receptor is also expressed in embryonic and adult cardiovascular tissues, including myocardial, endothelial and vascular smooth muscle cells. Targeted inactivation of the 5-HT_{2B} receptor gene results in lethality at various stages of development [39]. Of note, heterozygous 5-HT_{2B} receptor mutant intercrosses resulted in a frequency of homozygous pups in newborns of only 16.7%, significantly different from the expected 25% suggesting embryonic lethality. In parallel, in human cord blood cells, it was also demonstrated that TPH1, 5-HT_{2A}, and SERT-SLC6a4 genes were specifically expressed at the pro-erythroblast stage of differentiation [38]. At physiological dose of EPO, addition of 5-HT or of a 5-HT_{2A} receptor agonist significantly enhanced the expansion of $CD36^{+}$ cord blood cells revealing this highly conserved system where 5-HT stimulates proerythroblast proliferation and is necessary for EPO proliferative and anti-apoptotic effects.

Regarding circulating RBC, binding studies showed that no 5-HT receptors were expressed on isolated murine RBC, even though RBC lifespan was affected by 5-HT. Indeed 5-HT does not act by direct signaling, but as an anti-oxidant that preserves RBC from oxidative damage and hemolysis [36, 40]. Membrane senescence markers are increased in RBC from *Tph1*$^{-/-}$ mice and induce increased phagocytosis by macrophages [36]. A different macrophage phenotype in *Tph1*$^{-/-}$ mice is however not excluded and should be expected considering the work by the group of Angel Corbi [41] (see Chap. 6).

2.3 Immune Cells

Monocytes and macrophages are part of the innate immune system and can trigger and orientate natural killer (NK) and T cell responses. These cells have been showed to express various 5-HT receptors and SERT [41–44]. Controversial activity of 5-HT on monocytes/macrophages has been show, probably because of (1) a differential expression of 5-HT receptors at distinct states and in different tissues, (2) the effect is dose dependent and (3) the effect possibly synergizes with other cytokines activities. For instance, 5-HT_{1A} receptor signaling on murine monocytes has been showed to trigger NK activity while under high doses of Interferon gamma (INFγ), 5-HT_2 receptor signaling has been shown to suppress murine macrophage inflammatory cytokines production. In human macrophages, 5-HT_2-receptor signaling inhibits TNFα and IL-12 production but increases Il-10 and PGE2 [45]. Accordingly, it was shown that 5-HT_{2B} receptor signaling in murine monocytes skew their differentiation towards a M2-macrophage phenotype [46].

Dendritic cells (DCs) are professional antigen presenting cells essential to initiate and polarize T cell response. Different maturation states are obtained by various stimuli, in different locations [47] and are correlated to differential expression of 5-HT receptors. In inflammatory settings, 5-HT_{1B} and 5-$HT_{2A:2B}$ receptors have been shown to promote the chemotactic activity of 5-HT in vitro and immature murine DC migration towards lymph nodes in vivo [48].

Neutrophils, polymorphonuclear lymphocytes, are innate immune phagocytes that circulate and patrol the whole body in order to release lytic enzymes and generate oxidative burst, acting as first line effectors against pathogens. Beyond this activity, neutrophils are involved in inflammatory responses, macrophages recruitment, type M2 differentiation, tissue regeneration and angiogenesis [49]. Platelet derived or mast cell derived 5-HT have been described to promote neutrophils recruitment at the site of inflammation through 5-HT_2 receptor signaling [50] and enhance phagocytosis in animal models [51]. It is still under debate whether 5-HT action is playing a direct role or not on neutrophils [52]. In addition, several data from the literature remain controversial, as 5-HT has been also described as an antioxidant and a reactive oxygen species (ROS) scavenger depicting the complexity of the immune system [53, 54].

Eosinophils act to provide a defense response against parasitic infections and an immune response in allergy. However, given the wide range of interactions with other immune cells, platelets and ECs, eosinophils have been implicated in immune homeostasis, organ functional integrity and coagulation cascade [55]. These cells express many 5-HT receptors, among which 5-HT_{2A} receptor has been involved in trafficking and cytoskeletal reorganization in vitro [56], contributing to development of allergy induced asthma and inflammatory diseases in animal models [57, 58].

Lymphocytes are a heterogeneous population of adaptive cell immunity that orchestrate and execute many of immune response. T lymphocytes have been shown to express Tph1, SERT and MAO. The 5-HT_{1B}, 5-HT_{1A}, and 5-HT_{2A} receptors are expressed on activated T lymphocytes where 5-HT_{1A} receptors may have a role in proliferation. In a *Tph1*$^{-/-}$ mice model of collagen-induced arthritis, 5-HT deficiency was associated with a relative increase in Th17 lymphocytes in the lymph nodes, while Foxp3^{+} Treg cells were dampened suggesting that 5-HT could modulate the orientation of the inflammatory immune response [59]. In addition, activation of 5-HT_{2A} receptors in an allergic asthma mice model, has been showed to repress Th2 gene expression in activated T cells [60]. B lymphocytes were shown to express SERT, and 5-HT_{1A} and 5-HT_{3A} receptors but the implication of the serotonergic system in these cells remains to be understood [61, 62].

3 The Serotonergic System and the Hematopoietic Niche

Several cellular components that constitute the niche, may affect hematopoiesis. For instance, ECs are susceptible to local changes in 5-HT level. 5-HT has been shown to modulate systemic angiogenesis through EC migration and proliferation. Tph1, SERT, MAO and 5-$HT_{2A/2B}$ receptors have been described on peripheral endothelial and vascular smooth muscle cells in animal models [63, 64]. In addition, 5-HT has been shown to synergize with VEGF signaling, a known growth factor involved in angiogenesis. It is therefore tempting to hypothesize that EC within the HSCs niche may be equally affected. In 5-HT_{2B} receptor transfected mouse fibroblasts, 5-HT provided a mitogenic activity [27]. In addition, 5-HT was found to synergize with FGF and PDGF [22] that stimulate colony-forming unit fibroblasts formation and thus, enhance bone marrow microenvironment.

4 5-HT in Hematologic Diseases and Therapeutic Perspectives

4.1 Tryptophan, 5-HT and Anemia

Several published studies indicate a link between the serotonergic system and hematopoietic diseases. Anemia is one of the most prevalent symptoms in hematology and is associated with a high rate of morbidity and mortality. The etiologies of anemia are numerous and may result from an acquired defect in hematopoietic stem cells. One such example is low risk myelodysplastic syndromes (MDS), a clonal hematopoietic disease of the elderly, where anemia is the main symptom. MDS are characterized by dysplastic changes in the bone marrow especially a macrocytic anemia possibly due to an asynchronous proliferation and maturation of erythroblasts [65]. From this point of view, MDS could result from a cell cycle anomaly of erythroid progenitors inducing multinuclearity and enhanced intra-medullary cell death.

In a report, patients with MDS presented with lower tryptophan (trp) concentrations than healthy controls of similar age, and a significantly higher kynurenine (kyn) to trp ratio, suggesting enhanced trp degradation. The study implied that elevated levels of kynurenine could be involved in inhibiting hematopoietic progenitor amplification in patients with MDS related anemia [66] Enhanced tryptophan breakdown has also been observed in patients with chronic anemia, indicating that enhanced trp catabolism and as a consequence, trp depletion, might affect hematopoiesis [67]. Furthermore, to a link between anemia and trp metabolism toward the serotonergic pathway and with reference to 5-HT and its well-known effect on mood—low levels being associated with the onset of depression—a study published in 1998 suggested a relationship between low RBCs count and clinical response to fluoxetine in depressed elderly patients [68]. Along that line, the recent publication by Vulser and colleagues demonstrating a robust association between depression and anemia, brings further support to the emerging link between decreased levels of 5-HT and anemia [69]. At last, the results of a retrospective analysis showed that lower-risk MDS patients with anemia who received treatment with selective serotonin reuptake inhibitors (SSRIs) had significantly longer overall survival times than patients who did not receive SSRIs [70].

4.2 Hematological Malignancies

Given its mitogen and angiogenic properties, 5-HT has been associated with progression of numerous solid tumors [71]. 5-$HT_{1A/1B}$ receptors have been reported to be differentially expressed on leukemia stem cells (LSCs) as compared to their normal counterpart [72]. Inhibition of 5-HT_1 receptor in primary acute myeloid leukemia (AML) cells induced differentiation and apoptosis in vitro and in vivo, suggesting that 5-HT signaling might be targeted in AML. This effect was enhanced in combination with azacitidine and decitabine that are approved chemotherapy for AML [72].

Chronic myelomonocytic leukemia (CMML) is a heterogeneous clonal myelodysplastic/myeloproliferaive malignancy that may evolve toward AML. HSCs transplantation is the only curative treatment but is associated with high treatment related mortality. As observed in AML, 5-HT_1 receptor are expressed on CMML cells and inhibition of 5-HT_{1B} receptor reduced CMML primary cells survival in vitro [73]. In contrast to above mentioned action of 5-HT in AML and CMML, proapoptotic and antiproliferative effect of 5-HT has been suggested in various lymphomas. Fluoxetine, a commonly used SSRI in depressive disorders, is known to increase circulating levels of serotonin. Several lines of malignant B, Burkitt and T lymphomas showed a decrease in proliferation rate and viability when exposed in vitro to fluoxetine in a dose and time dependent manner [74–76]. The pro apoptotic effect of SSRI was independent of their ability to inhibit serotonin reuptake and an indirect immune-modulatory mechanism trough inflammatory cytokines and tumor infiltrating T cells was proposed in vivo [75, 77, 78]. Even though the mechanisms of action need to be further investigated, it is tempting to propose the use SSRI as potentials chemosensitizers.

4.3 Hematopoietic Stem Cell Transplantation (HSCT) and Allogeneic Immune Responses

Allogenic Hematopoietic Stem Cell Transplantation (HSCT) is a major therapeutic strategy for various non-malignant and malignant pathologies of the hematopoietic system (more than 80,000/year worldwide and 16,000 in Europe in 2018), in particular for acute leukemias, myeloproliferative or myelodysplastic syndromes, lymphomas, bone marrow failures and hemoglobinopathies. The efficacy of this treatment relies on the graft content of immune cells, especially T lymphocytes that recognize host tumor cells and induce a graft versus tumor (GVT) effect. However, T cells also recognize normal host tissues and induce the graft versus host disease (GVHD) that is source of high mortality. A major goal of HSCT is to obtain a persistent GVT without the deleterious GVHD. Controlling activation of Th1/Th17 lymphocytes and increasing Treg activity is one of the explored possibilities to decrease GVHD. As mentioned, uptake of 5-HT induces T cells proliferation and inflammatory cytokines secretion. SSRI, by inhibiting 5-HT uptake, may have an anti-proliferative effect [79–81]. Accordingly, fluoxetine was able to reduce circulating alloreactive T cells and GVHD symptoms and improve survival in a mice model of HSCT.

5 Conclusion

HSCs are present in most tissues including the hematopoietic system where they have the capacity to self-renew and to give rise to a defined set of mature differentiated progeny to maintain or repair their host tissue. During hematopoietic differentiation, the HSCs will give rise to progressively committed progenitors, including the common myeloid progenitor and the megakaryocyte-erythroid progenitors (Fig. 4.1). During the past years, several components of the serotonergic system

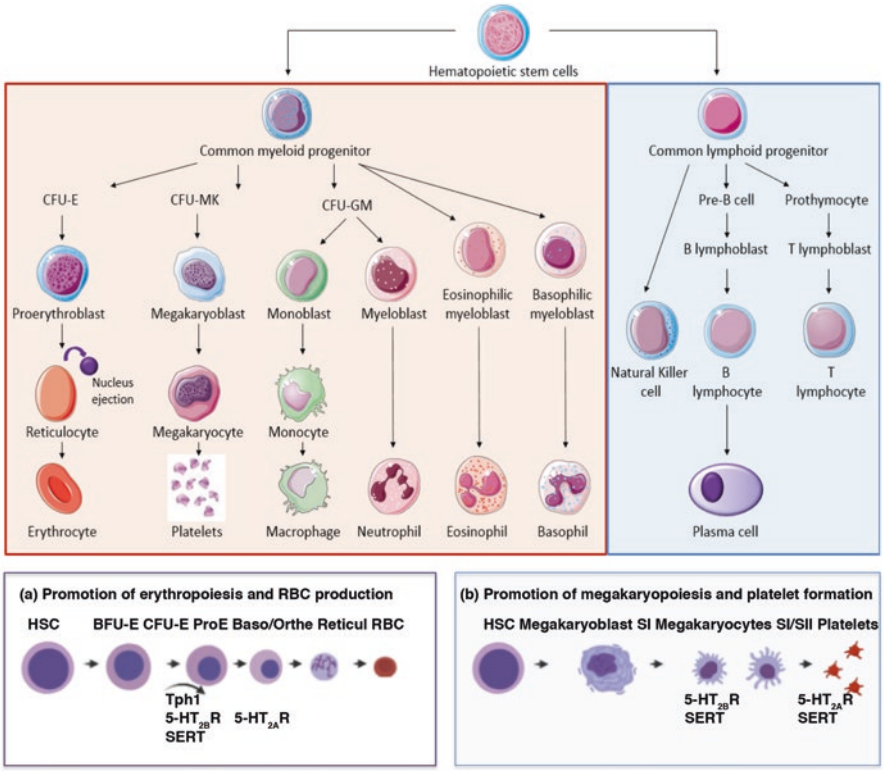

Fig. 4.1 Components of the serotonergic system in the hematopoietic system including the megakaryocyte-erythroid progenitors. (**a**) promotion of erythropoiesis and RBC production and (**b**) promotion of megakaryopoiesis and platelet formation. Adapted from [82]. Created in BioRender.com

have been characterized in the hematopoietic system. It is of interest that Tph1, the 5-HT_{2A} and 5-HT_{2B} receptors and SERT appear as hematopoietic regulators for the activities of the common myeloid and megakaryocyte-erythroid progenitors. The findings further support the concept of a local serotonergic network expressed in cells of the bone marrow. Taken together, serotonin alone or in combination with other factors of the hematopoietic environment might stimulate the cells to follow a certain path of differentiation. Further research on how this local system promotes HSCs and bone marrow proliferation to regulate key processes of normal and pathologic hematopoiesis from early development to adult life is currently investigated and should be of high value for regenerative medicine.

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Chapter 5
The 5-HT_{2B} Receptor, the Immune System and Neuroinflammation

Anne Roumier and Catherine Béchade

Abbreviations

5-HT	Serotonin
AGM	Aorta-gonad-mesonephros
ALS	Amyotrophic lateral sclerosis
DC	Dendritic cells
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinases
MHC	Major histocompatibility complex
PI3K	Phosphatidylinositol-3-OH kinase
SERT	Serotonin transporter
SOD	Superoxide dismutase
TLRs	Toll-like receptors
TPH	Tryptophan hydroxylase

1 Peripheral Serotonin and Immune Cells

Although serotonin is largely studied as a neurotransmitter, most of the body's serotonin is produced by enterochromaffin cells of the gut. These cells express tryptophan hydroxylase (TPH)1, the rate-limiting enzyme for serotonin production [1]. A second TPH isoform, TPH2, synthesizes serotonin in the central nervous system and gut enteric nerves [1]. Free serotonin concentrations in blood and tissues are normally kept in low nanomolar range. Immune cells, however, may also encounter serotonin released in the gut mucosa or from platelets which accumulate it via its transporter, SERT (*SLC6A4* gene) and store serotonin in granules expressing

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the vesicular monoamine transporter (VMAT)2. In turn, platelets can release this stored serotonin at sites of injury and inflammation. Platelet-derived serotonin is important for attracting innate immune cells such as neutrophils to inflamed tissue [2]. In addition to platelets, other immune cells such as dendritic cells (professional antigen-presenting cells) and B lymphocytes express SERT and thus, can accumulate and release serotonin. Interestingly, recent studies indicate that some immune cells are also capable of serotonin biosynthesis. Mast cells express TPH1 and levels of serotonin are elevated in patients with mastocytosis, who have greatly elevated mast cell numbers [3, 4]. Further, T lymphocytes [5–7] express TPH1 upon mitogen or T-cell receptor activation and can synthesize serotonin [8].

1.1 Serotonin, Immune Cells, and Hematopoiesis

Hematopoiesis includes different steps in which peripheral serotonin through 5-HT_{2B} receptors appears to be involved. In mice, hematopoietic stem cells initially derive from endothelial progenitor cells (hemangioblasts) present in blood islands on the yolk sac at E7.5. Hematopoiesis then occurs in the aorta-gonad-mesonephros (AGM) region at E10.5 in mice and moves to fetal liver at E12.5 before migrating to bone marrow around birth. Hematopoietic stem cells give rise to blood cells, including platelets, erythrocytes, myeloid and lymphoid cells. It has been proposed that serotonin via the 5-HT_{2B} receptor acts at hematopoietic stem cell progenitors directly or via modulation of the bone-marrow microenvironment [9]. Indeed, mice deficient in peripheral serotonin (*Tph1*$^{-/-}$) display morphological and cellular features reminiscent of ineffective erythropoiesis [10, 11]. In mouse embryos at E9, the expression of 5-HT_{2B} receptors was found to be located at the distal end of the embryo and along the neural tube similar to AGM [12]. Moreover, at E12.5, *Htr2b* gene knock out (*Htr2b*$^{-/-}$) embryos are smaller and paler than controls embryos with light pink liver [13] suggesting that the 5-HT_{2B} receptor may mediate some function of 5-HT in embryonic hematopoiesis. Later, in adult mice, the absence of 5-HT_{2B} receptors generates permanent changes in the composition of the blood and bone-marrow lineage, particularly in myeloid lineage and in endothelial cell progenitors. In particular, *Htr*$_{2B}$$^{-/-}$ mice displayed a significant increase in granulocyte precursors represented by CD11b+/GR+ cells and a significant reduction in immature endothelial progenitor cells corresponding to CD11b-/CD31+ population [14]. Serotonin participates also in pulmonary hypertension [15] and valvulopathies through the 5-HT_{2B} receptor [16]; we and others have shown that the initial functions of this receptor in these diseases are restricted to bone-marrow precursor cells [14, 15, 17–19] (see Chaps. 10–12). Thus, serotonin via 5-HT_{2B} receptors has the ability to regulate hematopoietic lineages including the myeloid lineage.

1.2 Serotonin and the Innate Immune Response

Innate immune system function involves monocytes, macrophages, dendritic cells, neutrophils, eosinophils, mast cells and natural killer cells that act immediately in the area of infection, leading to the destruction of pathogens. Innate immunity is primarily responsible for recognizing and eradicating "non-self" molecules presented by pathogens, and is therefore confined to recognizing extracellular pathogens (bacteria vs. viruses). This response is nonspecific with respect to particular invaders, but provides immediate host defense against pathogens via pattern recognition by toll-like receptors (TLRs). Pathogen-associated molecular patterns (e.g. peptidoglycans, bacterial lipopolysaccharides-LPS, double-stranded viral RNAs) bind TLRs on antigen-presenting cells, namely, dendritic cells and macrophages. These antigen-presenting cells then phagocytose pathogens and display pathogen-derived peptides via the major histocompatibility complex (MHC) on their cell surface for recognition by leukocytes of the "adaptive" immune system. They also secrete pro-inflammatory cytokines (e.g., IL1β, IL-6, TNFα), prostaglandins, and histamine, which further activate physiological responses, alerting the body to infection/invasion. Innate immunity also functions to communicate the presence of pathogens to cells involved in adaptive immune responses, see for review [20]. Several studies have shown the expression of the 5-HT$_{2B}$ receptor in cells from the innate immune system, including macrophages, mast and dendritic cells.

Gene expression profiling of macrophages revealed that 5-HT$_{2B}$ receptor mRNA is preferentially expressed by anti-inflammatory M2 (M-CSF) macrophages, whereas the 5-HT$_7$ receptor is expressed in both anti-inflammatory M2 and pro-inflammatory M1 (GM-CSF) macrophages [21]. The 5-HT$_{2B}$ receptor is also detected *in-vivo* in liver resident macrophages, the Kupffer cells, and in tumor-associated macrophages. Moreover, LPS, the archetypal macrophage-activating stimulus that signals via TLR4 upregulates the expression of 5-HT$_{2B}$ receptors 20-fold in murine thioglycollate-elicited peritoneal macrophages [22]. Serotonin was also shown to inhibit the LPS-induced release of pro-inflammatory cytokines, to upregulate expression of macrophage M2 polarization-associated genes and to reduce the expression of M1-associated genes. In addition, blockade of both 5-HT$_{2B}$ and 5-HT$_7$ receptors during *in-vitro* monocyte-to-macrophage differentiation preferentially alters the acquisition of M2 polarization markers [21] identifying both receptors as mediators of the anti-inflammatory skewing effect of serotonin (see Chap. 6).

Dendritic cells (DC) originating from hematopoietic stem cells are specialized in activating naive T lymphocytes to initiate primary immune responses. DCs are crucial players in immune defense by bridging innate and adaptive immune responses via their vast repertoire of pattern recognition receptors and antigen-presenting capability. Depending of their state of maturation, dendritic cells express different types of serotonin receptors: immature dendritic cells preferentially express 5-HT$_{1B}$, 5-HT$_{1E}$ and 5-HT$_{2B}$ receptors, while mature dendritic cells mostly express 5-HT$_4$ and 5-HT$_7$ receptors. However, the role of serotonin in DC function is poorly known.

It has been shown that serotonin is able to induce oriented migration in immature but not in LPS-matured dendritic cells via activation of 5-$HT_{1B/1E}$ and 5-$HT_{2A/2B}$ receptors [23]. This work has revealed a specific expression of 5-HT_{2B} receptor in a human dendritic subset cells: the inflammatory $CD1a^+$ monocyte-derived DCs [23]. In these cells, specific 5-HT_{2B} receptor activation inhibited the inflammatory response more specifically TLR2, TLR3, and TLR7/8-induced cytokine and chemokine expression (TNF-α, IL-6, IL-8, IP-10, IL-12) but not the type I interferon-β response. Moreover, specific activation of 5-HT_{2B} receptors also inhibits the polarization of $CD1a^+$ monocyte-derived DC-primed $CD4^+$ T cells towards inflammatory Th1 and Th17 effector lymphocytes, supporting the hypothesis of an immunomodulatory function of this receptor.

Mast cells have the capacity to synthesize and accumulate serotonin [3]. In turn, this stored serotonin can be released upon IgE cross-linking. Further, mast cells express mRNAs for multiple 5-HT receptors, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_6, and 5-HT_7 receptors [24]. Several roles of 5-HT in mast cell function have been shown. Serotonin can induce mast cell adherence to fibronectin and stimulates cell migration. Serotonin also attracts mast cells to sites of inflammation and its injection into the skin enhances the accumulation of mast cells. However, there is no evidence that serotonin degranulates mast cells or modulates their activation by IgE.

1.3 Serotonin and Adaptive Immunity

The response of the adaptive, or specific, immune system, occurs within hours of an infection and involves antigen-specific recognition and destruction of pathogens by T and B lymphocytes. Antigen presenting cells that migrate to lymph nodes will prime and educate T cells as to the nature of the pathogen. T cells then proliferate and differentiate into for example $CD4^+$ T-helper inflammatory cells (Th1) that activate macrophages, $CD4^+$ Th2 cells that aid antibody responses, or $CD8^+$ cytotoxic cells that target cells infected with intracellular microbes. The second component of adaptive immunity involves the contributions of B cells, located in lymph tissue, spleen and in the circulation. Upon stimulation, B cells become plasma cells (with or without the help of Th2 cells) that produce and secrete antibodies (immunoglobulins). Adaptive immunity is triggered at the immune synapse, where major histocompatibility complex peptides and co-stimulatory molecules expressed by dendritic cells are physically presented to T cells [20].

There is long standing evidence that serotonin can influence T-cell activation. Notably, mice treated with an irreversible inhibitor of TPH, para-chlorophenylalanine that leads to the depletion of intracellular stores of 5-HT exhibit a reduction in the number of activated T cells (IL-2Rα-CD25-positive) [6, 25], suggesting that serotonin contributes physiologically to T cell activation. Several 5-HT receptors:

5-HT_{1B}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_6 and 5-HT_7 receptors were found to be expressed in rat lymphoid tissues, *ex-vivo* isolated spleen, thymus, and peripheral blood lymphocytes. Additionally mitogen-stimulated spleen cells expressed mRNAs corresponding to the 5-HT_3 receptor [26]. A screen for serotonin receptor subtypes expressed in murine T cells revealed that naïve T cells selectively express 5-HT_7 receptors and that, upon T-cell activation, there is a strong upregulation of 5-HT_{1B} and 5-HT_{2A} receptors [6]. Interestingly, the 5-HT_{2B} receptor is found expressed in immature human T cells. Gene expression profiles during human $CD4^+$ T cell differentiation, identified the 5-HT_{2B} receptor with ten-fold greater expression in SP4 thymocytes over intrathymic T progenitor cells, double positive thymocytes (ITTP), naive T cells from cord blood (CB4) and naive T cells from adult blood (AB4) [27]. Further, 5-HT_{2B} receptors are differentially expressed among human T helper subsets cells. 5-HT_{2B} receptor expression was found to be increased in T helper cells cultured in the presence of cytokines promoting Th2 cell differentiation [28].

The role of serotonin in T cell immune response has also been reported *in-vivo* in chronic inflammatory diseases such as rheumatoid arthritis. Rheumatoid arthritis is a chronic disease that results in a disabling and painful condition as it progresses to destruction of the articular cartilage and ankylosis of the joints. Studies have shown that serotonin released by platelets can have a role in rheumatic diseases. In patients with rheumatoid arthritis, IL-1-containing platelet-derived vesicles called microparticles are abundant in arthritic joint fluid. Platelets also serve as a source of prostaglandins that contribute to synovial inflammation. Furthermore, serotonin released by platelets helps drive the persistent vascular permeability that characterizes the microvasculature of the inflamed synovium. Therefore, platelets have a distinct role in autoimmunity [29]. In mice, induction of arthritis triggers a robust increase in serotonin content in the paws combined with low inflammation. The absence of serotonin in arthritic *Tph1*$^{-/-}$ mice leads to a significant increase in osteoclast differentiation and bone resorption with an increase in IL-17 levels in the paws and in Th17 cells in the lymph nodes. In *ex-vivo* cultures of *Tph1*$^{-/-}$ splenocytes, addition of serotonin or agonists of the 5-HT_{2A} and 5-HT_{2B} receptors restored IL-17 secretion and Th17 cell differentiation supporting a direct action of 5-HT on lymphocytes through 5-HT_{2A} and 5-HT_{2B} receptors. Serotonin plays thus a fundamental role in arthritis through the regulation of the Th17/T-regulatory cell balance and osteoclastogenesis [30].

In conclusion, peripheral serotonin regulates inflammation and immunity by acting on 5-HT_{2B} receptors that are differentially expressed on immune cells, both in rodents and humans. Serotonin acts as a potent chemoattractant, recruiting innate immune cells to sites of inflammation, and also alters the production and release of cytokines and cell activation/proliferation.

2 Immune Cells in the Nervous System and 5-HT_{2B} Receptors

Serotonin has been widely involved in neuropsychiatric diseases, including depression, impulsivity or schizophrenia, yet mechanisms underlying their involvement in neurological diseases remains unclear. It is unknown whether and how serotonin might regulate brain inflammation, either acute, such as during medical illness, or chronic during neurodegenerative diseases. Increasing evidence suggests the involvement of microglia, the brain immune cells in neuropsychiatric disorders [31]. It has been established that serotonin through the 5-HT_{2B} receptor is an important regulator of microglia.

2.1 Microglia and 5-HT_{2B} Receptors

Microglia, the brain resident macrophages, are derived from yolk sac mesodermal hematopoietic stem cell precursors, primitive myeloid progenitors common to all myeloid cells. These progenitors enter the CNS through the blood stream at embryonic day E8.5 until the blood barrier is closed [32]. Microglia constitute 5–15% of adult brain cells depending on the brain regions and represent by far the largest population of immune cells in the brain. Pioneering *in-vivo* 2-photon imaging studies showed that, in resting physiological conditions, microglia have highly mobile and ramified processes, continuously monitoring the brain microenvironment [33, 34]. Upon local injury, microglia move rapidly their processes towards the lesion site. This chemotactic response can be mimicked by extracellular stimuli such as ATP [33]. It was reported that serotonin increases process motility of adult microglia in acute slices in response to acute injury. Moreover, serotonin enhances the chemotactic response of cultured microglia to ATP [35]. Analysis of the microglial phagocytic activity determined by the uptake of fluorescent microspheres reveals that serotonin application decreases phagocytic activity of amoeboid and cultured microglia [35]. Additionally, serotonin was able to induce a transient intracellular calcium (Ca^{2+}) signals in a subpopulation of cultured newborn and adult microglia [36] whereas most microglial cells responded to ATP [37].

Expression of microglial 5-HT_{2B}, 5-HT_{5A} and 5-HT_{7} receptors was shown by qPCR analysis of RNA isolated from primary cultured and from acutely isolated adult microglia [35]. The presence of functional 5-HT_{2B} receptors was confirmed by patch-clamp experiments in cultured and amoeboid microglia. Importantly, the 5-HT_{2B} receptor was found to be expressed in microglia as early as postnatal day 3 in mice [38]. We recently confirmed by 2-photon microscopy that microglial processes moved rapidly towards the source of serotonin via activation of the 5-HT_{2B} receptor [38, 39]. Moreover, serotonin was also shown to stimulates the release of exosomes in cultured primary microglia through the 5-HT_{2B}receptor activation [40].

2.2 Developmental Role of Serotonin on Microglia via the 5-HT$_{2B}$ Receptor

Modulation of microglial functions like phagocytosis and motility, by serotonin through the 5-HT$_{2B}$ receptor, is fundamental for the central nervous system since microglia can influence the balance of synaptogenesis and neuronal death during development and in pathology [31]. Microglial cells have been shown to modulate synapse formation, and thus to shape neuronal circuits [41]. The mouse retinogeniculate system is a classic model for studying developmental synapse elimination. During the first postnatal weeks, a massive synaptic pruning eliminates redundant synapses and evokes a segregated phenotype of the axonal projections from each retina [42]. A role for microglia in activity-dependent synaptic pruning has been demonstrated in the post-natal retinogeniculate system [43]. Eye-specific segregation of retinal projections in the thalamus also depends on an appropriate serotonin level [42, 44]. Thus, both microglia and serotonin participate in segregation of retinal projections. This *in-vivo* model of synaptic refinement during early brain development was used to investigate if serotonin through the 5-HT$_{2B}$ receptor participates to the maturation of the retinal projections to the thalamus. Analysis of retinal projections in the thalamus revealed that *Htr2b*$^{-/-}$ mice present anatomical alterations of the ipsilateral projecting area of retinal axons into the thalamus [38]. In addition, in primary microglia cultures from neonates, inflammatory markers are upregulated in *Htr2b*$^{-/-}$ microglia compared to *Htr2b*$^{+/+}$control [38]. These data support the possibility that microglia, serotonin, and 5-HT$_{2B}$ receptors participate in postnatal brain developmental events.

2.3 Neurodegenerative Diseases and 5-HT$_{2B}$ Receptors

Accumulating evidence suggests that the link between serotonin, microglial activation and neuroinflammation play a role in the pathogenesis of neurodegenerative diseases.

Alzheimer's Disease Soluble Aβ oligomers that accumulate with time in the brain are thought to initiate Alzheimer's disease and to trigger synapse failure and memory loss [45]. Recent data showed that exposure to Aβ oligomers triggers depressive-like behavior in mice and is associated with microglial activation, aberrant TNF-α signaling, and decreased brain serotonin levels. Conversely, serotonin blocked Aβ oligomers-induced microglial activation and elevated TNF-α production and release. Furthermore, inactivation or ablation of microglia blocked the increase in brain TNF-α and abolished depressive-like behavior induced by Aβ oligomers [46]. These findings support a key role of serotonin in preventing microglial activation in the context of Alzheimer's disease. In addition, Aβ oligomers failed to induce depressive-like behavior in TLR4-deficient mice and in mice harboring a

nonfunctional TLR4 variant in myeloid cells. These results establish that Aβ oligomers trigger depressive-like behavior via a double impact on brain serotonin levels and on microglial activation, revealing a cross-talk between brain innate immunity and serotonergic signaling as a key player in mood alterations in Alzheimer's disease [46]. It was independently reported that treatment with Aβ promoted neuronal-like differentiation of bone marrow-derived mesenchymal stem cells without toxic effects. The effect of Aβ was mediated by the 5-HT_{2B} receptor associated Gq-protein signaling, via activation of ERK1/2 signaling pathways [47]. Together, these findings support the hypothesis that serotonin may participate via 5-HT_{2B} receptors in preventing microglia-dependent activation and change in mood during Alzheimer's disease progression.

Amyotrophic Lateral Sclerosis Amyotrophic lateral sclerosis (ALS), also known as Charcot or Lou Gehrig's disease, is a disease that causes the death of motoneurons which control voluntary muscles. ALS is characterized by gradually worsening weakness due to muscles decreasing in size, leading to difficulty speaking, swallowing, and eventually breathing. Microglia are strongly activated in both ALS patients and animal models of ALS expressing mutant forms of superoxide dismutase (SOD)1 (mSOD1 mice). L Dupuis's laboratory has demonstrated that central serotonergic neurons degenerate during ALS in both patients and mSOD1 mice [48]. Although serotonergic neurons degenerate during ALS, the 5-HT_{2B}-receptor expression was found to be strongly increased at disease onset *i.e.* the time point of maximal microglial activation [49]. This upregulation was observed in both spinal cord and brainstem of different models of ALS, SOD1(G86R) and SOD1 (G37R) mice, expressing ALS-linked SOD1 mutations, and p150(G59S) mice, expressing a mutant form of dynactin linked to ALS cases and this upregulation was restricted to cells positive for CD11b, a marker of microglia (see Chap. 22).

Most importantly, ablation of 5-HT_{2B} receptors in transgenic ALS SOD1 (G86R) mice shortened survival of mSOD1 mice by 30% [49]. Disease onset was merely not changed, while disease progression after onset was potently accelerated. This resulted in increased degeneration of microglia, as evidenced by fragmentation of cellular processes that are positive for the microglial marker, Iba1 [49]. This complete loss of 5-HT_{2B} receptors was accompanied at end-point by decreased expression of key neuroinflammatory genes but also loss of expression of homeostatic microglial genes. Furthermore, the C allele of the SNP rs10199752 in *HTR2B* gene was associated with a longer survival in a large cohort of ALS patients. Patients carrying this allele showed increased 5-HT_{2B} receptor mRNA expression in spinal cord and displayed less pronounced degeneration of Iba1+ microglial cells than patients carrying two copies of the more common A allele. Thus, the 5-HT_{2B} receptor seems able to limit degeneration of spinal cord microglia, and slows disease progression in ALS [49]. In summary, the lack of 5-HT_{2B} receptors exacerbates ALS symptoms and progression of the disease. Considering the expression and upregulation of 5-HT_{2B} receptors in microglia, its protective effects likely results from a regulation of microglial functions.

3 Outlook and Prospects

In this review, we have summarized data showing that serotonin interacts with various immune cells and that these interactions may participate in myeloid cell maturation and inflammation through 5-HT_{2B} receptors. Serotonin via the 5-HT_{2B} receptors expressed by microglia may prevent microglia-dependent change in mood during Alzheimer's disease progression, and reduces ALS symptoms and microglial alterations. This set of data revealed that serotonin via 5-HT_{2B} receptors expressed in microglia participates in developmental/differentiation process, limits neuroinflammatory events and may have protective effect in neurodegenerative diseases.

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Chapter 6
5-HT_{2B} Receptor on Macrophages: What for?

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Abbreviations

ALS	Amyotrophic lateral sclerosis
AhR	Aryl hydrocarbon Receptor
DAMP	Danger-associated molecular patterns
EC	Enterochromaffin cells
GSEA	Gene Set Enrichment Analysis
GPCR	G-protein coupled receptor
IDO1	Indoleamine 2,3 dioxygenase
IFN	Interferon
MDD	Major depressive disorder
MCTO	Multicentric carpotarsal osteolysis
NK	Natural killer
NF-κB	Nuclear factor-κB
PAMP	Pathogen-associated molecular patterns
PI3K	Phosphatidylinositol-3 kinase
PLA2	Phospholipase A2
PLC	Phospholipase C
PDGFR	Platelet derived growth factor receptor
PAH	Pulmonary arterial hypertension
SSRI	Selective serotonin reuptake inhibitors
SERT	Serotonin transporter
TAM	Tumour-associated macrophage
TGF	Transforming growth factor
TPH-2	Tryptophan hydroxylase-2

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1 Peripheral Serotonin

The role of serotonin (5-hydroxytryptamine, 5-HT) as a neurotransmitter has been known for more than a century, although only a small percentage of the total body's serotonin is produced by Tryptophan Hydroxylase-2 (TPH-2)-expressing neurons. In exchange, 90% of body's serotonin is primarily produced by TPH-1-expressing enterochromaffin cells (EC) in the digestive tract [1]. This "peripheral serotonin" is then taken up and stored by platelets through the action of the serotonin transporter (SERT, encoded by *SLC6A4*), and subsequently released upon platelet activation [2–4]. In this manner, peripheral serotonin functions as a hormone, and platelets act as major regulators of plasma serotonin concentration, maintaining millimolar concentrations of serotonin in their dense granules while keeping plasma serotonin levels within the nanomolar range.

Details about the mode of action and pleiotropic effects of serotonin are still being gathered. Peripheral serotonin is involved in numerous physiologic processes (embryonic development, vasoconstriction, angiogenesis, temperature control, liver regeneration, mammary gland development, osteoclastogenesis, inflammation and fibrosis) [1, 2, 4, 5], and altered serotonin levels are linked to cardiovascular disorders, respiratory diseases and osteoporosis. The ability of peripheral serotonin to affect this diverse range of biological processes has inspired comments like "…. *at once implicated in virtually everything*, *but responsible for nothing*" (original [6]), "*Myriad effects of serotonin outside the central nervous system*" [7] or "*The problem ….. to determine what serotonin actually does for the gut has been that it is able to do too much*" [8]. The ability of serotonin to trigger epigenetic changes [9] and to modify histones (histone serotonylation) [10] might contribute to explain its widespread and tissue-specific effects.

Peripheral serotonin also contributes to haematopoiesis, and significantly modulates the effector functions of granulocytes, lymphocytes, monocytes, dendritic cells and tissue-resident macrophages. Since macrophages and dendritic cells are major drivers of immunity and inflammation, serotonin directly impinges on immune and inflammatory responses. Indeed, serotonin influences the development or resolution of inflammatory pathologies and is now known to contribute to Pulmonary Arterial Hypertension (PAH) [11, 12], atopic dermatitis [13], allergic asthma [14], systemic sclerosis [15, 16], inflammatory gut disorders [17–22], cancer angiogenesis [23], neuroendocrine neoplasms proliferation [24], collagen-induced arthritis [25] and amyotrophic lateral sclerosis [26]. In fact, and considering that neuronal serotonin participates in the control of anxiety and stress, it is not unexpected that peripheral serotonin functions as a stress sensor or adapter [27] and, consequently, controls and modulates immune and inflammatory responses. Regarding immune cells, the use of *Tph1*-defective mice has evidenced that mast cells and T-lymphocytes are capable of synthesizing and releasing serotonin, and that many other immune cell types might store, respond to and/or transport serotonin (T cells, macrophages, mast cells, dendritic cells and platelets).

All the physiologic and pathologic functions of brain and peripheral serotonin are mediated by seven families of serotonin receptors (5-HT$_1$ to 5-HT$_7$), encoded by 17 different genes that give rise to alternative splicing variants [4]. Except for 5-HT$_3$, all 5-HT receptors are G protein–coupled receptors (GPCRs) [4] whose intracellular domains activate various intracellular signalling cascades and lead to distinct functional outcomes [4]. The unique tissue distribution of each 5-HT receptor subclass partly explains the amplitude and tissue-specificity of serotonin activities, and serves to fine-tune physiological and cellular responses to serotonin, what has led to the suggestion that each 5-HT receptor type is linked to a specific biological response [4]. The availability of KO mice for *Tph1* and the various 5-HT receptor genes has provided definitive proof for the relevance of the actions exerted by peripheral serotonin on the skeleton (bone mass and osteoblast proliferation), cardiovascular system (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_7$ receptors), smooth muscle (5-HT$_{2A}$ receptor), liver [28], gastrointestinal tract (5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_{3A}$, 5-HT$_{3B}$, 5-HT$_4$, 5-HT$_7$ receptors), platelet aggregation (5-HT$_{2A}$ receptor), and even metabolic state (5-HT$_{2C}$, 5-HT$_6$ receptors) [29, 30].

2 Serotonin Effects on Inflammatory and Immune Responses

In adulthood, haematopoiesis takes place in the bone marrow and gives rise to all immune cell lineages. Although much remains to be learned about the underlying molecular mechanisms, it is now clear that peripheral serotonin functions as a growth factor for hematopoietic progenitor cells, as it contributes to proliferation and survival of erythroid progenitors [31] and megakaryocytes [32], increases the proliferation and mobility of G-CSF-mobilized CD34+ cells, enhances the amount of early and multilineage committed progenitors (erythroid, myeloid, megakaryocyte lineages), and promotes erythroid and myeloid lineage formation and monocyte/macrophage release [11]. The action of serotonin in haematopoiesis supports, in fact, the existence of "micro-serotonergic systems", where local serotonin production supersedes the hormonal function of gut-derived serotonin (reviewed in [27]).

2.1 Serotonin Effects on Immune Cells

A close relationship exists between serotonin, platelets and inflammation. The physiological concentration of plasmatic/vascular serotonin, maintained at nanomolar levels through uptake, storage or monoamine oxidase-mediated degradation [1], increases to micromolar levels around sites of inflammation, thrombus formation and fibrosis [5, 15, 33, 34], mainly due to its release from activated platelets exposed to pro-inflammatory stimuli [35, 36]. As an example, elevated plasma serotonin concentration is seen in cardiovascular disorders like coronary artery disease

and myocardial infarction [37, 38]. Enhanced levels of peripheral serotonin influence the development of immune and inflammatory responses by modifying the effector functions of lymphocytes, natural killer (NK) cells and monocyte/macrophages/dendritic cells. Indeed, platelet-derived serotonin is needed for leukocyte interaction with endothelial cells and trans-endothelial migration into sites of inflammation [39, 40], and directly affects endothelial cells to regulate leukocyte trafficking [41]. Serotonin also promotes survival and proliferation of mitogen-activated T and B lymphocytes [42, 43], enhances the proliferation, interferon (IFN)γ production and cytolytic function of NKs in vitro [44–46], and enhances migration towards inflammatory sites of mast cells [47], eosinophils [48, 49] and neutrophils [39]. Further, serotonin influences dendritic cell morphology, and controls dendritic cell chemotaxis towards draining lymph nodes [50] by controlling the expression of CCR7 [51], the chemokine receptor that guides migration towards lymph nodes for initiation of immune responses. In addition, serotonin can be shuttled from dendritic cells to T lymphocytes to modulate their activation, proliferation, and differentiation [52] (Table 6.1).

2.2 Serotonin Effects on Cytokine Production by Myeloid Cells

A timely coordinated disbalance between pro- and anti-inflammatory cytokines is absolutely required for promotion and suppression of immune responses as well as for initiation and resolution of inflammation. Stressing the importance of peripheral serotonin, the elevated levels of serotonin found at inflamed sites contribute to regulate cytokine production during inflammation promotion and resolution. Thus, serotonin increases IFNγ production of NK cells [46], but suppress IFNγ [56], TNF and IL-6 production [58, 60] in whole blood.

Dendritic cells are bone marrow-derived cells that exert potent antigen-presenting ability and display the unique capacity to initiate adaptive immune responses upon maturation. The modulatory actions of serotonin on dendritic cells depend on their state of functional maturation because immature and mature dendritic cells differ in their serotonin receptor profile. Immature dendritic cells (with homeostatic, protolerogenic and immunosuppressive functions) express *HTR1B*, *HTR1E* and *HTR2B* mRNA, while mature dendritic cells (characterized by a potent immune-stimulatory ability) express mRNA for 5-HT_4 and 5-HT_7 receptor [19, 66]. Serotonin alters the expression of IL-1β, IL-8, IL-12 and TNF only in mature monocyte-derived dendritic cells [66], while it modifies the production of IL-6, IL-10 and CCL22 in both immature and mature dendritic cells [50, 66]. All these effects on cytokine production drastically modify the T cell-polarizing ability of serotonin-treated dendritic cells [66].

Thus, and secondary to its ability to modulate IL-10, IL-12, CXCL10 (pro-Th1) and CCL22 (pro-Th2) production, serotonin favours the generation of Th2-type immune responses both in vivo and in vitro [50]. In line with this effect, serotonin reduces the expression of CD1a and co-stimulatory molecule expression in 5-HT_7

Table 6.1 Effects of serotonin on immune cells

Biological process	Cell type	Receptor	Reference
Phagocytosis and cytotoxicity	Macrophages/PMN		[200]
	Macrophages		[53]
	NK cells	5-HT1A	[201]
	Leukocytes		[202]
	Peritoneal macrophages	5-HT1A	[54]
Adhesion and migration	Mononuclear cells		[203]
	Monocytes	5-HT1A	[204]
	Eosinophils		[49]
	Mast cells		[47]
	Dendritic cells		[50]
	Microglia		[55]
	Neutrophils		[39]
	Eosinophils		[48]
	Dendritic cells	5-HT7	[51]
Oxidative burst	Macrophages		[205]
	Phagocytes		[206]
Cytokine production TNF IFNγ IL-1β IL-6 IL-10 TGFβ1 PGE2 IL-12 IL-17 CCL2	Monocytes		[207]
	NK cells		[46]
	Whole blood		[56]
	Vascular smooth muscle cells		[208]
	Aortic valve interstitial cells.		[57]
	LPS-treated PBMC	5-HT2A	[58]
	Microglial MC-3 cells	5-HT7	[59]
	Whole blood		[60]
	Monocyte/dendritic cells	5-HT1-7	[61]
	Macrophages		[62]
	Monocytes	5-HT1-7	[209]
	Macrophage-like synovial cells	5-HT2A-3	[63]
	Dendritic cells		[50]
	Alveolar macrophages	5-HT2C	[64]
	Dendritic cells		[22]
	Peritoneal macrophages	5-HT3	[210]
Proliferation survival	T lymphocytes	5-HT1A	[211]
	B lymphocytes	5-HT1A	[42]
	Lymphocytes	5-HT1A	[43]
	Megakaryocytes	5-HT2B	[32]
	Erythroid progenitors	5-HT2A	[31]

(continued)

Table 6.1 (continued)

Biological process	Cell type	Receptor	Reference
T cell priming T cell polarization	Macrophages		[65]
	Monocyte/dendritic cells	5-HT1-7	[61]
	Monocytes	5-HT1-7	[209]
	T lymphocytes	5-HT7	[52]
	Dendritic cells		[50]
	Dendritic cells		[22]
		5-HT2A-B	[25]
Radical scavenger	Mononuclear phagocytes		[212]
	Microglia		[213]
Immunomodulation Inflammation Fibrosis	Mononuclear cells		[36]
	Monocytes	5-HT3	[214]
	Aortic smooth muscle cells	5-HT2A	[215]
	Muscularis macrophages	5-HT4	[216]
	Whole blood	5-HT2A	[217]
	Macrophages	5-HT7	[16]
Serotonin uptake	Macrophages		[218]
	Dendritic cells		[219]
GPIbα shedding	Platelets	5-HT2A	[220]
Experimental colitis	Peritoneal macrophages		[21]
Bone resorption	Splenocyte/Osteoclast?	5-HT2A-B	[25]

receptor-expressing dendritic cells [61], reducing their antigen-presenting ability and skewing their naïve CD4 T cell-polarizing capacity [22, 50] (Table 6.1). Therefore, serotonin conditions the T cell-stimulatory capacity and cytokine-production ability of dendritic cells in a maturation-dependent manner.

3 Serotonin Effects on Macrophages

3.1 Macrophage Polarization

Like dendritic cells, macrophages participate in the coordination of innate and adaptive immune responses. Macrophages constitute a first line of defence against pathogens and harmful stimuli but also exhibit the functional plasticity required to initiate and resolve inflammatory processes, to maintain tissue homeostasis [67–69], to orchestrate tissue repair and angiogenesis, and to promote or inhibit tumour progression [70]. The functional versatility of tissue-resident macrophages (e.g., microglia, osteoclasts, Kupffer cells) arises from their various ontogenic origins (yolk sac, foetal liver, peripheral monocytes) and their capacity to variably differentiate in response to environmental and tissue-specific endogenous cues [71–79]. The existence of tissue-specific macrophage functions is exemplified by the distinct

primary functions of osteoclasts (bone macrophages with potent bone-degrading functions), microglia (brain macrophages that contribute to neural circuitry development, synaptic pruning, and modulation of angiogenesis and fluid balance) [80, 81], and Kupffer cells (liver macrophages primarily specialized in scavenging) [82]. On top of this heterogeneity, macrophages can acquire a wide variety of activation ("polarization") states when exposed to pathogen-associated molecular patterns (PAMP), danger-associated molecular patterns (DAMP), cytokines, chemokines and growth factors. The acquisition of a discrete macrophage polarization state ultimately depends on the integration of all the intracellular signalling pathways and transcription factors activated by the surrounding PAMP, DAMP, cytokines and growth factors [83], but also on their metabolic adaptation [84] and their developmental origin [71–74]. Thus, the huge plasticity of macrophage polarization relies on a combination of transcriptional, epigenetic, post-transcriptional and metabolic mechanisms, whose combination allows the acquisition of almost unlimited states of polarization (reviewed in [68, 79, 85]). In addition, and like other cells of the innate immunity, macrophages exhibit immunological memory (now coined as "trained immunity" or "innate immune memory"), by which they display long-term changes in their functional programs in response to stimulation [86]. These changes enable macrophages to display enhanced (*training*) or reduced (*tolerance*) responsiveness towards any secondary stimulation [86]. Macrophage immune memory ultimately depends on metabolic and epigenetic reprogramming, with histone modifications playing a central role in the process [86], and is now known to operate at the level of myeloid progenitors [87, 88].

Illustrating the importance of their functional plasticity, deregulated macrophage polarization triggers or contributes to chronic inflammatory diseases like cancer, atherosclerosis, rheumatoid arthritis and obesity [81, 89]. Excessive pro-inflammatory polarization in adipose tissue is linked to metabolic disease [90, 91], whereas uncontrolled anti-inflammatory/reparative polarization gives rise to fibrosis [89] and protects tumours from immune-surveillance [92, 93]. Because of this, "macrophage reprogramming" has become an attractive therapeutic alternative for inflammatory pathologies [94, 95]. As an approach to the analysis of macrophage heterogeneity, "an operationally useful but simplified conceptual framework" has been established by which two extremes of the macrophage polarization spectrum have been defined: macrophages with potent bactericidal, anti-tumoral or pro-inflammatory activities are commonly referred to as "M1 macrophages", whereas "M2 macrophages" refers to macrophages with pro-tumoral, immunosuppressive, pro-angiogenic or anti-inflammatory activities [79]. In general, pro-inflammatory/M1 macrophages are characterized by a TNF^{high}, IL-12^{high}, IL-6^{high}, IL-23^{high} and IL-10^{low} cytokine profile and potent production of reactive oxygen and nitrogen intermediates, whereas pro-fibrotic/anti-inflammatory/M2 macrophages usually exhibit an IL-10^{high}, IL-12^{low}, IL-23^{low} and TNF^{low} phenotype [70]. From the transcriptional point of view, STAT1, IRF5 and Nuclear factor-κB (NF-κB) (p65/p50) are major transcriptional drivers of the pro-inflammatory/M1 polarization, whereas diverse M2-type polarization states can be acquired through the action of NFκB (p50/p50), MAF/MAFB, STAT3, CREB, PPARγ and STAT6 (reviewed in [68, 79,

85]). At this point, it is worth mentioning that numerous studies have emphasized "difficulties of mouse-to-human extrapolation" regarding human macrophage polarization markers [76]: although the functions attributed to M1 and M2 macrophages are usually common in both species, only 26% of the polarization-associated genes are conserved between monocyte-derived human macrophages and bone marrow-derived mouse macrophages [96].

3.2 *Influence of Serotonin Receptors on Macrophage Effector Functions*

Macrophages are equipped with a large array of serotonin receptors whose presence is dependent on the functional state of macrophages. Human monocytes display *HTR1E*, *HTR2A*, *HTR3*, *HTR4* and *HTR7* mRNA, and monocyte-derived macrophages express mRNA for 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, and 5-HT_7 receptors [16, 97, 98]. However, and like dendritic cells, the range of serotonin receptors varies with the macrophage origin. For example, mouse microglia contain high amounts of *Htr2b* and much lower levels of *Htr1f* and *Htr2a* [99], functional expression of *Htr7* is found in a microglial cell line [59], and monocyte-derived human macrophages exhibit different serotonin receptor profiles depending on whether they are primed by M-CSF (*HTR2B*$^+$, *HTR7*$^+$) or GM-CSF (very low levels of all serotonin receptor subtypes) [98]. In addition, and at least in the case of human macrophages, activation by PAMPs like LPS results in an almost complete loss of the expression of the predominant serotonin receptors, namely 5-HT_{2B} and 5-HT_7 receptors [98], evidencing that changes in the profile of serotonin receptor expression take place during macrophage activation.

As stated above, macrophages are extremely sensitive to the tissue environment and promote and coordinate the initiation and the resolution of inflammatory responses, both of which are associated with changes in serotonin levels. In fact, serotonin modifies a plethora of macrophage functions, exerting a net anti-inflammatory effect. In monocytes, serotonin increases the production of LPS-stimulated IL-1β and IL-8, but it decreases TNF levels [97]. In the case of macrophages, serotonin appears necessary for optimal macrophage accessory function [65], and physiological concentrations of serotonin suppress IFNγ induced MHC class II expression [53, 100, 101], and increase tumour-associated macrophage (TAM)-mediated angiogenesis by reducing MMP12 and angiostatin expression [23]. In addition, serotonin increases microglia motility [55], alters phagocytosis [54, 55], decreases the LPS-induced production of TNF and IL-6 in murine peritoneal macrophages [102] and up-regulates the expression of CCL2 in 5-HT_{2C} receptor-expressing alveolar macrophages [64]. Similarly, serotonin stimulation leads to overexpression of anti-inflammatory PGE2 [62, 63], enhances LPS-stimulated IL-10 production and reduces LPS-induced TNF secretion in human alveolar macrophages and macrophage-like synovial cells [62] (Table 6.1). All these

changes reveal a clear anti-inflammatory action of serotonin on macrophages. These functional effects are compatible with the fact that exposure of human macrophages to serotonin leads to preferential activation of ERK and CREB [98], which are directly linked to anti-inflammatory responses [103, 104], and with the ability of serotonin to inhibit LPS-induced STAT1 activation [16]. Since serotonin causes epigenetic modifications [9] and even direct histone serotonylation [10], and considering the importance of epigenetic mechanisms for the establishment of the macrophage memory (*training*) [86], it is reasonable to assume that serotonin exposure might condition ("educate") macrophages for subsequent stimulation, and that this effect might also take place in bone marrow myeloid progenitors, on which serotonin is already known to have an effect [11].

The importance of the serotonin influence on macrophage effector functions is also supported by gene ontology analysis of the transcriptome of serotonin-treated cells. Gene Set Enrichment Analysis (GSEA) of the transcriptome of human macrophages exposed to 10μM serotonin for 6 hours not only points to a significant enrichment of genes upregulated by serotonin receptor agonists (co-dergocrine mesylate, pergolide) and prostaglandin, dopamine and adrenergic receptors ligands, but also of genes involved in chemotaxis, cAMP catabolic process, and regulation of cytokine production [16]. Along this line, 10 μM serotonin inhibits the production of pro-inflammatory cytokines in human macrophages primarily via 5-HT$_7$ receptor [16], and shifts the transcriptional profile of M-CSF-dependent human macrophages towards the anti-inflammatory side via 5-HT$_{2B}$ and 5-HT$_7$ receptors [16, 98]. At the gene expression level, serotonin reduces the expression of genes associated with the acquisition of the pro-inflammatory capabilities of GM-CSF-primed macrophages like *INHBA*, *CCR2*, *MMP12*, *ALDH1A2*, *CD1B* and *SERPINE1* [105, 106]. Conversely, serotonin potentiates the expression of genes associated with the M-CSF-driven anti-inflammatory differentiation of human macrophages (*SERPINB2*, *COL23A1*, *THBS1*, *STAB1*), an effect that can be prevented by 5-HT$_{2B}$- and 5-HT$_7$-specific antagonists [98]. Therefore, in vitro studies indicate that serotonin skews macrophages towards an anti-inflammatory phenotype [98].

More recently, whole transcriptomic analysis has demonstrated that serotonin rapidly modifies the macrophage gene signature, as it alters the mRNA levels of a significant number of genes within six hours of treatment [16]. This effect is primarily mediated by 5-HT$_7$ receptor, which leads to PKA activation, and causes upregulation of genes involved in chemotaxis and regulation of cytokine production, as well as augmented expression of genes positively regulated by IL-10 and Transforming growth factor TGFβ1 and negatively regulated by type I IFN [16]. In line with this transcriptional information, serotonin inhibits the LPS-stimulated release of IL-12p40 and TNF, reduces LPS- or IFNβ1-induced STAT1 activation and CXCL10 chemokine expression, and leads to enhanced expression of TGFβ1 [16]. Therefore, serotonin (10 μM) has a considerable impact on the human macrophage transcriptome and effector functions, and promotes the acquisition of a profibrotic and anti-inflammatory functional profile primarily via the 5-HT$_7$ receptor-PKA axis [16].

The functional and transcriptional effects of serotonin on macrophages has relevant therapeutic implications, because several antidepressants display anti-inflammatory effects and limit M1-macrophage polarization. Specifically, rolipram shifts macrophage away from M1 polarization through an increase in cAMP levels and subsequent PKA-CREB activation [107]. Regarding Selective Serotonin Reuptake Inhibitors (SSRI), sertraline and citalopram increase serotonin extracellular levels, and reduce the production of pro-inflammatory cytokines from LPS-activated monocytes while increasing IL-10 release [108–111], an effect that might also involve an elevation of cAMP levels. The specific serotonin receptor responsible for these effects is not clear, and although 5-HT_{2B} receptor appears required for the antidepressant action of SSRI [112], it seems reasonable to assume that 5-HT_4 or 5-HT_7 receptor might also mediate the action of SSRI [97, 98]. Along the same line, it is tempting to speculate that monoamine-oxidase inhibitors (that also increase serotonin levels) may also affect macrophage polarization through their ability to promote CREB phosphorylation. Importantly, the anti-inflammatory activity of SSRI is compatible with the fact that antidepressant treatment reduces inflammatory markers [113] and increases IL-10 levels [114] in depressed patients. The implications of the potential contribution of macrophage 5-HT receptors to the anti-inflammatory action of SSRI will be discussed later.

4 Expression of the 5-HT_{2B} Receptor on Macrophages

5-HT_{2B} receptor was first identified as a 5-HT_1-like receptor that mediates 5-HT-induced contraction of rat stomach fundus [115, 116]. Human 5-HT_{2B} receptor, encoded by the *HTR2B* gene at chromosomal position 2q36.3–2q37.1 [117], is evolutionary related to 5-HT_{2A} and 5-HT_{2C} receptors, with whom it shares a similar pharmacological profile [118], and is expressed during embryogenesis and mediates essential serotonin actions for normal development [119, 120]. In adults, 5-HT_{2B} receptor is mainly distributed in peripheral tissues but it is also detected in restricted areas of the brain [4], with microglia expressing 5-HT_{2B} receptor among other 5-HT receptors [55]. The growing list of pathophysiological effects of serotonin mediated by the 5-HT_{2B} receptor in various organs and tissues is presented in Table 6.2.

In the case of myeloid cells, the expression of the 5-HT_{2B} receptor is cell type-specific and depends on the prevailing macrophage-differentiating factors in the extracellular environment [98]. In human monocyte-derived dendritic cells, 5-HT_{2B} receptor expression is restricted to the $CD1a^+$ subset [121]. Similarly, 5-HT_{2B} receptor is preferentially expressed by monocyte-derived macrophages generated in the presence of M-CSF, which primarily function in maintenance of homeostasis and exhibit anti-inflammatory and immunosuppressive functions. Conversely, *HTR2B* mRNA is virtually absent in macrophages generated under the influence of GM-CSF, which are primed for potent pro-inflammatory and immuno-stimulatory activities [98]. Indeed, macrophage *HTR2B* expression in vitro is inhibited by pro-inflammatory agents like GM-CSF and LPS [98], and the loss of *HTR2B* mRNA

Table 6.2 Effects of 5-HT2B on immune cells and other cell lineages

	Effects	Cell type	Receptor	Reference
Heart and vessel wall	Valve disease (Ergot)			[221]
	Valvular abnormalities			[222]
	Valvular heart disease			[223]
	Morphogenetic functions	Myocardiac cells	5-HT2B	[119]
	Valvular heart disease (SSRI)		5-HT2B	[224]
	Heart development		5-HT2B	[128]
	Cardiac hypertrophy		5-HT2B	[129]
	Right ventricular failure	Cardiac fibroblasts	5-HT2B	[130]
	Mitral valvulopathy	Endothelial progen.	5-HT2B	[225]
	Vascular restenosis	Smooth Muscle	5-HT2B	[226]
Proliferation	Cell-cycle progression	Fibroblasts	5-HT2B	[131]
	Proliferation	Endothelial cells	5-HT2B	[227]
	Proliferation	Interstitial cells of Cajal	5-HT2B	[132]
	Proliferation	Fibroblasts/ neuroend tumors	5-HT2B	[133]
	Proliferation	Pancreatic beta cells	5-HT2B	[134]
	Proliferation	Hepatocytes	5-HT2B	[135]
	Proliferation	Hepatic stellate cells	5-HT2B	[28]
	Proliferation	Megakaryocytes	5-HT2B	[32]
	Proliferation	Hepatocytes	5-HT2B	[136]
	Proliferation	Hepatocytes	5-HT2B	[137]
	Proliferation/TGFα production	Hepatocytes	5-HT2B	[138]
	Proliferation	Lung fibroblasts	5-HT2B	[139]
Pulmonary arterial hypertension (PAH)	PAH (anorexic agents)			[228]
	PAH		5-HT2B	[140]
	PAH	Myeloid? progenitors	5-HT2B	[11]
Fibrosis	Tissue fibrosis	Fibroblasts	5-HT2B	[15]
	Myofibroblast differentiation	Lung fibroblasts	5-HT2B	[229]

(continued)

Table 6.2 (continued)

	Effects	Cell type	Receptor	Reference
Non peripheral effects	Neonatal respiratory activity	Medullary breathing center	5-HT2B	[230]
	Severe impulsivity		5-HT2B	[231]
	Behavioral/neurogenic effects		5-HT2B	[112]
	Antinociceptive effect (BW723C86)	Sensory neurons/ macrophage	5-HT2B	[232]
	Schizophrenic-like phenotype		5-HT2B	[233]
	Resistance to SSRI (fluoxetine)		5-HT2B	[234]
	Cocaine responses		5-HT2B	[235]
	Regulation of raphe 5-HT neurons	Serotonin neurons	5-HT2B	[236]
	Aggression-related cannabis resp.		5-HT2B	[237]
	Phrenic motor facilitation		5-HT2B	[238]
	Regulation of raphe 5-HT neurons	Serotonin neurons	5-HT2B-1A	[239]
	Cocaine-crack		5-HT2B	[240]
Asthma and allergic asthma mouse model	Eosinophilia		5-HT1-2	[14]
	Airway remodelling		5-HT2	[241]
	Prevention of allergic asthma		5-HT2	[242]
Cytokine production	Control of IL6, TNF, IL1α production	Ventricular fibroblasts	5-HT2B	[243]
	Cytokine release	Dendritic cells	5-HT4-7	[66]
	Cytokine/chemokine production	LPS-primed monocytes	5-HT3-4-7	[97]
	TGFβ1 production	Hepatic stellate cells	5-HT2B	[28]
	Inhibition of TGFβ1 production	Kupffer cells	5-HT2B	[28]
	Macrophage polarization	Macrophage	5-HT2B-7	[98]
	Cytokine release/T cell polarization	Dendritic cells	5-HT2B	[121]
	Motility and phagocytic activity	Microglia	5-HT2B	[55]

(continued)

Table 6.2 (continued)

	Effects	Cell type	Receptor	Reference
Other effects	Suppression of Ia expression	Macrophages	5-HT2	[100]
	Angiogenesis/MMP12	Macrophage		[23]
	Microglia develop./ synaptic refinement	Microglia	5-HT2B	[99]
	ALS progression	Microglia	5-HT2B	[26]
	Insulin/PGC1α–PPARγ mRNA	Pancreatic β cells	5-HT2B	[141]
Signaling	Ras activation	Fibroblasts, carcinoid tumors	5-HT2B	[142]
	ERK1/2, eNOS production	Endothelial cells	5-HT2B	[227]
	Inhibition of oxidative burst	Leukocytes	5-HT2	[244]
	Alkaline phosphatase activity	Osteoblasts	5-HT2B	[245]
	ERK, JunD	Hepatic stellate cells	5-HT2B	[28]
	Prostacyclin and PPAR-ß/δ	Osteoblasts	5-HT2B	[246]
	ERK1/2 activation	Megakaryocytes	5-HT2B	[32]
	pJNK, p21, STAT3 activation	Hepatocytes	5-HT2B	[136]
	PLC/PI3K/ERK2/ mTOR	Hepatocytes	5-HT2B	[137]
	β-Arrestin2/mTOR/ p70S6K	Smooth muscle cells	5-HT2B	[226]
	p21, pAkt	Lung fibroblasts	5-HT2B	[139]
	EGF/TGFα/p70S6K	Hepatocytes	5-HT2B	[143]

expression is a characteristic feature of activated macrophages and dendritic cells [98, 122]. Macrophage *HTR2B* mRNA expression is also controlled by anti-inflammatory cytokines (IL-4, IL-10) and, surprisingly, the LPS-induced downregulation of *HTR2B* mRNA can be prevented by blocking anti-IL-10 antibodies [98]. In any event, 5-HT$_{2B}$ receptor expression on macrophages might be also regulated through post-transcriptional mechanisms, as suggested in the case of uveal melanoma cells [123].

At the transcriptional level, *HTR2B* gene expression in human uveal melanoma cells is controlled by NFI and RUNX1 [124]. In the case of macrophages, the expression of *HTR2B* is critically regulated by the MAFB transcription factor, whose knockdown leads to a significant downregulation of *HTR2B* mRNA [125]. Moreover, *HTR2B* mRNA expression is upregulated in macrophages from patients with Multicentric Carpotarsal Osteolysis (MCTO), a disorder caused by mutations in the *MAFB* gene that lead to abnormally high levels of MAFB protein [125]. The link between MAFB and 5-HT$_{2B}$ receptor expression fits well with pro-fibrotic

action of 5-HT_{2B} receptor and the enhanced expression of MAFB in Dupuytren's disease, a fibroblastic proliferation of the palmar fascia [126]. Considering the critical role of MAFB in macrophage differentiation [127], the control of *HTR2B* gene expression by MAFB provides a clue about the molecular mechanism underlying the developmentally-regulated expression of 5-HT_{2B} receptor, and is in line with a prominent role for 5-HT_{2B} receptor at the initial stages of myeloid cell differentiation [11]. Whether MAFB also controls 5-HT_{2B} receptor expression in other cell lineages (e.g., fibroblasts) remains to be determined.

In vivo, the expression of the 5-HT_{2B} receptor is also primarily detected in human macrophages with anti-inflammatory and immunosuppressive activity, including tissue-resident alveolar and colonic macrophages, human liver Kupffer cells and even TAM [98]. In the case of mouse microglia, studies by the group of Maroteaux have demonstrated the functional expression of the 5-HT_{2B} receptor on postnatal microglia, and discovered that 5-HT_{2B} receptor mediates the movement of microglial processes towards serotonin [99]. The expression of the 5-HT_{2B} receptor in macrophages under homeostatic conditions is especially relevant because 5-HT_{2B} receptor is required for the therapeutic actions of SSRI [112], and because off-target effects on 5-HT_{2B} receptor are displayed by anti-migraine drugs like methysergide and ergotamine [144, 145], general anaesthetics [146], fenfluramine and conventional SSRIs [112, 147–149], and even by the dopamine agonists and anti-parkinsonian drugs pergolide and cabergoline [145, 150, 151]. Therefore, determination of the consequences of 5-HT_{2B} receptor activation in macrophages is required to fully evaluate the potential inflammatory effects of SSRI and the set of drugs mentioned above.

5 Consequences of 5-HT_{2B} Receptor Engagement in Macrophages: Gene Signature

5-HT_{2B} receptor engagement results in cell-specific functional outcomes as a consequence of its ability to variably trigger intracellular signals including Phospholipase A2 (PLA2) (endothelial cells, neuroendocrine and bone mesoblastic cells) and Phospholipase C (PLC) activation (astrocytes, lung vasculature, cardiomyocytes). Besides, 5-HT_{2B} receptor-dependent mitosis takes place during development and in many physiological settings. The robust link between 5-HT_{2B} receptor activation and cellular proliferation and fibrosis (neuro-endocrine tumours, fibroblasts, hepatocytes, Ito cells, cardiomyoblasts, Cajal interstitial cells, pancreatic β-cells, lung fibroblasts) [28, 128, 132–134, 139] derives from its capacity to activate $p21_{Ras}$, $p60_{Src}$, Phosphatidylinositol-3 kinase (PI3K) and ERK [28, 131, 139, 142], to promote the release of EGF, TGFα [137, 138, 143] or TGFβ1 [15, 28, 130], and to transactivate the Platelet-derived growth factor receptor (PDGFR) [128] (Table 6.2). Further, 5-HT_{2B} receptor activation inhibits JNK activation and HIF-1α expression, and activates pro-regenerative STAT3 phosphorylation in

hepatocytes [136], activates ERK and JunD in hepatic stellate cells [28], and reduces glucose-induced insulin secretion and mitochondrial activity in pancreatic β-cells through enhanced PGC1α and PPARγ expression [141] (Table 6.2). In fact, *Htr2b*$^{-/-}$ mice exhibit embryonic and neonatal lethality secondary to increased apoptosis and reduced cellularity in the ventricular *trabeculae*, as well as reduced bone density [129, 152]. Thus, the signalling capability of the 5-HT$_{2B}$ receptor is cell type-specific, as further illustrated by its ability to promote or inhibit TGFβ1 release in murine hepatic stellate cells or mouse Kupffer cells, respectively [28].

The analysis of the signalling/transcriptional effects of the 5-HT$_{2B}$ receptor in human macrophages has been complicated by the concomitant expression of the 5-HT$_7$ receptor, which mediates a large percentage of the signalling and transcriptional effects of serotonin on macrophages [16, 98]. To overcome this issue while addressing the signalling and transcriptional actions of 5-HT$_{2B}$ in macrophages, we have recently taken advantage of the availability of BW723C86, a 5-HT$_{2B}$ receptor agonist with 10-times higher selectivity for the 5-HT$_{2B}$ receptor *vs* 5-HT$_{2A/2C}$ receptor, and the SB204741 antagonist, which is 20-to-60-fold more selective for 5-HT$_{2B}$ receptor than for other 5-HT$_2$ receptors [153]. Using both agents, we have determined the transcriptional signature of macrophages exposed to 10μM BW723C86 in the presence or absence of SB204741 (Gene Expression Omnibus, GSE68061). As suggested by the transcriptional analysis, exposure to 10μM BW723C86 reduces the LPS-stimulated pro-inflammatory cytokine profile, albeit to a lower extent than the 5-HT$_7$ receptor-PKA axis [16]. This result is reminiscent of the effect of BW723C86 on human dendritic cells, where high concentrations of the agonist (300μM) are capable of abrogating the LPS-induced cytokine production and T cell-stimulatory activity of CD1a+ monocyte-derived dendritic cells [121]. The in vivo analysis of mouse microglia has provided definitive support for the contribution of 5-HT$_{2B}$ receptor to macrophage polarization, because *Htr2b*$^{-/-}$ microglia is characterized by a more pro-inflammatory profile and exhibit elevated levels of inflammatory genes directly related to chemotaxis (*Ccr2*, *Ccr3*, *Ccr5*, *Cxcr5*) [99].

In agreement with the physio-pathological significance of the 5-HT$_{2B}$ receptor (*see below*), gene ontology analysis of the transcriptome of BW723C86-treated macrophages has revealed that BW723C86 modifies the expression of gene sets related to "Amyotrophic Lateral Sclerosis" "Heart Valve Development" and "Regulation of Myeloid Leukocyte Differentiation". Although BW723C86 and serotonin share the capacity to modify the acquisition of polarization-specific genes during macrophage differentiation (*SERPINB2*, *THBS1*, *ALDH1A2*, *STAB1*, *COL23A1*, *MMP12*, *CD1B*) [98], a low overlap exists between the serotonin- and BW723C86-dependent transcriptomes, suggesting that engagement of 5-HT$_{2B}$ or 5-HT$_7$ receptors on human macrophages leads to different transcriptional outcomes. Unexpectedly, BW723C86 and serotonin activate the Aryl hydrocarbon Receptor (AhR), an effect prevented by 5-HT$_{2B}$ receptor antagonists. AhR is a ligand-dependent transcription factor that regulates biological responses to xenobiotics [154] and modulates immune and inflammatory responses [155], and whose activity in intestinal epithelial cells can be triggered by serotonin in a manner dependent on

SERT and independent on serotonin receptors [156]. The activation of AhR by BW723C86 not only extends the range of signalling pathways initiated upon 5-HT_{2B} receptor engagement but suggests a potential role for AhR in the pathophysiological processes involving 5-HT_{2B} receptor (see Sect. 7).

Comparison of the transcriptomes of macrophages exposed to BW723C86 in the presence and absence of SB204741 has also evidenced that a significant percentage of the BW723C86-induced gene changes could not be blocked by the 5-HT_{2B} receptor antagonist, suggesting that the widely used 5-HT_{2B} receptor agonist BW723C86 might exhibit 5-HT_{2B} receptor-independent effects. Interestingly, the BW723C86-triggered transcriptional effects that are insensitive to the presence of SB204741 are related to osteoclastogenesis and, in fact, BW723C86 modulates the expression of osteoclast-specific genes and regulators of monocyte-to-osteoclast differentiation. The actual involvement of 5-HT_{2B} receptor in the SB204741-insensitive BW723C86-induced deserves to be further clarified, especially because *HTR2B* gene expression in human macrophages is dependent on MAFB [125], which negatively regulates osteoclast generation through inhibition of FOS, MITF, and NFATc1 [157].

6 Physio-Pathological Consequences of 5-HT_{2B} Receptor Engagement in Macrophages

In agreement with its ability to alter the macrophage transcriptome and limit the production of inflammatory cytokines, solid proofs of the functional consequences of 5-HT_{2B} receptor engagement in macrophages have been obtained through the analysis of *Htr2b*-deficient microglia. Using two-photon microscopy, the group of Maroteaux has demonstrated that 5-HT_{2B} receptor mediates the rapid motility of microglial processes towards serotonin in postnatal microglia, and that the lack of 5-HT_{2B} receptor determines the appearance of anatomical alterations of the ipsilateral projecting area of retinal axons into the thalamus, thus implying that 5-HT_{2B} receptor participates in brain maturation [99]. The use of *Htr2b*-deficient mice has also revealed the pathological relevance of 5-HT_{2B} receptor expression in microglia. The expression of 5-HT_{2B} receptor is elevated in mononuclear phagocytes in animal models of amyotrophic lateral sclerosis (ALS) [26], and is required for activation of mononuclear phagocytes during ALS. The elimination of 5-HT_{2B} receptor leads to increased degeneration of mononuclear phagocytes and fragmentation of cellular processes, together with enhanced disease progression and reduced life span [26]. Importantly, these changes are accompanied by an altered gene expression profile, including genes associated with both pro-inflammatory (*Nox2*, *Ccl4*, *Mhc2*) and anti-inflammatory (*Ym1*, *Tyrobp*) microglial polarization, while the expression of the *Trem2* gene, associated with neurodegeneration, is enhanced [26]. Even more relevant, the capacity of the 5-HT_{2B} receptor to limit degeneration of spinal cord microglia and slow ALS progression has been confirmed in a large cohort of ALS patients, where the presence of a specific polymorphisms in the *HTR2B* gene (C allele, rs10199752) associates with increased *HTR2B* mRNA levels in spinal cord, reduced mononuclear phagocyte degeneration and longer survival [26].

7 Potential Implications of the Expression of 5-HT$_{2B}$ Receptor in Macrophages

7.1 5-HT$_{2B}$ Receptor, Cell Proliferation and Wound Healing

The link between serotonin and cell proliferation has been known for a long time, and is more evident in organs like heart and liver, where high serotonin levels have pathological outcomes. Activation of the 5-HT$_{2B}$ receptor improves survival in small liver grafts transplantations [135] and reverses age-associated impairments in regenerative capacity [158]. Indeed, 5-HT$_{2B}$ receptor mediates, at least partly, the capacity of serotonin to promote cell proliferation in numerous cell types and to upregulate the expression of growth factors like TGFβ1 [57, 159, 160], IGF-1 and HGF [24]. Given these antecedents, it is formally possible that the link between the 5-HT$_{2B}$ receptor and growth factors production might be also operative in macrophages. Macrophages are capable of secreting various serotonin-inducible proliferative factors, a capacity that is especially prominent upon anti-inflammatory/profibrotic polarization. In fact, gene expression profiling has revealed that serotonin triggers the expression of growth factors in M-CSF-polarized macrophages [98]. Therefore, the presence of 5-HT$_{2B}$ receptor on pro-tumoral TAM might contribute to the ability of serotonin to favour tumour growth [161–163], although the extent of the latter is still a matter of debate [164].

Wound healing is a second aspect that is worth mentioning regarding the influence of serotonin on macrophage polarization. Macrophages actively participate in wound healing [89], a process where platelets and serotonin are well-defined players [165]. During wound healing, platelet aggregation and serotonin release are rapidly followed by macrophage recruitment and release of pro-inflammatory cytokines in response to potentially damaging exogenous or altered endogenous products [165]. Therefore, it could be hypothesized that during wound healing, and acting via macrophage 5-HT$_{2B}$ receptor, platelet-derived serotonin might impair pro-inflammatory cytokine production and, concomitantly, enhance the release of growth factors fostering fibroblast/endothelial cell proliferation to restore tissue integrity and functionality.

7.2 5-HT$_{2B}$ Receptor-Initiated Signalling and Pulmonary Arterial Hypertension (PAH)

PAH is a progressive disorder caused by increased pulmonary artery pressure due to vasoconstriction, and characterized by pulmonary endothelial dysfunction and aberrant proliferation of various cell types (endothelial cells, smooth muscle cells, and pulmonary fibroblasts), all of which lead to right ventricle pressure overload, vascular remodelling and hypertrophy [166]. Although increased 5-HT$_{2B}$ receptor expression in pulmonary arteries associates with PAH in humans and animal models of

PAH [140], 5-HT_{2B} receptor expression in bone marrow progenitors is needed for the development of both hypoxia or monocrotaline-induced PAH [11, 140]. In fact, blockade of 5-HT_{2B} receptors with specific antagonists (SB204741, terguride, PRX08066, C-122, RS-12744) impairs pulmonary pressure and arterial wall thickening in hypoxia- or monocrotaline-induced rodent models of PAH [11, 140, 167–170]. Similarly, rat models of PAH induced by either monocrotaline or hypoxia and Sugen 5416 (SU5416, a potent inhibitor of VEGFR1 and VEGFR2) [171] can be prevented by RP5063 [172, 173], an antipsychotic drug with high affinity for $DA_{2/3/4}$ dopamine receptors and 5-$HT_{2A/2B/7}$ receptor and moderate affinity for SERT.

Interestingly, the analysis of lungs from SU5416/hypoxia-treated animals has evidenced upregulation of AhR and increased expression of the AhR target gene CYP1B1 [174]. Besides, the AhR antagonist CH223191 can reverse both the elevated right ventricular systolic pressure and the pulmonary vascular remodelling seen in SU5416/hypoxia-treated animals [175]. These results have led to the proposal that AhR has a pathologic role in SU5416/hypoxia-induced PAH [175]. Since 5-HT_{2B} receptor is required for PAH development, our recent finding that 5-HT_{2B} receptor agonists trigger AhR activation is in line with a pathological role of the 5-HT_{2B}-AhR link in PAH.

7.3 5-HT_{2B} Receptor and Psychiatric Diseases

Although the underlying mechanisms are not completely understood, evidence now supports the implication of systemic inflammation in the aetiology of depression. Usually accompanied by increased pro-inflammatory cytokine levels (IL-2, TNF) and reduced levels of anti-inflammatory IL-4 and TGFβ1, Major Depressive Disorder (MDD) is triggered by IFN therapy in HCV-infected patients, and MDD symptoms improved during blockade of TNFα in rheumatoid arthritis patients [176]. Indeed, pro-inflammatory agents like LPS induce “sickness behaviour” [177, 178], a set of depression-like symptoms (fatigue, anhedonia and sleep disturbances) which are secondary to increased levels of pro-inflammatory cytokines and alteration of brain function by resident and blood-derived immune cells [179]. Similarly, vaccinations against virus [180] prompts depression-like symptoms and neuroinflammation that are also seen in diseases associated to excessive levels of TLR ligands (e.g., obesity/long-chain saturated fatty acids) [181, 182]. Interestingly, as major promoters of inflammatory responses, macrophages have long been linked to inflammation-induced depression (“Macrophage theory of depression”) [183] and, in fact, some forms of depression are now considered as a microglial disease [184, 185].With this in mind, the modulatory effects of serotonin on macrophages during inflammatory responses might also be operative in the case of the behavioural and physiological depression-like symptoms that take place during the “sickness behaviour”. In fact, all the above-mentioned results suggest that PAMP receptor activation in peripheral macrophages triggers a low-grade inflammation and the subsequent acquisition of a partial pro-inflammatory polarization which would lead to the

appearance of "sickness behaviour" symptoms. Further, and in line with the current views of innate immunity memory [86], it is tempting to conjecture that the appearance of "sickness behaviour" symptoms would be facilitated by macrophages previously "trained" by agents that promote a "low-grade" systemic inflammation.

At the molecular level, several facts support a role for macrophage polarization in the onset of depression-like symptoms and MDD. The cytokines produced after infection or TLR activation, specially type I IFN, enhance the expression and activity of the indoleamine 2,3-dioxygenase (IDO1), an enzyme that drives the production of pro-depressive kynurenines and lowers tryptophan and serotonin levels [178]. Of note, IDO1 is preferentially expressed in response to agents (TLR ligands) or cytokines (TNF, type I IFN) that promote pro-inflammatory (M1) macrophage polarization, whereas factors that favour the acquisition of an anti-inflammatory macrophage polarization state (IL-10, IL-1Ra, IGF1) concomitantly downregulate the levels of pro-depressive brain proinflammatory cytokines. In this regard, anti-inflammatory M-CSF-conditioned macrophages exhibit a high MAFB-dependent expression of IGF1 [125], which suppresses inflammatory signalling [186], inhibits LPS- and TNFα-induced sickness behaviour, and exhibits antidepressant and anxiolytic activity [187, 188], thus suggesting that the IGF1´s antidepressant activity is associated to its anti-inflammatory ability. Very recently, "cAMP signalling pathway" (which leads to CREB activation) and "Insulin secretion" (related to IGF1) have been identified as two of the gene sets associated to both MDD and response to antidepressants [189]. Based on these antecedents, it can be envisaged that serotonin-conditioned macrophage polarization would limit brain inflammation and the appearance of depression-like symptoms. On this subject, and further stressing the differences between human and mouse macrophages, murine macrophages do not induce the enzymes associated with tryptophan catabolism in response to proinflammatory signals [190], and even responses to glucocorticoids appear to be different between human and mouse macrophages [191, 192].

The above predictions also fit with the known anti-depressive actions of SSRI and their ability to correct cytokine imbalance during inflammatory responses. SSRI, commonly used to treat depression and other psychiatric illnesses, exert a modulatory effect on the peripheral immune system, affecting cell proliferation, apoptosis and cytokine production [110, 111]. In fact, SSRI exhibit immunosuppressive effects in inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis (reviewed in [193]), and can even alter the pro-inflammatory Th1:Th2 ratio, reduce the Th17:Treg ratio, and increase TGFβ1 levels in patients with MDD [194] and in a mouse model of multiple sclerosis [102, 195, 196]. All these observations have led to increasing support for the therapeutic use of SSRI in inflammatory diseases. Interestingly, the 5-HT$_{2B}$ receptor appears to mediate the therapeutic activity of SSRI, whose long-term behavioural and neurogenic effects are abolished by inactivation of 5-HT$_{2B}$ receptors [112]. In fact, agonist stimulation of 5-HT$_{2B}$ receptor mimics SSRI responses [112]. By analogy to MDD, and since tissue-resident microglia also play a crucial role in certain psychiatric pathologies (e.g. schizophrenia, bipolar disorders, autism) [178, 197–199], the ability of serotonin to modify the effector functions of macrophages poses the question of whether modulation of

microglia polarization by 5-HT_{2B} receptors might also modulate neurodegenerative and neuropsychiatric pathologies.

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Part II
Peripheral Actions

Chapter 7
Bone and Serotonin Receptor Type 2B

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Abbreviations

5-HTR	Serotonin receptors
BMD	Bone mineral density
HBM	High bone mass
MSC	Mesenchymal stem cell
NET	Neuroendocrine tumor
OPG	Osteoprotegerin
PAH	Pulmonary artery hypertension
PGI2	Prostacyclin
PPAR-ß/δ	Proliferator activated receptor ß/δ
RANK	Receptor activator of NF-κB
RANKL	RANK ligand
SERT	Serotonin transporter
SSRI	Serotonin selective reuptake inhibitor
TPH	Tryptophan hydroxylase

1 Introduction

Bone remodeling is a cyclic and continuous physiological process ensuring the maintenance and renewal of the bone matrix. The bone mineralization is the process of laying down minerals on the protein matrix of bone. Calcium and phosphorus are

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chief minerals found in the bone along with small amount of carbonate and magnesium. Osteoblasts are the responsible cells for bone deposition. They derive from mesenchymal stem cells and also regulate osteoclasts. Osteoclasts are multinucleated cells responsible for bone resorption. Osteocytes are the most numerous cells present in bone. They are formed from osteoblasts trapped in matrix, called osteoids, and derive from hematopoietic precursors common to the monocyte/macrophage lineage. Osteoblasts have a crucial role in maintaining the balance of bone formation and resorption. Osteoblasts secrete RANK ligand (RANKL), which binds to the Receptor Activator of NF-κB (RANK) receptor on pre-osteoclasts and thus induces their differentiation. Osteoblasts also secrete osteoprotegerin (OPG), a RANKL decoy receptor, which prevents RANK/RANKL interaction by binding to RANKL; hence avoiding osteoclast differentiation. Thus, the balance between RANKL/OPG production by osteoblasts determines osteoclast differentiation and activity.

2 Serotoninergic Receptors and SERT in Bone

First *in vitro* studies have reported the expression of serotonin receptors (5-HTR) and/or of a functional serotonin transporter (SERT) in primary bone cells or in bone cell lines [1]. Osteoblast cell lines express mainly 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2B}R protein and/or specific binding sites [1]. A proliferative action of serotonin on chicken osteoblasts, mimicked by a 5-HT_{2B}R agonist, has been described [1]. This study was performed with a physiological concentration of serotonin (1–1000 nM) in medium that had not been serotonin depleted. In this model, the 5-HT_{2B}R mRNA expression was demonstrated in osteocytes, osteoblasts, and periosteal fibroblasts, a population containing osteoblast precursor cells [2]. Besides, the analysis of the clonal murine osteocytic cell line, MLO-Y4, demonstrates expression of the SERT, the 5-HT_{1A}R and 5-HT_{2A}R by real-time RT-PCR and immunoblot analysis [3]. In addition, 5-HT regulates osteoclast differentiation through SERT and 5-HTRs [4, 5]. In RAW cells, Battaglino et al. [4] detected mRNA expression of 5-HT_{1B}R, 5-HT_{2B}R and 5-HT_{4}R. However, in osteoclast derived from murine spleen, 5-HT_{1B}R, 5-HT_{2A}R and 5-HT_{2B}R were found [5].

3 Serotonin Receptors and Osteoporosis

Osteoporosis is due to an imbalance between bone formation by osteoblasts and resorption by osteoclasts. Deciphering factors controlling bone formation is therefore of utmost importance for understanding and treatment of osteoporosis [6]. *In vivo*, the bone phenotype of mice with a global invalidation of the serotonin 2B receptor gene (*Htr2b*$^{-/-}$) presented osteoporosis from 4 months of age [7]. These mice displayed significantly reduced bone formation, which was intensified in

18-month-old mice as it can be observed in senile human osteoporosis. In addition, osteoblasts of *Htr2b*$^{-/-}$ mice have reduced proliferation rate in the presence or absence of serotonin in the medium. These data signify that this receptor is able of both constitutive and paracrine activities. These results were in accordance with the data obtained by Locker et al. [8] on a mesoblastic cell line. The other 5-HT_{2A}R and 5-HT_{1A}R binding sites on murine osteoblasts have no detectable effect on bone [8]. However, the administration of MDL11939, a selective 5-HT_{2A}R antagonist, impaired bone formation in mice with no resorption modification, and are responsible for reduced bone mass. *In vitro*, the pharmacological inhibition of 5-HT_{2A}R signaling significantly decreased alkaline phosphatase activity in osteoblastic cells. These observations were confirmed by siRNA treatment against 5-HT_{2A}R in MC3T3-E1 cells suggesting that 5-HT_{2A}R signaling plays also a role in osteoblast differentiation [9].

To develop new drugs targeting serotonin/5-HT_{2B}R in order to treat osteoporosis, the signaling pathways responsible for the osteoblast defect in *Htr2b*$^{-/-}$ mice was studied. The phospholipase A2-arachidonic acid pathway was found involved in this phenotype. Among different eicosanoids studied, osteoblasts without 5-HT_{2B}R were associated with a ten-fold over-production of prostacyclin (PGI2). Also, a specific prostacyclin synthase inhibitor (U51605) rescued totally osteoblast aggregation and matrix mineralization in *Htr2b*$^{-/-}$ osteoblasts without having any effect on WT osteoblasts. Prostacyclin is the endogenous ligand of the nuclear peroxisome proliferator activated receptor ß/δ (PPAR-ß/δ), and its inhibition in *Htr2b*$^{-/-}$ cells rescued totally the alkaline phosphatase and osteopontin mRNA levels, cell-cell adhesion, and matrix mineralization. These findings revealed a coupling between PPAR-ß/δ and 5- HT_{2B}Rs in bone that might also occur in other tissues, since the plasma level of PGI2 was also increased in *Htr2b*$^{-/-}$ mice [10]. In heart, 5-HT_{2B}Rs regulate cardiac development [11] and function and PPAR-ß/δ is an essential transcription factor in the myocardial metabolism [12]. Moreover, prostacyclin treatment improves pulmonary artery hypertension (PAH) patients [13], suggesting that the 5-HT_{2B}R-prostacyclin/PPAR-ß/δ coupling could also be involved in PAH. 5-HT_{2B}R activation appears to play a major role in this disorder, and *Htr2b*$^{-/-}$ mice do not develop PAH after hypoxia [14].

4 SSRIs and Osteoporosis

Central serotonin signaling is a frequent therapeutic target because of the beneficial role of pharmaceutical agents that antagonize SERT, such as serotonin selective reuptake inhibitors (SSRIs), as fluoxetine or paroxetine, in major depressive disorders and other affective conditions. Serotonin needs its membrane transporter, SERT, to enter the cells. Several studies have revealed the existence of a functional SERT in both osteoblast and osteoclast cell lines [4, 15]. The first *in vivo* study to investigate the serotoninergic system and bone was that of Warden et al. [16]. They reported a significant deficit in bone formation in SERT knockout mice and also in

mice treated with fluoxetine. This bone phenotype was only related to a decrease in bone formation. Furthermore, fluoxetine inhibited osteoblast differentiation and mineralization in two different mouse models of bone repair. Cessation of the fluoxetine treatment led to complete reversion of the repair process. In conclusion, fluoxetine negatively impacts fracture healing [17]. This observation was also reported with the SSRI, Sertraline, that also impairs bone wound healing through disruption of bone repair and regeneration [18]. Clinically relevant doses of fluoxetine induce only moderate bone architecture changes in rats [19]. A slightly diminished bone quality of femurs was found that was reflected in a lower bone tissue strength, compensated by changes in bone geometry [18]. Besides, fluoxetine has a negative effect on osteoprogenitor cells derived from mesenchymal stem cells (MSCs) extracted from rat adipose tissue and led to apoptosis independently of serotonin levels in the culture supernatant. Fluoxetine exerted a direct inhibitory effect on bone cells via an apoptosis-dependent pathway. Furthermore, in the presence of fluoxetine, expression levels of serotonergic genes, including 5-HT_{1B}R, 5-HT_{2A}R and 5-HT_{2B}R and SERT, were down-regulated [20]. Alongside these experimental works, several studies have evaluated the impact on osteoporosis of SSRIs, widely used to treat depression. In contrast to the undoubted phenotype observed in growing mice, studies evaluating bone density in adult patients treated with SSRIs have provided less conclusive [21–23] or inconclusive [24] studies. The recent objective meta-analyze showed that the use of SSRIs was not associated with lower or higher bone mineral density (BMD) [25]. Also, with regard to fractures, most of the data showed a significant increase in odd ratio for fractures in patients treated with SSRIs [26]. Moreover, study evaluating bone remodeling markers in patients treated with an SSRI suggested that this treatment reduces bone formation but not resorption [27]. In fact, the role of SSRI in osteoporosis needs more longitudinal studies in various treated patients to be conclusive.

5 Serotonin and Bone Remodeling

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in serotonin biosynthesis. There are two isoforms of this enzyme: TPH2 mainly expressed in brain, and TPH1 expressed elsewhere in the body. Serotonin is synthesized by TPH1 in the enterochromaffin cells of the gastrointestinal tract and accumulates in platelet-dense granules, which constitute the body's main serotonin reservoir. There are no serotonergic nerves in the bone, nor in any other peripheral tissue except the gut. Serotonin stored in the platelets is not biologically active in the absence of platelet activation. Platelets do not synthesize serotonin, but it is internalized by the SERT, stored in dense granules, and released during platelet activation. The action of serotonin in bone has been the object of controversies.

In one hand, the Yadav et al. [28] paper was principally based on TPH1 gene invalidation in the gut of mice in which the different serotonin receptors in osteoblasts had been invalidated. These authors observed an increase in bone density in

mice in which the 5-HT_{1B}R had been specifically inactivated in the osteoblasts, whereas binding sites for this receptor could not be detected in osteoblast primary cultures. For these authors, the gut-derived serotonin contributes to 'free' circulating serotonin levels and is a modulator of bone mass and quality; serotonin produced in the gut decreased bone formation via the osteoblast 5-HT_{1B}Rs [29]. Besides, there is an association between serotonin and fractures, especially hip fractures in adult men. The authors found that high levels of serotonin predict an increased risk for nonvertebral osteoporotic fractures To note that the study was realized from a large cohort of 950 men (aged 69 to 81 years) excluding SSRI users from analysis [30]. Their results were in accordance to Yadav et al. publication. Moreover, Yadav et al. [31] also showed that a specific TPH1 inhibitor, LP533401, could increase bone density through an anabolic action that was as potent as that of teriparatide in ovariectomized mice and rats. Cui et al. [32] observed that the inhibitor of TPH1, LP923941, which is an enantiomer of LP533401 lowering circulating serotonin, did not change the bone density of the mice. Concretely, it is not convincing to use a specific TPH1 inhibitor to treat osteoporosis due to the large effects of serotonin on other tissues.

In other hand, in growing *Tph1*$^{-/-}$ mice, bone mass was increased, but this phenotype was resolved at maturity; the adult phenotype was in accordance with the data of Cui et al. [32] although the authors did not study bone remodeling in these mice. In works of Chabbi-Achengli et al. [5], bone formation was unchanged in growing mice and reduced in mature mice, which explains the elevated bone density seen in the growing mice that had returned to normal at maturity. In both juvenile and mature mice, there was evidence of decreased bone resorption, as evaluated by both bone histomorphometry and D-pyridinoline, a biochemical marker of bone resorption. In a functional study, bone-marrow of *Tph1*$^{-/-}$ mice transplanted at birth with wild-type cells retarded the deficit in bone resorption and proved that an intrinsic osteoclast defect was responsible for the osteoclast phenotype [5]. Moreover, osteoclast differentiated with M-CSF and RANKL from primary spleen mouse cells were able to synthesize serotonin and this was the sole source of serotonin in the bone microenvironment. Serotonin has direct stimulatory effect on bone formation pathways and TPH1 is expressed in mouse osteoblasts and osteoclasts, indicating their ability to produce serotonin.

Besides, serotonin enhanced the proliferation of human mesenchymal stem cells and primary osteoblasts and 5-HT_{2A}R expression. Serotonin increased OPG and decreased RANKL secretion from osteoblasts, suggesting a role in osteoblast-induced inhibition of osteoclast differentiation [5]. The local serotonin synthesis was increased by RANKL. Consequently, serotonin increases osteoclastogenesis by a paracrine/autocrine mechanism. Furthermore, osteoclast synthesis of serotonin is sufficient to induce an increase in osteoblast proliferation. However, this hypothesis cannot explain why normal bone formation was maintained in growing mice despite the decreased bone resorption in *Tph1*$^{-/-}$ mice [5]. In addition, the intracellular concentration of serotonin depends on two mechanisms. On one hand, osteoclasts can produce serotonin from active TPH1 and, on the other hand, the role of SERT is to uptake the serotonin from extracellular area. The function of intracellular serotonin

is unknown but these cells possess the enzyme transglutaminase, as pancreatic β-cells [33] or platelets, that can activated by serotonin [34]. This enzyme can covalently link serotonin to the glutamine residue of small GTPase to form a glutamyl-amide bond (serotonylation). This process allows the activation of these G proteins. Activated small GTPases have been shown to regulate many processes in intracellular trafficking, such as vesicle formation, movement, and membrane fusion [35]. Thus, there is a direct role for serotonin, independent from its contributions to cellular signaling, in the mediation of permissive gene expression [36]; it has been proposed that serotonylation plays a major role in osteoclast.

6 Controversial Role of LRP5

The Wnt LRP5/β-catenin signaling pathway plays an essential role in the regulation of osteoblast progenitor proliferation, differentiation and survival [37]. Humans with specific missense gain-of-function mutations in the Wnt co-receptor *LRP5* have a high bone mass (HBM) whereas loss-of-function mutations in this gene cause osteoporosis idiopathic even osteoporosis pseudoglioma [38]. Karsenty et al. [28] hypothesized that the major role of LRP5 is not owing to the expression of this gene in cells of the osteoblast lineage, but it is dependent on the synthesis of serotonin by the gut, which is regulated by LRP5. They reported that duodenal Tph1 expression and blood serotonin are both lower in mice carrying HBM-allele than in wild-type mice. At opposite, in *Lrp5*$^{-/-}$ mice, TPH1 was overexpressed in the duodenum and bones leading to an elevated circulating levels of serotonin. Moreover, the authors found that *Lrp5*$^{-/-}$ mice treated with a selective and irreversible inhibitor of TPH (parachlorophenylalanine), exhibited reduced circulating serotonin levels and normalization of their skeletal phenotype. These drastic treatments provide preliminary evidence that elevated circulating serotonin contributes to the decreased bone formation and mass observed in *Lrp5*$^{-/-}$ mice [28]. These authors pointed out that the TPH1 enzyme is regulated by the *Lrp5* gene [29]. However, it remains unknown how LRP5 affects TPH1 expression in enterochromaffin cells of the gut, with no identified ligand for gut, LRP5 mediates a currently unknown molecular pathway leading to altered TPH1 expression. It also remains to be shown how serotonin synthesized in the gut reaches bone cells to activate 5-HTRs.

Part of the controversy was based on the measurement of the serum levels of serotonin that mainly reflects the number of platelets as the concentration of serotonin in individual platelets is fairly constant. The amount of free serotonin in platelet-poor plasma is extremely low, between 2 and 15 nM in mice, whereas serotonin content in whole blood is 50,000 fold higher. As expected, the authors found that high serotonin concentration (50 μM) inhibited the proliferation of osteoblasts from both *Lrp5*$^{-/-}$ and wild-type mice. However, the concentrations used were

largely higher than physiological concentrations, which led normally to osteoblast proliferation and osteoclast differentiation [10]. Besides, according to Cui and colleagues [32], the HBM-causing *Lrp5* alleles in mice did not alter TPH1 mRNA expression in the duodenum or serotonin levels in the blood. These results were confirmed in human with the p.Gly171Val and p.Asn198Ser high-bone mass mutation [39]. At opposite, Karsensty's team obtained a low level of serotonin in presence of HBM-causing Lrp5 alleles in mice [29] and human [40]. The difference between the results of the two teams is owing to the techniques of serotonin measurements and the analysis of the expression pattern of the HBM-allele. Interestingly, the patients with neuroendocrine tumors (NETs) frequently have markedly elevated serotonin levels. The small number of patients who presented NETs with higher serotonin metabolites had a lower BMD at the hip as compared to controls [41]. These data need to be confirmed with a large cohort including for example fractures as a clinical outcome.

7 Zebrafish Model

Interestingly, the zebrafish model has led to various information about the role of serotonin in bone [42]. Indeed, the zebrafish model is a vertebrate organism that can regenerate its fins after amputation [42]. This regenerative process involves a local synthesis of serotonin in the wound. The intracellular accumulation of serotonin was only induced at the initiation of the fin regeneration. After a transient synthesis of serotonin, it was no longer present in the growing tissue. The expression of two serotonin synthesizing enzymes, TPH1a and TPH1b in the blastema suggested the local production of this monoamine. However, neither the depletion of serotonin by chemical inhibition of TPH, nor the ectopic administration of this monoamine affected fin regeneration, indicating that serotonin was not the limiting actor of the fin regeneration. Moreover, it seems that the presence of serotonin during regeneration depends on fibroblast growth factor and retinoic acid signaling [42]. Nevertheless, physiological serotonin production is favorable for bone [42].

8 Conclusion

In conclusion, serotonin, its receptors (5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2B}R) and its transporter SERT play a major role in bone remodeling at osteoclast and osteoblast levels. In osteoclast, a probable complete serotoninergic system exists but further experiments are requested to define the role of each actors. In osteoblast, proliferation and differentiation could be dependent or independent of serotonin. Taken together these data suggest is the existence of a closed communication between osteoclast and osteoblast around the serotoninergic system.

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Chapter 8
Roles of 5-HT_{2B} Receptor in Pain

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Abbreviations

AA	Arachidonic acid
AEA	Anandamide
AP	Action potential
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
DRG	Dorsal root ganglia
ER	Endothelium reticulum
GDNF	Glial cell-derived neurotropic factor receptor
IB_4	Isolectin B_4
NMDAR	N-methyl-D-aspartate receptor
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKCγ	Protein kinase Cγ
PLCβ	Phospholipase Cβ
5-HT	Serotonin 5-hydroxytryptamine
SNL	Spinal nerve ligation
SP	Substance P
TRPV1	Transient vanilloid receptor 1
TTX-R	Tetrodotoxin-resistant

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1 Introduction

Serotonin, or 5-hydroxytryptamine (5-HT) is known as a classical monoamine neurotransmitter; its primary release in the enteric nervous system regulates intestinal movement. However, it is also found in the central nervous system (CNS), where it plays various physiological and psychological functions involved in mood, appetite, sleep, learning and memory. Its deficiency is associated with many psychiatric disorders [1]. Researchers have paid much attention to its function in the CNS, but only 1–2% of the total amount of serotonin is produced by neurons in the brain, so most serotonin is detected in peripheral tissues and has other important functions [2].

About 90% of serotonin is secreted from the enterochromaffin cells of the gastrointestinal tract [3]. 5-HT is also released from many different types of immune cells and platelets and is related to inflammatory responses. Growing evidence has suggested that 5-HT is a pro-inflammatory and pro-nociceptive agent that can cause pain and hyperalgesia by activating various subtypes of 5-HT receptors present in primary afferents [4–8].

The 5-HT receptors are classified into seven subtypes, some with more than one receptor; for example, 5-HT_1 has 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptors and 5-HT_2 has 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors [9]. All 5-HT receptors are G-protein coupled receptors, except for 5-HT_3, which is an ionotropic receptor. Different subtype receptors on nociceptors have distinct mechanisms in regulating 5-HT–induced pain or hyperalgesia [3, 5, 6, 10, 11]. In this chapter, we focus on the roles of the 5-HT_{2B} receptor in various types of pain and how it regulates pain.

2 5-HT_{2B} Receptor Participates in Different Pain Models

The role of the 5-HT_{2B} receptor in nociception has been rarely investigated and discussed, mainly because the presence of 5-HT_{2B} receptor in dorsal root ganglia (DRG) is controversial. No 5-HT_{2B} or 5-HT_{2C} receptor mRNA was detected in rat DRG [12], whereas Nicholson et al. [13] found 5-HT_{2B} but not 5-HT_{2C} receptor present in rat DRG. Lin et al. [14] confirmed the presence of 5-HT_{2B} receptors in mouse DRG. Reflecting its presence in DRG, later studies demonstrated the involvement of the 5-HT_{2B} receptor in various types of pain, probably with distinct roles.

Peripheral administration of the 5-$HT_{2B/2C}$ receptor antagonist SB206553 and 5-HT_{2B} receptor antagonist RS127445 inhibited 5-HT–induced mechanical hyperalgesia but not thermal hyperalgesia in the mouse hind paw [14, 15]. Peripheral injection of the 5-HT_{2B} receptor agonist BW732C86 induced a similar nocifensive behavior as 5-HT, thus confirming that the peripheral 5-HT_{2B} receptor mediates 5-HT–induced mechanical hyperalgesia. In a formalin model, intrathecal or peripheral injection of the 5-HT_{2B} receptor antagonist RS127445 reduced

formalin-induced flinching behavior in the second phase, which suggests that both peripheral and spinal 5-HT_{2B} receptors may participate in the later phase of pain transduction [16]. Oral delivery of the 5-HT_{2B} receptor antagonist FRI0011 reduced pain induced by systemic lipopolysaccharide injection [17]. FRI0011 also reduced production of inflammatory cytokines (interleukin 6 and tumor-necrosis factor α) [17]. Therefore, 5-HT_{2B} receptor may regulate inflammatory cytokines to affect pain.

5-HT_{2B} receptor was also found involved in neuropathic pain. In L5/L6 nerve injury, 5-HT_{2B} receptor expression was enhanced in the ipsilateral dorsal part of the spinal cord. Intrathecal injection of the 5-HT_{2B} receptor antagonist RS127445 reduced the increased expression of 5-HT_{2B} receptor and attenuated tactile allodynia [18]. Spinal superfusion of the 5-HT_{2B} receptor selective antagonist SB204741 bilaterally reduced thermal and mechanical allodynia at day 2 after spinal nerve ligation (SNL) [19], which suggests that spinal 5-HT_{2B} receptor, unlike peripheral 5-HT_{2B} receptor, facilitates pain transduction induced by both mechanical and thermal stimuli. After SNL, protein kinase C γ (PKCγ) was upregulated and the phosphorylation of the N-methyl-D-aspartate receptor (NMDAR) subunit was enhanced in rat dorsal horn neurons. Blockage of the 5-HT_{2B} receptor by SB204741 decreased PKCγ upregulation and NMDAR phosphorylation [19]. The 5-HT_{2B}–Gq–PLCβ pathway may activate PKCγ to promote NMDAR phosphorylation in the spinal cord. In contrast, Urtikova et al. [20] found that intrathecal injection of the 5-HT_{2B} receptor agonist BW723C86 induced mechanical and cold allodynia caused by chronic constriction injury of the sciatic nerve, which could be reversed by co-injection of RS127445. Whether the 5-HT_{2B} receptor plays a pro- or anti-nociceptive role in neuropathic pain remains debated.

In addition, oral administration of the 5-HT_{2B} receptor antagonist RS127445 inhibited visceral hypersensitivity provoked by restraint stress [21]. The 5-$HT_{2B/2C}$ receptor agonist meta-chlorophenylpiperazine induced dural plasma protein extravasation, an animal model of migraine, in guinea pigs [22]. The 5-HT_{2B} receptor antagonist could inhibit meta-chlorophenylpiperazine–induced dural plasma protein extravasation, which suggests that 5-HT_{2B} receptor may have an important role in generating migraine pain [23].

These lines of evidence suggest that both peripheral and spinal 5-HT_{2B} receptor is involved in transduction of nociceptive signals in different types of pain models. 5-HT_{2B} receptor seems to play a nociceptive role in different pain models, except for a certain type of neuropathic pain. Although 5-HT_{2B} receptor activation controls the Gq/G11–phospholipase Cβ (PLCβ) pathway, peripheral and spinal 5-HT_{2B} receptor–mediated signaling targets different types of PKC and downstream molecules to regulate pain sensation.

3 Roles of 5-HT_{2B} Receptor in the Periphery

Lin et al. [14] previously demonstrated that 5-HT–induced mechanical hyperalgesia was attributed to 5-HT_{2B} receptor activation. The later study by Su et al. [15] provided detailed mechanisms that 5-HT_{2B} receptor activation controls the Gq/G11–PLCβ–PKCε pathway to modulate mechanical hyperalgesia in both IB_4-negative or -positive neurons.

Two classes of small unmyelinated C-fiber nociceptors are responsible for transduction of noxious stimuli. The peptidergic C-fiber expresses the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP), and the non-peptidergic C-fiber binds isolectin B_4 (IB_4-positive) and expresses glial cell-derived neurotropic factor receptor (GDNF) and P_2X_3 receptor [24]. IB_4-negative neurons have a lower action potential (AP) threshold and shorter AP duration than IB_4-positive neurons [25, 26]. 5-HT injection enhanced evoked intracellular calcium signals in IB_4-negative but not IB_4-positive neurons [15]. Thus, IB_4-negative neurons may be the major neurons that respond to 5-HT and transduce the 5-HT signal to induce mechanical hyperalgesia. Blocking 5-HT_{2B} receptor, PLCβ or PKCε before 5-HT injection inhibited the enhancement of calcium signals in IB_4-negative neurons. This finding agreed with behavioral results showing that blocking the 5-HT_{2B}–PLCβ–PKCε pathway inhibited mechanical hyperalgesia.

IB_4-positive neurons with increased density of tetrodotoxin-resistant (TTX-R) Na^+ channels and longer AP could result in more efficient calcium influx into the presynaptic terminal, for increased transmitter release [27]. IB_4-positive neurons mediating a more reliable synaptic connection may participate in maintenance of hyperalgesia. Despite no increase in intracellular calcium signals in IB_4-positive neurons after 5-HT injection, the number of the IB_4-positive neurons responding to 5-HT was increased [15]. As expected, blocking 5-HT_{2B} receptor, PLCβ or PKCε before 5-HT injection also inhibited the calcium signals in IB_4-positive neurons.

3.1 Induction of Mechanical Hyperalgesia: Involvement of Transient Vanilloid Receptor 1 (TRPV1) in 5-HT_{2B} Receptor–Mediated Signaling in IB_4-Negative Neurons

In IB_4-negative neurons, 5-HT–induced calcium signals were completely inhibited by removal of extracellular calcium, which suggests that calcium signals are mainly due to calcium influx through channels. Su et al. [15] confirmed the participation of TRPV1 in 5-HT signaling transduction in IB_4-negative neurons. TRPV1, a capsaicin, heat and proton receptor, is widely expressed in sensory neurons, especially in peptidergic C-fibers [28–30]. After 5-HT injection, the capsaicin-evoked calcium signals were significantly enhanced in IB_4-negative neurons. Administration of a TRPV1 antagonist before 5-HT injection in mice inhibited 5-HT–induced

mechanical hyperalgesia [15]. Consistent with the results, 5-HT–induced mechanical hyperalgesia was absent in mice lacking the TRPV1 gene.

We have no evidence demonstrating that TRPV1 can be activated by 5-HT directly, although TRPV1 function is enhanced by PKA and PKC phosphorylation [31, 32]. How 5-HT_{2B} receptor activates TRPV1 is unclear. Besides proton, heat, and capsaicin activation, TRPV1 can also be activated by anandamide (AEA), an endogenous fatty-acid neurotransmitter generated from N-acylphosphotidylethanolamides via PLC-mediated hydrolysis [33]. Thus, activation of a 5-HT_{2B} receptor may mediate PLC leading to AEA formation to activate TRPV1. In addition, the arachidonic acid (AA) metabolite products 12- and 15-HEPETE and 5-HETE are TRPV1 agonists [34]. 5-HT_{2B} receptor activation activates phospholipase A2, thus leading to the neuronal secretion of AA. Alternatively, TRPV1 is activated or inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2) [35], so 5-HT_{2B} receptor activation likely causes PIP_2 cleavage, relieving PIP_2-dependent inhibition on TRPV1. Accordingly, 5-HT_{2B} receptor activation may relieve PIP_2-dependent inhibition of TRPV1 and generate the endogenous ligands AEA or AA to activate and regulate TRPV1 function, thereby resulting in mechanical hyperalgesia in the periphery.

C-fibers are excited by noxious stimuli and also by pruritic compounds [36]. 5-HT can induce pain and itch sensations in mice and humans [37]. A subset of 5-HT–sensitive neurons is sensitive to histamine and chloroquine, involved in itch perception [38]. 5-HT_2 receptors respond to 5-HT–induced itch by activating the Gq/G11–PLC pathway, which leads to mitogen-activated protein kinase and PKC activation [39]. GPCR–TRP channel pathways are the major pathways for itch responses. 5-HT–induced mechanical hyperalgesia is also mediated by the 5-HT_{2B}–TRPV1 pathway. The similarity of the GPCR–TRP channel axis between pain and itch sensations suggests that mechanisms used in pain sensations are possibly involved in itch sensations.

3.2 Maintenance of Mechanical Hyperalgesia: Involvement of 5-HT_3 in 5-HT_{2B} Receptor–Mediated Signaling in IB_4-Positive Neurons

In the study by Su et al. [15], 5-HT–induced calcium signals in IB_4-positive neurons were partially sensitive to removal of extracellular calcium, which suggests that the calcium signals may be from channels in both the plasma membrane and endothelium reticulum (ER). The 5-HT_3 receptor antagonist granisetron specifically inhibits 5-HT–induced calcium signals in a small set of IB_4-positive population, which explains the sensitivity of these neurons to removal of extracellular calcium. Thus, IB_4-positive neurons have two distinct pathways for response to 5-HT stimulation: one is the 5-HT_{2B}–PLCβ–PKCε pathway and the other is the 5-HT_{2B}–PLCβ–PKCε/5-HT_3 receptor pathway.

5-HT_3 receptor is found in pain-related regions and is involved in pain processing [40, 41]. Administration of a 5-HT_3 receptor antagonist in mice did not inhibit mechanical hyperalgesia but shortened the duration of mechanical hyperalgesia [14], which suggests that 5-HT_3 receptor may affect modulation of the maintenance of hyperalgesia. Given that IB_4-positive neurons can exhibit sustained responses but not transient or mixed responses to low pH [42], the responses in granisetron-sensitive IB_4-positive neurons are thought to be responsible for extending the duration of 5-HT–induced mechanical hyperalgesia.

3.3 5-HT_{2B}–Gq–PLCβ–PKCε Pathway Modulates Sodium Channels

Although calcium signals regulated by 5-HT_{2B} receptor are critical for 5-HT–induced mechanical hyperalgesia, sodium currents may also have important roles in mechanical hyperalgesia. $Na_v1.8$ (a TTX-R channel) is related to inflammatory mechanical hyperalgesia [43, 44]. 5-HT increases TTX-R I_{Na} currents [45] and PKC can modulate these currents [46, 47]. Therefore, 5-HT_{2B}–Gq–PLCβ–PKCε signaling could regulate voltage-gated Na^+ channels to affect mechanical hyperalgesia.

4 Roles of 5-HT_{2B} Receptor in the Spinal Cord

Peripheral inputs or abnormality and also central neuroplasticity contributes to the establishment and maintenance of chronic pain. Once the central sensitization occurs, painful sensations are generated even in the absence of the noxious stimulus [48]. The central sensitization in the spinal dorsal horn can be attributed in part to an excitatory amino acid, glutamate [49, 50]. Peripheral injury or inflammation sensitizes dorsal horn neurons and increases their responsiveness to glutamate application [51, 52], which was reduced after the administration of glutamate receptor antagonists [53, 54]. Intrathecal injection of NMDA leads to hyperalgesia, which can be reversed by application of an NMDA antagonist [55]. The NMDA antagonist MK-801 can reduce the hyperalgesia that develops in rats with adjuvant-induced inflammation [56] or reduce the inflammation-induced expansion of the receptive field of spinal nociceptive neurons [57]. Peripheral inflammation elevates levels of phosphorylated NMDA receptors in the spinal dorsal horn [58, 59].

Aira et al. [19] demonstrated that spinal 5-HT_{2B} receptors are involved in SNL-induced mechanical and thermal hyperalgesia. PKCγ was upregulated and the phosphorylation level of the NMDAR subunit enhanced in rat dorsal horn neurons after SNL. Spinal superfusion of the 5-HT_{2B} receptor antagonist SB204741 decreased the PKCγ upregulation and NMDAR phosphorylation level. Peripheral injury likely releases 5-HT to activate 5-HT_{2B}–Gq–PLCβ signaling, which activates

PKCγ to further phosphorylate NMDAR, leading to hyperalgesia. Unlike peripheral 5-HT_{2B} receptor controlling mechanical hyperalgesia, spinal 5-HT_{2B} receptor regulates both mechanical and thermal hyperalgesia. 5-HT_{2B} receptor targeting a distinct PKC in the periphery and spinal cord may explain the differences. Alternatively, in the periphery, 5-HT_{2B} receptor may be located in neurons that receive mechanical stimuli.

5 Outlook and Prospects

Although 5-HT_{2B} receptor may mediate distinct mechanisms in various types of pain, the study of 5-HT-induced pain provides several insights for the role of 5-HT_{2B} receptor in the periphery: (1) 5-HT_{2B} receptor mediates mechanical hyperalgesia but not thermal hyperalgesia; (2) 5-HT_{2B} receptor mediates distinct mechanisms in peptidergic and non-peptidergic nociceptors; (3) the 5-HT_{2B}–Gq–PLCβ–PKCε–TRPV1 axis in peptidergic neurons contributes to inducing mechanical hyperalgesia; (4) the 5-HT_{2B}–Gq–PLCβ–PKCε–5-HT_3 axis in non-peptidergic neurons participates in maintenance of hyperalgesia; and (5) the GPCR–TRP channel axis is also used in pain sensation. 5-HT_{2B} receptor located at peripheral or spinal loci may activate distinct types of PKCs to regulate nociceptive transduction: (1) in the periphery, 5-HT_{2B} receptor mediates mechanical hyperalgesia only but in the spinal cord, it regulates both mechanical and thermal hyperalgesia, and (2) in the periphery, 5-HT_{2B}–Gq–PLCβ signaling activates PKCε to regulate TRPV1 or 5-HT_3. In the spinal cord, 5-HT_{2B}–Gq–PLCβ signaling activates PKCγ to regulate NMDAR. Accordingly, these lines of evidence support 5-HT_{2B} receptor as a potential pain target and can facilitate the development of therapeutic drugs.

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Chapter 9
5-HT_{2B} Receptor, the Heart and Blood Vessels

Laurent Monassier

Abbreviation

5-HT	5-hydroxytryptamine
Ang II	Angiotensin II
ANT-1	Adenine nucleotide translocator
BMP	Bone morphogenic protein
BNP	Brain natriuretic peptide
CGRP	Calcitonin gene-related peptide
DOCA/salt	Deoxycorticosterone acetate and salt
EGFR	Epidermal growth factor receptor
EMT	Endothelial-mesenchymal transformation
ERK1/2	Extracellular signal-regulated kinase
MMPs	Metalloproteases
NOS	NO synthase
NOX	NAD(P)H oxidases
PI3K	Phosphatidylinositol-3 kinase

1 Introduction

The first description of serotonin effects was in the cardiovascular field, a long time before its identification as 5-hydroxytryptamine (5-HT). In fact, physiologists observed at the end of nineteenth century, that a substance present in serum was acting on heart and vessels. The German scientist Weiss showed in 1896 that intravenous injection of serum into an animal caused an increase in breathing and heart rate, followed by a rapid decrease in blood pressure leading eventually to death by

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shutdown of respiratory system [1]. In 1918, some others pointed out that uncoagulated blood or citrated plasma were not vasoconstrictors, while citrated serum or blood platelet extracts (but not from leucocytes or erythrocytes) were clearly vasopressors [2]. In the early 1930s, a substance contacting smooth muscles was isolated from intestinal enterochromaffin cells and called enteramine [3]. Finally, in late 1940s, Page, Green and Rapport isolated a vasoconstrictor substance that they called serotonin before it was identified as 5-HT [4, 5].

Serotonin can activate numerous receptors. This large diversity of targets explains the complexity of the cardiovascular effects of this transmitter [6]. Concerning the 5-HT_{2B} receptor, its low level of expression in healthy cardiovascular tissues explains that its physiological and pathophysiological functions are still misunderstood. Nevertheless, new insights were recently provided using mice knockout for this receptor, *Htr2b*$^{-/-}$, that revealed the crucial contribution of this receptor to cardiac development (see Chap. 2), cardiac remodeling and coronary vasomotion. In this chapter, we will review the current knowledge concerning the role of the 5-HT_{2B} receptor in adult heart and vessels.

2 Serotonin, the 5-HT_{2B} Receptor in Cardiac Hypertrophy and Fibrosis

2.1 Left-Ventricular Hypertrophy

5-HT_{2A} and 5-HT_{2B} receptors are both expressed by cardiomyocytes. If their role in myocardial physiology is largely unknown, they are implicated in cardiac hypertrophy and failure. Surprisingly, despite a similar canonical coupling, only the 5-HT_{2A} receptor activation affects cardiac contractility by triggering positive inotropic responses [7]. In fact, 5-HT_{2B} receptors do not affect hemodynamics but appear involved in myocardial hypertrophy. Patients with congestive heart failure demonstrate an overexpression of 5-HT_{2B} receptors that is positively correlated with plasma cytokine and norepinephrine concentrations [8]. This receptor could be a major target of circulating 5-HT, plasma concentrations of the messenger being increased in heart failing patients and in animal studies with cardiac hypertrophy induced by aortic banding. Both 5-HT_{2A} and 5-HT_{2B} receptors are expressed at the cardiomyocyte cell surface and were implicated in cardiac hypertrophy and failure. The stimulation of 5-HT_{2A} receptors induces positive inotropic responses [7], but 5-HT_{2B} receptor activation does not elicit any contractile response. An impaired positive inotropic response to the β-adrenergic receptor agonist dobutamine was identified in *Htr*$_{2B}^{-/-}$ cardiomyocytes as a probable consequence of chronic left-ventricular dysfunction in these animals [9].

If the absence of 5-HT_{2B} receptors leads to ventricular hypoplasia, Nebigil et al. [10] hypothesized that 5-HT via this receptor could act as a surviving factor for cardiomyocytes. These authors demonstrated that 5-HT via the Gq-coupled 5-HT_{2B}

receptor is antiapoptotic. It protects cardiomyocytes against serum deprivation-induced apoptosis as manifested by DNA fragmentation, nuclear chromatin condensation, and TUNEL labeling and prevents cytochrome c release and caspase-9 and -3 activation via cross-talks between phosphatidylinositol-3 kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK1/2) signaling pathways. The mechanisms underlying this protection involves an activation of ERK kinases to inhibit Bax expression induced by serum deprivation and a stimulation via PI3K/Akt of NF-kB that is required for the regulation of the mitochondrial adenine nucleotide translocator (ANT-1). In parallel to these biochemical studies, ultrastructural analysis in *Htr2b*$^{-/-}$ mice heart revealed pronounced mitochondrial defects in addition to altered mitochondrial enzyme activities (cytochrome oxidase and succinate dehydrogenase) and ANT-1 and Bax expressions. Serotonin acts thus as a surviving factor implicated in myocardium homeostasis, targeting, via 5-HT$_{2B}$ receptors, mitochondria in cardiomyocytes [10] (see Fig. 9.1).

A new role for the cardiac 5-HT$_{2B}$ receptor has been identified in heart hypertrophy and failure. In cardiac explants obtained from human adults suffering from heart failure, 5-HT$_{2B}$ receptors were found to be overexpressed. This overexpression was positively correlated with cytokine and norepinephrine plasma concentrations [8]. Moreover, 5-HT plasma levels are also increased in animals with a cardiac hypertrophy induced by aortic constriction. All these observations argue in favor of a role of 5-HT in cardiac hypertrophy or heart failure through a stimulation of the 5-HT$_{2B}$ receptor. This receptor is expressed in various cells of the heart including

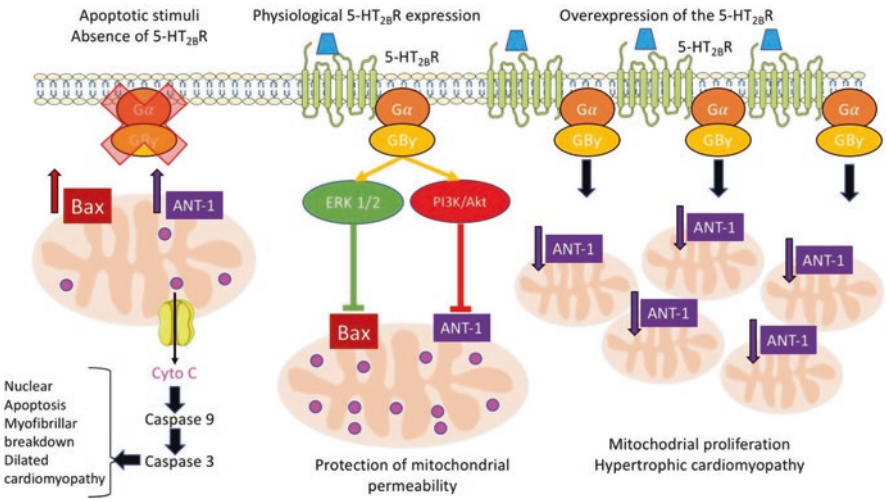

Fig. 9.1 Role of 5-HT cytoprotective signaling pathways in cardiomyocytes. 5-HT binding to 5-HT$_{2B}$ receptors activates both PI3K/Akt and ERK kinases, which inhibit ANT-1 and Bax expression, respectively, to control mitochondrial membrane permeability (middle). In the *Htr2b*$^{-/-}$ mice, the lack of receptor signaling triggers apoptosis by altered signaling to mitochondrial (left). In 5-HT$_{2B}$ receptor overexpressing mice, the receptor by acting at ANT-1 expression regulates mitochondria number, thereby leading to hypertrophic heart (right)

cardiomyocytes and fibroblasts of the extracellular cell matrix. Serotonin could therefore trigger ventricular hypertrophy by acting directly on cardiomyocytes or by a local paracrine manner through fibroblast activation. The first hypothesis was investigated in rats where a two-week-long aortic banding surgery induced a significant increase in mRNA and protein expression of the 5-HT_{2B}. Blocking the receptor with the 5-$HT_{2B/2C}$ receptor antagonist, SB215505, significantly reduced the increase in heart weight, heart wall thickness, left ventricular mass and the expression of the brain natriuretic peptide (BNP). Conversely, it did not affect the up-regulation of 5-HT_{2B} receptor protein expression. The authors addressed the question of the target cell of these effects by *in-vitro* mechanical stretch of cardiomyocytes and incubation with 5-HT. In these conditions, BNP protein expression increased time-dependently in parallel with the 5-HT_{2B} receptor. The downregulation of the receptor following transfection with a specific siRNA blocked the increase of NF-κB translocation and BNP protein [11]. All together these results support the idea that 5-HT and 5-HT_{2B} receptors act as triggers of cardiomyocyte hypertrophy independently of the extracellular cell matrix. A combinatory role of mechanical stress and the humoral activation is also pointed-out. The second hypothesis involves fibroblasts of the myocardial matrix. In mice lacking 5-HT_{2B} receptors, a chronic stimulation with the adrenergic agonist isoproterenol produced an important tachycardia but was completely unable to induce left ventricular hypertrophy [12]. Similarly, a 14 day-long infusion of angiotensin II (Ang II) increased blood pressure but no hypertrophy in the same animals [13]. Moreover, the 5-HT_{2B} receptor blockade by SB215505 prevented the increase in cardiac superoxide generation in the same infusion models [13]. The cardiac cell type expressing 5-HT_{2B} receptors (cardiomyocytes versus non-cardiomyocytes) involved in this pathological heart hypertrophy was addressed *in-vivo*. By crossing *Htr2b*$^{-/-}$ mice with mice overexpressing the receptor in cardiomyocytes, we generated mice expressing the 5-HT_{2B} receptor solely in cardiomyocytes. Similarly to *Htr2b*$^{-/-}$ animals, these mice were shown as fully resistant to isoproterenol-induced cardiac hypertrophy, dysfunction and increase in plasma cytokines concentrations [8]. This work emphasized the contribution of non-cardiomyocytes in adrenergic-induced left-ventricular hypertrophy. The 5-HT_{2B} receptor blockade was shown to prevent cytokines release as well as NAD(P)H oxidases (NOX) activation in cardiac fibroblasts. We identified a functional interaction between AT1 and 5-HT_{2B} receptors *via* a transinhibition mechanism that involves heterodimeric receptor complexes. This phenomenon is implicated in cytokines release by cardiac fibroblasts [8], (see Fig. 9.2).

Based on all these data, our group investigated the effects of a chronic 5-HT_{2B} receptor blockade by the selective antagonist, RS127445, in old spontaneously hypertensive male rats. These animals show a left ventricular hypertrophy and a chronic left-ventricular dysfunction with an apparently normal ejection fraction [14]. RS127445 effects were studied with or without a blood pressure reduction with the calcium channel antagonist nicardipine. In this model, the 5-HT_{2B} receptor is overexpressed in the left ventricle but, despite this overexpression, the antagonist failed to improve cardiac function and hypertrophy. A likely explanation seems to be an action on coronary arteries (see below).

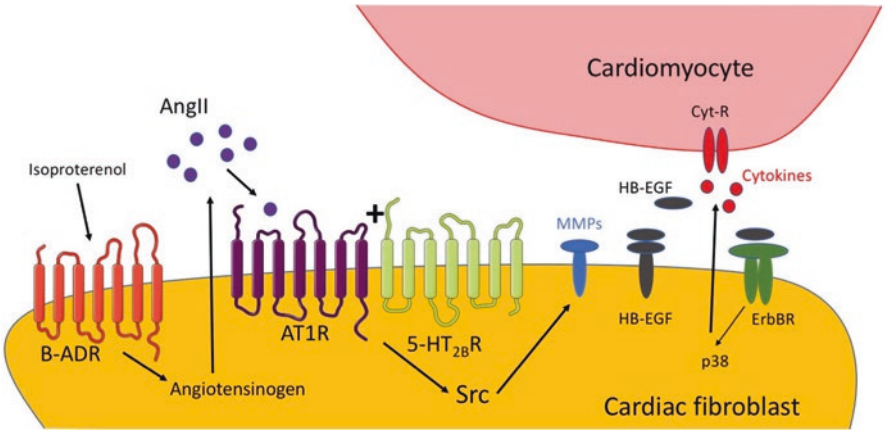

Fig. 9.2 Hypertrophic signaling pathway for cytokine production after 5-HT or Ang II stimulation in adult cardiac fibroblasts. Stimulation of β-Adrenergic receptors on cardiac fibroblasts leads to the release of Ang II that stimulates the AT1–5-HT2B receptor complexes activating MMPs, which induces the pro-HB- EGF cleavage via Src. Soluble HB-EGF activates ErbB-receptors to induce cytokine release via p38 mitogen-activated protein kinase activation. Released cytokines and HB-EGF bind their receptors in cardiomyocytes to activate hypertrophy in a paracrine way

2.2 Cardiac Fibrosis

If the 5-HT$_{2B}$ receptor acts as a co-receptor to AngII in cardiac hypertrophy by stimulating ventricular fibroblasts, it is tempting to speculate that it could also regulate extracellular matrix deposit and fibrosis. The treatment of neonatal cardiac fibroblasts with 5-HT increases the expression of smooth muscle α-actin, a marker of fibroblast differentiation into myofibroblasts, stimulates their migration, and enhances secretion of TGF-β1 and expression of metalloproteases (MMPs). All these effects were initially assumed to be mediated through 5-HT$_{2A}$ receptors [15]. But, in mice, 5-HT- or AngII-stimulated TGF-β1 release in adult cardiac fibroblasts is sensitive to 5-HT$_{2B}$ receptor blockade [8]. Treatments with epidermal growth factor receptor (EGFR, ErbB1/4)-selective inhibitors or with selective inhibitors of MMPs also abolish AngII- and 5-HT-induced cytokine release. Finally, the use of *HB-EGF*$^{-/-}$ cardiac fibroblasts confirms that EGFR transactivation is absolutely required for AngII- and 5-HT-dependent cytokine release. All these results point-out that the 5-HT$_{2B}$ receptor acts as a co-receptor of Ang II to cardiac fibrosis.

Another aspect of fibrosis is endothelial-mesenchymal transformation (EMT). Inducers of EMT during valvulogenesis include VEGF, TGF-β1, and Wnt/β-catenin, which are regulated in a spatiotemporal manner. Serotonin can initiate TGF-β signaling and recent evidences suggest that degenerative valvular disease may be mediated by developmental pathways including bone morphogenic protein (BMP), Wnt and Notch signaling, nitric oxide, and Ang II [16]. Wnt2 acts as an angiogenic factor for endothelium *in-vitro* and *in-vivo* whose target genes undergo complex regulation by the tissue microenvironment [17]. Gene profiling identified the 5-HT$_{2B}$

receptor as a down-regulated target gene of Wnt2 signaling in HUVEC. Valve interstitial cells are made from cells of various origins i.e. embryonic epicardium and endocardial cushions and the adult bone marrow. This opens the interesting possibility that these populations of fibroblasts are functionally different and could differ in their susceptibility to and/or participation in fibrotic pathological processes [18] (see Chaps. 13 and 14).

3 Serotonin, the 5-HT_{2B} Receptor and the Regulation of Vasomotor Tone

3.1 Hypertension

In the late 1970s, the 5-HT_2 receptor antagonist ketanserin was clinically used as an antihypertensive compound but the reduction of blood pressure was attributed to its affinity for α_1-adrenergic receptors, excluding a role for 5-HT in systemic pressure control. This postulate was confirmed by the absence of effect of ritanserin, a non-selective 5-HT_2 receptor antagonist that lacks α-adrenergic receptor affinity. Finally, the *Htr2b*$^{-/-}$ mouse was characterized for resting blood pressure and did not demonstrate any difference compared to controls. Similarly, the selective 5-HT_{2B} receptor antagonists SB215505 and SB206553 neither affected basal blood pressure nor response to a 14 days long AngII infusion [13]. In fact, due to the heterogeneous expression of serotonergic receptors in the vascular wall, 5-HT plays a complex role in regulating vasomotor tone. In arterial wall, endothelial cells express 5-HT_{1B}, 5-HT_{2B} and 5-HT_4 receptors, whereas smooth muscle cells express 5-HT_{1B}, 5-HT_{2A}, 5-HT_7 receptors and in some cases 5-HT_{2B} [19]. Indeed, receptors expressed on endothelial cells can induce vasodilation, while stimulation by 5-HT of smooth muscle cells elicits vasoconstriction. Interestingly, this vasoconstriction is much greater in experimental or genetic models of hypertension than in normotensive animals [20] and smooth muscle from rats, which were made hypertensive by a combination of deoxycorticosterone acetate and salt (DOCA/salt) are more responsive to 5-HT, opening the question of 5-HT involvement in the pathophysiology of hypertension and suggesting a shift in receptor population expressed by large arteries. Concerning 5-HT_{2B} receptors, different studies [21, 22] provided pharmacological and molecular evidences showing that 5-HT_{2A} (ketanserin-sensitive) receptors are primarily responsible for contraction of arteries of normotensive rats, whereas 5-HT_{2B} receptors (relatively insensitive to ketanserin) are primarily responsible for contraction of DOCA/salt arteries of rats. This switch could explain a higher contribution of 5-HT in hypertension than the physiological state because 5-HT has higher affinity for 5-HT_{2B} than for 5-HT_{2A} receptors; a low concentration being enough to trigger hypertension through 5-HT_{2B} receptor activation. These *in-vitro* data were confirmed *in-vivo* because the 5-HT_{2B} receptor selective antagonist LY-272015 reduces blood pressure of hypertensive DOCA/salt rats [23].

5-HT_{2B} receptors can also be involved in drug-induced hypertension. Fenfluramine belongs to a family of anorectic compounds that were withdrawn from the market due to the induction of pulmonary hypertension and valvulopathy during the treatment. The fenfluramine active metabolite is nordexfenfluramine known to induce systemic hypertension in rats by activating 5-HT_{2A} receptors. This compound has a higher affinity for 5-HT_{2B} receptors whose expression is increased in DOCA/salt hypertensive rats. Thus, the 5-HT_{2B}-mediated increase in blood pressure by norfenfluramine can be revealed in the context of high blood pressure making hypertensive patients at risk when using such compounds. This pressure effect seems to take place in small arteries because, in aorta of normal or hypertensive rats, contractile effect of norfenfluramine is mediated by 5-HT_{2A} but not 5-HT_{2B} receptors. 5-HT_{2A} and 5-HT_{2B} receptors seem therefore to play complementary roles depending on vascular beds and/or physiological state of vessels [24].

In summary, 5-HT weakly contributes to basal vasomotor tone but its contribution is reinforced in hypertension mainly due to changes in expression from 5-HT_{2A} to 5-HT_{2B} receptors, which display higher affinity to 5-HT.

3.2 Coronary Arteries

In-vitro studies on endothelial cells isolated from human coronary arteries have shown that 5-HT, by activating 5-HT_{1B} and 5-HT_{2B} receptors, is responsible for nitrites production from NO metabolism [25]. In conscious dogs, the 5-HT_2 receptor high affinity ligand, ergonovine, induces a biphasic response i.e. a vasodilation depending on endothelial NO synthase (NOS-3) activity followed by vasoconstriction. Similarly, in rats, 5-HT increases coronary flow in a dose-dependent manner. These effects are obtained on vessels with an intact endothelium. In our model of chronic left ventricular dysfunction with preserved ejection fraction in aging spontaneously hypertensive rats [14], we observed an increase in subendocardial ventricular fibrosis and ECG troubles corresponding to myocardial ischemia. We then explored a possible contribution of the 5-HT_{2B} receptor to coronary artery vasodilation in wild-type and *Htr2b*$^{-/-}$ mice. First, 5-HT injections induced myocardial fibrosis in *Htr2b*$^{+/+}$ animals that was amplified in knockout mice. Secondly, we observed an augmentation of coronary artery resistance in *Htr2b*$^{-/-}$ mice, a reduction of cGMP content in coronary vessels and a potentiation (≈60%) of the vasoconstriction induced by the 5-HT_2 receptor agonist α-methyl-5-HT. These data argue in favor of the role of the endothelial 5-HT_{2B} receptor in coronary artery vasodilatation [26]. The presence of endothelial dysfunction or partial suppression of endothelial cells could therefore amplify the coronaro-constricting effects of 5-HT released in vascular lumen [27]. These data open the possibility that interactions between activated platelets and vascular wall contribute to acute ischemic or ischemic/reperfusion lesions.

3.3 Migraine

Migraine is a syndrome that affects 15–18% of women and about 6% of men. It is characterized by intense, pulsatile headaches, classically unilateral and often accompanied by nausea, vomiting and photo/phonophobia. Briefly, the origin of migraine is a decrease in cerebral flow by vasoconstriction, followed by a reactive vasodilatation responsible for headache. The vasodilation is associated with release of vasodilators such as NO and 5-HT. It has long been known that 5-HT receptors are involved in pathophysiology of migraine because of its strong contribution to regulation of cerebral vasomotricity [28]. Moreover, the use of serotonergic antimigraine drugs such as triptans (5-HT_{1B} agonists), reveals some of the pathophysiological mechanisms underlying this pathology. Indeed, numerous clinical trials have shown efficacy of compounds such as sumatriptan in the treatment of migraine crisis [29]. Triptans act by producing a cerebral vasoconstriction and also at sensory trigeminal ganglia. Thus, anti-migraine compounds, such as dihydroergotamine and triptans, in addition to their vascular action, prevent activation of trigeminal-vascular complex and release of calcitonin gene-related peptide (CGRP) associated thereto [30, 31]. They also prevent possible generation of retrograde nerve impulses (axon reflex) in nociceptive circuits in response to local meningeal excitation. Overall, this dual action, both vascular and neuronal, through stimulation of 5-HT_{1B} receptors, most likely accounts for efficacy of these drugs in stopping migraine attacks [28]. Nevertheless, methysergide and pizotifen are 5-HT_2 receptor antagonists [32, 33], and their preventive effects of migraine may thus result from 5-HT_{2B}-receptor blockade, which expressed by endothelial cells of meningeal vessels [34]. Their activation induces relaxation by synthesis of NO in cerebral arteries and jugular vein and concomitant activation of sensory trigeminovascular afferents [35]. They are also expressed in rat basilar artery where their activation triggers a contraction that is prevented by ergometrine [36]. In addition, a recent genetic study identified 5-HT_{2B} receptors as a susceptibility gene to migraine [37]. Endothelial 5-HT_{2B} receptors may thus trigger dilation of meningeal blood vessels, which by activating sensory trigeminovascular afferents induces head pain.

3.4 Tumor Angiogenesis

In tumor-infiltrating macrophages, 5-HT does not enhance colon cancer tumor cell proliferation but may act as a regulator of angiogenesis by reducing the expression of MMP-12, entailing lower levels of angiostatin—an endogenous inhibitor of angiogenesis [38]. 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 NOS-3 in human endothelial cells. In SB204741-treated mice, the selective blockade of the 5-HT_{2B} receptor resulted in the reduction of tumor angiogenesis and growth through the inhibition effect of ERK1/2 and NOS-3 [39].

Therefore, the possibility that 5-HT_{2B} receptors participate in tumor angiogenesis is a likely possibility that remains to be evaluated in other tumors subtypes.

4 Outlook and Prospects

The 5-HT_{2B} receptor is a fascinating target, which contributes to the normal adult cardiovascular physiology despite its low expression; however, its overexpression is observed in many pathological situations. Why is this receptor overexpressed and what is the contribution of 5-HT in these events? A common feature could be a reaction of the tissue to cell loss and/or increase in mechanical stress. The 5-HT_{2B} receptor would then be the favorite target of 5-HT to trigger cell hypertrophy, growth, neoangiogenesis and fibrotic scaring. Therefore, the 5-HT_{2B} receptor would appear as part of the re-expression of a fetal program making a link between development, tissue repair and scaring.

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Chapter 10
5-HT_{2B} Receptor in Cardiopulmonary Disease

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Abbreviations

5-HT	Serotonin
5-HT_{2B}R	Serotonin receptor 2B
AngII	Angiotensin II
AT_1R	Angiotensin II type 1 receptor
BMPRII	Bone morphogenetic protein receptor II
GAG	Glycosaminoglycan
GPCR	G protein-coupled receptor
IL	Interleukin
MCT	Monocrotaline
PAC	Proangiogenic cell
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary artery smooth muscle cell
PH	Pulmonary hypertension
ERK	Extracellular regulated kinase
ROS	Reactive oxygen species
RS127445	5-HT_{2B}R antagonist
RVSP	Right ventricular systolic pressure
SB204741	5-HT_{2B}R antagonist
SERT	Serotonin transporter
SHR	Spontaneously hypertensive rat
SuHx	Sugen-hypoxia
TFGβ	Transforming growth factor β
WT	Wild type

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1 Introduction

Cardiopulmonary disease is widely appreciated to be a leading cause of death worldwide. Such diseases have a vast range of etiologies and extensive research efforts have been focused on understanding disease initiation and progression. Serotonergic dysfunction has long been understood to contribute to cardiopulmonary pathology, and recent discoveries have elucidated unique roles for serotonin—or 5-hydroxytryptamine (5-HT)—receptors, such as the 5-HT_{2B} receptor (5-HT_{2B}R), in the pathophysiology of various cardiopulmonary diseases. This chapter focuses on three main areas of research involving 5-HT_{2B}R: (1) heart valve disease, (2) pulmonary hypertension, and (3) cardiac hypertrophy. The following studies have motivated the investigation of 5-HT_{2B}R as a chief mediator of cardiopulmonary disease.

2 Heart Valve Disease

The discovery that 5-HT plays a causal role in cardiopulmonary disease can be traced back to 1931 when the Dutch pathologist A. J. Scholte documented thickened tricuspid valves in a deceased carcinoid syndrome patient [1]. Carcinoid syndrome occurs following oncogenic transformation of enterochromaffin cells, which are the primary synthesizers of 5-HT in the gastrointestinal tract. If a carcinoid tumor metastasizes to the liver, tumor cells will release vasoactive 5-HT into the systemic circulation via the hepatic veins. Increased plasma 5-HT level leads to carcinoid heart disease, indicated by the characteristic development of a plaque-like, fibrous thickening of the heart valves found in over 65% of carcinoid syndrome patients [2–4]. Further, several classes of drugs that target 5-HT signaling were found to contribute to the onset of heart valve disease and ultimately led to the investigation of the specific 5-HT receptor subtype responsible for the development of heart valve disease.

2.1 Initiators of Heart Valve Disease

Discovery of the role of 5-HT_{2B}R in heart valve disease can be attributed to the observation of diseased valves in patients taking medications for non-valve related conditions. Several classes of medications have metabolites now known to activate 5-HT_{2B}R, resulting in compromised cardiac valves.

2.1.1 Anorexigens

Imbalance between energy intake and expenditure can result in eating disorders and obesity, which are important health concerns in developed countries. Medications for excessive eating disorders inadvertently aided in the discovery of 5-HT$_{2B}$R as a key mediator of heart valve disease. The anorexigen combination regimen of *fen*fluramine and *phen*termine ('Fen-Phen') became widely prescribed starting in 1984. While both drugs had been previously prescribed with minimal success, the combination of the two drugs resulted in sustained weight loss with fewer adverse side effects and improved appetite control [5]. Phentermine primarily functions through the release of norepinephrine to reduce perception of hunger [6]. However, fenfluramine is an amphetamine derivative that stimulates serotonin release while simultaneously inhibiting the function of 5-HT uptake transporters, increasing 5-HT which signals through the hypothalamus to suppress appetite [7]. Fen-Phen was highly popular until 1997 when it was found that it increased both left- and right-sided heart valve defects after 12 months of use [8]. The incidence of heart valve disease was later reported to be as high as 25% in patients treated on average for 20 months [9]. These studies were followed by a seminal report which comprehensively tested 15 molecules at 11 distinct 5-HT receptor subtypes and systematically determined that the fenfluramine metabolite, norfenfluramine, exhibited high potency and high affinity for the 5-HT$_{2B}$R. All other 5-HT receptor subtypes were ruled out based on pharmacological differences from heart valve disease-associated molecules and negative control molecules. Interestingly, phentermine did not display agonism at the 5-HT$_{2B}$R, providing an explanation as to why the use of phentermine for decades prior to Fen-Phen did not result in the emergence of heart valve disease and further connected serotonin signaling through 5-HT$_{2B}$R to disease [10].

Prescribed to patients with hypertriglyceridemia or diabetes, benfluorex functioned as an appetite suppressant due to its close structural relationship with amphetamines. This drug, like Fen-Phen, metabolizes into nordexflenfluramine, now known to be a high affinity 5-HT$_{2B}$R agonist. A 2012 study documented that a 40-year-old woman on benfluorex therapy underwent a mitral valve replacement and resumed the therapy after the operation. Upon examination 4 years after valve replacement, the woman presented with mitral valve bioprosthesis hypertrophic scarring and similar histopathological lesions on the aortic valve. These lesions were formed by smooth muscle α-actin- and vimentin-positive cells which deposited plaques in the glycosaminoglycan (GAG) matrix [11]. This case further validates the hypothesis that norfenfluramine activation of 5-HT$_{2B}$R causes valve disease.

2.1.2 Ergot-Derived Therapeutics

Migraine headaches are believed to be transmitted through the blood vessels in the brain associated with the meninges. Several human meningeal tissues express *HTR2B* – the gene encoding 5-HT$_{2B}$R—and circulating 5-HT levels fluctuate during

the phases of a migraine. In an animal model exposed to a known migraine-inducing agent, 5-HT_{2B} activation is required for the release of neuroinflammatory peptides which mediate trigeminal nerve activation and the sensation of pain. It is known that 5-HT_{2B}R activation can induce the release of nitric oxide as well as induce the relaxation of cerebral vessels, suggesting a causative role of 5-HT_{2B}R activation in neuroinflammation, endothelium-dependent relaxation, and activation of sensory trigeminovascular afferents [12, 13].

Ergot-derived therapeutics were the standard treatment for migraines until the mid-1960s when a strong link was discovered between the ergot agents – methysergide and ergotamine – and heart valve disease. A later study determined that these compounds possessed a high affinity for 5-HT_{2B}R in heart valve tissue [14]. It is even suggested that the similar structure of 5-HT and ergot agents could point to a common pathology with carcinoid valve disease [15].

The non-selective dopamine agonists pergolide and cabergoline were popular therapeutics prescribed to patients with Parkinson's disease and are also ergot-derived compounds. Unsurprisingly, the interference of these compounds with the dopamine/5-HT signaling axis resulted in decidedly similar echocardiographic and histopathological findings as observed in the valves of patients with carcinoid heart disease [16]. Patients receiving either pergolide or cabergoline were reported to have moderate-to-severe regurgitation in at least one heart valve more frequently compared to patients who received non-ergot therapeutics or controls. Furthermore, the incidence of heart valve disease has been found to be as high as 28% in patients receiving ergot-derived dopamine agonists where no increase in prevalence is associated with other dopamine agonists [17, 18].

Pergolide and cabergoline induced fibrotic changes in cardiac valves are due to their high affinity for the 5-HT_{2B}R. The association of ergot-derived compounds with fibrotic pathologies as well as the structural and functional similarity to 5-HT acting upon the 5-HT_{2B}R reveal yet another pathological function of 5-HT_{2B}R signaling.

2.1.3 Amphetamines

While amphetamine derivatives have been prescribed as anti-obesity drugs, the amphetamine 3,4-methylenedioxymethamphetmaine—MDMA ("ecstasy")—is a psychostimulant drug of abuse used recreationally throughout Europe and North America. This drug reverses the function of the 5-HT reuptake transporter, resulting in a concentrated release of 5-HT and psychostimulatory effects [19]. MDMA and its metabolite 3,4-methylenedioxyamphetamine—MDA—preferentially bind and activate human recombinant 5-HT_{2B}R. Similarly to fenfluramine, these drugs induce and prolong mitogenic signaling through 5-HT_{2B}R *in vitro* which suggests that MDMA would induce valvular heart disease with continued use [20, 21].

2.2 Heart Valve Disease Mechanisms

HTR2B and *HTR2A* mRNAs are highly expressed in human heart valves with no *HTR2C* mRNA detectable. 5-HT$_{2B}$R signaling is of particular interest since the above-mentioned prescribed drugs and their active metabolites activate this receptor and result in valve disease whereas chemically similar drugs which do not bind the 5-HT$_{2B}$R (e.g., lisuride and terguride) do not result in valve pathology. This highlights the public health implications of therapeutics aimed at 5-HT signaling. In order to avoid mistakes of the past, 5-HT$_{2B}$R screening is a necessity to identify potential drug-induced valvular heart disease.

Despite the litany of disease-causing agents described, mechanistic characterization of 5-HT$_{2B}$R-induced valve disease is lacking. It is known that medications acting through serotonergic mechanisms, specifically 5-HT$_{2B}$R, are likely to result in valvulopathy. While it appears that the valve interstitial cells are responsible for valve fibroplasia, it is unclear if they are the direct target of 5-HT$_{2B}$R agonists. Valvular regurgitation and insufficiency can result from subtle, non-destructive thickening and the associated hemodynamic overload can lead to myocardial dysfunction and heart failure, highlighting the need to understand 5-HT$_{2B}$R signaling mechanisms and limit erroneous receptor activation.

Several reports have described valvular lesions arising after treatment with anorexigens, ergot alkaloids, or carcinoid syndrome as "glistening, superficial plaque-like thickenings" that present on the surface of the leaflets and cusps [22]. This valve phenotype is recapitulated in Sprague-Dawley rats by continuous 5-HT administration over a three-month period. Morphological and echocardiographic alterations of the rat aortic valve mimic those observed in carcinoid heart disease. Aortic valve leaflets are thickened and retracted with evident carcinoid-like plaques made of collagen rich tissue in rats treated with 5-HT. These changes are thought to be due to increased proliferation of cardiac valvular subendocardial cells which concomitantly increased expression of the 5-HT$_{2B}$R [23]. Another study observed similar changes as early as 7 days following 5-HT administration. Modified Movat's pentachrome staining revealed significantly thicker mitral and aortic valves due to an expansion of GAG content resulting in loss of valve compliance and function. The GAG network was also more vascularized following 5-HT administration indicating a loss of quiescence and increased remodeling. These changes faithfully mimic anorexigen-associated valvulopathy. 5-HT treated rats had transcription of the gene encoding the 5-HT transporter (SERT or 5-HTT) down regulated, indicating a decrease in 5-HT receptor recycling; 5-HT$_{2B}$R gene transcription was also increased [22]. SERT is critical to protect against the adverse effects of 5-HT overactivity, so valve remodeling is thought to be a combined effect of its downregulation combined with increased mitogenic 5-HT$_{2B}$R signaling.

Similar to six other classes of 5-HT receptors, 5-HT$_{2B}$R is a G protein-coupled receptor (GPCR) that follows a well characterized signaling cascade. The Gαq subunit is released upon receptor activation which proceeds to activate the downstream effectors phospholipase C-β and protein kinase C through release of intracellular

calcium and diacylglycerol liberation. 5-HT_{2C} receptors are absent in the cardiovascular system while 5-HT_{2A}R and 5-HT_{2B}R are presumed to have similar cardiovascular functions due to the significant sequence homology. In the context of valve disease, specific activation of the 5-HT_{2B} GPCR also activates second messenger signals identified by the phosphorylation and activation of both the tyrosine kinase Src and extracellular regulated kinase (ERK) pathways. These pathways may synergize with signaling of transforming growth factor β (TGFβ) leading to enhanced mitogenesis [4].

Valve interstitial cells are thought to be the drivers of valve remodeling. In the context of calcific aortic valve disease, quiescent cells are activated into a myofibroblast-like phenotype, leading to increased extracellular matrix deposition and eventual formation of bone-like calcific nodules. Nodule formation can be modeled *in vitro* by treating valve interstitial cells with TGFβ1 resulting in simultaneous phosphorylation of the tyrosine kinase Src and expression of the contractile marker SM22α. Interestingly, treatment with the specific 5-HT_{2B}R antagonist SB204741 mitigates the formation of nodules by preventing SM22α upregulation, but Src phosphorylation is still increased. Time-lapse microscopy revealed Src is sequestered upon administration of the 5-HT_{2B}R antagonist, arresting its motility to restrict the phosphorylation and activation of the type II TGFβ receptor [24].

The 5-HT_{2B}R may also play a role in the progression of mitral valve disease. In a canine model of myxomatous mitral valve disease, a significant increase in the expression of 5-HT_{2B}R was observed in symptomatic valves compared to control animals while there was no change in 5-HT_{2A}R expression in either group. Staining of this receptor co-localized with α-smooth muscle actin expression, indicating a potential role for the 5-HT_{2B}R in valve remodeling by contractile interstitial cells [25]. A subsequent study investigated 5-HT_{2B}R in the context of mitral valve prolapse in human, canine, and murine tissue and observed an association between mitral valve prolapse and leaflet 5-HT_{2B}R expression in humans. The same was observed in canine tissue along with an increase in ERK phosphorylation, which is known to increase interstitial cell activation and remodeling. This effect was prevented with the 5-HT_{2B}R antagonist LY272015. Finally, in mice given chronic angiotensin II (AngII) infusion, valve leaflet area was significantly increased in response to AngII but was mitigated in the presence of a 5-HT_{2B}R antagonist; neither of the 5-HT_{2A}R antagonists terguride or ketanserin had an effect on leaflet area [26]. These studies sufficiently describe the specific role of 5-HT_{2B}R, but not 5-HT_{2A}R, in the remodeling of mitral valves.

Due to the lengthy nature of valve disease onset, the scarcity of available tissue, and a variety of disease triggers, animal models of valve disease are lacking and have limited studies focused on perturbating and probing specific molecular mechanisms behind valvulopathies. However, observational studies have made it clear that 5-HT_{2B}R signaling plays an extensive role in disease development—most likely through the activation of normally quiescent valve cells—resulting in valve remodeling, loss of compliance, and ultimately, loss of valve function (Fig. 10.1).

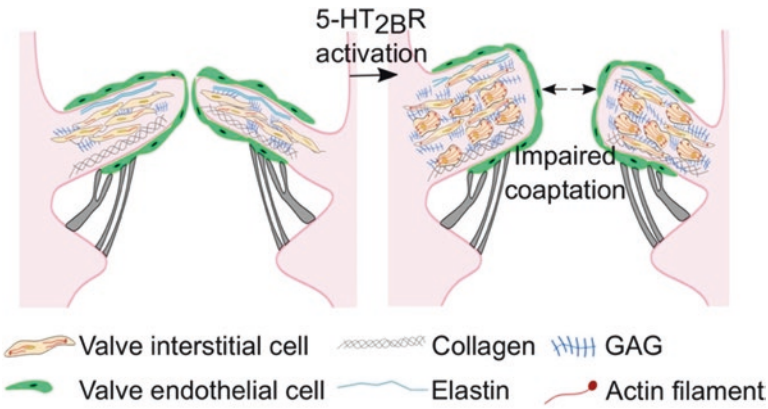

Fig. 10.1 Impact of 5-HT$_{2B}$R activation on valve structure and function. Interstitial cell expansion and GAG deposition decreases valve compliance and coaptation

3 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a deadly disease of the pulmonary vasculature that is incompletely understood in terms of its cellular and molecular mechanism. Over 200,000 people are hospitalized annually in the United States with some form of pulmonary hypertension (PH) with an associated mortality rate approaching 10% [27]. PH is clinically defined as a mean pulmonary arterial pressure exceeding 25 mmHg, measured by right ventricle catheterization [28]. Group 1 PH encompasses PAH which can be idiopathic, heritable, or acquired. Idiopathic PAH has no known cause with 2–3 new cases per million annually, whereas over 75% of heritable PAH is due to a mutation in the bone morphogenetic protein receptor II (BMPRII) gene and is about 10 times less prevalent than idiopathic PAH. Acquired PAH is commonly associated with exposure to other risk factors such as human immunodeficiency virus, scleroderma, or anorexigen use [29]. While PAH incidence is rare, the mortality rate is striking, with merely 67% of patients surviving 3 years after diagnosis. This number may even be artificially high due to a strong influence of survival bias introduced from the method of data collection [30, 31].

3.1 Models of PAH

Increased pulmonic blood pressure, the hallmark of PAH, is due to a progressive increase in pulmonary vascular resistance and remodeling associated with vasoconstriction. It is histologically characterized by neomuscularization of small pulmonary arteries and intimal thickening, medial hypertrophy, adventitial proliferation, and abnormal extracellular matrix deposition. The culmination of these remodeling events is irreversible lumen narrowing, increased pulmonary artery resistance,

hypoxia, right heart hypertrophy, and eventually, right heart failure and death [32]. Multiple experimental models of PH in rodents have been utilized to capture different aspects of the disease, and interpretation of results should consider the method used to induce disease. Small rodents do not develop disease that completely recapitulates human PAH. While many experimental models are characterized as PH models and do not further designate a PH group, for the sake of simplicity in this chapter, the discussed experiments will be referred to as models of PAH. The classical model of PAH is chronic hypoxia exposure. This model has led to understanding of hypoxia-induced vascular remodeling, however, the obliterative lesions observed in human patients with severe PAH are not replicated with chronic hypoxia. The monocrotaline (MCT) lung injury model attempts to address the limitation of chronic hypoxia by causing pulmonary arterial endothelial cell dysfunction and inflammatory cell infiltration. Pulmonary vasoconstriction and right heart hypertrophy are faithfully modeled with MCT; however, this model is restricted to rats as mice do not develop disease in response to MCT which is yet to be understood. While MCT injury is simple and reproducible, it is an acute injury that fails to fully capture the evolving nature of human PAH [33]. In order to recapitulate heritable PAH, mouse models harboring BMPRII mutations have been developed to study the disease. Smooth muscle- and vascular endothelial cell-specific knockouts of BMPRII have independently been shown to generate pulmonary vascular remodeling and increases in right ventricular systolic pressure (RVSP). However, this model is highly variable, incompletely penetrant, and complex vascular lesions do not form [34]. Toward this end, the vascular endothelial growth factor receptor 1 and 2 blocker, SU5416 (“Sugen”), combined with chronic hypoxia (SuHx) has served as a preclinical drug model. SuHx results in angioobliterative pulmonary lesions and increased RVSP which is not reversible upon returning to normal air. This model has been useful for studying the reversibility of PAH and uncovering the immunological mechanisms behind the pathobiology of PAH [34]. While no one method completely emulates human PAH, these models have increased the understanding of disease progression and characteristics.

The cellular mechanism behind PAH hinges upon an interplay between the vascular endothelium, pulmonary artery smooth muscle cells (PASMCs), and bone marrow-derived cell populations. Nitric oxide is a key mediator of vasodilation, as well as downregulation of leukocyte adhesion and vascular proliferation. Reduced nitric oxide bioavailability has been reported in PAH and contributes to increased PASMC migration and proliferation in the distal arteries of the lungs [35]. Disruption of vascular homeostasis in the context of PAH is commonly due to an imbalance of prostacyclin and endothelin. Prostacyclin is produced by endothelial cells and induces PASMC relaxation and vasodilation; endothelin is produced by vascular endothelium, PASMCs, and lung fibroblasts and induces calcium release by the sarcoplasmic reticulum as well as PASMC proliferation and vasoconstriction. As these factors become imbalanced, vascular resistance and remodeling occurs, leading to right ventricle hypertrophy [36]. On a molecular level, receptor tyrosine kinases play a critical role in modulating cell proliferation, migration, and differentiation. In humans and experimental models of PAH, platelet-derived growth

factor receptor, epidermal growth factor receptor, fibroblast growth factor receptor, and c-kit receptor have all been investigated for their pathogenic role in contributing to excessive vascular remodeling. Ample evidence exists for the tyrosine kinase Src being abnormally activated in PAH and mediating the effect of receptor tyrosine kinases [37]. Evidence for inflammatory infiltrate in the onset and progression of PAH pathology is growing. Known increases in cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor-α are elevated in patients with PAH. Elevated circulating cytokine levels are associated with inflammatory cell recruitment and accumulation, and they have been correlated with worse clinical outcomes [38].

Therapies aimed at treating PAH are lacking and often focus on mitigating symptoms of the disease but are yet unable to address the underlying pathophysiology due to the limited understanding of disease mechanisms. Three classes of medications currently used include: prostacyclin analogues to restore deficient endogenous prostacyclin levels, phosphodiesterase type 5 inhibitors to compensate for down-regulated nitric oxide pathway, and endothelin antagonists to inhibit the up-regulated endothelin pathway [39]. These medications were adopted from treatments for other illnesses, and therefore are not ideal for managing PAH. They modestly improve disease symptoms through transitory vessel dilation without addressing the underlying pathophysiological hypertensive agents of vessel stiffening and remodeling [40, 41]. This highlights the need for a more specific class of therapies aimed at directly targeting molecular pathways relevant to PAH.

3.2 Serotonin in PAH

Identification of the role of 5-HT in PAH pathogenesis goes back to the 1960's when an epidemic of PAH arose in a Swiss population taking the anorexigen aminorex fumarate; a second serotonergic aneroxigen-induced outbreak of PAH accompanied the use of Fen-Phen in the 1990s [42]. These drugs are SERT substrates responsible for modulating the bioavailable plasma serotonin levels. SERT inhibitors abrogate 5-HT-induced mitogenesis, and mice deficient for SERT have partially reduced pulmonary vascular remodeling under hypoxic conditions [43]. A correlation between high plasma 5-HT levels and total pulmonary resistance was established in the 1980s when a patient was diagnosed with PH while carrying a familial platelet storage deficiency [44]. 5-HT is released by pulmonary neuroendocrine cells and neuroepithelial bodies in response to hypoxia and is sustained in PH patients 5-HT mediates a myriad of functions in the vasculature, most notably smooth muscle cell hypertrophy and hyperplasia [45]. PASMCs and endothelial cells both express mRNA encoding 5-HT$_{1B}$R, 5-HT$_{2A}$R, 5-HT$_{7}$R, and 5-HT$_{2B}$R. Being a potent pulmonary vasoconstrictor and capable of inducing vascular remodeling, 5-HT has a dual role in response to hypoxia and acting through its cognate receptors [46].

3.3 5-HT$_{2B}$R Mechanism in PAH

The anorexigens fenfluramine and dexfenfluramine increase the risk of developing PH by a factor of 3.7- to 23-fold depending on the study referenced [47, 48]. The primary dexfenfluramine metabolite, nordexfenfluramine, is a high affinity 5-HT$_{2B}$R agonist. Furthermore, 5-HT$_{2B}$R overexpression (but not 5-HT$_{2A}$R) is observed in PAH [46]. This led to the discovery of 5-HT$_{2B}$R activation being necessary for the development of hypoxia-induced increases in RVSP and vascular muscularization. In mice, 5 weeks of exposure to 10% O_2 causes a pathologic increase in RVSP that is completely nullified in the presence of the 5-HT$_{2B}$R antagonist RS-127445. Compared to normoxic mice, hypoxia significantly increases the number of fully muscularized arteries, an effect prevented with RS-127445 administration. These results were reproduced in 5-HT$_{2B}$R knockout mice, and the utility of 5-HT$_{2B}$R ablation was further strengthened when the dual insult of hypoxia and dexfenfluramine was unable to increase RVSP. Hallmarks of lung-remodeling associated with PAH progression are cell proliferation and increased serine elastase, which activates stores of growth factors such as TGFβ. Cell proliferation, elastase activity, and TGFβ levels are all normalized in hypoxic animals treated with RS-127445 and in 5-HT$_{2B}$R knockout animals. Interestingly, repeated acute exposure to hypoxic conditions increases RVSP independent of 5-HT$_{2B}$R activity, but 5-HT$_{2B}$R knockout mice do not have increase elastase activity compared to wild type (WT) mice after repeated acute hypoxia [46]. This indicates the function of 5-HT$_{2B}$R is not at the level of acute vasoconstriction but rather at the level of downstream signaling mechanisms that govern vascular remodeling.

Following the discovery that 5-HT$_{2B}$R is necessary for hypoxia induced PAH, the experimental inflammatory model of PAH using MCT administration was investigated in rats in order to parse out the individual contributions of SERT and 5-HT receptors. It was demonstrated that SERT inhibitors were able to prevent an increase in RVSP and pulmonary artery muscularization but 5-HT$_{2B}$R antagonism was not. SERT inhibitors abrogate the production of inflammatory cytokines characteristic of MCT-induced injury as well as reverse right ventricle hypertrophy with no effect of 5-HT$_{2B}$R antagonism reported [49]. These findings point back to the original serotonin hypothesis of PAH that peripheral 5-HT availability is the key driver of disease. However, the MCT model of PAH has received fair amounts of criticism for the acute, destructive nature of disease, and since it has been shown that 5-HT$_{2B}$R does not confer protection over acute disease onset [46], these data should be considered carefully. In contrast, subsequent studies in the MCT experimental model of disease have shown the utility of 5-HT$_{2B}$R antagonism in treating PAH in rats. Multiple 5-HT$_{2B}$R-specific antagonists have been shown to reduce RVSP, vascular remodeling, and right ventricle hypertrophy in MCT-injected rats [50, 51]. The discordant results in the discussed MCT experiments demonstrate a need for a better experimental model of PAH that captures disease progression more faithfully.

Mice expressing the patient-derived R899X BMPRII mutation develop PAH within a few weeks with about 50% disease penetrance. These mice have been a

useful model for exploring the cellular mechanism behind the heritable form of PAH for patients harboring a BMPRII mutation. 5-HT$_{2B}$R antagonism with SB204741 normalizes RVSP to control levels and decreased the stiffness of distal pulmonary vessels, consistent with other models of PAH. In this model, 5-HT$_{2B}$R antagonism was observed to work through the tyrosine kinase Src. Antagonism simultaneously decreased Src phosphorylation and trafficking leading to decreased activation of downstream effectors and subsequent transcription of genes encoding contractile proteins such as RhoA, gamma actin, and myosin light chain 12a. Isolated BMPRII smooth muscle cells displayed significantly enhanced contractile behavior which was abrogated with 5-HT$_{2B}$R antagonism. Interestingly, administration of a 5-HT$_{2B}$R antagonist to WT animals increased immune cell infiltrate and slightly increased vessel stiffening, findings opposite from the treated BMPRII mutants [37]. These data indicate a protective role of 5-HT$_{2B}$R in heritable PAH most likely through direct control of signaling downstream of BMPRII through Src. Notably this model does not require exposure to hypoxic conditions or endovascular injury, and as such is able to isolate direct signaling mechanisms that could influence disease.

Platelets are a primary source of peripheral 5-HT, but do not express 5-HT$_{2B}$R. Therefore, a link between 5-HT levels and 5-HT$_{2B}$R must be established to understand the mechanism behind 5-HT$_{2B}$R's control over the development of PAH. Hypoxia-induced increase in plasma 5-HT can be prevented in 5-HT$_{2B}$R knockout mice or with 5-HT$_{2B}$R antagonism. Plasma 5-HT strongly correlates with RVSP, lung 5-HT$_{2B}$R expression, and the plasma 5-HT metabolite 5-HIAA, but not blood 5-HT, indicating a platelet-independent mechanism [52]. While SERT expression levels are not altered by treatment of dexfenfluramine or RS-127445, SERT uptake of plasma 5-HT is significantly increased with 5-HT$_{2B}$R ablation. 5-HT$_{2B}$R agonism causes the reverse effect, and pretreatment with the SERT inhibitor paroxetine prevents 5-HT$_{2B}$R mediated increase in plasma 5-HT [52]. These findings indicate that the hypoxia-induced increase in plasma 5-HT is completely dependent on both 5-HT$_{2B}$R and SERT and is carried out in a platelet-independent manner. The molecular regulators guiding this effect are uncertain, however 5-HT$_{2B}$R-dependent SERT phosphorylation has been observed in a mouse stem cell line providing a potential molecular link between the two [53].

3.4 5-HT$_{2B}$R Controls Bone-Marrow Contribution to PAH

While early studies have focused on the form and function of 5-HT and its availability, a shift in focus to bone marrow-derived cell contributions to differentiating/proliferating smooth muscle cells has gained significant attention recently. Striking evidence using bone marrow transplants combined with chronic hypoxia showed that bone marrow 5-HT$_{2B}$R expression is required to develop pathologic RVSP measurements. WT mice receiving 5-HT$_{2B}$R null bone marrow were immune to RVSP increases following 3 weeks of exposure to hypoxic conditions. Prior to hypoxia

exposure, 5-HT_{2B}R null mice have altered bone marrow composition. Most notably, there are fewer CD45+CD11b-CD31+ proangiogenic precursor cells which is preserved following exposure to hypoxia and may constitute the endothelial/PASMCs responsible for PAH [32]. Bone marrow-derived proangiogenic cells (PACs) are a subset of myeloid lineage cells thought to directly contribute to small-vessel remodeling. While they are poorly characterized, they are commonly identified by a combination of endothelial and hematopoietic or stem cell markers, and their presence in peripheral blood has been correlated with PAH through indirect mechanisms of promoting pathologic vascular remodeling in neighboring cells. In the SuHx model of PAH, ablation of PACs prevents any RVSP increase and vessel stiffening, indicating the direct contribution of PACs to experimental PAH with enhanced endovascular injury. Administration of the 5-HT_{2B}R antagonist SB204741 decreases the number of PACs (CD45+CD11b-CD31+) in peripheral blood as well as the number of PACs that have taken residence in lung tissue, leading to normalized RVSP values and vessel wall stiffness, as measured by atomic force microscopy. PAC ablation following 3 weeks of SuHx is also sufficient to reverse disease. This phenomenon is potentially conserved in humans as the presence of at least one bone marrow-derived CD31+ cell in pulmonary vessels enhances vessel wall stiffness [54]. This study relied upon enhanced endovascular injury, indicating endothelial injury response is integral for PAC function. This cell population is also broadly defined and translation beyond mouse models will be aided by further characterization of the cell type. While the mechanism driving PAC-induced vessel stiffening and RVSP elevation has yet to be investigated, the discovery that 5-HT_{2B}R-driven PAC recruitment promotes the development of PAH provides further impetus to pursue 5-HT_{2B}R as a driver and potential therapeutic target for PAH.

The data presented above clearly show that 5-HT_{2B}R contributes to the development and progression of disease in experimental models of PH mimicking human PAH. The myriad of experimental procedures model different contributors to disease, with strengths and weaknesses evident for each one. Convincing evidence has been put forth regarding 5-HT_{2B}R affecting unique aspects of PAH in various disease models (Fig. 10.2). While the exact mechanism is not fully understood and current understanding must be viewed in a context-dependent manner, encouragement can be taken from the successful application of 5-HT_{2B}R ablation in mitigating pulmonary hypertensive disease across the board.

4 5-HT_{2B}R in Cardiac Development, Vascular Injury and Hypertrophy

5-HT is an active signaling molecule throughout early embryogenesis. 5-HT signaling can be impaired even before neurogenesis, indicating an even wider role beyond neurotransmission. 5-HT's suspected role in cardiovascular morphogenesis was confirmed when embryos grown in high concentrations of 5-HT or 5-HT-specific

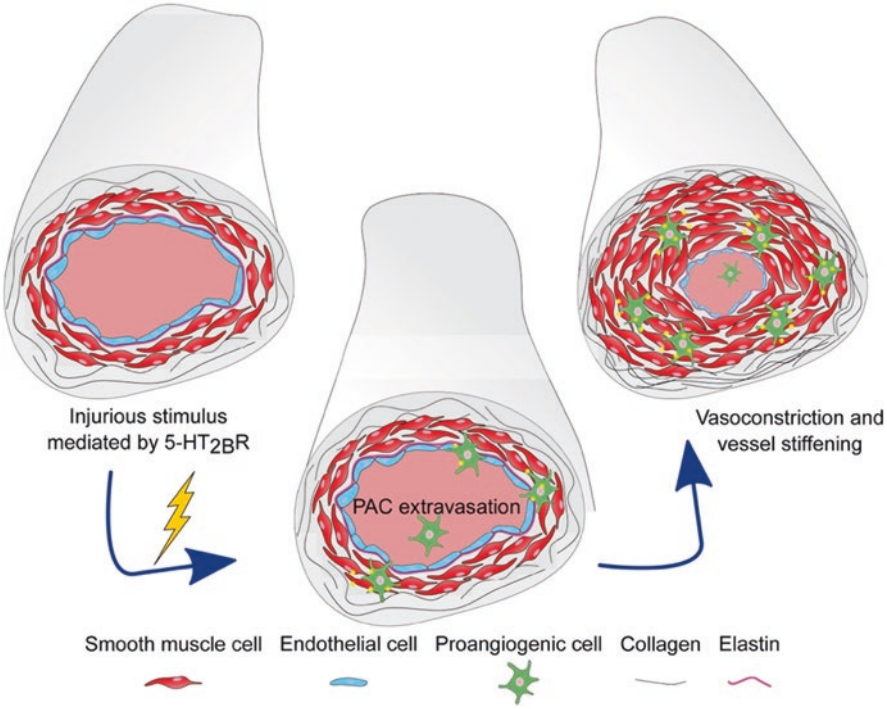

Fig. 10.2 PAH is mediated by 5-HT$_{2B}$R via recruitment of PACs that drive arterial muscularization and stiffening

reuptake inhibitors were observed to decrease the proliferation of myocardium, cardiac mesenchyme, and cardiac endothelium [55]. Prior to the generation of 5-HT$_{2B}$R-knockout mice, no obvious developmental defects (other than behavioral) had been attributed to 5-HT receptors. The generation of mice harboring a specific genetic mutation deleting *Htr2b* uncovered the contribution of 5-HT$_{2B}$R to cardiac development.

4.1 5-HT$_{2B}$R Is Required for Normal Cardiac Development

In 2000, the existence of cardiovascular abnormalities associated with the generation of 5-HT$_{2B}$R mutant mice were first documented. The expected frequency of mutant pups was higher than the observed frequency, indicating a non-negligible rate of midgestational lethality. At 10.5 days postcoitum, fewer trabecular cells and decreased myocardial thickness are observed in mice lacking 5-HT$_{2B}$R. Thinner myocardial walls lead to myocardial rupture as the cause of death, explaining the observed accumulation of blood in the pericardium [56].

Neuregulin, a protein playing a significant role in neural and cardiac development, functions through binding its receptor ErbB-2. ErbB-2 is localized to the ventricular wall of the myocardium, and interestingly, mice lacking neuregulin exhibit a similar embryonic cardiac phenotype as mice lacking 5-HT_{2B}R. There is a significant reduction of ErbB-2 expression in 5-HT_{2B}R mutant mice, providing a potential mechanism for abnormalities in cardiac development. The signaling cascade of ErbB-2 is transactivated by GPCRs, suggesting the Gq-coupled 5-HT_{2B}R transactivates the ErbB-2 pathway to regulate cardiac morphogenesis. This hypothesis is further supported by data showing that cardiomyocytes in newborn mice will proliferate in response to 5-HT or neuregulin, but 5-HT_{2B}R mutant cardiomyocytes do not display a mitogenic response to these signals [56].

The developmental defects of 5-HT_{2B}R mutant mice manifest themselves in impaired cardiac structure and function in adult hearts. Adults exhibit noticeable left ventricle dilatation with increased left ventricle end-diastolic and end-systolic diameters, consistent with persistent tissue-level remodeling in response to impaired cardiac morphogenesis. Functionally, 5-HT_{2B}R mutant hearts have a 20% decrease in fractional shortening compared to WT mice. These changes coincide with a 15% decrease in the number of cardiomyocytes, and the existing cardiomyocytes are 12% shorter, resulting in decreased ventricular mass. Upon ultrastructural analysis, myofilaments are misaligned, I bands are not observed, and Z bands are wider than expected, resulting in decreased sarcomere length. Despite increased preload condition (indicated by increased left ventricle end-diastolic diameter), mutant hearts treated with the adrenergic stimulus isoproterenol generate significantly less force upon contraction than WT hearts [57]. Decreased force generation resulting from fewer cardiomyocytes formed into shorter sarcomeres is the ultimate functional consequence of developmental deficiency of 5-HT_{2B}R and is typical of what is observed in dilated cardiomyopathy. Of note, myocardial apoptosis or immune cell infiltrate is not observed in response to 5-HT_{2B}R deletion. Additionally, there appears to be a partially sex-dependent phenomenon as male mice had more pronounced biological changes than age-matched females [57]. While cardiomyocyte defects are the most pronounced and easily observable, fibroblasts have been specifically shown to transduce mitogenic signals in a 5-HT_{2B}R-dependent manner [58]. Note that these developmental studies were all performed in the context of global 5-HT_{2B}R deletion.

Cardiomyocyte-specific overexpression of 5-HT_{2B}R was shown to have the inverse effect of global 5-HT_{2B}R deletion. Mice exhibiting cardiomyocyte-restricted 5-HT_{2B}R overexpression displayed an increase in left ventricular free wall thickness as well as approximately 11% more cardiomyocytes, resulting in an overall increase in cardiac mass. The effect is not accompanied by a concomitant decrease in systolic performance indicative of compensated left-ventricular hypertrophy. Similar to 5-HT_{2B}R knockout animals, 5-HT_{2B}R overexpression does not lead to myocardial apoptosis, fibrosis, or notable inflammatory cell infiltration. Contrary to the knockout model, sarcomeric structure is normal, but differences in mitochondria can be observed. They appear rounded, irregular, and more abundant. Functionally, the mitochondria are significantly more enzymatically active with decreased expression

of the mitochondrial defect marker adenine nucleotide translocator. These data suggest 5-HT_{2B}R signaling increases metabolic activity and oxidative phosphorylation in mitochondria [59]. 5-HT signaling through 5-HT_{2B}R acts as a survival signal to cardiomyocytes by inhibiting serum withdrawal-induced apoptosis [60]. This effect could potentially transition from a hypertrophic to cardiomyopathic phenotype, controlled by the signaling of 5-HT_{2B}R.

4.2 5-HT_{2B}R Mediates Vascular Function and Remodeling

Due to the peripheral storage of 5-HT in platelets and its vasoactive function, 5-HT likely plays a pathologic role in low-flow conditions such as thrombosis, ischemic injury, and hypertension. In autoperfused rat hindquarters, 5-HT results in conflicting vasoactive functions, causing vasodilation at low concentrations and vasoconstriction at high concentrations. In this investigation of healthy vessels, the contractile response was mimicked with the non-selective 5-HT_2 agonist α-methyl-5-HT, but not the selective 5-HT_{2B} agonist BW723C86 indicating a 5-HT_{2B}R independent mechanism [61]. However, 5-HT_{2B}R expression does increase in the context of injury. In the small arteries of deoxycorticosterone acetate (DOCA)-salt-hypertensive rats, 5-HT causes contraction, exacerbating the hypertensive phenotype. mRNA levels of 5-HT_{2B}R are increased in the mesenteric arteries suggesting this receptor begins to contribute to the disease phenotype. Endothelium-denuded isolated superior mesenteric arteries of DOCA-salt rats have a substantial increase in maximum contraction in response to the 5-HT_{2B}R agonist BW723C86 compared to normotensive rats indicating a smooth muscle cell-mediated effect. The 5-HT_{2B}R antagonist LY272015 effectively reduced mean blood pressure in DOCA-salt rats with no effect on normotensive rats [62]. These results indicate that in the context of injury, 5-HT_{2B}R influences vessel contraction contributing to hypertension.

The use of percutaneous interventions such as balloon angioplasty and stenting has widened for the treatment of occluded vasculature. A concern accompanying these types of interventions is the restenosis of the vessel through infiltration of smooth muscle cells. 5-HT_{2B}R influences vascular restenosis modeled through wire injury of the femoral artery. Administration of BW723C86 intensifies restenosis by increasing the degree of neointima formation. Wire injury denudes the vascular endothelium, inducing a strong smooth muscle cell response. Smooth muscle cells respond to 5-HT_{2B}R agonism by increasing their proliferation and migration. This adverse response contributes to neointima formation and is blocked in 5-HT_{2B}R knockout mice. The intracellular signaling of 5-HT_{2B}R in this context is not through the canonical Gαq protein but through β-arrestin2 mediated activation of mammalian target of rapamycin/p10S6K signaling [63].

These findings reveal a smooth muscle cell-mediated role of 5-HT_{2B}R in exacerbating arterial contraction in hypertensive patients and vascular restenosis following percutaneous intervention which could potentially be leveraged to therapeutically reduce injurious vascular remodeling.

4.3 Hypertrophic Response to 5-HT$_{2B}$R Stimulation

Cardiac hypertrophy is a physiological adaptation in response to increased workload, whether it is through increased chronotropic or ionotropic effects. While this can be an advantageous adaptation, such as following exercise by an athlete, prolonged and extensive hypertrophic remodeling can lead to cardiomyocyte death and cardiac fibrosis. 5-HT$_{2B}$R signaling influences cardiac hypertrophy in a context-dependent manner wherein different experimental models yield results contingent upon the method of hypertrophy induction and cell populations influenced.

Chronic adrenergic stimulation of cardiomyocytes through β-adrenergic receptors is a strong predictor of morbidity and mortality in cases of congestive heart failure. Models using prolonged dosing with norepinephrine and the specific β1/β2 adrenergic agonist isoproterenol have been instrumental in elucidating the mechanisms underlying cardiac hypertrophy in response to sympathetic stimulation. 5-HT levels are also associated with sympathetic overstimulation providing a potential mechanism for 5-HT$_{2B}$R overexpression-induced myocardial hypertrophy [64]. Isoproterenol administration causes an increase in heart mass, heart rate, and cardiomyocyte size. Despite cardiomyocytes expressing 5-HT$_{2B}$R, activation of the receptor does not elicit a contractile response [19]. One reported function of 5-HT$_{2B}$R antagonism in norepinephrine induced cardiac hypertrophy is through the downregulation of Bax, decreasing cardiomyocyte apoptosis and partially reversing established cardiac hypertrophy [65].

An important hallmark of cardiac hypertrophy is an increase in the inflammatory milieu within the myocardium. In response to *in vitro* isoproterenol induction, cardiac fibroblasts will secrete the inflammatory cytokines IL-6, IL-1β, and tumor necrosis factor-α. This increase is prevented in 5-HT$_{2B}$R null cardiac fibroblasts or when treated with a 5-HT$_{2B}$R antagonist. This same cytokine response is observed *in vivo*, and the 5-HT$_{2B}$R antagonist SB206553 prevents increases in plasma levels of these inflammatory cytokine and the subsequent cardiac hypertrophy, indicating a deleterious role of cardiac fibroblast 5-HT$_{2B}$R signaling [64]. The contribution of cardiac fibroblasts was further magnified in a model of cardiomyocyte-driven overexpression of 5-HT$_{2B}$R in mice on a 5-HT$_{2B}$R null background, thus relegating 5-HT$_{2B}$R expression strictly to cardiomyocytes. In response to chronic isoproterenol infusion, these transgenic animals do not develop cardiac hypertrophy or exhibit a decrease in cardiac function.

The transgenic animals also do not exhibit an increase in IL-6, IL-1β, or TGFβ1. It was reported in human left ventricle tissue that 5-HT$_{2B}$R co-localizes and co-precipitates with another GPCR, the angiotensin II type 1 receptor (AT$_1$R). These two receptors work in concert to initiate cytokine release that drives ventricle dilatation, wall thinning, and hypertrophy (Fig. 10.3). Activation of both receptors by their respective ligands is required to achieve cytokine release. An investigation of 16 patients diagnosed with congestive heart failure revealed that 5-HT$_{2B}$R expression is significantly elevated irrespective of cardiomyopathy etiology, disease severity, or treatment. 5-HT$_{2B}$R expression significantly correlated with expression of the

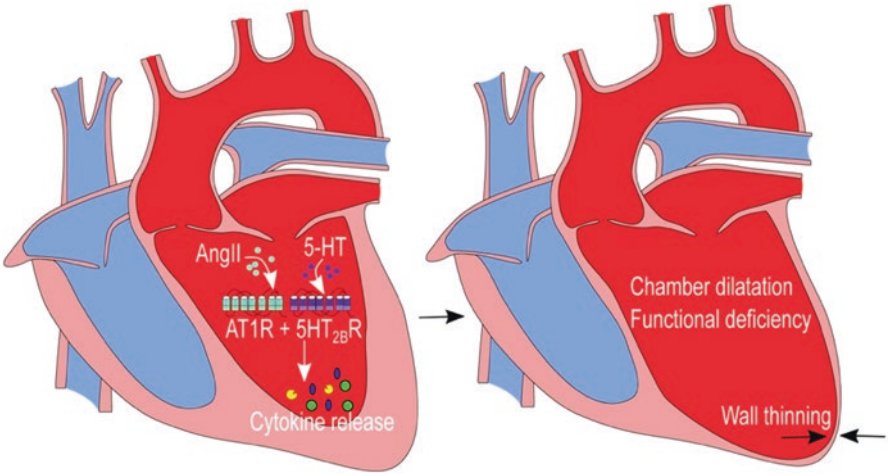

Fig. 10.3 Cardiac hypertrophy is driven by concomitant activation of AT_1R and 5-HT$_{2B}$R. Inflammatory and fibrotic cytokines lead to left ventricular remodeling as seen by wall thinning and chamber dilatation

cytokines IL-6, tumor necrosis factor-α, and TGFβ1, further highlighting its contribution in cardiac hypertrophy [20].

Left ventricular hypertrophy has been linked with excessive formation of reactive oxygen species (ROS). Cardiac ROS is triggered by AngII as well as isoproterenol, both of which elicit a hypertrophic cardiac response. Administration of the 5-HT$_{2B}$R antagonist SB215505 is sufficient to prevent cardiac dilatation and increased mass in a load-independent manner as it acts without cardiodepression or lowering blood pressure. The effect of treatment can be attributed to a normalization of the superoxide anion of oxygen by abolishing NAD(P)H oxidase over-activation. The *in vivo* findings were replicated in left ventricle fibroblasts, further supporting the idea that 5-HT$_{2B}$R acts through cardiac fibroblasts [66]. In addition, ROS generation could potentially be linked to a functional role of 5-HT$_{2B}$R in mitochondria. This study highlights the interplay between the 5-HT$_{2B}$R and AT_1R, in regulating cardiac hypertrophy through production of hypertrophic cytokines and ROS.

Arterial banding is another methodology to induce cardiac hypertrophy by artificially increasing loading immediately distal to the ventricles. The tissue responds through cardiomyocyte hypertrophy, fibrosis, and cardiomyocyte apoptosis. Banding of the pulmonary artery causes a decrease in cardiac output due to TGF-β1-induced collagen deposition which can be mitigated through 5-HT$_{2B}$R antagonism [67]. Wistar rats that have undergone an aortic banding procedure increase the expression of 5-HT$_{2B}$R in cardiomyocytes. Administration of a 5-HT$_{2B}$R antagonist prevents the hypertrophic characteristics of increased heart weight and decreased wall thickness. Cardiomyocyte hypertrophy in response to mechanical stress was found to be mediated by nuclear factor-κB and blocked through 5-HT$_{2B}$R

antagonism [68]. These data point to 5-HT_{2B}R directly influencing cardiomyocyte hypertrophy in response to mechanical load.

Spontaneously hypertensive rats (SHR) progressively develop diastolic dysfunction with preserved ejection fraction without any exogenous stimuli. After a few weeks of hypertension, diastolic dysfunction develops without a deterioration of systolic function, similar to essential hypertension in humans. Administration of the highly selective 5-HT_{2B}R antagonist RS-127445 during the natural course of hypertensive cardiomyopathy in SHRs did not reduce left ventricular dilatation despite increased *Htr2b* mRNA, but instead exacerbated left ventricle dilatation and thinning of the septal and posterior walls, resulting in a severe eccentric hypertrophic phenotype. Brain natriuretic peptide levels, a cardiac hormone correlated with hypertension and hypertrophy, are decreased by RS-127445 despite worsened cardiac hypertrophy, pointing to an intracellular mechanism independent of the pathological state. Interestingly, 5-HT_{2B}R antagonism causes an increase in subendocardial interstitial fibrosis in SHRs. In the same study, aortic rings isolated from WT and 5-HT_{2B}R knockout mice demonstrated different vasoactive responses when stimulated with the general 5-HT_2R agonist α-methyl-5-HT. WT samples respond in a dose dependent manner, where 5-HT_{2B}R mutants vasoconstrict with an increased tension, indicating a potential vasodilating role of 5-HT_{2B}R [69]. These confounding results to aforementioned studies highlight the context-dependent action of 5-HT_{2B}R signaling in the realm of cardiac hypertrophy, and its actions must be understood in a variety of environments to characterize its pathologic or therapeutic mechanisms.

Cardiac hypertrophy is a complex condition associated with a wide variety of initiating factors. 5-HT_{2B}R plays a dual function in both cardiac fibroblasts and cardiomyocytes regulating the *in vivo* response to these factors.

5 Future Considerations

Findings throughout the literature show that multiple cardiopulmonary diseases can potentially be addressed through therapeutic manipulations of 5-HT_{2B}R signaling (Fig. 10.4). The tissue distribution and pharmacologies of 5-HT_{2B}R in rodents and humans are similar, emphasizing the translational importance of the findings described in rodent models [70]. Importantly, drugs (and their metabolites) targeting serotonergic signaling should be screened for activation of 5-HT_{2B}R, as it has been well documented in the cases of valve disease and PAH that 5-HT_{2B}R activation significantly increases risk of disease incidences. Multiple conditions mediated by vascular and interstitial cell dysfunction have been reported to be facilitated through signaling of 5-HT_{2B}R. In particular, valve hyperplasia, arterial remodeling leading to pathologic RVSP and subsequently PAH, and inflammatory cytokine secretion upstream of cardiac hypertrophy all have contributions from 5-HT_{2B}R. Evidence has been presented for the influence of 5-HT_{2B}R signaling over both resident and recruited cells, indicating a utility in both early and chronic stages

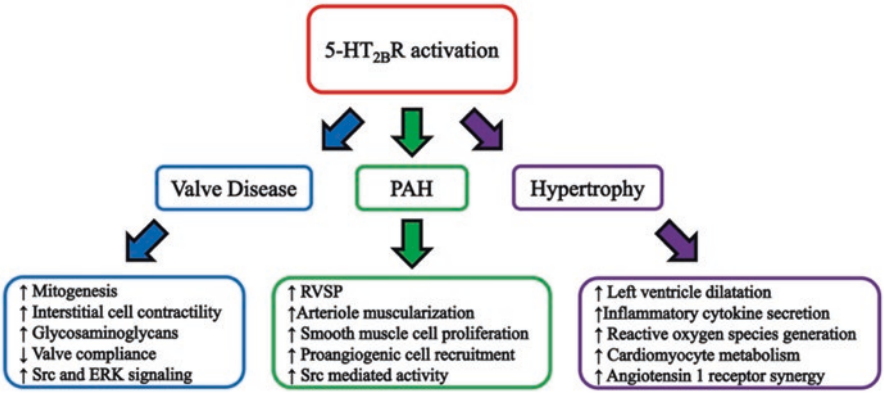

Fig. 10.4 Cardiopulmonary diseases facilitated by 5-HT$_{2B}$R activity

of disease. Further investigation into disease- and tissue-specific 5-HT$_{2B}$R-targeted treatment paradigms is warranted based on the therapeutic potential supported by strong evidence put forth throughout years of research and current lack of effective therapies for cardiopulmonary disease.

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Chapter 11
Serotonin and Cardiac Valves Degeneration in Dog

Jérôme Guyonnet

Abbreviations

ACE	Angiotensin converting enzyme
ACVIM	American College of Veterinary Internal Medicine
CKCS	Cavalier King Charles Spaniel
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase
HF	Heart failure
MVD	Mitral valve disease
Ndf	Nordexfenfluramine
SERT or alternatively 5-HTT	Serotonin transporter
SNP	Single nucleotide polymorphism
TGFβ	Transforming growth factor beta
TPH1	Tryptophan hydroxylase-1
VHD	Valvular heart disease
VICs	Valve interstitial cells

1 Introduction

Chronic valvular heart disease (VHD), characterized by progressive myxomatous degeneration and thickening of the mitral valve leaflets, is the most common heart disease in dogs. The disease is typically found in many small breeds that are predisposed. Mitral valve disease (MVD) is the leading cause of death of small breeds and has been found to be 20 times more prevalent in Cavalier King Charles spaniels (CKCS) than in the average dog breed. It is estimated to affect 10% of the entire dog population, but at a much older age of onset than for CKCSs. The prevalence of the disease in CKCS animals older than 10 years is greater than 90%. MVD, the most common cardiovascular disease in dogs, represents 75% of all cardiovascular

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diseases in this species [1]. MVD is a polygenetic disease and currently, there is no etiologic treatment for this pathology.

Serotonin is a monoamine neuro-transmitter that is produced in the central nervous system as well as in enterochromaffin cells in the gastrointestinal tract. Upon release into the circulation, serotonin is rapidly taken up by platelets via SERT and virtually all circulating serotonin is stored in the dense granules of platelets. Platelet serotonin release is triggered by a variety of stimuli, including endothelial damage, platelet aggregation, and serotonin receptor agonists. High concentrations of SERT also are found in the pulmonary and coronary endothelium and likely contribute to regulation of local vascular tone. Interestingly, SERT is expressed in rat and mice embryonic myocardium and is specifically involved in valvulogenesis. The precise function of serotonin in valve tissue is unknown, but based on its role in valve formation and its expression on mature valves, it is likely that both serotonin and SERT play a physiologic role in maintaining healthy valve tissue.

Although the exact causes of MVD are not known, some hypotheses explaining its onset include platelet dysfunctions, mechanical stretch, increased serotonin and 5-HT_{2B} receptor signaling, and/or inherited genetic disorders.

2 Pathology, Pathogenesis

Mitral valve regurgitation is attributable to myxomatous MVD, also known as chronic mitral valve insufficiency. The disease is caused by progressive myxomatous degeneration of the mitral valve [2] leading to incomplete coaptation of the leaflets and valvular regurgitation. Myxomatous MVD has been shown to be associated with valve thickening and abnormal motion of the mitral leaflets observed by echocardiogram analysis in both humans and dogs. Canine myxomatous MVD has a strong resemblance to primary mitral valve prolapse in man, [3] with a strong correlation existing between the endothelin receptor density and the degree of valvular changes. In human, an age older than 50 years associated with a depressed left ventricular function, moderate to severe mitral regurgitation, mitral valve thickness >5 mm, and atrial fibrillation have often been reported to represent significant risk factors for cardiovascular events.

In dogs, the disease is characterized by a slow progression over years. Many affected dogs that never progress to reveal clinical signs of overt heart failure (HF) because of the age of onset. The disease consistently is characterized by changes in the cellular constituents as well as the intercellular matrix of the valve apparatus (including the valve leaflets and chordae tendineae). Classically, mitral valves lose their flexibility, increase their surface and appear convex; thus, these lesions are characterized as myxomatous. Chordae tendineae often appear thickened and elongated, thus leading to prolapse imaging by echocardiography and inducing mitral regurgitation diagnosed by left apical systolic murmur [2]. Chordae tendineae rupture is classically observed in advanced degenerative stages and leads to complications, such as ventricular hypertrophy, followed by HF and death, despite treatments [4].

Anatomically, degenerative MVD is characterized by changes that involve both the collagen content and the alignment of collagen fibrils within the valve. Expansion of the spongiosa layer is characterized by changes in the proteoglycan content of this layer. Dysregulation of the extracellular matrix appears to be central to these changes. Valve interstitial cells (VICs) acquire properties of activated myofibroblasts that include high proteoglycan /glycosaminoglycan deposits, and degradation of the fibrillar matrix, which lead to collagen disorganization and elastin fiber fragmentation, as previously described in human chronic heart disease [5]. Several effectors, such as matrix metalloproteinase collagenase, elastases, proteoglycans (decorin, versican, and biglycan) or markers of activated VIC phenotype (alpha smooth muscle actin), were identified in myxomatous mitral valve tissues [6]; however, there is limited knowledge regarding the mechanisms that initiate or progressively destroy mitral valve tissues.

It has been reported that some small-breed dogs are predisposed to degenerative MVD, including CKCS, Miniature Poodles, Miniature Schnauzers, Chihuahuas, Pomeranians, Fox Terriers, Cocker Spaniels, and Pekingese breeds. The prevalence of chronic valvular disease was studied in 494 CKCSs with a mean age of 3 years [7]. Cardiac murmurs were detected in 65 (13.2%) of the dogs. Among 61 CKCSs with a mean age of 6.4 years, cardiac murmurs were detected in 32 (52%). In both groups of dogs the prevalence of cardiac murmurs was low among dogs younger than three years (1.9%) but increased with age. The estimated ages at which 50% of the dogs had developed murmurs were 7.5 and 6.2 years, respectively. When 39 of the 61 dogs were re-examined three years later, cardiac murmurs were detected in 28 (72%), and the intensities of the murmurs had generally increased. Nine (28%) of the dogs which had previously had murmurs had been euthanized for signs of congestive HF whereas none of the dogs which had been free of murmurs had died from congestive HF. In another study on 207 Dachshunds dogs of Poland [8], 172 animals (83%) had chronic valve disease with the mitral valve affected most often (130 dogs), both mitral and tricuspid valves infrequently (39 dogs), and only 3 dogs with the tricuspid valve. Lesions described in small-breed dogs, like CKCS or Dachshunds are usually myxomatous. Another team characterized the composition and distribution of components in the extracellular matrix of mitral valves in 50 dogs predisposed for chronic valve disease, compared to healthy ones [9]. Alterations of extracellular matrix, activation of stromal cells and modifications in endothelial layer were observed in mild, moderate and marked chronic VHD, and were described as myxomatous.

3 Mitral Valve Degeneration

Although abnormal serotonin signaling is unlikely to be the sole primary cause of myxomatous MVD in dogs, alterations in the serotonin signaling system might be involved in the pathological process of valvular deformation. However, further investigations should be conducted to evaluate the potential role of serum and cardiac tissue serotonin, respectively, in the pathogenesis of myxomatous

MVD. Interactions of platelet, valvular, and myocardial serotonin signaling warrant further investigation.

3.1 Involvement of Serotonin to Mitral Valve Degeneration

It is well known that high circulating serotonin or serotonergic drugs can induce valvulopathy in humans and rats. In carcinoid syndrome (functional neoplasia of enterochromaffin cells), patients with the highest levels of plasma serotonin are at greatest risk to develop valvulopathy [10]. Fenfluramine or ergot derivatives were linked to mitral and aortic valve dysfunction and share in common the pharmacological property of being 5-HT_{2B} receptor agonists [11]. The contribution of serotonin and its receptors to cardiovascular tissue remodeling, with a particular emphasis on cardiac hypertrophy, fibrosis and valve degeneration are summarized in Fig. 11.1. The canine cardiovascular system is rich in serotonin-signaling components. In particular, canine mitral valve tissue expresses 5-HT_{2A}, 5-HT_{2B}, 5-HT_{1B} receptors, as well as SERT and the limiting serotonin synthetic enzyme, tryptophan hydroxylase-1 (TPH1) [13]. 5-HT_2 receptors are secondarily linked to Gq proteins, and through activation of phospholipase C these receptors mediate downstream mitogen activated protein kinase signaling pathways such as the extracellular signal-regulated kinase (ERK1/2) systems. Phosphorylation of ERK1/2 initiates processes of cellular differentiation and proliferation.

Importantly, in valve tissue, ERK1/2 transforms normally quiescent VICs into the more active myofibroblast phenotype. The serotonin pathway also is closely linked to the transforming growth factor beta (TGFβ1) system. Serotonin increases TGFβ1 expression and is closely associated with differentiation and proliferation of VIC. Thus, both the serotonin and TGFβ1 systems are found in the canine mitral valve, and by their ability to activate myofibroblasts, these systems may play an important role in the development and progression of degenerative MVD. Some valve degenerative processes such as the carcinoid heart disease, drug-induced valvulopathy are clearly linked to serotonin as degenerative MVD in inbreed dogs [14].

In dogs, several studies with naturally occurring myxomatous MVD, serotonin concentrations in mitral valve leaflets and left ventricular myocardium have been shown to be higher than in healthy control. These findings suggest that differences in serum serotonin concentrations between dogs with or at risk of developing myxomatous MVD likely reside within the platelet serotonin pool or other causes. In a study, 483 healthy dogs of nine breeds aged 1–7 years were examined at five European centers [15]. The absence of cardiovascular, organ-related, or systemic diseases was ensured by thorough clinical investigations including echocardiography. Results shown median serotonin concentration was 252.5 ng/ml. Overall breed difference was found ($p < 0.0001$), and 42% of pairwise breed comparisons were significant. Among the included breeds, Newfoundlands, Belgian Shepherds and CKCS had highest serotonin concentrations. Fifty dogs affected with degenerative MVD, 34 dogs predisposed to degenerative MVD but without cardiac murmur or

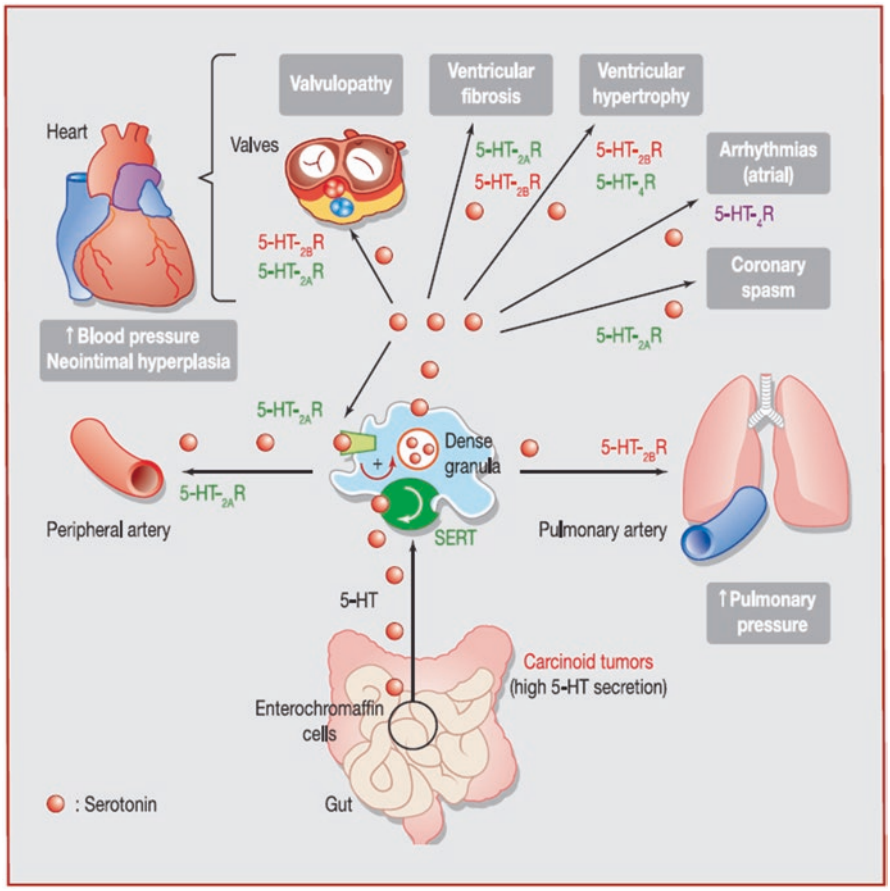

Fig. 11.1 Deleterious cardiovascular effects of 5-hydroxytryptamine (5-HT; serotonin) according to [12]

echocardiographic evidence of degenerative MVD, and 36 healthy large breed control dogs had median serotonin concentrations greater than the upper limit of the human reference range (40–450 ng/mL) [16]. Dogs with degenerative MVD had significantly higher serum serotonin concentrations when compared with large breed control dogs. In another study, 92 dogs prospectively recruited were classified by standard echocardiography into healthy dogs of breeds predisposed to myxomatous MVD, but without echocardiographic evidence of the disease, mild, moderate, or severe myxomatous MVD groups [17]. Serum serotonin concentration was found to decrease with increasing severity of myxomatous MVD, and left atrial to aortic root ratio was the variable most strongly associated with serotonin. In a study on forty-five dogs, mitral valve serotonin concentration from dogs with degenerative MVD was ninefold greater than in dogs with non-cardiac disease, and 13.5-fold greater than in dilated cardiomyopathy [18]. Tissue serotonin concentration was

highest in mitral valve and left ventricle of myxomatous MVD-affected dogs, suggesting altered serotonin signaling as a potential feature of myxomatous MVD. Findings showed that platelet serotonin was elevated in CKCS compared to other breeds, and that serotonin in left ventricular myocardial and mitral valve leaflet tissue of myxomatous MVD-affected dogs.

These results led to the investigation of the interactions between platelets and valvular serotonin signaling in dogs. An overexpression of the 5-HT_{2B} receptor was subsequently identified in CKCS mitral valve tissue by RT-qPCR [19] and immunohistochemical analysis [20], whereas a downregulation of the serotonin transporter (SERT, or alternatively 5-HTT) was identified in the late stage of degenerative MVD by immunohistochemical analysis [21]. Therefore, the 5-HT_{2B} receptor chronic stimulation by free serotonin could activate remodeling pathways and be autoamplified by the reduction of serotonin capture as a consequence of the reduction of SERT expression. An inhibition of SERT function is also possible in valves because 5-HT_{2B} receptor stimulation has been shown, in primary neurons, to inhibit SERT function/phosphorylation [22]. This central role of SERT function and expression in degenerative MVD is reinforced by the recent finding of three polymorphisms in the SERT gene of Maltese dogs that were predicted to induce damage to protein function [23].

A team of human and veterinary researchers studied the relationship between serotonin receptors and mitral VICs and leaflet remodeling in humans and CKCSs [24]. Canine mitral valve prolapse leaflets showed 5-HT_{2B} receptor upregulation. Mitral valve RNA was used for microarray analysis of mitral valve prolapse patients versus control, highlighting genes that indicate the involvement of serotonin receptor pathways and extracellular matrix remodeling in mitral valve prolapse. Human mitral valve leaflets were also studied in-vitro and ex-vivo with biomechanical testing to assess remodeling in the presence of a pure 5-HT_{2B} receptor antagonist (LY272015). Antagonism of 5-HT_{2B} receptor mitigates mitral VIC activation in-vitro and mitral valve remodeling in-vivo. In humans, mitral valve prolapse is associated with an upregulation in 5-HT_{2B} receptor expression and increased 5-HT receptor signaling in the leaflets. Antagonism of 5-HT_{2B} receptor mitigates mitral VIC activation in-vitro and mitral valve remodeling in-vivo. These observations support the view that serotonin receptor signaling is involved not only in previously reported serotonin-related valvulopathies, but it is also involved in the pathological remodeling of mitral valve prolapse.

In conclusion, CKCS dogs, which are predisposed to an early onset of myxomatous MVD, had higher serum serotonin concentrations compared with dogs of other breeds (Table 11.1). Mitral valve serotonin concentrations seem more appropriate to follow the relation between increasing of serotonin and valvulopathy but not easy to measure. In addition, systemic concentrations are the sum of several sources of serotonin and are not completely representative of local concentrations contributing to myxomatous MVD. This is illustrated in the following table where there is a tenfold to 100-fold difference between the serotonin concentrations at the valve and the serum. Serum serotonin concentration results from equilibrium between serotonin synthesis, platelet uptake and storage, and metabolism. Hence, it can be questioned whether altered circulating serotonin concentrations in dogs with myxomatous

Table 11.1 Serum and mitral valves serotonin concentrations (measured by an ELISA kit and HPLC) in several breeds of healthy dogs and those with various stages of myxomatous MVD

Years	Breed	Tissues	Serotonin	Kit	References
2009	Myxomatous MVD (CKCS, mixed breed, Yorkshire Terriers, Pomeranians, Maltese, Brittany Spaniel, Dachshund, Miniature Doberman Pinscher, Rat Terrier)	Serum	765.5 ng/mL	ELISA	[16]
	Predisposed myxomatous MVD (CKCS, 2 mixed breeds, miniature Poodles, Cocker Spaniel, English Springer Spaniel, Jack Russell Terrier, Teacup Poodle, Fox Terrier)		774.9 ng/mL		
	Healthy (mixed breed, Great Danes, Labrador Retrievers, Weimaraner, Golden Retrievers, American Pit Bull Terrier, Bull Mastiff)		509.8 ng/mL		
2013	Control (mostly CKCS, Dachshund, mixed breed, Jack Russell, Shih-tzu)	Serum	657 ng/ml	ELISA	[17]
	Mild myxomatous MVD (mostly CKCS, Dachshund, mixed breed, Jack Russell, Shih-tzu)		645 ng/ml		
	Moderate myxomatous MVD (mostly CKCS, Dachshund, mixed breed, Jack Russell, Shih-tzu)		585 ng/ml		
	Severe myxomatous MVD (mostly CKCS, Dachshund, mixed breed, Jack Russell, Shih-tzu)		513 ng/ml		
2014	Myxomatous MVD (CKCS, Jack Russell Terrier, Chihuahua, Cocker Spaniel, Toy Poodle)	Mitral valve	32.4 ng/mg	HPLC	[20]
	Other heart disease (Doberman, mixed breed, Boxer, Great Dane)		2.4 ng/mg		
	Non-heart disease (Beagle, mixed breed, Bassett hound, Cane Corso, Welsh Terrier, CKCS, German Shepherd)		3.6 ng/mg		
2015	Control (CKCS)	Serum	591 ng/ml	ELISA	[18]
	Mild myxomatous MVD (CKCS)		746 ng/ml		
	Moderate myxomatous MVD (CKCS)		638 ng/ml		
	Severe myxomatous MVD (CKCS)		388 ng/ml		

MVD are caused by alterations in the peripheral production and handling of serotonin or by alterations in local serotonin production in the mitral valve (or both).

3.2 Involvement of Mechanical Stress/Mechanotransduction to Mitral Valve Degeneration

In soft-tissue biomechanics, the presence of normal tissue stress is considered to be closely related to the regulation of tissue homeostasis [25]. Cardiac valves are living tissues with the ability to repair and remodel in response to damage. Valve biology

can be modeled in these extreme hemodynamic conditions. Several studies have shown that pathophysiological alterations in mechanical loading lead to stress changes and subsequent tissue adaptations that affect tissue structure and composition [26]. The fundamental question common to all soft collagenous tissues is the relationship between tissue remodeling and cell-level deformations [27]. The above is especially true in heart valve leaflet tissues, where alterations in tissue stresses due to physiological conditions, disease, and surgical repair have long been suspected to play a major role in valvular remodeling. The average adult heart beats at approximately 2.21 billion times during the lifetime, and 0.53 billion for a small dog with blood flow directed within its chambers by the four heart valves. Heart valves function within a highly demanding intrinsically mechanical environment; the movement of their structures is coordinated by their complex geometry and the underlying, intricate, and highly organized extracellular matrix. Of the four heart valves, the mitral valve is subjected to the greatest hemodynamic forces.

At tissue level, induced deformation is a major driver for mitral VIC mechanoregulation [27]. Findings on collagen fiber alignment showed that the collagen/VIC coupling and micromechanical interactions are major drivers for mitral VIC deformation and subsequent phenotypic activation. These results indicated that cellular compression occurs in the physiological range, whereas cellular elongation drives mechanobiological response at higher strain levels, suggesting that compression and stretching could lead to different mechanotransduction pathways. Results at different length scales revealed that normal responses are observed only within a defined range of tissue deformations, whereas deformations outside of this range lead to altered responses, evidenced by changes in a-smooth muscle actin, type I collagen, and other extracellular matrix and cell adhesion molecule regulation. Thus, cell responses have a delimited range of in-vivo deformations that maintain a homeostatic response, suggesting that deviations from this range may lead to deleterious tissue remodeling and failure (Fig. 11.2).

Most of studies are performed on cultured VIC try to mimic the valve tissue, which is subjected to considerable mechanical stress. Several laboratories have developed systems to subject native or bioengineered valve tissues to mechanical properties, such as high blood pressure, alternative flow and frequency, mimicking physiological conditions [28].

These approaches enable the investigation of mechanical strain and the relationship between mechanics and serotonin. Although serotonin alters the mitral valve microenvironment and global valve mechanics, it may also be a direct mechanomodulator.

Lacerda et al. [29], developed an in-vitro valve model system capable of applying static and cyclic tensile strain to canine mitral valves in culture, in which static and cyclic strain increased the expression of myxomatous effector proteins, chondrogenic markers and the myofibroblastic phenotype compared to unstrained controls in canine mitral valves. Mitral valves were subjected to 30% static or cyclic tensile strain and compared to cultured mitral valves subjected to 0% strain for 72 h. The expression of TPH1, was significantly increased in canine mitral valves subjected to 30% static and 30% cyclic strain compared to unstrained mitral valves. Serotonin levels were higher in media of cyclically strained valves compared to unstrained

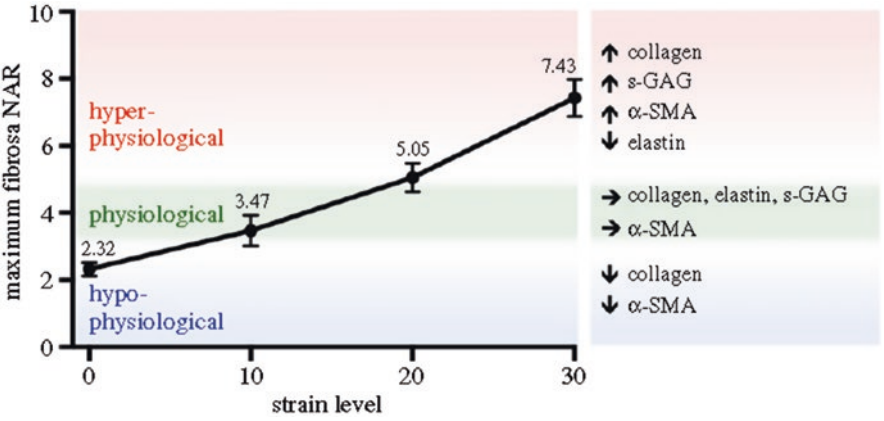

Fig. 11.2 Mitral VIC deformation is a major driver for cellular mechanoregulation. A maximum fibrosa nuclear aspect ratio (NAR) that is less than 3.28 is bracketed as hypophysiological, an NAR between 3.28 and 4.92 is bracketed as physiological and an NAR above 4.92 is hyper-physiological. This suggests that there is a narrow physiological range of mitral VIC NAR. From ref [27]

valves after 72 h of culture. The expression of TPH1 increased with higher serotonin levels suggesting local serotonin synthesis modulated by mechanical strain. All these results support that serotonin acts as a local modulator required for the adaptation of the valve matrix to increased constraints and to maintain homeostasis.

Cell proliferation, collagen synthesis, and tissue stiffness in response to cyclic stretch seem to be specifically modulated by the 5-HT_{2A} receptor in the aortic valve [30] while in-vitro experiments in mitral valves implicate the 5-HT_{2B} receptor. This strongly suggests that the 5-HT_{2A} receptor subtype is sensitive to elevated stretch. Conversely, in mitral valve tissues, in-vitro static experiments implicated the 5-HT_{2B} receptor. At the tissue scale, treating VIC with a 5-HT_{2B} agonist acutely decreases tone generation of the cells, tissue alignment, and increases the tensile modulus along the primary fiber alignment axis [31]. Similar mechanisms may be at play in serotonin-related MVD. In both aortic banded rats and neonatal rat cardiomyocytes, mechanical stress can enhance 5-HT_{2B} signaling in ventricular models of pressure induced cardiomyopathy [32]. Cyclic stretch upregulates 5-HT_{2A} and 5-HT_{2B} receptor expression in porcine aortic valve cusps causing VIC proliferation and extracellular matrix remodeling [33]. Myofibroblastic phenotype markers, and matrix catabolic enzymes, cathepsins, matrix metalloproteases, increased with increasing cyclic strain in cultured sheep mitral valves with serotonin present in the media [34].

Orton et al. [5], proposed a pathway (Fig. 11.3) whereby degenerative MVD is mediated through a local serotonin signaling mechanism and raised several questions as identification of the initiating triggers for degenerative valve disease, identification of mechanosensor and mechanotransduction mechanisms in heart valve cells, and understanding the complex interactions of signaling mechanisms.

The team reported increased 5-HT_{2B} receptor protein and decreased SERT protein in canine degenerative mitral valves, increased phosporylated ERK (pERK) without change in total ERK in canine degenerative mitral valves, consistent with

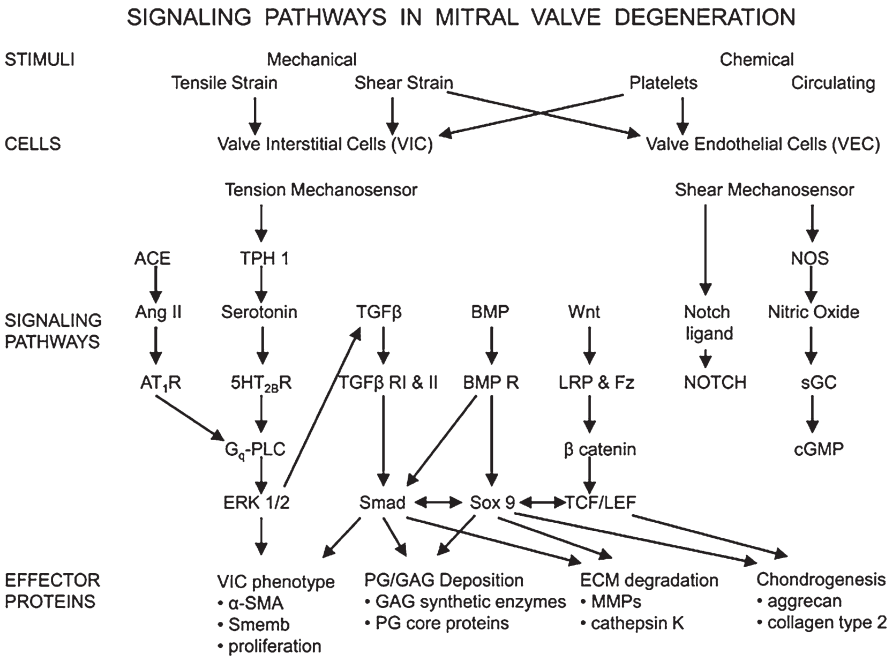

Fig. 11.3 Signaling pathways in mitral valve degeneration. *ACE* angiotensin converting enzyme , *Ang II* angiotensin II, *AT1R* angiotensin II receptor type 1, *TPH1* tryptophan hydroxylase 1, *5-HT$_{2B}$ receptor* serotonin type $_{2B}$ receptor, *PLC* phospholipase C, *TGFβ* transforming growth factor β, *BMP* bone morphogenetic protein, *LRP* lipoprotein receptor-related protein, *Fz* frizzled receptor, *NOS* nitric oxide synthase, *sGC* soluble guanylate cyclase, *αSMA* α smooth muscle actin, *SMemb* non-muscle embryonic myosin, *GAG* glycosaminoglycan, *PG* proteoglycan, *MMPs* matrix metalloproteinases [5]

active serotonin signaling. Interestingly, it was observed several-fold increase in expression of TPH1 in both human and canine degenerative mitral valves. Increased TPH1 expression in both early- and late-stage disease in dogs can be consistent with a possible initiating role for serotonin. Based on these findings, a hypothesis was proposed whereby degenerative MVD is mediated through a local serotonin signaling mechanism (Fig. 11.4). In this issue, static and cyclic tensile strain could induce increased expression of TPH1 and increased local serotonin synthesis in cultured canine mitral valves. This study supports a local serotonin mechanism in degenerative MVD that is initiated by tensile strain.

While valvulopathic effects of serotonin and angiotensin-II (Ang-II) are individually known, their interactions specifically in the context of the mechanobiological responses of altered valve were not clear. In this context, increased serotonin levels would result in accelerated progression toward disease in the presence of Ang-II. After 3 weeks, average systolic blood pressure was significantly increased in the serotonin, Ang-II and combination groups compared to control [35]. Echocardiographic analysis demonstrated significantly reduced ejection fraction in Ang-II and the combination groups. VIC orientation, cellular contractility and

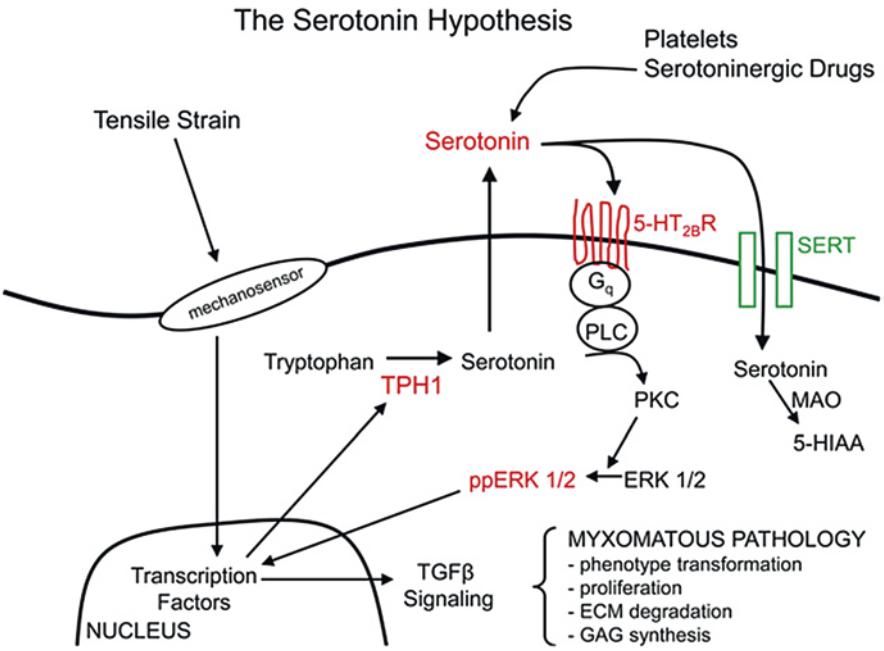

Fig. 11.4 The serotonin hypothesis for degenerative MVD. Tensile strain on heart valve cells initiates upregulation (red) of the rate-limiting serotonin synthetic enzyme, *TPH1* tryptophan hydroxylase 1, through an unknown mechanosensor mechanism. TPH1 mediates the local synthesis and release of serotonin. Serotonin interacts with the upregulated serotonin type $_{2B}$ receptor (5-HT$_{2B}$ receptor) leading to activation of *PKC* protein kinase C and phosphorlyation of ERK 1/2. *pERK 1/2* Phosphorylated ERK translocates to the nucleus to induce *TGFβ* transforming growth factor β signaling and transcription of effector genes mediating myxomatous pathology. The *SERT* serotonin transporter is downregulated (green) leading to decreased uptake and metabolism of serotonin by *MAO* monoamine oxidase [5]

collagen gene expression was highest for the serotonin + Ang-II combination treatment compared to all other groups. Serotonin and Ang-II thus interact to result in significantly detrimental alteration of function and remodeling in the valve. Mechanomodulation affects mitral valve disease through serotonin, upregulates serotonin synthesis through a mechanosensory mechanism.

3.3 Involvement of Platelet Dysfunction to Mitral Valve Degeneration

In all species, VHD has the potential to be affected by platelet activation or function as a result of turbulent high-velocity blood flow and fluid shear stress. Increased platelet activation and reactivity would be expected initially; however, continuous stimulation and stress may lead to structural and biochemical changes associated with decreased platelet function. Alteration of platelet function in humans with

heart disease contributes to the development of vascular remodeling, thromboembolic events, and fatalities.

Former work in human [36] addresses the indirect relationships between mitral valve prolapsed, thromboembolic disease and hemodynamic irregularities (platelet activation, serotonin liberation). The incidence of platelet coagulant hyperactivity in patients with mitral valve prolapse was 76% compared with 6% in control patients. Further, the frequency of abnormal results of platelet coagulant activities was significantly higher in all patients with mitral valve prolapse than in control patients with non-thrombotic diseases. The demonstration of these abnormalities is consistent with the view that exposure of flowing blood to the abnormal hemodynamics or valve surfaces in mitral prolapse gives rise to in-vivo platelet stimulation resulting in intravascular thromboembolism. These results suggested that platelets play a critical role in the purported association of thromboembolism and mitral valve prolapse. Additional studies were performed to investigate potential platelet activation in dogs with clinical heart disease.

Platelet activation, as assessed on the basis of mean platelet component concentration and platelet component distribution width, was not a feature of subclinical chronic VHD in CKCSs [37]. Increased platelet closure time in CKCSs with a regurgitant jet size >50% may reflect quantitative and qualitative changes in von Willebrand factor. Significant differences in several platelet variables, including platelet count, mitral valve prolapse, and platelet component distribution width, were compared between CKCSs and dogs of other breeds. Such interbreed variation must be considered when interpreting results. Similar conclusions were reported in human [38], where mitral regurgitation in MVD was associated with systemic platelet activation. Mitral valve prolapse itself was not associated with increased platelet activation. The degree of platelet activation was positively correlated with the severity of mitral regurgitation and was independent of the underlying etiology of MVD, age and left atrial size.

4 Genetics

The small breeds' especially CKCS are susceptible to numerous inherited disorders. Domestic dogs (Canis familiaris) have an intimate connection with human society and are valued as workers, hunters, herders, and companions. Recently, their importance has been augmented by their value as a model organism for human disease [39]. Hundreds of genetic disorders have been described in dogs and more than half of them resemble specific human disorders [40]. This is mainly due to the unique evolutionary history of dogs are thought to be descended from a common ancestor with wolves. The dog was the first mammal to be domesticated, and its relationship with humans began more than 15,000 to 100,000 years ago [41]. The second phase involved selective breeding, which predominantly occurred in the last few centuries and has resulted in more than 400 recognized breeds [42]. In addition to a variety of

genetic diseases, dogs exhibit huge variation in size, shape, physiology, and behavior [43, 44].

A team examined the effect of domestication on the dog genome by comparison with its wild ancestor, the gray wolf [45]. Considering that dogs arose from a small gene pool relatively recently in evolutionary time and that selection for diversity in breeds is even more recent, the huge phenotypic variation observed in modern day breeds is striking. They observed a variation in dog and wolf genes using whole-genome SNP data. The rate of non-synonymous substitutions and synonymous substitutions, was around 50% greater for SNPs found in dogs than in wolves, indicating that a higher proportion of non-synonymous alleles segregate in dogs compared with non-functional genetic variation. Authors suggested that the majority of these alleles are slightly deleterious and that two main factors may have contributed to their increase. The first is a relaxation of selective constraint due to a population bottleneck and altered breeding patterns accompanying domestication. The second is a reduction of effective population size at loci linked to those under positive selection due to Hill–Robertson interference. A comparison in dog and wolf lineages indicated that dogs appear to have been accumulating non-synonymous mutations in mitochondrial DNA genes at a greater rate than wolves [45].

Since the year 1922, Write raises the debate about the breeding of domestic where animal's consanguineous mattings are frequently made and the importance of having a coefficient by means of which the degree of inbreeding may be expressed has been brought [46]. Inbreeding reduces the genetic variation within a breed, and tends to accentuate the expression of diseases that are due to recessive genes. In a work, inbreeding and genetic diversity in dogs were also assessed from DNA analysis [47]. On average, dog breeds currently retain approximately 87% of the available domestic canine genetic diversity. These authors concluded that global exchange of genetic material may hasten the loss of alleles and this practice should be discussed in relation to the current effective population size of a breed and its expected future popularity, and that genomic data do not always support the results from pedigree analysis.

It has been suggested that degenerative MVD has a strong genetic component and the inheritance of mitral valve prolapse is believed to be polygenic. A genome wide association study compared Single Nucleotide Polymorphism (SNP) in more than one thousand CKCS separated into two groups, degenerative MVD-affected or degenerative MVD-unaffected dogs, after cardiac auscultation and echocardiography. Despite the identification of two regions which included 20 and 11 genes, respectively, associated with development of degenerative MVD, no genes were identified [48]. Thus far, different researchers have identified different chromosomes as possible regions for genes affecting mitral valve deterioration in MVD-affected dogs. A study [49] reported that MVD in CKS breed is a polygenic threshold trait and that sex of the offspring influences threshold levels. In a 2016 a team of researchers [50] investigated variations of the COL1A2 gene in 50 MVD-affected and 80 control Poodles. The authors found that the allele T of the rs22372411 variant was over-represented in myxomatous MVD patients compared with healthy

controls. They concluded about an association of the rs22372411 COL1A2 gene variant with susceptibility to canine myxomatous MVD.

Another question is the relation between genetic and body size [51]. Multiple studies addressed the genetics of size variation in dogs from different viewpoints. One of the most interesting studies related to signaling pathways in mitral valve degeneration [52] showed that high mobility group HMGA2 affects cardiomyocyte differentiation and that a morpholino mediated knock-down of the gene leads to improper heart formation in frog embryos. Perhaps the most interesting gene in this group is SMAD family member 2 (SMAD2) which interacts directly with TGFβ. Recent studies show that TGFβ2, working through SMAD2/3, is required to achieve mature valve structure. In addition, an increase in TGFβ signaling, identified by the correlated increase in SMAD2 expression, contributes to mitral valve degeneration in a mouse model of Marfan's syndrome in which the mitral valves show increased leaflet length and thickness and folding conformation. Given this correlation, a dog with altered SMAD2 expression due to selection for its growth retarding properties may also experience problems with cardiac valve development.

Other authors [53, 54] suggest that familial occurrence of mitral valve murmur in the CKCS breed is not due to a single major gene effect, indicating that breeding strategies to eliminate the disease cannot be based on genotype information at this time. A role of SERT function and expression in degenerative MVD was reinforced by the finding of three polymorphisms in the SERT gene of Maltese dogs that were predicted to induce damage to protein function [23]. More recently, an abstract from North Carolina State veterinary researchers [55], hypothesized that genetic variants in the serotonin receptor signaling pathway would be associated with the development of mitral valve prolapse in dogs and add insight into this complex disease. In humans, mitral valve prolapse is a common heritable condition. Dogs serve as a spontaneous animal model of familial mitral valve prolapse, with several breeds genetically predisposed. As in humans, previous studies have suggested that canine mitral valve prolapse may be polygenic and may be associated with alterations in the serotonin pathway. DNA was isolated from blood samples from 51 dogs with mitral valve prolapse and whole genome sequencing was performed. No high, moderate, or high impact modifying variants in canine genes orthologous to the human genes known to be associated with the serotonin receptor signaling pathway were shared in all dogs used for this study. High impact variants were inconsistently identified in the serotonin receptor signaling pathway in this canine model. No single high impact variant, gene, or gene family accounted for all dogs. Although serotonin may play a role in mitral valve prolapse development, it may not be the primary genetic cause of the disease.

These genetic investigations, along with other approaches, indicated the need of investigating therapeutic opportunities around serotonin-related valvulopathy.

5 Treatments in Asymptomatic Stages

5.1 Current

Asymptomatic dogs with left apical systolic murmurs characteristic of mitral regurgitation should undergo baseline diagnostics to establish the etiology of the murmur. If a diagnosis of stage B1 degenerative valve disease is confirmed by echocardiography or presumed (in an older small breed dog) based on the presence of a normal radiographic vertebral heart size, then no therapy is indicated. Recommendations for stage B1 degenerative valve disease remain unchanged [56]. There is no historical or new evidence to support intervention with a specific therapy at this stage. Emphasis in this stage should include client communication concerning the value of scheduled follow-up evaluations to assess the presence and severity of any disease progression. In addition, comorbidities, such as systemic hypertension, that may impact the rate of degenerative valve disease progression should be intermittently screened for during stage B1. No drug or dietary treatment is recommended by the American College of Veterinary Internal Medicine (ACVIM) consensus [57].

Stage B2 is defined as dogs with degenerative valve disease that have evidence of heart enlargement but have never suffered from signs or symptoms attributable to chronic HF. Dogs are typically identified in this stage based on the presence of a moderately loud systolic heart murmur characteristic of mitral regurgitation and heart enlargement, specifically left atrial enlargement, with or without left ventricular dilation. Definitive diagnosis of degenerative valve disease and assessment of heart enlargement can be confirmed by echocardiography or presumed (in an older small-breed dog with a mitral regurgitation murmur) based on the presence of an increased radiographic vertebral heart size. Because stage B2 includes all dogs with degenerative valve disease and any magnitude of heart enlargement that have never suffered from chronic HF, it is a heterogeneous population. Dogs at this stage may be days away from developing chronic HF, or may never develop chronic HF in their lifetime. This is reflected in the long median time to onset of chronic HF, approximately 27 months, that has been reported in stage B2 degenerative valve disease. However, it is important to recognize that all stage B2 dogs have a risk of going into chronic HF. Many studies have provided important information on the natural progression of stage B2 degenerative valve disease and reported factors that can be used to identify which stage B2 dogs have higher versus lower risks of developing chronic HF. Known risk factors for development of chronic HF and a poor outcome in dogs with stage B2 degenerative valve disease include larger heart size as measured by echocardiography or vertebral heart size, rapid progression of heart enlargement based on repeat evaluations, and high levels of cardiac biomarkers, such as N-terminal pro B-type natriuretic peptide.

However, despite the wide body of knowledge concerning degenerative valve disease, there has historically been no consensus with respect to therapeutic recommendations for stage B2 degenerative valve disease. This is a consequence of the

lack of data confirming that initiation of any treatment in stage B2 degenerative valve disease can significantly delay the onset of chronic HF. However, treatment with an angiotensin converting enzyme (ACE) inhibitor has been historically advocated by some cardiologists for the treatment of some stage B2 dogs and is based predominantly on the results of the VETPROOF (Veterinary Enalapril Trial to Prove Reduction in Onset Of HF) study in combination with their well-known safety profile. Pimobendan is recommended by ACVIM consensus [57] at a dosage of 0.25–0.3 mg/kg PO q12h. ACE inhibitors: For patients in Stage B2 on either initial examination, or in which the left atrium has increased markedly in size on successive monitoring examinations, 5 (of 10) panelists recommend treatment with ACE inhibitors [57].

5.2 Future

Ideally, future therapies will be developed that focus on prevention or early termination of progressive valve degeneration in stage B1 dogs, rather than focusing exclusively on treatment options for dogs that have already progressed to more advanced stages of the disease. Frustratingly, despite the common nature of degenerative valve disease in both humans and dogs, the pathophysiologic triggers that underlie the development of this disease remain largely unknown. One important structural transformation that has been associated with the development of degenerative valve disease pathology involves the transformation of VICs, predominant cell types present in the mitral valve, from a typical quiescent cell to an activated myofibroblast phenotype. Triggers for this transformation have been associated with both the serotonin and TGFβ1 pathways. Research into these lines of investigation suggest that clinical trials studying serotonin antagonists or serotonin receptor antagonists [58] and serotonin receptor inverse agonists [62] may be the next step forward in degenerative valve disease research in dogs.

5.2.1 Receptor Inverse Agonist

Inverse agonist can be helpful when receptors (5-HT_{2A} and/or 5-HT_{2B}) are constitutively active [59]. Indeed, it has been found that receptors may exist in a constitutively active state, particularly when expressed in high amounts in cultured cells. Such receptors, “active on their own”, may be calmed down by so-called inverse agonists, compounds that were originally classified as antagonists. It is interesting to note that sarpogrelate, a selective 5-HT_{2A} antagonist, was prescribed for more than 30 and 20 years without significant adverse effects [60] and without a single known report of VHD. It was demonstrated that sarpogrelate improved left ventricular systolic function in acute myocardial infarction [61]. A publication [62] reported that sarpogrelate showed a potent inverse agonist activity, to constitutively active mutant (C322K) of human 5-HT_{2A} receptor. A recent work [66] of constitutive

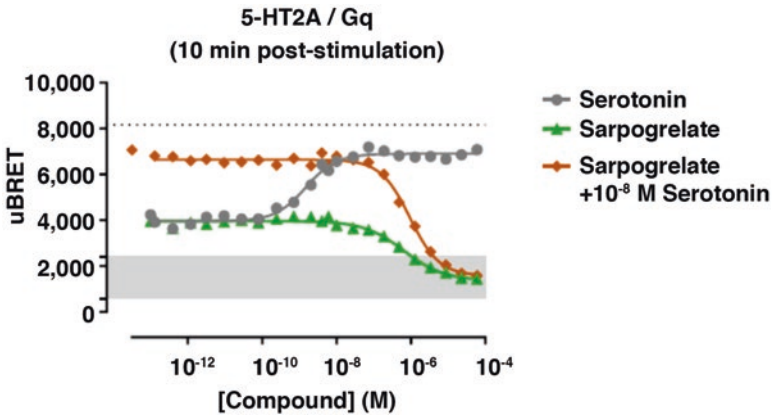

Fig. 11.5 Effect of ligands on 5-HT2A -mediated Gαq activation. HEK293 cells were co-transfected with 50 ng of plasmid encoding one of the 5-HT2A orthologue and plasmids coding for GAPL-Gαq. Increasing amounts of test compounds were added and the BRET assay was performed 10 min later. Data are expressed as uBRET. Unpublished Ceva report [66]

activity of Gq signaling pathway was observed in study of functional activity using canin 5-HT_{2A} (Fig. 11.5) where sarpogrelate reverse the constitutive activity of heterotrimeric Gαq protein. In conclusion, these types of molecules, inverse agonists of 5-HT receptors, should be tested for efficacy in preventing or treating VHD as has been suggested [63].

Using mice models and bone-marrow transplantation experiments, early heart valve degeneration was shown to involve the mobilization of endothelial progenitor cells due to 5-HT_{2B} receptor stimulation. The identification of endothelial progenitors sharing this receptor in human mitral valve prolapse tissues reveals the relevance of this mechanism in human heart valve remodeling [12]. Recently, a French team [64] developed a murine model of drug-induced valvulopathy by continuous subcutaneous infusion of nordexfenfluramine (Ndf) at 1 mg/kg/day for 28 days to test the contribution of the 5-HT_{2A} and or 5-HT_{2B} receptors. NdF effects were evaluated in WT mice co-treated with sarpogrelate or in *Htr2a*$^{-/-}$ mice. Sarpogrelate treatment prevented the NdF-induced increase in urinary 5-Hydroxy-Indole-Acetic-Acid (5-HIAA) and blood serotonin levels. This treatment leads to the development of valve lesions characterized by cushions with a high density of endothelial progenitor cells (CD34+/CD31+) originated from bone-marrow. Valve lesions were completely prevented by the inhibition of both 5-HT_{2A} and 5-HT_{2B} receptors by antagonists and in transgenic *Htr2b*$^{-/-}$ or *Htr2a/2b*$^{-/-}$ mice. This work also highlighted the initiating mechanisms of valve lesions by indicating serotonin as an important determinant in valvular cell recruitment that leads to valve remodeling. Many other proposed molecular mechanisms for heart valve degeneration are also probably implicated.

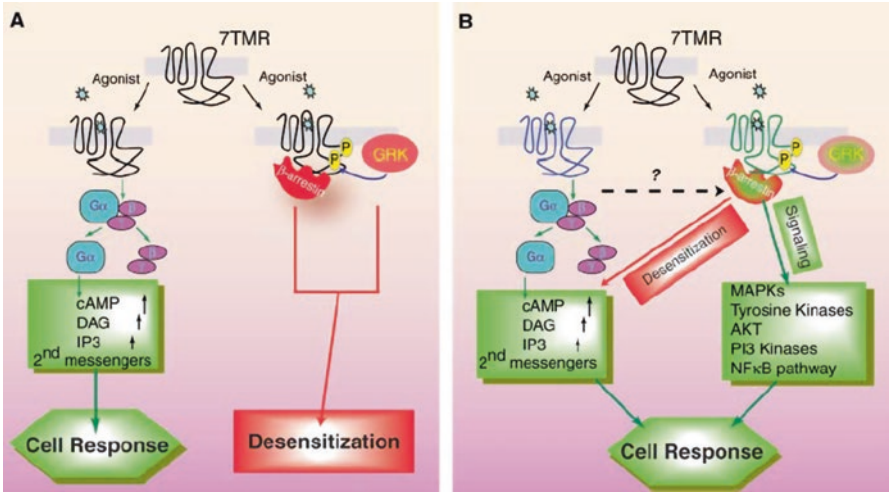

Fig. 11.6 Signal transduction by seven transmembrane receptors. (**a**) Classical paradigm. The active form of the receptor (R*) stimulates heterotrimeric G proteins and is rapidly phosphorylated by G protein–coupled receptor kinases (GRKs), which leads to β-arrestin recruitment. The receptor is thereby desensitized, and the signaling is stalled. (**b**) New paradigm. β-arrestin not only mediate desensitization of G protein–signaling but also act as signal transducers themselves. According to [65]

Drugs targeting serotonin receptors are approved treatments for a diverse array of indications. Serotonin receptors also frequently mediate serious drug side effects via unanticipated 'off-target' actions. A notable example is the now-banned appetite suppressant fenfluramine, which exerts its potent anti-obesity actions by activating 5-HT_{2C} receptors. Fenfluramine was ultimately withdrawn from the market because of a high incidence of drug-induced VHD, which occurs as a result of off-target activation by fenfluramine and its active metabolite Ndf at the closely related 5-HT_{2B} receptor. Consequently, candidate medications are routinely screened for 5-HT_{2B} receptor agonist activity before progressing to clinical trials. Not unexpectedly, 5-HT_{2B} receptor antagonists have been proposed as potential therapeutics for VHD and other fibrotic disorders, including carcinoid syndrome. Thus, understanding the action of drugs at 5-HT_{2B} receptor is clearly important for future drug development. In conclusion, inverse agonists may be useful in the treatment of cardiovascular diseases and of diseases caused by constitutively active receptors such as 5-HT_{2B} receptors.

5.2.2 β-arrestin-2

5-HT_{2B} receptor in addition to activation via Gq/11 of phospholipase C activation, inositol phosphate (IP) accumulation, intracellular calcium release and protein kinase C activation, can also recruit β-arrestin-2 (also known as arrestin-3; encoded by ARRB2 in humans) and downstream effector activation. Drugs such as lysergic acid diethylamide (LSD) and ergotamine (ERG) prefer arrestin recruitment and are considered 'arrestin-biased agonists. The discovery of β-arrestin–mediated signaling highlights the concept "biased agonist" [65]. In the simplest classical models, receptors exist in two states, active and inactive, with agonists stabilizing the active state, thereby driving activation of effectors (Fig. 11.6). However, the conformation of a receptor that interacts with a G protein can be distinct from that which interacts with another effector such as β-arrestin. The ability of ligands to differentially favor one or the other conformation suggests significantly greater diversity and fine-tuning of signaling possibilities for a single receptor than previously imagined. Moreover, such putative β-arrestin- or G protein-specific ligands might have valuable therapeutic properties and perhaps more restricted side effects.

In a surprising way, an unpublished study [66] showed that canine 5-HT_{2B} receptor was completely inactive at engaging β-arrestin-2 in response to serotonin. This observation is supported by primary amino acid sequence of canine 5-HT_{2B} receptor. Interestingly, canine 5-HT_{2B} receptor lacks a long C-terminal tail segment that contains a highly conserved (in mouse, rat and human receptors) stretch of S/T residues. Such residues, when phosphorylated by G-protein-coupled receptor kinases (GRKs), bind to β-arrestin with high affinity (Fig. 11.7).

The question that remains unanswered is the putative relationship between this inability in β-arrestin coupling and the predisposition of certain breeds of dogs to the myxomatous MVD. The 5-HT_{2B} receptor is required for heart development regulating differentiation and proliferation of cardiac tissue; SERT deficient mice develop cardiac fibrosis, and valvulopathy [68]. Further studies are needed to determine whether β-arrestin-2 is "silent" in all population of dogs or particularly in predisposed breeds. These researches should be performed in comparison with the native breed (wolf), as well as to further elucidate the molecular mechanisms by which β-arrestin mediates this pathology and its potential protector effect.

The absence of β-arrestin in certain dog breeds could explain a continuous activation of the G protein signaling pathway without the "braking" process. This could explain 5-HT_{2B} receptors constantly activated in dog.

The integration of all these new data (genetic, pharmacological …) about serotonin pathways and especially 5-HT_{2B} receptor contribution should allow a better understanding of the strong predisposition of small breeds of dogs to develop myxomatous MVD and lead to more appropriate treatments.

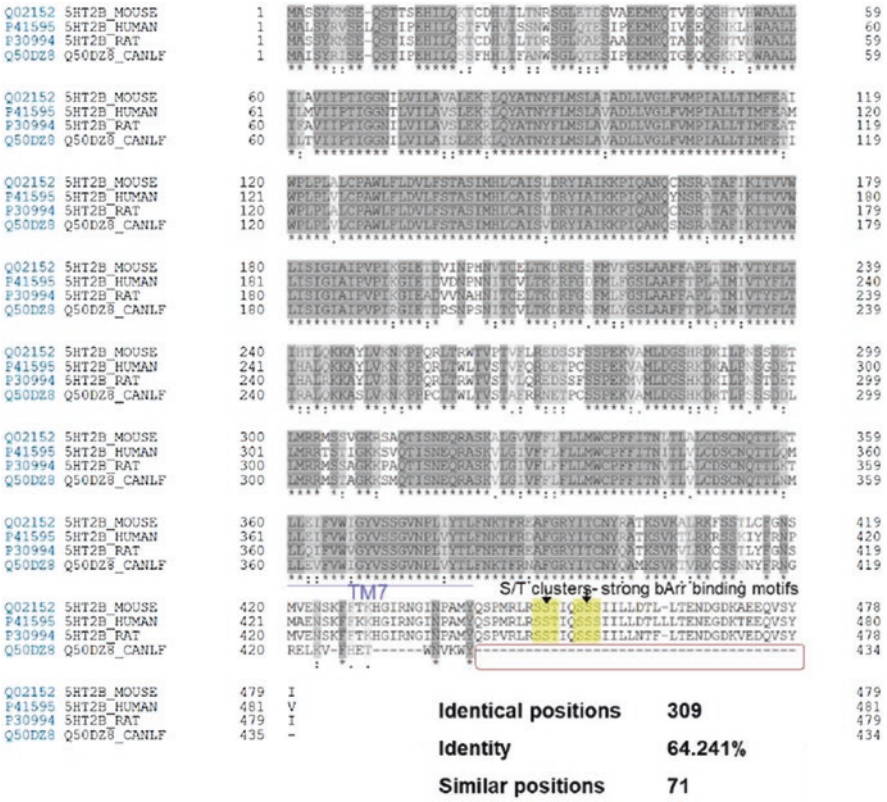

Fig. 11.7 Amino acid sequence alignment of 5-HT_{2B} receptor orthologue (rat, mouse, canine and human). Sequence alignment performed using UniProt's Align function (https://www.uniprot.org/). Canine 5-HT_{2B} receptor lacks a long C-terminal tail segment (red box) that contains a highly conserved (in mouse, rat and human receptors) stretch of S/T residues (yellow highlight). Such residues, when phosphorylated by G-protein-coupled receptor kinases (GRKs), bind to β-arrestin with high affinity. Unpublished Ceva report and [67]

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Chapter 12
The Discovery of 5-HT_{2B} Receptor Pharmacology Through the Understanding of Drug-Induced Valvulopathy

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1 From the Serotonin Hypothesis to the Serotonin 5-HT_{2B} Receptor in Valvular Degeneration

The "serotonin hypothesis" in the development of cardiopulmonary disease follows the use of phentermine-fenfluramine association (Phen-Fen) as a treatment for obesity. The first case reports described unusual valvular morphology and regurgitation in 24 young women and pulmonary hypertension in 8 of them, after initiation of Phen-Fen therapy [1]. Quickly, the histopathological signature associated with fenfluramine-induced valvulopathy was compared with right-sided heart valve lesions induced by carcinoid syndrome, this disease caused by serotonin-secreting neuroendocrine tumors [2]. The serotonin releasing agent fenfluramine (Pondimin®, Ponderal®) and its potent stereoisomer, (+)-fenfluramine, also called dexfenfluramine (Redux®, Isomeride®), are substituted phenylethylamine analogs used as anorectic agents, and withdrawn from the market in 1997 due to the occurrence of cardiac valve disease and pulmonary hypertension. Although fenfluramines increase neuronal serotonin concentrations by interacting with the serotonin transporter (SERT) and VMAT proteins [3], Rothman and Baumann [4] demonstrated that chronic fenfluramine at clinically-relevant doses decreased blood serotonin levels, and increased plasma serotonin only two-fold to four-fold above baseline, in

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catheterized rats undergoing *in vivo* microdialysis [5, 6]. MAO inhibitors treatment produces comparable increments in plasma serotonin [7], without increasing the risk of cardiac valve disease. These data challenge the "serotonin hypothesis" based on the fact that chronic fenfluramine administration increases plasma or blood serotonin to a level sufficient to cause valvulopathy in humans. Interestingly, fenfluramine and its stereoisomers, but also their de-ethylated metabolites norfenfluramine and its stereoisomers, share the common pharmacological property to be potent serotonin releasers. In *in vitro* binding assays, (+)-fenfluramine releases [^{3}H]5-HT with an EC50 value of 51.7 nM, and (+)-norfenfluramine releases [^{3}H]5-HT with an EC50 value of 59.3 nM [8]. Continued interest in the pharmacology of these drugs indicated that fenfluramines exhibit very low (>4 μM) binding affinity for human 5-HT$_{2B}$ receptors. To confirm this 5-HT$_{2B}$ receptors hypothesis, Rothman et al. [9] evaluated in radioligand binding assays, the affinity of drugs-inducing valvulopathy (i.e. methysergide, its active metabolite methylergonovine, and ergotamine) vs. negative drug controls (i.e. fluoxetine and its metabolite norfluoxetine, and trazodone and its metabolite m-chlorophenylpiperazine) on several G protein coupled-receptors, including serotonin receptors but also adrenergic, histaminergic, GABAergic receptors and catecholamine transporters. They demonstrated that all drug inducing valvulopathy or their metabolites share the pharmacological property to have high affinity for the 5-HT$_{2B}$ receptor and to display partial or full agonism at this receptor, using phosphoinositide hydrolysis assays, in HEK-293 cells transiently transfected with human 5-HT$_{2B}$ receptor [9]. The pharmacological notion that 5-HT$_{2B}$ receptor agonists induce valvulopathy was recently confirmed *in vivo*. Our laboratory developed a pharmacological model of valve degeneration in mice, by chronically infusing nordexfenfluramine (NdF), the main metabolite of dexfenfluramine and of benfluorex. We demonstrated that blocking 5-HT$_{2B}$ receptor by antagonists (SB204741 and SB206553) or in transgenic *Htr2b*$^{-/-}$ mice lacking the 5-HT$_{2B}$ receptor gene, successfully prevents NdF-induced valve lesions [10]. Moreover, we also disclaimed the "serotonin hypothesis" by treating mice simultaneously with para-chlorophenylalanine, a tryptophan hydroxylase inhibitor that decreases blood serotonin by about two thirds, but fails to prevent NdF-induced cardiac valve lesions [10].

This "serotonergic" heart valve disease concerns essentially the left-sided heart, and leads to regurgitation because of incomplete coaptation of the valve leaflets. Among all the diagnoses of chronic valve insufficiency (degenerative, rheumatic, post-endocarditis, congenital, ischemic, secondary to left ventricular failure, post-radiation, valvular tumor), drug-induced valvulopathy is attributable to progressive myxomatous degeneration, affecting more frequently the mitral valve [11]. These fibromyxoid cardiac valve lesions are characterized by valve thickening and abnormal motion of the leaflets observed by echocardiography. Not only the leaflets, but also the subvalvular apparatus may be affected. In atrio-ventricular valve disease, prominent subvalvular thickness and shortening of the chordae tendinae also contribute to the regurgitation [12]. Macroscopically, these myxoid valvular changes do not induce drastic modifications in the valve architecture, but appear as glistening white plaque, deposited on the surface of the valves [1]. Histologically, these

deposits look like a very dense cellular cushion, in an abundant extracellular matrix (ECM) [12]. These degenerative lesions involve both the collagen content and the alignment of collagen fibrils within the valve, as well as the fragmentation of elastin fibers. The spongiosa layer is expanded by proteoglycan content. Dysregulation of the extracellular matrix appears to be central to these changes and results from myofibroblasts activation [13].

2 Cellular Mechanisms, Involved in Valvular Degeneration, Regulated by Serotonin Stimulation

To understand the pharmacological mechanisms involving the 5-HT$_{2B}$ receptor in valve degeneration, we must first look for its cellular location. Manivet et al. [14] demonstrated that the tissue distribution of 5-HT$_{2B}$ receptor protein is similar in rodents and humans, as well as its pharmacology, allowing easier extrapolation of animal model results in humans. Although this receptor is weakly expressed in healthy tissues, it is found in the heart valves of different species, such as dogs [15], rats [16, 17], pigs [18], monkeys [16] and humans [18, 19], by PCR or immunohistochemistry. The expression of the 5-HT$_{2A}$ receptor is also found in human, pig [18], dogs [20] and sheep [21] valves, leading to the use of highly selective ligands to interpret the effects induced by the stimulation of 5-HT$_2$ receptors. However, it should be noted that only one study isolated 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtype messenger RNA from human valve interstitial cells [22], leading to the study of the involvement of the serotonergic system in valvular degeneration focused on 5-HT$_2$ receptors. When trying to identify the cellular location of these 5-HT$_2$ receptors, there's a lack of data. Only our team has identified, after FACS sorting of CD34+ progenitor cells from pathological human mitral valves, the expression of the 5-HT$_{2B}$ receptor, by qPCR and immunofluorescence [10].

Valvular tissue is made up of extracellular matrix (ECM), which is the essential component guaranteeing the function and viability of the tissue. ECM is laminated in three layers composed of proteoglycans, collagen types I, III and IV, and elastin, each imparting distinct biomechanical property. For example, the degree of sulfation pattern of glycosaminoglycans (GAGs) gives them different biomechanical properties: 4-sulfated GAGs are located in tensile loading regions, and may sequester transforming growth factor (TGF-β) whereas the 6-sulfated GAGs are located in compressive loading regions [23]. The quality and quantity of valvular ECM depend on function/activation of valvular interstitial cells. Two types of cells form the heart valve tissue: valvular endothelial cells (VECs), that cover the valvular leaflet surface, and valvular interstitial cells (VICs), located below the surface. This VICs population is heterogeneous with five different phenotypes identified: embryonic progenitor endothelial/mesenchymal cells, quiescent cells, activated VICs (aVICs), progenitor VICs and osteoblastic VICs [24]. VICs share morphologic and functional properties of fibroblasts, smooth muscle cells and myofibroblasts. These

"phenotypes" exhibit plasticity and may convert from one form to another. These most prevalent cells in all three layers (atrialis, spongiosa and fibrosa), maintain the integrity of normal valves and regulate repair processes after valve injury, such as remodeling, synthesis or degradation of ECM. Indeed, matrix metalloproteinases (MMPs), matrix-degrading enzymes, are synthetized by VICs as inactive zymogens, that could be activated by interleukin-1β or tumor necrosis factor-α, or inhibited by tissue inhibitors of metalloproteinases (TIMPs) through the formation of irreversible complexes [25]. MMPs and theirs inhibitors play important role in the homeostasis of ECM, as well as in the pathogenesis of heart valve diseases, including myxomatous mitral valve disease [26] and drug-induced valvulopathy [27]. In normal leaflet, the marker of activated VICs, alpha smooth muscle actin (αSMA) is approximatively expressed by 57% of cells, but increased during injury and according to culture conditions [28]. VICs density, proliferation and apoptosis are markedly higher in fetal valves than in adult valves, then these features increase again during degenerative processes, implying a re-induction of an embryonic mechanism at the pathological stage [29]. About valvular endothelial cells (VECs), this cell population is also heterogeneous but not yet fully characterized. They have a different phenotype from vascular endothelial cells isolated from aorta. In response to mechanical stress, porcine aortic VECs align perpendicular to blood flow, whereas endothelial cells from the aorta align parallel to the flow [30]. Porcine aortic valvular endothelial cells express more genes associated with chondrogenesis, where porcine aortic endothelial cells express mostly osteogenic genes [30], suggesting a protective role against valve calcification. Moreover, VECs respond differently to injuries and display different transcriptional profiles, according to aortic versus ventricular sides of the valve [31]. Some authors argue that some VECs are progenitor cells, which may serve to replenish valvular cells during homeostatic cellular turnover and in response to injury [32].

The heterogeneity and plasticity of the valvular cells make it difficult to study the cellular and molecular mechanisms responsible for valvular degeneration. Although the cell type expressing the 5-HT_2 receptor(s) remains questionable, recent studies, using microarray technology, highlighted the contribution of 5-HT_{2A} and 5-HT_{2B} receptors in pathological conditions in canine [33] and human myxomatous mitral valve tissues [34, 35], compared to healthy tissues. The differential expression of genes involves serotonin receptors pathway, but also extracellular matrix remodeling, TGF-β pathway, and antioxidant system. We will summarize the cellular mechanisms involved in valvular degeneration induced by the stimulation of 5-HT_2 receptors.

2.1 Valve Interstitial Cell Proliferation

As already described in ventricular fibroblasts [36, 37], 5-HT_{2B} receptor has been shown to elicit mitogenic pathway and secretion of ECM components in heart valve fibroblasts [18, 19, 38]. The mitogenic effect of serotonin on human VICs *in vitro*

was first described following the incorporation of [^{3}H] thymine into the DNA of these cells and stimulated for 48 h by serotonin (0.1 μM) [38]. Collagen synthesis response to serotonin of VICs was observed with a more modest effect (15% incorporation of proline [^{3}H]) after stimulation by serotonin at 1 μM [38]. These data, obtained on valve interstitial cells from transplant recipient hearts, are the first to observe cellular effects-dependent on serotonin and resulting from an increase in the intracellular concentration of calcium.

In human embryonic kidney 293 cells transfected with three subtypes of human 5-HT_2 serotonin receptor genes, Fitzgerald et al. [18] confirmed the high affinity of nordexfenfluramine for 5-HT_{2B} and 5-HT_{2C} receptors and highlighted the transduction pathway activated: hydrolysis of inositol triphosphate (IP3), increase in intracellular Ca^{2+} concentration and phosphorylation extracellular signal-regulated kinase (ERK1/2) supporting the hypothesis of activation of the 5-HT_{2B} receptor canonical pathway. The same mitogenic response was identified in human VICs after treatment with potent 5-HT_{2B} receptor agonists, MDMA (3, 4-methylenedioxymethamphetamin, also known as ecstasy), MDA (its metabolite) and norfenfluramine ([^{3}H] thymidine deoxyribose incorporation, via ERK1/2 phosphorylation) and was prevented by pretreatment with a 5-HT_{2B} receptor antagonist [19].

This biological result of activating 5-HT_{2B} receptors as responsible for valve fibromyxoid lesions by proliferation of myofibroblasts and activation of smooth muscle cells leading to thickening and retraction of the valvular leaflet, is essentially based on cellular data from heterogeneous populations of healthy VICs or transfected cells, and with high doses of serotonin. When we sought to evaluate the proliferation of VICs by KI-67 labelling on histological sections of mitral valves from our mouse model of drug-induced valvulopathy, infused for 28 days with nordexfenfluramine, we observed *ex vivo* only few nuclei labelled indicating a very low rate of proliferation identical to that of adult control mice [10]. These results suggest that other cellular effects due to stimulation of the 5-HT_{2B} receptor are involved in valve degeneration. On the other hand, Connolly et al. [39] observed on healthy and pathological mitral VICs, human and dog, in culture, that the incorporation of [^{3}H] thymine was prevented when adding ketanserin (5-HT_{2A} receptor antagonist) and GR55562 (5-HT_{1B} receptor antagonist). These results suggest the involvement of other serotonergic receptors, inducing the serotonin-dependent mitogenic effect.

2.2 Secretion of Extracellular Matrix (ECM) Components

Regardless of the etiology of valve degeneration, cardiac valve lesions show histopathological alterations in ECM organization, leading to functional impairments such as insufficiency or stenosis. As soon as the valve interstitial cells are activated, they produce several constituents of the extracellular matrix, leading to fibrosis and valve remodeling. *In vivo*, an increase in the production of glycosaminoglycans and a decrease in the amount of collagen (Movat's pentachrome staining) were observed

in valvular tissue, after chronic subcutaneous administration of serotonin in rats [17] or after treatment with pergolide, a dopaminergic agonist for 20 weeks intraperitoneally in the treatment of Parkinson's disease responsible for valvulopathy [40]. This compositional alteration of ECM, similar to fibromyxoid lesions and also observed in carcinoid heart, was associated with increased expression of 5-HT_{2B} receptor in aortic and mitral valves [40], whereas the expression of serotonin transporter (SERT) gene was down regulated [17]. In another study, the same team observed that the histological alterations (significant valvular thickening, high amount of glycosaminoglycans and low amount of collagen) of a Sprague Dawley rat spontaneously affected by mitral valvular degeneration, were similar to that of a Fisher rat treated with dl-amphetamine for 103 weeks and found over-expression of the 5-HT_{2B} receptor in the mitral leaflet by immunohistochemistry [41]. These results suggest that 5-HT_{2B} receptor play a crucial role in VICs activation and in the maintenance of the structural homeostasis of valve tissue, but also that the serotoninergic system is involved in spontaneous valvular degeneration. Balachandran et al. [42] demonstrated that cyclic stress of the valve, using a bioreactor, increased proliferation, collagen and GAGs production in porcine aortic valve leaflets. This ECM remodeling effect was increased under serotonin treatment, as cyclic stretch increases the expression of 5-HT_{2A} and 5-HT_{2B} receptors by a factor 4.5 in DNA microarray study. On the other hand, only the blockade of the 5-HT_{2A} receptor by ketanserin reduces collagen synthesis and proliferation in pathological stretch conditions (15% dynamic stretch), while the blockade of the 5-HT_{2B} receptor by SB204741 is effective on the remodeling of ECM in physiological conditions (10% dynamic stretch) [43]. This *ex vivo* model highlights the sensitivity of resident VICs to cyclic stretch, increasing basal expression of 5-HT_2 receptors, and suggesting that ECM remodeling of cardiac valves could be exacerbated to any conditions where serotonin is increased. *In vitro*, treatment of VICs with serotonin (1 μM) increases modestly the incorporation of [^{3}H] proline into collagen compared to untreated cells [38]. This low increase in the production of extracellular matrix (collagen, glycans, hyaluronic acid), by serotoninergic stimulation of VICs, through ERK phosphorylation, has been found in other species, such as sheep [44], dogs and humans [39]. The different effects in terms of remodeling lead to evoke the presence of cofactors *in vivo*, absent *in vitro*, or a paracrine regulation between different types of VICs.

2.3 TGF-Beta Signaling: Multicellular Contribution to Cardiac Valve Degeneration

Both 5-HT_{2A} and 5-HT_{2B} receptors have been shown to elicit secretory responses in ventricular and heart valve fibroblasts [45–47]. In sheep aortic valvular interstitial cells culture, serotonin stimulation induces the secretion of TGF-β1, via activation of phospholipase C (PLC) [44]. This effect is inhibited by MDL100907, a selective 5-HT_{2A} receptor antagonist [47]. In humans, TGF-β receptors I and II have been

identified, by immunohistochemistry, in tricuspid and pulmonary valve samples from patients with carcinoid heart disease, as well as in healthy tricuspid valve samples [44]. While TGF-β1 has been identified endothelially only in healthy valve specimens, and the latent form of TGF-β1 is present only in significant amounts in pathological valve specimens [44].

The cytokine TGF-β is a central regulator of cellular processes such as cell differentiation, proliferation, migration, and cell-to-cell communication, that control valve function during embryonic development and in adult injury responses. Among congenital mitral valve disease, there are two syndromic pathologies (affecting multiple organs as the heart, blood vessels, lungs, skin and bones) related to the TGF-beta pathway. These syndromes result from the structural weakness of connective tissue. Marfan syndrome results from heterozygous mutations in *FBN1*, the gene that encodes fibrillin-1, which is the principal component of microfibrils in the ECM [48]. Affected individuals have mitral valve elongation and myxomatous leaflet thickening, leading to mitral valve prolapse. Mutated fibrillin-1 in Marfan syndrome leads to failed sequestration of TGF-β, and then ensuing overactivity of TGF-β signaling cascades [49]. Mice with a cysteine-substitution mutation in an epidermal growth factor–like domain in fibrillin-1 have longer and thicker mitral valves than wild-type control mice [50]. In addition, TGF-β antagonism *in vivo*, using TGF-β neutralizing antibody, prevents the pathological prolongation and thickening of mitral valves in this model [50]. The TGF-β signaling is up regulated in aortic tissues of Marfan syndrome patients. Loeys–Dietz syndrome, which has similar phenotypic features with Marfan syndrome is caused by mutations in *TGFBR1/2*, *SMAD2/3*, or *TGFBR2/3*, all coding for components of the TGFβ-signaling pathway [51]. Hutcheson et al., [52] demonstrated that TGF-β1-mediated myofibroblasts activation in porcine aVICs can be arrested by 5-HT_{2B} antagonism. In these cultures of isolated porcine aVICs, both 5-HT_{2B} receptor antagonists did not affect cell viability or proliferation, but physically sequestered pSrc, preventing non-canonical TGF-β1 signaling [52]. Moreover, Merryman's team [53] demonstrated that 5-HT_{2B} receptor antagonist, SB204741, reduces SRC phosphorylation and activity, induced in *Bmpr2* mutant mice and so prevents heritable pulmonary arterial hypertension (PAH) in this model. In these works, the blockade of 5-HT_{2B} receptor, reducing SRC phosphorylation and activity, prevents both side effects, PAH and valvulopathy, induced by 5-HT_{2B} receptor agonists (see Chap. 10).

2.4 Mobilization of Progenitor Cells

The plasticity of valve cells makes it difficult to determine their origin and renewal. Visconti et al. [54] demonstrated that hematopoietic stem cells, originated from bone-marrow, migrated within host cardiac valves, after lethally mice irradiation combined with transplantation of individual clonal populations. Later, this same team characterized these bone-marrow hematopoietic stem cells, migrating into cardiac valves and, established that the engraftment of bone-marrow-derived cells

occurs as part of normal valve homeostasis [55]. Our team pointed the contribution of circulating progenitor cells activated by 5-HT_{2B} receptor agonists in the development of drug-induced heart disease by publishing the first case report of mitral bioprosthesis degeneration during benfluorex therapy [56]. In a 40-year-old woman treated with benfluorex, her mitral bioprosthesis, an initially acellular tissue, was completely degraded and numerous myofibroblasts were found [56]. Later, we demonstrated, in lethally irradiated wild-type mice engrafted with a *Htr2b*$^{-/-}$ bone-marrow and perfused chronically with NdF, that restricted ablation of 5-HT_{2B} receptors to bone-marrow prevents the NdF-induced mitral valve lesions, supporting the contribution of 5-HT_{2B} receptors to the mobilization of bone-marrow-derived endothelial progenitors [10]. The same mechanism was identified in pulmonary arterial hypertension: mice with restricted expression of 5-HT_{2B} receptors in bone-marrow cells was protected by hypoxia or monocrotaline-induced increase in pulmonary pressure and vascular remodeling [57]. The understanding of 5-HT_{2B} receptor agonists adverse effects permitted to highlight an early mechanism of valvular degeneration and pulmonary arterial hypertension, and to identify a new therapeutic target.

3 Pharmacology of 5-HT_{2B} Receptor Agonists

For safety pharmacology purposes, after the various "fenfluramine scandals", the researchers tried to develop a "screening the receptor agonist valvulopathogen compounds. Huang et al. [58] screened 2200 compounds that were FDA approved or investigational medications to identify 5-HT_{2B} receptor agonists, using calcium-based high-throughput screening. More than an assessment of specific binding and selectivity of compounds, it is the evaluation of their efficacy on the 5-HT_{2B} receptor and their potency in several pathways that should be sought. Of the 2200 compounds tested, only 27 were identified as 5-HT_{2B} agonists, including seven valvulopathogens. Among the set of pathways associated with stimulation of the 5-HT_{2B} receptor, calcium flux-based screening is suitable to the initial identification of 5-HT_{2B} receptor agonists but not for the discrimination of valvulopathogen risk. Differences were observed between efficacies and potencies (Emax and pEC_{50}) for the five pathways tested: calcium flux, transcription factor NFAT activity, ERK2 phosphorylation, arrestin translocation, inositol phosphates accumulation. Among the seven valvulopathogens compounds tested by Huang et al. [58], we can dissociate two families: ergot and fenfluramine derivatives. To date, no specific pharmacological pathway has been linked to VHD development. However, some hypothesis can be made, due to the characteristics of valvulopathogen compounds, getting serotonergic agonism properties.

3.1 Receptor Oligomerization

Already demonstrated with other GPCR, receptor oligomerization allows the modulation of the signaling pathways under agonist stimulation and could also modulate the ligand's binding capacity. However, it is difficult to discriminate pharmacological modulation resulting from an indirect interaction (e.g. via adaptor protein) or from a direct interaction (e.g. via the formation of an oligomer complex), as well as the pharmacological relevance of such interactions.

The first interaction between 5-HT_{2B} receptor and other GPCR to be described was with 5-HT_{1B} receptor, in 2007 by Janoshazi et al. [59]. Using binding experiments, they demonstrated that each receptor cross regulates the other one, notably on their internalization pathways, in an agonist dependent manner. When expressed alone, internalization of 5-HT_{1B} receptor is Cav1-dependent, and internalization of 5-HT_{2B} receptor is β-arrestin 2-dependent. However, co-expression of both receptors leads to a switch of internalization pathways. When co-expressed with 5-HT_{2B}, 5-HT_{1B} receptor agonist-induced internalization is Cav1- independent but PKC-dependent. As well, when co-expressed with 5-HT_{1B} receptor, a portion of 5-HT_{2B} receptor internalizes through a Cav1-dependent pathway. Moreover, in the presence of 5-HT_{2B} receptor, kinetic of agonist-induced internalization of 5-HT_{1B} receptor is greatly increased, and in addition, the agonist also triggers the internalization of 5-HT_{2B} receptor. But application of a 5-HT_{2B} receptor agonist does not induce 5-HT_{1B} receptor internalization. However, under agonist stimulation and when co-expressed with 5-HT_{1B} receptor, the kinetic of internalization of 5-HT_{2B} receptor is slightly increased. As the authors rightly discuss, these changes in pharmacological properties of both receptor in the presence of the other is probably due to an indirect interaction, and not to the formation of an oligomer. Indeed, using confocal imaging and FRET experiments, they found little to no colocalization between the two receptors. Moreover, despite one RT-qPCR analysis, which identified the 5-HT_{1B} receptor in human valve interstitial cells, no colocalization with the 5-HT_{2B} receptor was observed in native cardiac valve cells.

Oligomerization of the 5-HT_{2B} receptor has however been demonstrated with several other receptors. The first report of such complex is from 2009 by Jaffré et al. [60] between 5-HT_{2B} and AT1 receptors. They showed that both receptors are needed *in vivo* for the adrenergic-dependent cytokine production and subsequent cardiac hypertrophy, and that each one is capable of blocking cytokine release mediated by the other. Using immunoprecipitation assay and confocal imaging, they also demonstrated that both receptors colocalize and interact in the same cell compartment. These data support the hypothesis that 5-HT_{2B} and AT1 receptors interact together and can modulate the signals mediated by stimulation of either one of the protomers. This functional interdependence between both receptors is named transinhibition. In a recent study Perez et al. [61] investigated serotonin-induced effects, in the presence of angiotensin II-induced hypertension, in the context of the mechanobiological responses, due to altered valve mechanics. After three weeks of treatment with serotonin (2.5 ng/kg/min), angiotensin II (400 ng/kg/min), or a

combination of both, in C57Bl/6 J mice, they compared hemodynamic effects, left ventricular function, and valve thickness and remodeling between groups. A reduced ejection fraction, thick leaflets, large proportion of thick collagen fibers were observed in the combination group, leading to phenotype a new model of cardiac valve degeneration in mice [61]. These authors suggest that serotonin and angiotensin II interact to result in significantly detrimental alteration of function and remodeling in the valve, without any interaction studies being realized on cardiac valve cells. Recently, a long acting selective angiotensin 1 receptor inhibitor, irbesartan, was associated with a reduction in the rate of aortic dilatation in patients with Marfan syndrome [62]. Although the effect of this treatment has not been evaluated on valvular damage, it remains of interest, particularly in the inhibition of the endothelial to mesenchymal transformation in mitral VECs. Wylie-Sears et al. [63] demonstrated that losartan or MEK 1/2 inhibitors block endothelial-to-mesenchymal transformation, in response to TGF-β, in mitral sheep VECs. This therapeutic target has yet to be evaluated in a model of cardiac valve lesions but seems promising for preventing the reconstitution of VICs and fibrosis.

Oligomerization between 5-HT_{2A} and 5-HT_{2B} receptors has not been extensively studied. In BRET experiments, Moutkine et al. [64] obtained hyperbolic curves when these receptors were co-expressed in COS-7 cells, suggesting an interaction. However, such interaction has not been validated *in vivo* and its pharmacological relevance is yet to be described. Interestingly, in mice treated with Sarpogrelate (a 5-HT_{2A} receptor antagonist) and NdF, we showed that the blockade of 5-HT_{2A} receptor prevented NdF-induced valvular thickness, compared as wild type mice treated with NdF. These results were confirmed with the use of knock-out mice, *Htr2a*$^{-/-}$, treated with NdF. These mice displayed only a partial response to NdF [10], indicating a possible role of 5-HT_{2A} receptor in VHD development. As such it would be of interest to further study and validate the existence and pharmacological relevance of an oligomer between 5-HT_{2B} and 5-HT_{2A} receptors.

Overall, these studies demonstrate that 5-HT_{2B} receptor agonist-mediated signaling can be modulated by interaction and even by simple co-expression with other receptors, in a particular cell type. Given the heterogeneity of the valve cellular organization, oligomers formation studies could help decipher VHD mechanism through the understanding of interactions between 5-HT_{2B} and other receptors in homeostatic conditions, and under stimulation of fenfluramine or ergot derivatives.

3.2 Biased Agonism

Some agonists are able to modulate the conformation of receptor upon binding and thus modify several downstream pathways. It is the case of biased agonists. An agonist is considered biased or to have “functional selectivity”, when it preferentially activates one pathway over the other. Works demonstrated that serotonin receptors can engage in differential signaling that is determined by the chemical nature of the ligand [65]. Some ergot derivatives such as ergotamine are biased

5-HT_{2B} receptor agonists, increasing the recruitment of β-arrestin by a factor 228 compared to a classical full agonist [66]. Ergotamine also binds to 5-HT_{1B} receptor but does not induced a biased response. Thus, resolution of 5-HT_{2B} and 5-HT_{1B} receptors structure in complex with ergotamine via crystallography allowed the study of conformational differences between these receptors, and thus modulation upon agonist binding [67]. Changes in signaling pathways on 5-HT_{2B} receptor are due to changes in conformation of highly conserved motifs and so called "micro-switches" [66, 68]. Differences between the shift and conformation of some highly conserved motifs between the two receptors such as DRY (Asp-Arg-Tyr), NPxxY and P-I-F motifs could be predictive of a β-arrestin bias on 5-HT_{2B} receptor. However, this bias for β-arrestin pathway on 5-HT_{2B} receptor has not been linked to valvulopathy development.

3.3 Receptor Binding Kinetic and Pharmacokinetic

What could be interpreted as a bias on the 5-HT_{2B} receptor might result from a difference in kinetic of binding. Unett et al. [69] showed that a number of ergot derivatives are slow binders at 5-HT_{2B} receptor. These ligands also displayed slower receptor dissociation rates, associated with persistent signaling. As such, Unett et al. [69] hypothesized that the ligand could be internalized with the receptor, rending them unable to dissociate properly and thus keeping the receptor in an activated state.

Finally, another element to be taken into account in assessing the risk of drug-induced valvulopathy is the notion of treatment duration and the dose received [70]. The risk was high among patients who had taken daily doses of pergolide that exceeded 3 mg for at least 6 months [71]. The same dose-to-toxicity relationship was observed with fenfluramine derivatives. In a large cohort study, including more than 1 million diabetic patients, using data from two large national linked databases, health insurance system (SNIIRAM) and hospitalization (PMSI), Weil et al. [11] observed a threefold increase in the risk of cardiac valvular insufficiency and that this valvular risk increases even more with benfluorex treatment of 3 months and more. More than the quantity of active drugs present in the plasma, it is the level of metabolites that needs to be evaluated. As previously described in the serotonin hypothesis part, the potent 5-HT_{2B} receptor agonist-induced valvulopathy is the common metabolite of fenfluramine, dexfenfluramine and benfluorex: nordexfenfluramine [9]. Even if the metabolic pathways are poorly identified, the plasma concentrations of nordexfenfluramine are similar (Cmax = 25–30 ng/ml) with daily recommended therapeutic doses of fenfluramine (60 mg/day), dexfenfluramine (30 mg/day) or benfluorex (450 mg/day), leading to their labeling of prodrugs and named this adverse event as off-target effect. The production of the toxic metabolite responsible for the valvulopathogen effect is also described with ergot derivatives: methylergonovine, which is the metabolite of methysergide, is a more potent 5-HT_{2B} receptor agonist that methysergide [9]. To screen for valvulopathogenic risk, the

affinity and function of metabolites on the 5-HT_{2B} receptor should therefore also be investigated, and whether the concentration obtained *in vivo* is sufficient to induce valvulopathy. Finally, the determination of a safety margin is difficult to establish and must be based on non-clinical (binding, agonism 5-HT_{2B} receptor, pathway) and clinical (metabolite, dose dependent echocardiography) data [72, 73].

4 Conclusion

Due to occurrence of VHD, the majority of drug presenting, their metabolites or themselves, agonist properties on 5-HT_{2B} receptor have been withdrawn from the market. Development of 5-HT_2 receptors agonists as therapeutic compounds is avoided by pharmaceutical companies. This raises a serious issue as the serotonergic system is involved in the pathogenesis of numerous diseases, notably 5-HT_2 receptors. Thus, these receptors represent potentially good therapeutic targets. Understanding the pharmacology associated with VHD development is fundamental to allow the development of new drugs devoid of cardiac side effects mediated by 5-HT_{2B} receptor stimulation (see Fig. 12.1). The canonical signaling of the receptor is associated with mitogenic effect on myofibroblasts *in vitro*, but it is not the only pathway associated with VHD. Indeed, as shown by the study of Huang et al. [58], screening on a calcium flux assay does not allow the discrimination between valvulopathogen and non-valvulopathogen 5-HT_{2B} agonists. However, 5-HT_{2B} receptor display interesting properties such as the ability to oligomerize with other GPCRs such as AT1 receptor. Moreover, 5-HT_{2B} agonists are able to modulate the pharmacology associated with stimulation of the receptor by preferentially activating one pathway over another (biased agonism or functional selectivity), or by

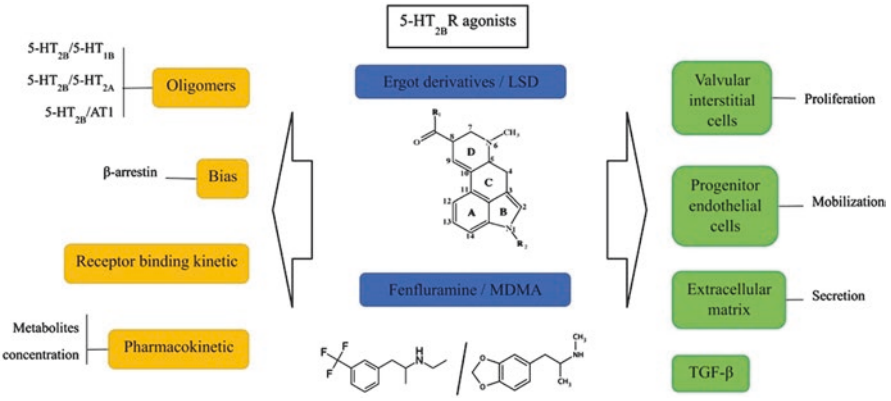

Fig. 12.1 Diagram representing the link between two families of valvulopathogen 5-HT_{2B} receptor agonists (in blue) and the cellular mechanisms related to VHD development (in green). The different pharmacological properties of 5-HT_{2B} receptor agonists (in orange) provide new insights in signaling pathway modulation, but that have not been linked to valvulopathy development yet

displaying different binding kinetics. These pharmacological properties give new insights in possible mechanisms linked to VHD development and highlight new therapeutic targets downstream of the valvulopathogen pathway. However, further studies are needed to find if these properties are relevant in regard of valvulopathy.

The withdrawal of fenfluramines follows the identification of 5-HT_{2B} receptor agonists properties of its metabolite, nordexfenfluramine. As such, notions of dose, treatment duration and quantity of metabolites have to be considered in the balance benefice/risk evaluation. For example, fenfluramine in association with phentermine was withdrawn in diabetic or obese adult patients, but their use is allowed again at low dose in rare pediatric disease. Thus, infants suffering from Dravet syndrome, a rare serious epilepsy condition notably hard to treat, were treated with low dose fenfluramine, without identification of the mechanism responsible of therapeutic effect. With a low dose treatment (mean of 0.3 mg/kg/day) for up to 28 years (1–28 years, mean of 9.2 years) on 19 patients, 5 patients present minor signs of valve remodeling, but no functional consequences have been reported [74]. These very limited data must be interpreted with the utmost caution, due to the age of the population, its size, the absence of data on norfenfluramine concentrations and the lack of knowledge about valvular mechanisms involved in cardiac growth. The fact that majority of the treatments began before the age of 2 years also raise the interrogation of the prevalence of VHD development in infants. As their heart is still growing and the remodeling mechanisms may thus still beneficial, we can hypothesize that bone marrow progenitor mobilization is less detrimental than in adults.

Thus, even with limited knowledge on 5-HT_{2B} receptor pharmacology, 5-HT_{2B} receptor agonism should not be systematically associated with VHD, as numerous factors must be taken into account (dose/duration of the treatment/pathway activated). Risk/benefice balance must also be assessed depending on the condition treated, as 5-HT_2 receptor agonists may be useful for treating rare and severe diseases (e.g. Dravet syndrome, amyotrophic lateral sclerosis) with very limited therapeutic solution to this date.

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Chapter 13
Serotonin and Fibrosis

Oliver Distler, Michel Neidhart, and Przemysław Błyszczuk

Abbreviations

COL1A1	Collagen 1α1
COL1A2	Collagen 1α2
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
FN1	Fibronectin
MDMA-"Ecstasy"	3,4-methylenedioxy-methamphetamine
SMA	Smooth muscle actin
SSRI/SNRI	Serotonin norepinephrine re-uptake inhibitors
SERT/5-HTT	Serotonin transporter SLC6A4
αSMA	Alpha smooth muscle actin
SSc	Systemic sclerosis
TGF-β	Transforming growth factor beta
TPH1	Tryptophan hydroxylase

1 Introduction

1.1 Fibrosis

Fibrosis can occur in many tissues within the body, including skin, liver, lung, heart, intestine and pancreas. It is a pathological process of the wound healing response that replaces damaged tissue with non-functional collagen-rich scar tissue to maintain the physical boundaries of the affected organ [1]. However, scar tissue can cause serious complications for organs whose capability depends on cellular function and/or mechanical deformation. The fibrotic extracellular matrix provides a

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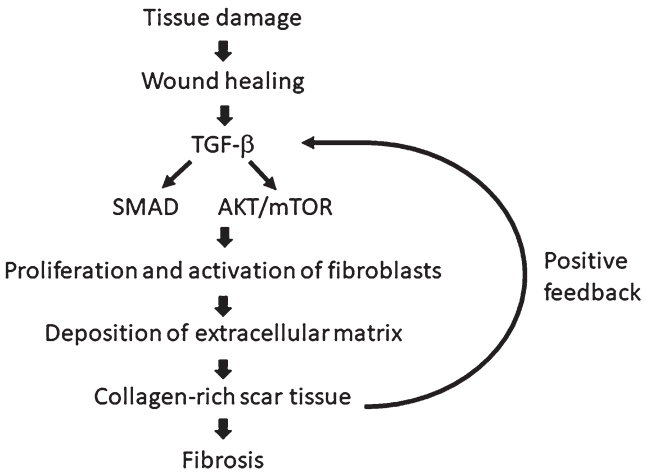

Fig. 13.1 Role of fibroblasts in wound healing and generation of fibrosis. An amplification is triggered by collagen-rich scar tissues that stimulate TGF-β and results in excessive deposition of extracellular matrix. AKT/mTOR is given as an example for non-canonical signaling pathways

positive feedback loop leading to progressive tissue stiffening, activation of profibrotic transforming growth factor beta (TGF-β) signaling, and to a self-perpetuating disease [2]. These mechanisms initiate signal transduction pathways such as the SMAD [3] and AKT/mTOR [4] pathways that ultimately lead to the proliferation and/or activation of fibroblasts, which deposit extracellular matrix into the surrounding connective tissue. The process of tissue repair is complex, with tight regulation of extracellular matrix synthesis and degradation ensuring maintenance of normal tissue architecture. However, the entire process, although necessary, can lead to a progressive irreversible fibrotic response, if tissue injury is severe or repetitive, or if the wound healing response itself becomes deregulated (Fig. 13.1).

1.2 Myofibroblasts

A ubiquitous characteristic of fibrosis is the presence of myofibroblasts, a contractile cell that deposits high amounts of fibrotic extracellular matrix including type I collagen and extra-domain A isoform of fibronectin. Most myofibroblasts are derived from activated resident mesenchymal cells [5], but can also arise from epithelial cells through epithelial-mesenchymal transition [6]. They usually stain for the intermediate filament vimentin, which is a general mesenchymal cells marker, and alpha smooth muscle actin (α-SMA, encoded by the ACTA2 gene). Myofibroblasts are responsible for wound closure. Failure of myofibroblasts to undergo apoptosis can lead to increased deposition of extracellular matrix proteins [7]. In turn, pathological stiffness facilitates release of TGF-β [8] and alters the

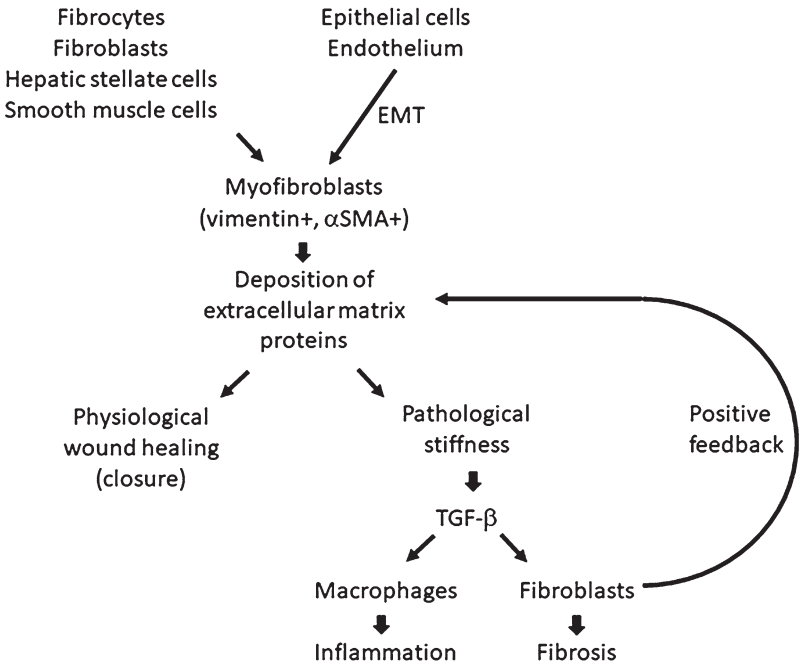

Fig. 13.2 Central role of myofibroblasts in the pathogenesis of fibrosis. Myofibroblasts generated from different cell types are responsible for the deposition of extracellular matrix. This can lead to pathological stiffness that triggers the release of TGF-β, which in turn stimulates fibroblasts to produce more extracellular matrix. This positive feedback loop can result in fibrosis

macrophage phenotype [9], both resulting in further activation of fibroblasts, excessive collagen deposition and fibrosis (Fig. 13.2).

1.3 Tryptophan Metabolites

Major metabolites of tryptophan including L-kynurenine and serotonin exert modulatory effects on both fibroblast activation in vitro and pathological fibrosis in vivo [2]. A major source of serotonin outside the nervous system is enterochromaffin cells of the intestinal epithelium. In those cells, L-tryptophan is enzymatically hydrolyzed and 5-hydroxytryptophan is subsequently decarboxylated into serotonin. In the circulation, serotonin is found in platelets. It is released upon platelet aggregation at sites of tissue damage, thereby modulating wound healing. Mice deficient for tryptophan hydroxylase (TPH1), the rate-limiting enzyme for serotonin production outside the central nervous system, showed reduced experimental skin fibrosis [10, 11]. Serotonin has potent vasoactive and chemotactic properties and promotes pro-inflammatory cytokines release by monocytes.

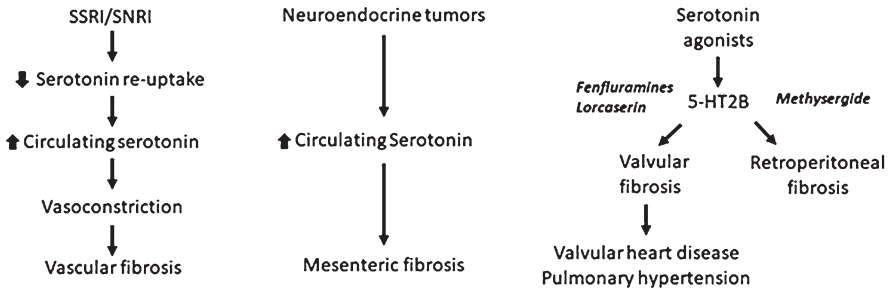

Fig. 13.3 Examples of clinical evidences for the role of serotonin in fibrosis: use of SSRI/SNRI, serotonin-secreting neuroendocrine tumors and use of serotonin agonists

1.4 Clinical Evidences

Some clinical evidences exist suggesting a role for serotonin and serotonin agonists in fibrosis (Fig. 13.3). A retrospective study [12] demonstrated a significant association between usage of selective serotonin re-uptake inhibitors and serotonin norepinephrine re-uptake inhibitors (SSRI/SNRI) and interstitial lung disease in the elderly. Neuroendocrine tumors are often associated with the development of local and distant fibrosis [13]. There is strong evidence implicating serotonin and the activation of 5-HT_{2B} receptors in the pathogenesis of carcinoid heart disease, which is characterized by the development of fibrotic endocardial plaques. A clinical study of patients with mid-gut neuroendocrine tumors showed a correlation between elevated platelet serotonin and the presence of a mesenteric mass [14]. Another study demonstrated a significant association of mesenteric fibrosis with urinary 5-hydroxyindoleacetic acid, the main metabolite of serotonin [15]. The serotonin pathway has been repeatedly implicated in anorexigens-associated pulmonary vascular disease. The increase of systemic serotonin levels results in vasoconstriction of the pulmonary arteries and initiates a cascade of pathologic remodeling leading to vascular fibrosis [16]. The 5-HT_{2B} receptor has the strongest association with valvular heart disease and pulmonary hypertension. Serotonin agonists, such as fenfluramines and lorcaserin, can cause valvular fibrosis [16]; this is also the case for the amphetamine derivative 3,4-methylenedioxy-methamphetamine (MDMA-"Ecstasy") [17]. The ergot methysergide can cause retroperitoneal fibrosis due to its in vivo conversion to a 5-HT_{2B} agonist [18]. All these drugs cause fibrosis via the 5-HT_{2B} receptor. Dopamine with structural similarities to serotonin, such as pergolide and cabergolide (used in the treatment of Parkinson disease), have also been associated with heart valve diseases involving 5-HT_{2B} agonism, thus limiting their clinical utility [19]. The headache drug methysergide, which has structural similarities to serotonin, can cause pleuro-pulmonary fibrosis.

2 Skin Fibrosis

Matrix biosynthesis and deposition are complex processes that are critical in development, maintenance of tissue homeostasis and repair of injured tissues. Disturbances in the regulation of these processes can result in a wide range of pathological conditions which are associated with tissue fibrosis, from excessive scarring to systemic sclerosis (SSc). In hypertrophic scars, serotonin as well as histamine are increased. The clinical symptoms of fibrosis can vary considerably with a broad range from isolated small areas to the involvement of the entire integument. Fibrosis is triggered by a multitude of different stimuli leading to activation of the immune and vascular system that then initiate fibroblast activation and formation of matrix depositing, as well as generation of myofibroblasts [20].

2.1 Myofibroblasts and Excessive ECM Production

Myofibroblasts are the primary extracellular matrix (ECM)-secreting cells during wound healing and fibrosis, and are largely responsible for the contractility of scar tissue as it matures. Serotonin may favor the generation of myofibroblasts [21]. Ultimately, myofibroblasts deposit excessive amounts of ECM inducing a pathological architecture and alterations in growth factor binding and biomechanical properties, which culminates in skin hardening and loss of mobility.

2.2 Dermal Fibroblasts and Increased Collagen Turnover

Fibroblasts are the predominant mesenchymal cell type and the main effector of ECM homeostasis, mediating its continuous turnover. In great part, the anti-fibrotic effects observed after inhibition of serotonin signaling resulted from the reduced direct effect of serotonin on fibroblasts. In human dermal fibroblasts, serotonin dose-dependently induced expression of collagen type I α-chains (COL1A1, COL1A2) and collagen deposition [22]; this was dependent on the activation of the 5-HT_{2B} receptor.

2.3 Animal Models

Animal models of human diseases represent an important experimental platform in preclinical research. Many experimental models of SSc have been generated to induce SSc phenotype in the skin and in internal organs. The cytotoxic agent bleomycin is commonly used to induce skin and lung fibrosis in mice and rats. Repetitive

Table 13.1 Pharmacological effects of serotonin receptor blockade in SSc models of bleomycin-induced skin and lung fibrosis

Serotonin receptor	Inhibitor	Effect	Organ	Reference
5-$HT_{2A/B}$	Terguride	Antifibrotic	Skin, lung	Königshoff et al. [31], Dees et al. [22], Elaidy and Essawy [32], Tawfik and Makary [34]
5-$HT_{2A/2B}$	Cyproheptadine	Antifibrotic	Skin	Dees et al. [22]
5-HT_{2A}	Ketanserin	Antifibrotic	Lung	Fabre et al. [24]
5-HT_{2B}	EXT5, EXT9	Antifibrotic	Lung	Löfdahl et al. [25]
5-HT_{2B}	SB-204741	Antifibrotic	Skin	Dees et al. [22]
5-HT_{2B}	SB-215505	Antifibrotic	Lung	Fabre et al. [24]
5-HT_{2C}	RS-102221	Antifibrotic	Lung	Elaidy and Essawy [32]
5-$HT_{3/4}$	Tropisetron	Antifibrotic	Skin	Stegemann et al. [48]
5-HT_{7}	SB-269970	Antifibrotic	Lung	Tawfik and Essawy [34]

intradermal deliveries of bleomycin induce a progressive thickening of the skin, whereas a single intratracheal injection of the cytotoxic drug is used to trigger lung injury, inflammation and subsequent pulmonary fibrosis. SSc-like phenotype can be also induced by bone marrow transplantation between specific mouse strains, which results in chronic graft-versus-host disease or it can develop spontaneously in the specific transgenic mice, such as TSK-1, TSK-2, Fra-2-tg and many others. Experimental data from bleomycin SSc mouse models confirmed a profibrotic role of serotonin-mediated signaling in the skin and lungs [23]. It has been shown that bleomycin injection induced serotonin production and expression of serotonin receptors [24]. A number of studies demonstrated that pharmacological inhibition of serotonin receptors with specific antagonists successfully ameliorated fibrotic changes in the skin and lungs of mice or rats treated with bleomycin (Table 13.1).

The relevance of profibrotic serotonin signaling in bleomycin SSc models has been further confirmed in mice lacking peripheral serotonin by genetic knockout of TPH1. Accordingly, TPH1-deficient mice showed reduced dermal and pulmonary fibrosis upon challenge with bleomycin [11, 22]. In bleomycin models, serotonin has been suggested to exacerbate inflammatory response, oxidative stress, collagen synthesis and myofibroblast formation leading to fibrotic phenotype (Fig. 13.4). At the molecular level, serotonin signaling was implicated in regulation of pAkt/p21 pathway [25].

2.4 Transforming Growth Factor Beta (TGF-β)

TGF-β is a multifunctional cytokine and a key regulator of ECM assembly and remodeling. Specifically, TGF-β has the ability to induce the expression of ECM proteins in mesenchymal cells, and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM. Recent data suggest that 5-HT

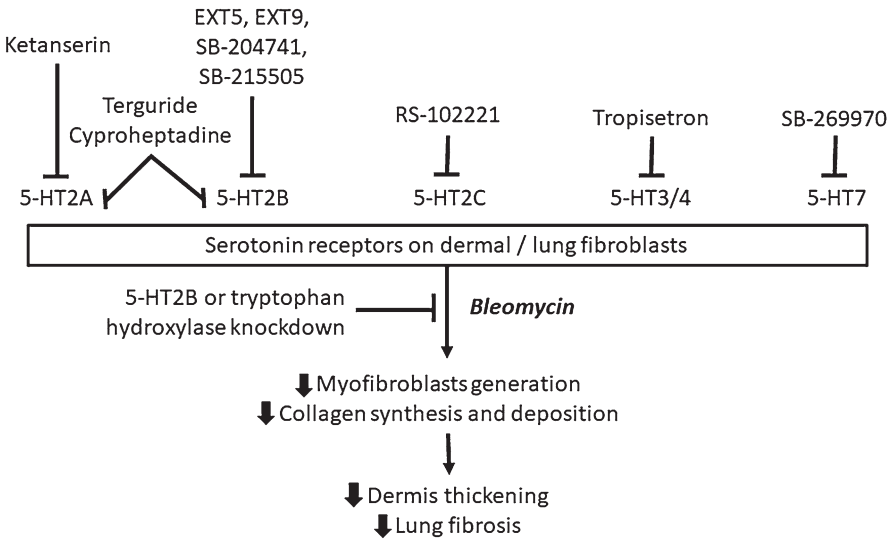

Fig. 13.4 The role of serotonin in skin fibrosis is revealed by the action of serotonin antagonists and in genetically 5-HT_{2B}/tryptophan hydroxylase knockout mice

inhibitors reduce fibrosis via suppression of TGF-β-mediated non-canonical signaling pathways in fibroblasts, involving ERK1/2 and STAT3 phosphorylation [26]. An interesting concept is that serotonin induces the production of TGF-β in skin macrophages, which in turn triggers the up-regulation of pro-fibrotic genes in dermal fibroblasts [21].

2.5 Formation of a Temporary Scar

Serotonin stimulates both vasoconstriction and vasodilation, influences inflammatory responses and promotes formation of a temporary scar which acts as a scaffold for normal tissue to be restored. However, in situations of chronic injury or damage, serotonin signaling can have deleterious effects and promote aberrant wound healing resulting in tissue fibrosis and impaired organ regeneration [27].

3 Fibrosis in Other Organs

The fibrosis process in other organs shows some similarities with the pathogenesis occurring in the skin. We will shortly review them.

3.1 Liver Fibrosis

Cirrhosis is a pathological condition in which the liver does not function properly due to long-term damage, which is characterized by the replacement of normal parenchyma by scar tissue. This scar tissue blocks the portal venous flow through the organ, raising the blood pressure and disturbing normal function. Hepatic serotonin signaling pathways help to regulate the growth and regeneration of parenchymal liver cells. Sinusoidal hepatic stellate cells, which are negative regulators of hepatocytes regeneration, are the major contributor to fibrogenesis in liver diseases, producing great amount of extracellular matrix proteins, as well as the collagenase inhibitor TIMP-1. They strongly up-regulate expression of 5-HT_{2A} and 5-HT_{2B} upon their trans-differentiation into myofibroblasts [28]. Serotonin synergizes with platelet-derived growth factor to stimulate their proliferation. In rat liver damaged by injection of CCl_4, 5-HT_{2B} receptors are localized to fibrotic tissues. 5-HT_2 receptor antagonists inhibit hepatic stellate cells proliferation and induce apoptosis. This implies serotonin signaling in the regulation of fibrosis, since the balance between proliferation and apoptosis is an important factor in its progression [27]. Signaling through the 5-HT_{2B} receptor on liver myofibroblasts is both anti-regenerative and pro-fibrotic [29]. Accordingly, in mice, 5-HT_{2B} knockout stimulates hepatocyte proliferation and suppresses fibrosis [27]; serotonin triggers the expression of TGF-β1 via ERK- and JunD pathways. In turn, TGF-β inhibits hepatocyte proliferation and up-regulates the expression of fibrogenic genes. In addition, serotonin increases the pro-fibrotic matricellular protein connective tissue growth factor (CTGF) from hepatic stellate cells [28]. Thus, in a pathological situation, important cross-talks occur between these serotonin-driven epithelial cell growth mechanism and serotonin signaling pathways that act on myofibroblasts to stimulate hepatic fibrosis. Similarly, in response to biliary injury, serotonin triggers the production of TGF-β by myofibroblasts; in turn, TGF-β acts on cholangiocytes and stimulates their proliferation [30]. In summary, sinusoidal hepatic stellate cells can differentiate into myofibroblasts that express more serotonin receptors. Serotonin triggers the release of TGF-β resulting in increased proliferation and deposition of extracellular matrix. The parenchymal tissue progressively progresses into a collagen-rich scar tissue. This can cause liver dysfunctions. More information regarding liver fibrosis is found in Chap. 14.

3.2 Lung Fibrosis

Lung fibrosis has been associated with different types of human chronic respiratory diseases, including pulmonary arterial hypertension and chronic obstructive pulmonary disease. In addition, lung fibrosis is a serious pathological component of SSc. Serotonin can stimulate the proliferation and fibrogenic actions of lung fibroblasts. Recently, serotonin was shown to increase p21/CDKN1A and phosphorylated Akt

in human lung fibroblasts [29]. Bleomycin-induced pulmonary fibrosis is a model used to evaluate pathological mechanisms and pharmacological interventions. In mice, the development of bleomycin-induced lung fibrosis is dependent on 5-$HT_{2A/2B}$ receptors [31]. In murine lungs, serotonin increased over the progression of fibrosis; this occurs in conjunction with increased expression of 5-$HT_{2A/2B}$ receptors [24, 31]. In genetically tryptophan hydroxylase knockout mice, bleomycin induced less inflammatory cytokines and fibrosis-associated proteins [11]. Pharmacological blockade of either 5-HT_{2A} or 5-HT_{2B} receptors reduced lung collagen content, as well as procollagen 1 and procollagen 3 transcript levels. In rats, 5-HT_{2C} and 5-HT_7 receptors also are involved in fibrosis [32–34]. Treatment of rat pulmonary artery adventitial fibroblasts with serotonin induced proliferation and activation, as shown by increased expression of smooth muscle actin (SMA), CTCF, collagen type I, fibronectin and TGF-β, as well by increased phosphorylated-SMAD3 [3]; this was mediated at least in part by 5-HT_{2A} receptors. Human lung fibroblasts exposed to a 5-$HT_{2A/2B}$ antagonist showed reduced responses to serotonin and TGF-β [32]. Thus, human lung fibroblasts treated with this 5-$HT_{2A/2B}$ antagonist showed reduced TGF-β-induced COL1A1 expression and collagen deposition [31]; they also have down-regulated plasminogen activator inhibitor-1 and JunD transcripts. In summary, serotonin increases the proliferation of human lung fibroblasts and type II alveolar epithelial cells. Together with TGF-β, serotonin favors the synthesis and deposition of extracellular matrix. This leads to lung fibrosis. Similarly, in the murine model of bleomycin-induced lung fibrosis, serotonin increased collagen synthesis and deposition. More information regarding the effect of serotonin on cardiopulmonary functions are found in Chap. 10.

3.3 Cardiac Fibrosis

Cardiac fibrosis refers to the excess deposition of extracellular matrix in the cardiac muscle. Fibrotic cardiac muscle is stiffer and less compliant; this can cause heart failure. Long-term treatments with serotonin agonists are associated with cardiac fibrosis, including weight loss drugs (fenfluramine, chlorphentermine), antiparkinson drugs (pergolide and cabergoline) and antimigraine drugs (ergotamine and methysergide). In mice, knockout for the serotonin transporter SLC6A4 (SERT/5-HTT) led to increased serotonin in the circulation and the development of focal fibrotic lesions in cardiac tissues [35]. On the other hand, pharmacological antagonists of 5-$HT_{2A/2B}$ or 5-HT_{2B} prevented TGF-β-mediated deposition of collagen in murine cardiac fibroblasts [36]; thus, the protective effect of 5-HT_{2B} antagonism in cardiac fibrosis models may be at least in part due to the inhibition of TGF-β signaling. Wildtype murine cardiac fibroblasts stimulated with serotonin showed increased release of pro-inflammatory cytokines (IL-6, TNFα and IL-1β); this response is inhibited by 5-HT_{2B} antagonists and can be restored by treatment with a 5-HT_{2B} agonist [37]. Treatment of rat cardiac fibroblasts with serotonin induced proliferation, migration and up-regulation of TGF-β and matrix metalloproteinases [38]; this

was mediated at least in part by 5-HT_{2A} receptors. Serotonin and 5-HT_{2B} receptors also synergize with angiotensin II-induced release of IL-1β and TGF-β from murine cardiac fibroblasts [39]. Interactions between the angiotensin II and 5-HT_{2B} receptors are the key liming events in cardiac fibroblast activation leading to cytokine and TGF-β releases. In summary, serotonin triggers the proliferation of cardiac fibroblasts. Together with angiotensin II, the action of serotonin leads to the release of pro-inflammatory cytokines and TGF-β. In turn, TGF-β increases the deposition of extracellular matrix in the heart. The increased stiffness and decreased compliance may result in heart failure. More informations regarding the effect of serotonin on cardiac structures and functions are found in Chap. 9.

3.4 Intestinal Fibrosis

Intestinal fibrosis in e.g. Crohn's disease has been associated with inflammation and involves multiple cell types, including fibroblasts, smooth muscle cells and epithelial cells. Expression of TGF-β is up-regulated in inflamed mucosa of inflammatory bowel disease patients [40]. Recently, a serotonin re-uptake transporter SLC6A4 gene polymorphisms (in intron 2) was associated with Crohn's disease [41]. Furthermore, patients with small intestinal neuroendocrine tumors, serotonin and other cytokines released from tumor cells may induce fibrosis, leading to carcinoid heart disease and abdominal fibrotic reactions [42].

3.5 Pancreatic Fibrosis

Chronic pancreatitis is characterized by ongoing inflammation of the pancreas that results in progressive loss of the endocrine and exocrine compartment owing to atrophy and/or replacement with fibrotic tissue. In rats, selective 5-HT_{2A} receptor antagonists inhibit the progression of acute and chronic pancreatitis [43]. Furthermore, an association between prominent stromal fibrosis in pancreatic neuroendocrine tumors and serotonin levels has been described by several studies [44]. Most of the so-called sclerozing variant of pancreatic neuroendocrine tumors produce serotonin.

3.6 Peripheral Blood

Serotonin skews human macrophage polarization through engagement of 5-HT_{2B} and 5-HT_7 receptors [33]; it primes macrophages for reduced pro-inflammatory cytokines production and IFN type I-mediated signaling. Serotonin upregulates TGF-β production in a 5-HT_7- and PKA-dependent manner. Thus, even in the

peripheral blood, it could promote an anti-inflammatory and pro-fibrotic process. More informations regarding the effect of serotonin on macrophages and hematopoiesis are found in Chaps. 6 and 4.

4 Systemic Sclerosis

SSc is characterized by a wide spectrum of microvascular and immunological abnormalities, leading to a progressive deposition of collagens in the skin, lungs, stomach, heart and the kidneys.

4.1 Platelet Aggregation

Platelet aggregation, the process by which platelets adhere to each others at sites of vascular injury, is critical for hemostatic plug formation and thrombosis. It is a common feature of SSc and results in increased serotonin release [23] (Fig. 13.5). In

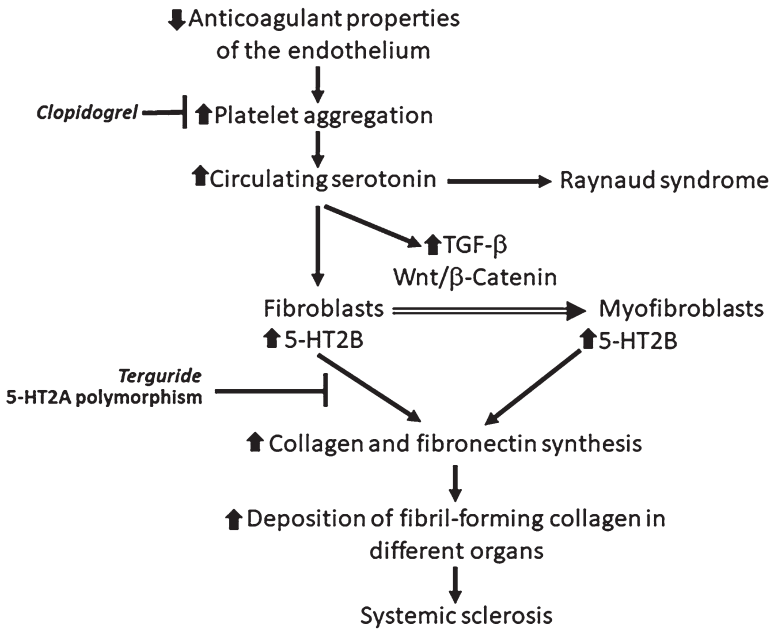

Fig. 13.5 Role of serotonin in SSc. More platelet aggregation results in increased circulating serotonin. Fibroblasts differentiate into myofibroblasts, both showing increased expression of serotonin receptors. They produce more collagen and fibronectin. This results in an increased deposition of collagen in various organs

SSc, it has been proposed that loss of antithrombotic properties of the endothelium may trigger platelet activation and the release of serotonin [28]. Accordingly, platelet inhibition by clopidogrel led to decreased serotonin content in the fibrotic skin [22].

4.2 Dermal Fibroblasts

Early experiments revealed that serotonin can stimulate the proliferation of fibroblasts and can cause remodeling of skin in a manner that resembles skin of patients with SSc. These patients have an increased expression of 5-HT_{2B} localized to fibroblasts, myofibroblasts, keratinocytes and within microvessel structures. The transition of fibroblasts to an activated myofibroblasts involves multiple pathways, including well known signaling cascades such as TGF-β and Wnt/β-Catenin signaling, as well as epigenetic reprogramming and a number of defined cellular pathways. Cultured dermal fibroblasts from SSc patients and healthy individuals respond to serotonin by increasing their production of collagen 1α1 (COL1A1), collagen 1α2 (COL1A2) and fibronectin (FN1) [45].

4.3 TGF-β

Serotonin upregulates TGF-β1 and triggers the nuclear translocation of phosphorylated SMAD3 (p-SMAD3) [47]. This suggests that serotonin-mediated pro-fibrotic effects in SSc fibroblasts proceeds at least in part through activation of the TGF-β1 signaling. Terguride and SB204741 reduce pro-fibrotic potential of human adult dermal fibroblasts and suppress TGF-β-mediated non-canonical pathways, ERK1/2 and STAT3, which have been implicated in the regulation of pro-fibrotic genes (i.e. TGF-β1, COL1A1, COL1A2, ACTA2, CTGF and FN1) and in the development of fibrosis [26].

Based on such observations, the clinical efficacy of terguride, a serotonin receptor antagonist, in SSc was planned to be assessed in a randomized placebo-controlled phase III trial [23, 46, 47]. However, this trial could not be initiated due to serotonin-independent potential cardiovascular adverse effects of terguride in phase 1 studies. The absence of selectivity of the compound can be suggested because some 5-HT receptor antagonists used in clinical trials have additional effects on other transmission systems, e.g. nicotinic acetylcholine receptors [48].

4.4 Genetics

Numerous HLA alleles/non-HLA polymorphisms, microsatellites and chromosomal abnormalities have been associated with SSc. A missense mutation in the serotonin 5-HT_{2A} receptor (His452Tyr) was found to be underrepresented in patients with SSc [49]; the mutated allele resulting in a threefold risk reduction of developing SSc. Possibly, this mutation reduced the sensitivity to platelet aggregation [48], as well as the responsiveness of fibroblasts to serotonin [50]. Thus, it has been hypothesized that mutations in 5-HT_{2A} receptor gene may desensitize fibroblasts and contribute to tissue fibrosis [2].

5 Conclusion

The pro-fibrotic activity of serotonin in vivo is due at least in part to its direct effect on fibroblasts and/or myofibroblast progenitors. Distinct serotonin receptors are involved in the fibrotic pathophysiology; thus, the activation effects of serotonin on fibroblasts are likely mediated through signaling via 5-HT_{2A} and 5-HT_{2B} receptors. An increased serotonin signaling via these receptors may exacerbate the fibrotic response. In skin, this includes increased collagen type I synthesis and deposition, as well as release of TGF-β and growth factors. In most other tissues, the pro-fibrotic effect of serotonin also is in great part due to TGF-β. Taken together, these studies proposed that pharmaceutical inhibition of specific serotonin receptors may be beneficial for the treatment of fibrotic disorders, such as SSc.

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Chapter 14
5-HT_{2B} Receptors in Liver

Lucy Gee and Fiona Oakley

Abbreviations

BDL	Bile duct ligation
BECs	Biliary epithelial cells
CHC	Chronic hepatitis C
CLD	Chronic liver disease
ECM	Extracellular matrix
FFA	Fatty acids
HM	Hepatic myofibroblasts
HSC	Hepatic stellate cells
HCC	Hepatocellular carcinoma
HFD	High fat diet
mTOR	Mammalian target of rapamycin
ERK1	Mitogen activated protein kinase 1
MAO-A	Monoamine oxidase A
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
PHx	Partial hepatectomy
PSC	Primary sclerosing cholangitis
SERT	Serotonin transporter
TGF-β1	Transforming growth factor β1
TG	Triglyceride
TPH1	Tryptophan hydroxylase 1
VLDL	Very low-density lipoprotein
WH	Wound healing

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1 Liver Biology and Function

The liver has many functions, mainly relating to digestion and detoxification, electrolyte/fluid balancing and haemostasis. The liver is made up of four lobes, the right and left lobules and smaller caudate and quadrate lobes. Uniquely, the liver has a dual blood supply from both the portal vein and hepatic artery. Each lobe contains lobules, the functional units of the liver [1]. These are composed of a portal triad formed of a portal vein and hepatic artery branches as well as a bile duct.

Hepatocytes constitute 60% of the cells within the liver. The parenchymal hepatocytes within a lobule have a distinct zonation [2] based on oxygenation and function and are comprised of three zones. The peri-portal zone (zone 1) is the best oxygenated and the most regenerative, and so modulates oxidative processes such as bile formation, cholesterol formation and gluconeogenesis. Since Zone III has the lowest oxidative capacity it is involved in detoxification processes such as biotransformation of drugs by cytochrome p450 enzymes, as well as glycolysis and glycogen synthesis.

The other major parenchymal cells within the liver are the biliary epithelial cells (BECs) /cholangiocytes. This cell type lines the bile duct, allowing for passage of bile from hepatocytes through to the gut for conjugation. There are both small intrahepatic bile ducts embedded throughout the liver, and large bile ducts which line the space between the liver and the intestinal exit. Each BEC has a primary cilium which is responsible for detecting and signalling changes in bile flow and osmolality [3].

There are also a number of nonparenchymal cell populations within the liver. For example, the hepatic stellate cells (HSC) and portal fibroblasts, both of which produce extracellular matrix (ECM) and maintain liver organization, and are activated in wound healing (WH). There are also resident immune cells in the liver such as Kupffer cells, the resident macrophages. These cell types have important role as they are the first immune population to come into contact with bacteria and endotoxins travelling through the portal vein to the liver from the gut [4].

1.1 Hepatic Wound Healing and Fibrosis

Hepatic WH is a tightly regulated multi-cellular response to liver injury that stimulates a rapid and efficient repair of the damaged tissue, replacement of lost epithelial cells and restoration of the normal liver architecture and function. Regardless of the disease aetiology, be it as a consequence of metabolic disease, alcohol, viral infection, autoimmune or genetic disease, the repair response to hepatic injury occurs through three overlapping phases of inflammation, repair (scar formation), and liver regeneration [5]. It is a normal physiological process that ensures tissue homeostasis and organ function is maintained. However, under conditions of repeated liver injury during chronic liver disease (CLD), the normal WH process becomes highly

dysregulated, inflammation becomes chronic, hepatic myofibroblasts (HM) persist and secrete excessive extracellular matrix, leading to the formation of collagen-rich scars which gradually thicken as the disease progresses [6]. Fibrosis is the pathological consequence of CLD and over years or decades the accumulation of fibrotic scar tissue impairs liver function, ultimately leading to advanced fibrosis/cirrhosis and increased risk of organ failure or developing liver cancer [7]. Another consequence of CLD and fibrosis is a decrease in the regenerative capacity of the remaining parenchymal tissue, as such these processes are intricately linked and there is a delicate balance of pro-fibrotic and pro-regenerative signalling within the WH niche that is vital to maintain normal liver function.

Hepatocytes (and cholangiocytes in biliary disease) are central to WH and when damaged function as initiators of wound repair, releasing alarmins and danger signals to promote immune cell recruitment and activation of HM [6]. HM also termed activated hepatic stellate cells (aHSC) are derived from quiescent hepatic stellate cells (qHSC), which reside in the Space of Disse, through a process known as transdifferentiation [8]. HM are the professional scar-forming cells of the liver and during normal WH, secrete ECM. This forms a temporary scar, which acts to retain tissue structure and provide a scaffold for hepatocytes to repopulate. Whereas, during CLD, HM become highly proliferative, migratory and contractile cells that secrete inflammatory cytokines and chemokines, and are responsible for the production of a dense network of collagen and fibronectin-rich fibrotic scar tissue.

Macrophages, in the liver are derived from two sources; either Kupffer cells, the resident liver macrophage or recruited monocytes. These cells are critical regulators of all stages of WH, playing a central role in both fibrogenesis and fibrolysis [9]. In response to specific stimuli in the WH niche macrophages adopt either an M1 inflammatory phenotype which drives fibrogenesis or an M2 restorative phenotype that facilitates matrix remodelling, reversion of fibrosis and stimulation of epithelial proliferation to promote restoration of tissue homeostasis [10].

1.2 Reversibility of Liver Fibrosis

It is now clear that CLD is a highly dynamic process and work from pre-clinical studies and clinical trials has revealed that fibrosis is not only progressive but also reversible [11, 12]. Cessation of the liver injury either through lifestyle modification e.g. weight loss or abstinence from alcohol, or successful treatment of the underlying cause of CLD, e.g. anti-viral therapy in hepatitis B or C or CCL2 inhibitors in clinical trials for metabolic disease can lead to resolution of fibrosis, even at an advanced stage [12–15]. Pre-clinical studies reported that clearance of HM by apoptosis or through dedifferentiation/deactivation stimulated ECM remodelling and reversal of fibrosis [12, 16, 17]. These were important discoveries and a paradigm shift in the field because, therapies which limit liver injury, target the cellular drivers of CLD or induce clearance of HM not only have the potential to halt the fibrogenic process, but could reverse it [18, 19].

2 The Role of Serotonin in Liver

Serotonin (5-Hydroxytryptamine, 5-HT) has long since been known to undergo storage in the dense granules of the platelets, exhibiting a role in platelet activation as evidenced (among other things) by the reduced platelet activation in cardiovascular disease patients when treated with serotonin reuptake inhibitors (SSRIs) [20]. Ordinarily platelets carry serotonin in the blood and release it at injured sites where it stimulates coagulation and the process of haemostasis [21]. This platelet activation and concurrent serotonin release plays an important role in the wound healing response and the unique regenerative capacity of the liver.

2.1 The Role of Serotonin in Wound Healing and Liver Regeneration

Data published in 2006 by Clavien's group [22] discovered an important role for serotonin in hepatocyte proliferation and liver regeneration. This data used the partial hepatectomy (PHx) model, removing 70% liver volume to investigate the role of serotonin in liver regeneration. Hepatic mRNA expression of 5-$HT_{2A/2B}$ receptors were both significantly upregulated (between three and fourfold increase) at 2 days post PHx, implicating 5-HT_2 receptors in the regenerative process.

In addition, it was also shown that this serotonin is derived from platelets. A large reduction in usual hepatocyte proliferation was seen following PHx in platelet deficient mice, induced either pharmacologically by busulfan or clopidogrel, which antagonises ADP in platelets and prevents platelet aggregation leaving them functionally bereft. However hepatic regeneration was restored by administration of the serotonergic 5-$HT_{2A/2B/2C}$ receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI)—highlighting the direct role of serotonin in liver regeneration following injury. In this paper 5-$HT_{2A/2B}$ receptor agonists were also employed to tease out the subtype specific effects, and both drugs caused Ki67 reduction compared with vehicle controls, again implicating 5-$HT_{2A/2B}$ receptor specific modulation in serotonin-dependent hepatic regeneration.

As well as wild type (WT) PHx experiments, Lesturel et al. [23] also used peripheral serotonin Tryptophan hydroxylase 1 (TPH1) knockout mice (*TPH1*$^{-/-}$) to further investigate the mitogenic potential of serotonin. These animals had reduced regenerative capacity and hepatocyte proliferation was significantly impaired at 2 days post PHx compared to WT controls, mirroring effects seen in previous experiments using serotonin receptor antagonists. This proliferative capacity was restored upon treatment with the serotonin precursor 5-HTP to restore platelet serotonin levels. These experiments provided the first evidence of direct serotonin dependent modulation of liver regeneration and identified a specific role for platelet derived serotonin in the wound healing response (Fig. 14.1).

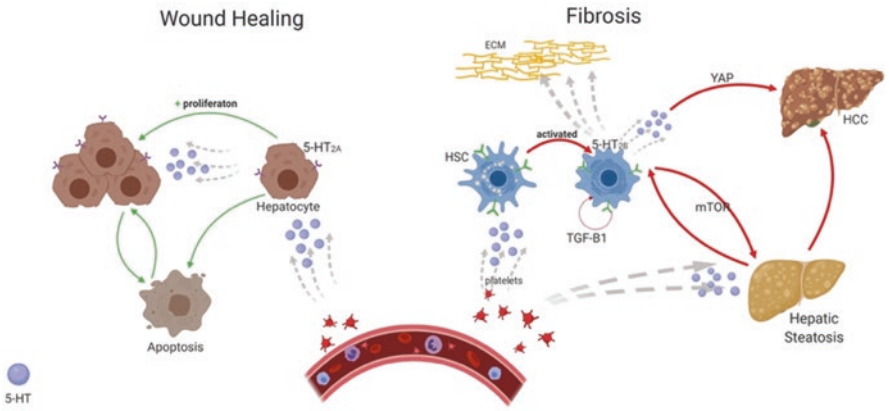

Fig. 14.1 The role of serotonin and 5-HT$_{2B}$ receptor signalling in liver disease, fibrosis, regeneration and liver cancer. Platelet derived 5-HT leads to hepatocyte proliferation through the 5-HT$_{2A}$ receptor as well as to hepatocyte apoptosis, an important process in liver regeneration. In hepatic myofibroblasts (aHSCs) platelet derived serotonin signalling through 5-HT$_{2B}$ receptors increases TGF-β1 pro-fibrogenic cytokine leading to further HSC activation and promotion of fibrosis. Signalling through 5-HT$_{2B}$ through the YAP transcription factor leads to tumorigenesis and promotes HCC development. It can also signal via the mTOR pathway to contribute to steatosis in the liver, leading to further fibrogenesis, a process which is fueled by further platelet 5-HT. This steatosis also contributes to HCC development. Figure created with biorender.com

Subsequent data from the same group confirmed a role for platelet derived serotonin using the same *TPH1*$^{-/-}$ mice [24]. When subjected to hepatic ischaemia for 60 min serotonin deficiency was not found to decrease tissue injury and necrosis was comparable after 7 days. However, when the proliferative capacity of hepatocytes was analysed it was reported that serotonin deficiency did lead to significantly reduced hepatocyte proliferation. In addition later published data on liver grafts, showed that the serotonin agonist DOI also enhanced hepatocyte proliferation in this context [25], cementing knowledge of the mitogenic capacity of serotonin. In this study DOI treatment led to 50% survival after 30% liver graft at 7 days compared to no survival in control animals. When treated with a 5-HT$_{2B}$ receptor antagonist the beneficial effects of DOI were lost, suggesting the regenerative effect is mediated largely through this receptor, and is unlikely to be a synergistic 5-HT$_{2A/2B}$ receptor effect as previously thought.

Despite striking data from the Clavien's group, other published studies have failed to find the same pronounced effect with platelet serotonin knockout models. A study from 2009 using serotonin transporter (SERT) knockout rats showed that despite having only 1–6% of normal platelet serotonin and the concurrent impaired haemostasis, Wistar rats were able to retain the normal regenerative capacities of the liver [26]. It is important to note however, differences in the models used—there is zero detectable blood serotonin in the *TPH1*$^{-/-}$ mice, whereas the SERT KO mice

have greatly reduced capacity but still retain small amounts of platelet serotonin capacity.

This could suggest therefore that platelet serotonin may not be the major factor modulating the proliferation of hepatocytes and their regenerative capacity. Serotonin may be provided from a number of additional sources (not limited to platelets) this could possibly include serotonin from resident cells within the liver. An alternative suggestion is that the small amount of platelet serotonin still present in the SERT KO model may suffice to still facilitate this effect.

The capacity for the liver to regenerate is decreased with age in both rodents and humans however, the mechanisms causing the impaired hepatocyte renewal are not fully resolved [27–29]. A role for serotonin signalling in aged liver regeneration was assessed by Furrer et al. [30] by performing 70% liver resection in young (7–8 weeks old) versus 2-year old mice either with or without the serotonin receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI). No mortality was observed in the young mice, whilst, 52% of the aged mice died post-hepatectomy and in the surviving animals the hepatic proliferative index was significantly lower than that of the young mice. Consistent with previous studies the 5-HT_{2B} receptor expressed was increased 48h post-hepatectomy in young mice but this response was blunted in the aged mice. Administration of DOI accelerated the regenerative response post-hepatectomy in the young mice. Notably, DOI increased survival from 52% to 86% in the aged hepatectomised mice, however, 5-HT_{2B} receptor levels or platelet serotonin counts were comparable between the DOI treated and untreated aged mice, excluding the possibility that increased serotonin availability or 5-HT_{2B} receptor levels/signalling were important factors in the therapeutic effects of DOI. The authors demonstrated that DOI, in a vascular endothelial growth factor (VEGF) dependant fashion, improved liver regeneration in the aged mice by stimulating the opening of sinusoidal fenestrae, increasing portal flow and facilitating platelet adhesion.

2.2 The Role of Serotonin in Liver Fibrosis

Serotonin signalling has long since been implicated in fibrogenesis in many organs such as kidney, heart, lung and the liver among others. The first evidence of this was published in 1964 in the Lancet and was related to observations in patients with Carcinoid syndrome. 5-HT_{2B} receptor agonism specifically has been associated with fibrosis under a range of conditions. These include the now discontinued anti-obesity medication fenfluramine, and Parkinson's treatment such as pergolide which were found to cause fibrosis and valvulopathy in the heart.

In the liver, the first indication of the role of serotonin in fibrosis was presented by Ruddell and colleagues in 2006 [31]. Rat HSCs were found to significantly increase expression of 5-HT_{2A} (~100-fold) and 5-HT_{2B} (~50-fold) receptor when undergoing transdifferentiation from quiescent HSC (qHSC) into activated hepatic stellate cells (aHSC), also termed HM. HSC differentiation is a particularly

important process in fibrosis as the equilibrium between HSC proliferation and apoptosis in this cell type is a key determinant of fibrosis advancement. It was hypothesised that HSCs may have an influence on hepatocyte proliferation, changes which have a known association with fibrosis and are a contributing factor in fibrotic disease. HSCs secrete many soluble factors that influence hepatocyte proliferation, including hepatocyte growth factor (HGF), transforming growth factor β1 (TGF-β1) and interleukin-6 (IL-6).

The second key finding from this work was that dosing with serotonin was found to inhibit HM/aHSC apoptosis in these cells. 5-HT$_2$ receptor antagonists showed varying apoptotic action in the aHSCs, with antagonists spiperone, LY53857, and methiothepin producing a dose dependent inhibition, with spiperone inducing apoptosis at a comparable level as the pro-apoptotic agent gliotoxin using caspase 3 assays. This was partially blocked upon incubation with serotonin, highlighting the capacity of serotonin to modulate the phenotype and survival of HM.

5-HT$_{2B}$ receptor expression was selectively induced in HM in fibrotic regions, strongly implicating 5-HT$_2$ receptor signalling in the regulation of fibrosis in the liver. This study provided the first evidence for the role of 5-HT$_2$ receptor (particularly 5-HT$_{2B}$) in the initiation and progression of fibrotic processes in the liver (Fig. 14.1).

However, whilst this study implicated 5-HT$_{2B}$ receptor signalling in HM survival and hepatic fibrosis, this work did not distinguish between serotonin signalling, via 5-HT$_{2B}$ receptors as a driver of fibrosis or whether activations of this pathways was merely a consequence of fibrogenesis. Therefore, follow up work used specific 5-HT$_{2B}$ receptor knockouts to assess if 5-HT$_{2B}$ receptor signalling modulates the fibrotic processes. Initial experiments showed that either selective depletion of HSCs using C1-3 gliotoxin, where the pro-apoptotic compound gliotoxin is conjugated to C1-3, a single-chain antibody that targets synaptophysin which is only expressed in HM in the liver [32, 33], or antagonism of 5-HT$_{2B}$ receptor by selective inhibitor SB-204741 stimulated hepatocyte proliferation in the bile duct ligation (BDL) model or when acutely CCl_4 injured [34]. The same results were not replicated with 5-HT$_{2A}$ antagonist Ketanserin indicating a specific regulatory role for 5-HT$_{2B}$ receptor in liver regeneration.

Previous work by Clavien's lab reported a regenerative role for 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor in the liver, therefore these data suggest that serotonin can exert pleiotropic effects within the liver, depending on the cell type and context of the liver injury. Subsequently, hepatocyte proliferation was compared in *Htr2b*$^{-/-}$ (5-HT$_{2B}$ receptor gene KO) vs WT mice undergoing partial hepatectomy. Proliferation was elevated after PHx at 36 and 72 h post-hepatectomy, and the excess proliferation was attributed to a failure to induce TGFβ1 expression in the KO mice-highlighting the pro-fibrotic influence of 5-HT$_{2B}$ receptor in this model. TGFβ1 is a potent profibrotic cytokine that inhibits hepatocyte proliferation and can stimulate the deposition of ECM by HSCs [35, 36].

Mechanistically, the pro-fibrotic and anti-regenerative actions of 5-HT$_{2B}$ receptor signalling was found to be mediated through the upregulation of TGFβ1. Upon stimulation of 5-HT$_{2B}$ receptors, the mitogen activated protein kinase 1 (ERK1)

becomes phosphorylated, which in turn activates the AP1 transcription factor family member JunD. Chromatin immuno-precipitation assays confirmed that serotonin promotes binding of JunD to the TGFβ1 promoter in rodent and human HSC in a 5-HT_{2B}-ERK-JunD dependant manner to directly regulate TGFβ1 expression.

In conclusion, it seems that the modulatory capacity (both negative and positive) of serotonin in the liver are entirely situation dependent. Historical studies into the regenerative pathways in the liver have been largely focussed on the healthy liver. However, in a diseased environment a complex signalling network occurs, regulated by HSCs expressing 5-HT_{2B} receptor. The original Clavien's papers showed that platelet derived serotonin stimulated regeneration due to 5-HT_{2A} receptors expressed on hepatocytes. This study showed that 5-HT_{2B} receptor is highly expressed in the diseased liver in fibrotic areas by HM. This in turn induces TGFβ1. Therefore, these two strands of work indicate pleotropic effects by 5-HT_2 class of serotonin receptors, with opposing effects being moderated by 5-HT_{2A} receptor (pro-proliferative, pro-regenerative) and 5-HT_{2B} receptor (pro-fibrotic, anti-regenerative). These data suggest that within a pro-fibrotic environment, this signalling feeds back to HMs via pro-fibrotic cytokine TGFβ1 to activate them and further stimulate fibrosis.

Treatment options for liver fibrosis are severely limited therefore improving our understanding of the molecular triggers that modulate cellular crosstalk during liver fibrosis could ultimately deliver more effective therapies [18].

3 The Role of Serotonin in Cholestatic Liver Disease

Cholestatic liver disease is defined as a stoppage or reduction of bile flow. This can be caused by autoimmune disease such as in Primary Biliary Cholangitis (PBC) and Primary Sclerosing Cholangitis (PSC), or by other causes such as disrupted blood flow (ischaemic Cholangiopathy) or drug or hepatitis induced.

Primary Biliary Cholangitis (formerly cirrhosis) is an intrahepatic autoimmune biliary disease characterised by an orchestrated immune response against anti-mitochondrial antigens (AMA). This leads to the destruction of the intrahepatic BECs, portal inflammatory infiltrates leading to the disruption of the normal bile homeostasis and exposure of hepatocytes to high levels of bile acids [37].

PSC is a disease of unknown pathogenesis. One idea is that impairments to bile acid toxicity sensing elements is important in the disease pathogenesis [38]. PSC leads to characteristic fibrotic structures, concentric layers throughout the bile ducts parallel to the BECs. These fibrotic 'onion skin' layers then lead to restricted movement of bile, causing cholestasis [39].

Serotonin signalling via 5-$HT_{1A/1B}$ receptors has been implicated in cholestatic disease and the development of biliary hyperplasia, whilst 5-HT_{2B} receptor signalling in the liver has pro-fibrogenic actions [31]. Therefore, Kyritsi et al. [40] investigated a potential role for activation of 5-HT_{2B} receptors in biliary disease and cholestasis. To test this, two models of cholestatic liver disease were used (1) Bile duct ligation model, where the common bile duct is surgically ligated, resulting in a

backflow of toxic bile into the liver, which causes biliary damage, portal inflammation and fibrosis. (2) A genetic model of cholestasis which develop features of PSC; the *mdr2*$^{-/-}$ mouse [41], which lacks the Mdr2/Abcb4 transporter, leading to a depletion of phospholipids in the bile, which in turn, causes bile regurgitation into the liver.

Immunohistochemical characterisation of the serotonin signalling/synthesis and degradation pathways revealed that 5-HT$_{2A/2B/2C}$ receptors and TPH1 (serotonin synthesis) were elevated in both BDL and *mdr2*$^{-/-}$mice. Conversely, monoamine oxidase A (MAO-A, serotonin degradation) levels were significantly reduced. Dual immunofluorescence staining confirmed HSC and cholangiocytes as the primary cellular source of 5-HT$_{2A/2B/2C}$ receptors in the liver of BDL and *mdr2*$^{-/-}$ mice [40]. Whereas gain of TPH1 and loss of MAO-A expression was seen in hepatocytes, HM and cholangiocytes. This suggests that during cholestatic liver injury there is an imbalance in serotonin production and degradation, which could result in an increase in local serotonin levels and subsequent paracrine activation of 5-HT$_2$ receptors. Expansion of the ductular epithelium, namely, a ductular reaction (DR) and fibrosis are histological features of these disease models. Administration of 5-HT$_{2A/2B}$ receptor agonists to either control WT or *mdr2*$^{-/-}$ mice or control or BDL rats exacerbated the ductular reaction and fibrogenic responses, however, this effect was blunted by administration of 5-HT$_{2A/2B}$ receptor antagonists [40].

Assessment of inflammatory cytokines, chemokine and growth factors in cholangiocytes isolated from *mdr2*$^{-/-}$ mice or BDL rats treated with or without 5-HT$_{2A/2B/2C}$ receptor antagonists confirmed that signalling via 5-HT$_2$ receptors promote a pro-inflammatory phenotype in these cells, which is attenuated by pharmacological blockade of 5-HT$_{2A/2B/2C}$ receptor signalling. In vitro studies using human immortalised HSC and cholangiocyte cell lines revealed that 5-HT$_{2A/2B/2C}$ receptor antagonists supressed the fibrogenic and inflammatory phenotype of these cells. The translational impact of these findings was further demonstrated when an increase in 5-HT$_{2A/2B/2C}$ receptors and TPH1 expression was found in PSC patient livers and raised levels of serotonin in PSC patient serum [40]. The authors concluded that molecules targeting the TPH1/MAO-A/5-HT/5-HT$_{2A/2B/2C}$ signalling axis could have therapeutic value for the treatment of cholestatic liver disease.

4 The Role of Serotonin in Metabolic Liver Disease

Non-alcoholic fatty liver disease (NAFLD), defined by simple steatosis (in >5% of hepatocytes), encompasses a spectrum of liver diseases characterised by increased triglyceride (TG) accumulation in the liver in the absence of excess alcohol consumption and is strongly associated with symptoms of the metabolic syndrome including obesity, insulin resistance/type 2 diabetes mellitus and dyslipidaemia. In a subset of patients, NAFLD is progressive and patients will develop non-alcoholic steatohepatitis (NASH), fibrosis, advanced fibrosis/cirrhosis and have an increased risk of developing hepatocellular carcinoma (HCC) [42]. As a consequence of the

obesity epidemic the global prevalence of NAFLD is reported to be 24% of the population [43] and this number is higher, exceeding 30% in some developed countries. Currently the molecular and cellular mechanisms driving the transition from NAFLD to NASH are unclear. Interestingly, clinical studies have revealed that fibrosis stage at time of diagnosis predicts both disease-specific and NAFLD-related disease mortality [44].

A link between serotonin production and pathogenesis of NAFLD-NASH was described by Nocito et al. [45] in a study showing that *Tph1* null mice develop less inflammation and hepatocellular injury in the methionine-choline deficient (MCD) diet to model NAFLD/NASH. Whilst elevated serotonin levels, induced by L-Tryptophan feeding, exacerbated hepatic steatosis and fibrosis in mice fed a high fat diet via activation of the mammalian target of rapamycin (mTOR) pathway [46].

The link between serotonin and NAFLD was further consolidated in a study by Li et al., which reported an increase in hepatic 5-$HT_{2A/2B}$ receptor expression and activation of mTOR in WT mice after high fat diet (HFD) feeding or administration of serotonin [47]. This was concomitant with an increase in hepatic TG production and circulating free fatty acids (FFA) and very low-density lipoprotein (VLDL). HFD or serotonin induced hepatic steatosis and NAFLD associated metabolic changes were supressed by sarpogrelate, a preferential 5-HT_{2A} receptor antagonist. To interrogate the mechanistic basis of the increased TG and VLDL, expression of GPAT1, the rate limiting enzyme in TG synthesis and MTTP, the key protein regulating VLDL production, were measured in HFD fed or serotonin treated animals. Both proteins were induced by HFD and serotonin but this response was diminished by sarpogrelate, confirming that 5-HT_2 receptor signalling is one pathway that mediates these processes in the liver. In vitro, exposure of the hepatocyte cell line, HepG2, to palmitic acid (PA), upregulated levels of TPH1, 5-$HT_{2A/2B}$ receptors, caused activation of mTOR and promoted lipid droplet (LD) formation, the latter being increased by co-stimulation with serotonin. Consistent with the in vivo studies, expression of 5-HT_2 receptors, GPAT1 and MTTP was induced in HepG2 cells treated with PA or serotonin and this was associated with increased lipid droplet formation and elevated levels of TG and VLDL. Both sarpogrelate or rapamycin therapy was sufficient to reduce lipid droplet formation and TG overproduction, providing evidence that under conditions of over-feeding, a 5-HT_2 receptor-mTOR signalling axis regulates hepatic steatosis and lipid metabolism. Whilst this study did not assess fibrosis or fibrotic gene expression in this model, hepatic inflammation (increased TNFα) was increased by HFD feeding or serotonin but supressed by sarpogrelate. Hepatic steatosis and inflammation are features of NASH, therefore it is feasible that increased serotonin in the context of obesity could create a pro-fibrotic environment and that increasing the longevity of HFD feeding, could have provided insights into the role of 5-HT_2 receptor signalling in the establishment of fibrosis in metabolic liver disease.

Cellular signalling and communication through the gut-liver axis has been reported by multiple groups to play a key role in the pathogenesis of NAFLD/NASH [48]. Enterochromaffin cells in the gut are the primary cellular source of peripheral serotonin, which is released in portal vein reaching the liver before being taken up by platelets and then delivered to other organs via the circulation. Serotonin produced in the gut could feasibly contribute to the pathogenesis of NASH. Suppression of serotonin synthesis in the gut, by selectively knocking out the Tph1 gene in the mouse intestine (*Villin-Cre*), reduced hepatic steatosis and inflammation after HFD feeding [49]. To determine if these effects were mediated by 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor signalling in hepatocytes, 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor floxed mice were crossed with albumin-Cre mice, to create hepatocyte specific 5-HT$_{2A}$ (*Htr2a* LKO) or 5-HT$_{2B}$ receptor (*Htr2b* LKO) knockout mice respectively and then fed a MCD diet for 8 weeks to induce NAFLD/NASH. *Htr2a* LKO but not *Htr2b* LKO mice were protected from developing hepatic steatosis and inflammation as well as lower serum TG, confirming that the 5-HT$_{2A}$ receptor mediated the gut derived serotonin-dependant progression of fatty liver disease [49].

Metabolic dysfunction in the liver such as increased hepatic TG production are associated with long-term stress (LTS) also termed hyperglucocorticoidemia-induced insulin resistance. Long-term stress induced in rodents under extreme stress conditions was associated with increased hepatic and visceral fat, raised serum levels of FFA, TG, VLDL and the stress hormones corticosterone, growth hormone and adrenaline as well as induction of hepatic TPH1 and 5-HT$_{2A/2B}$ receptor expression. The hepatic and metabolic manifestations of long-term stress were inhibited by sarpogrelate, whilst administration of serotonin exacerbated the disease phenotype [50]. Serotonin has been reported to regulate glucose uptake and hepatic energy production. Activation of mTOR, a critical regulator of metabolism, lipogenesis and autophagy, and its downstream target SK6 in the liver of long-term stress rats, was attenuated by sarpogrelate, suggesting that increased hepatic serotonin as a result of long-term stress activates the mTOR pathway in a serotonin dependant manner. Mechanistic studies in cultured hepatocytes combined with 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor knockdown using siRNA identified serotonin synthesis and signalling via the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors as critical for FFA induced TG production and the glucocorticoid dependant activation of de novo lipogenesis.

5 The Role of Serotonin in Liver Cancer

Hepatocellular carcinoma (HCC) is the primary form of liver cancer and the fastest rising cause of cancer death [51]. In western countries NAFLD is now the primary indication for liver cancer [52]. Typically, HCC emerges on the background of CLD and fibrosis/cirrhosis is a risk factor for the development of liver cancer. Serotonin is raised in the serum and in platelets of HCC patients. Serum serotonin correlates with tumour size, blood platelet count, survival and is a predictor of poor prognosis [53]. Similarly, data from a single-centre observational study showed that serum

serotonin levels were significantly higher in chronic hepatitis C (CHC) infected patients with cirrhosis or HCC than healthy controls or non-cirrhotic CHC infected individuals [54]. The authors also reported a positive correlation between α-fetoprotein protein (AFP) or prothrombin due to vitamin K absence-II (PIVKA-II) across the patient groups, suggesting that serum serotonin levels combined with vitamin K absence-II could provide a screening tool to identify CHC with HCC.

A rationale to study serotonin and 5-HT_2 receptor signalling in liver cancer is provided from studies that show serotonin signalling stimulates liver regeneration, and work published by Soll et al. [55] that reported that 5-$HT_{1A, 1B, 2B, 7}$ receptors are expressed in the liver of patients with HCC. Immunohistochemical staining of tissue-microarrays comprised of matched non-tumour and tumour tissue from 109 HCC patients revealed that 5-HT_{1A} and 5-HT_7 receptor were expressed at comparable levels between the tumour and non-tumour tissue. Whereas, levels of 5-HT_{1B} receptor are expressed in 32% of patients with HCC and 5-HT_{1B} receptor expression correlates with tumour size, whilst, 35% of HCC patients were positive for 5-HT_{2B} receptors. To further investigate the function of these receptors on cancer cell biology, Huh7 and HepG2 cancer cell lines were treated with the 5-HT_{1B} receptor antagonist SB216641 or 5-HT_{2B} receptor antagonist Ly272015. Blockade of either 5-HT_{1B} or 5-HT_{2B} receptor signalling reduced the proliferative capacity and survival of these cell lines. Furthermore, the mitogenic effects of serotonin signalling via these receptors on HepG2 cells was mediated through ERK activation, which controls cellular proliferation.

In a separate study, Soll et al. [56] confirmed the role of 5-HT_{2B} receptor signalling in HCC cell proliferation and survival. Stimulation of Huh7 or HepG2 cells with the 5-HT_{2B} receptor selective agonist α-ME-HTP induced their growth and metabolic activity, whilst, these effects were mirrored when the cells were treated with a 5-HT_{2B} receptor selective antagonist. Next, apoptosis was investigated in Huh7 cells, by exposure to TNF-α and Actinomycin D (a chemotherapy drug), or serum starvation. Caspase 3, the executioner caspase mediating cell apoptosis, was activated in TNF-α and Actinomycin D treated Huh7 cells but this was blunted by serotonin, however serum starvation failed to induce Huh7 apoptosis. Transmission electron microscopy of the serum-deprived cells revealed that Huh7 cells adopt the classical features of microautophagy, but serotonin abrogated these effects. Serum starvation dependant activation of autophagy in Huh7 cells was confirmed by an increase in LC3, p62 (autophagy proteins) and activation of the mTOR signalling pathway. Serotonin treatment was shown to significantly diminish these responses. To determine if 5-HT_{2B} receptor signalling promotes cancer growth, Huh7 tumour sub-cutaneous xenografts were grown in immunocompromised mice treated with vehicle or the 5-HT_{2B} receptor antagonist SB204741. Histological analysis of the xenografts confirmed that the tumours express 5-HT_{2B} receptor and phosphorylated p70S6K, a marker of mTOR signalling. The link between 5-HT_{2B} receptor signalling, activation of mTOR and subsequent cancer growth was further consolidated by a positive correlation between expression of 5-HT_{2B} receptor and levels of p-p70S6K and Ki67 positive cancer cells. The authors concluded that targeting the 5-HT_{2B}

receptor-mTOR-p70S6K pathway could offer a novel therapeutic approach for the treatment of HCC.

Further insights into the role of 5-HT$_{2B}$ receptor signalling in the development of hepatic steatosis and HCC was gained in a study by Niture et al. [57]. The serotonin dependant uptake of oleic acid (OA), a neutral lipid, was assessed in normal human hepatocytes and the cancer cell lines HepG2 and SK-Hep1. Uptake of oleic acid and lipid droplet accumulation were increased by serotonin in primary hepatocytes and the HepG2 and SK-Hep1 cancer cell lines. Serotonin dependant steatosis was concomitant with an elevation in mRNA levels of key proteins involved in binding, transport and synthesis of lipids as well as lipid inducible transcription factors e.g. sterol regulatory element-binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor (PParα and PParγ) as well as SIRT1, which can stimulate autophagy and modulate cellular metabolism. Similar to previous studies, Niture et al. [57] also reported that serotonin induced autophagy in HCC cells but importantly, demonstrated that this was essential for inducing the steatosis phenotype. The authors also showed that serotonin promotes cell survival of HCC cells by inducing expression of Notch and Jagged1, the ligand for Notch, and by activating this pathway. Pharmacological blockade of 5-HT$_{2B}$ receptor activation in five different HCC cell lines blunted Notch signalling and limited steatosis, conversely serotonin reuptake inhibitors had the opposite effect, confirming a role of 5-HT/5-HT$_{2B}$ receptor in the establishment of hepatic steatosis. Exposure of hepatocytes to ethanol to model excessive alcohol consumption, caused hepatic steatosis and co-treatment of HepG2 and SK-Hep1 cells with serotonin and ethanol induced Notch signalling, autophagy and steatosis. Serum serotonin levels were elevated in 8-week ethanol treated mice and this was associated with increased hepatic steatosis and autophagy, suggesting that 5-HT/5-HT$_{2B}$ receptor signalling may also have a pathological role in the development of alcoholic liver disease.

Multiple studies have shown that the balance between hippo and Yes-associated protein (Yap) protein signalling plays central roles in regulating cell fate decisions, cell survival and proliferation. Activation of Yap promotes liver regeneration, whilst aberrant Yap signalling is associated with the development of hepatocellular carcinoma [58]. Work by Liu et al. [59] investigated the potential cross talk between serotonin signalling and Yap in the context of modulating hepatoma cell phenotype. Serotonin signalling via the 5-HT$_{2B}$ receptor stimulated the growth, migration, metastatic and invasive potential of HepG2 and HCC liver cancer cell lines. At a molecular level, activation of the 5-HT$_{2B}$ receptor triggered activation of ERK and an increase in Yap and connective tissue growth factor (CTGF) expression. Yap siRNA silencing in the HCC cell lines, supressed the metabolic, proliferative and migratory effects of serotonin signalling. Whilst, pharmacological inhibition of the 5-HT$_{2B}$ receptor, supressed the serotonin-dependant ERK phosphorylation, subsequent induction of Yap expression and its nuclear translocation. Therefore, establishing a link between 5-HT/5-HT$_{2B}$/ERK/Yap signalling and the tumorigenic activities of liver cancer cells. The authors then performed a HepG2 xenograft experiment, where tumours were allowed to form under control (unstimulated) or serotonin stimulated conditions either with or without administration of a 5-HT$_{2B}$ receptor or

ERK small molecule inhibitor to ascertain if manipulating the 5-HT/5-HT_{2B}/ERK/Yap signalling axis accelerated cancer formation in the presence of serotonin but limited tumour formation and growth when the signalling axis was inhibited. Tumour size and weight was significantly greater in the presence of serotonin compared to the control group. Both the 5-HT_{2B} receptor antagonist or ERK inhibitor significantly reduced the serotonin dependant cancer growth, however, these tumours were still bigger than the control group. Importantly the administration of both inhibitors reduced ERK phosphorylation, Yap and connective tissue growth factor expression, cellular proliferation and the nuclear translocation of Yap, all of which were induced by serotonin. This study provided further evidence that the 5-HT/5-HT_{2B}/ERK/Yap signalling axis was a tractable target for HCC.

Fatima et al. [60] characterised the expression of serotonin receptors in metastatic and non-metastatic HCC cell lines and 33 paired non-tumour and HCC tumour tissues using qPCR. 5-HT_{1D}, 5-HT_{2B}, 5-HT_7 receptors were overexpressed in all of the cell lines as well as the tumour tissue in 63.6%, 36.4%, and 45.4% of cases respectively. Conversely, 5-HT_{2A} and 5-HT_5 receptor levels were reduced in the cell lines and in 51.5% and 90.1% of tumour cases respectively. Serotonin improved viability and stimulated the proliferation of HuH-7 and HepG2 cells cultured under serum deprived conditions. Consistent with other studies, this was associated with a decrease in levels of the autophagy markers microtubule-associated protein light chain 3 (LC3B) and p62. However, this group went on to demonstrate that in serum free conditions, serotonin induced expression of β-catenin and its downstream targets Axin1, cyclin D1, DKK1 and GSK3β. The mitogenic actions of serotonin via activation of β-catenin were reported to be mediated both in vitro and in vivo by 5-HT_7 receptor signalling, however, the authors did not discuss whether 5-HT_{2B} receptor contributed to the activation of this pathway.

Serotonin receptors are expressed on both HM and liver cancer cells and liver cancer is more prevalent and more aggressive in males compared to females. Therefore, work by Yang et al. [61] asked if there are differences in serotonin production/signalling between the two genders and if so, does this effect the cellular crosstalk between HM and cancer cells to drive liver cancer progression. Interestingly, the authors used a transgenic zebrafish model of HCC; where hepatocyte-specific $kras^{V12}$ is expressed in an inducible manner by administration of doxycycline, to allow temporal induction of liver cancer. Macroscopically, male kras+ fish liver had bigger livers than females, whilst, histological analysis revealed an increase in hepatocyte density and thickening of the hepatic plates in the male fish, consistent with an HCC pathology. Conversely, the histological appearance of dysplasia's in the female kras+ fish displayed features of adenomas. HM, fibrosis and cellular proliferation was increased in the male kras+ fish than the female kras+ fish, suggesting that the tumorigenic process was more advanced in the male fish. 5-HT_{2B} receptor was highly expressed in HM in both male and female kras+ fish, whereas 5-HT_{2B} receptor was absent in epithelial cells. Levels of serotonin and TPH1 were increased in male kras+ fish, suggesting that the sex differences in the cancer phenotype could be driven by increased availability of serotonin which signals via 5-HT_{2B} receptors.

To investigate this further, female kras+ fish were given either serotonin or the 5-HT$_{2B}$ receptor agonist BW723C86 for 7 days, while, PCPA (TPH1 inhibitor) or SB204741 (5-HT$_{2B}$ receptor antagonist) and cancer development was assessed. As predicted, activation of the 5-HT-5-HT$_{2B}$ receptor pathway in female fish resulted in hepatomegaly and the development of HCC, whilst, blocking serotonin synthesis or activation of the 5-HT$_{2B}$ receptor, supressed tumorigenesis, directly implicating serotonin-5-HT$_{2B}$ receptor signalling in liver cancer development and progression. A series of elegant experiments, which modulated the activation/inhibition of 5-HT$_{2B}$ receptor and TGFβ1 signalling in male and female kras+ fish, revealed that the cancer phenotype was driven by 5-HT$_{2B}$ receptor expressed on HM, stimulating excessive serotonin production by hepatocytes, which exacerbated liver cancer progression [61]. In a cohort of healthy control, CLD, cirrhotic and HCC patients, serotonin and TGFβ1, levels were high in males with liver disease or cancer. There was also a positive correlation with gender serum serotonin and TGFβ1 and hepatic inflammation or cirrhosis, suggesting that these pathways could contribute to the progression of CLD and HCC. A follow up study, added further mechanistic insight into the serotonin-dependent establishment of HCC in this inducible hepatocyte-specific kras+ zebrafish model [62]. Akin to the previous study, SB204741 limited cancer growth and HM activation, but the authors also showed a reduction in the fibrosis markers collagen I and laminin, and an increase in cancer cell apoptosis. These effects were mirrored by the serotonin agonist. Macrophages and oncogenic hepatocytes were shown to be the cellular source of serotonin, which is required for HM activation. In turn, the 5-HT/5-HT$_{2B}$ receptor dependant activation of HM stimulated the recruitment of tumour-associated macrophages, creating a positive pro-tumorigenic cellular crosstalk within the cancer niche.

6 Conclusion

In summary, 5-HT$_{2B}$ receptor play critical roles in the progression of CLD, fibrosis and cancer and are important regulators of hepatic regeneration. Therapeutic modulation of the 5-HT$_{2B}$ receptor pathway holds promise for the treatment of these diseases.

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Chapter 15
Metabolic Regulation: Insulin Secretion and Action

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Abbreviations

BAT	Brown adipose tissue
FFAs	Free fatty acids
HTR	5-HT receptor
5-HTP	5-Hydroxytryptophan
JAK2	Janus kinase 2
KO	Knockout
AMS	α-Methyl serotonin maleate
(MEK)1/2	Mitogen-activated protein kinase kinase
PI3K	Phosphoinositide 3-kinase
PRLR	Prolactin receptor
5-HT	5-hydroxytryptamine
STAT5	Signal transducer and activator of transcription 5
TPH1	Tryptophan hydroxylase-1
UCP1	Uncoupling protein 1
WAT	White adipose tissue

1 Pancreatic β-Cells

Pancreatic islets consist of several types of cells that produce various hormones. For example, α-cells produce glucagon, β-cells produce insulin, δ-cells produce somatostatin, ε-cells produce ghrelin, and PP cells produce pancreatic polypeptide. These cells interact with each other in orchestrating glucose homeostasis, while insulin-producing β-cells play a central role in regulating blood glucose levels. After a meal, the serum concentration of glucose increases, with glucose entering pancreatic β-cells through glucose transporter. Glucose is metabolized by glycolysis,

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providing mitochondria with pyruvate, which initiates the citric acid cycle (Krebs cycle). Mitochondrial respiration increases the intracellular ATP/ADP ratio, which leads to the closure of K_{ATP} channels. The accumulation of intracellular K^+ depolarizes β-cells, opening voltage-dependent Ca^{2+} channels to allow Ca^{2+} influx. This, in turn, induces exocytosis of insulin granules from the readily releasable pool near the plasma membrane, a process called first-phase secretion. After readily releasable pool secretion for a few minutes, sustained insulin secretion is triggered from a reserve pool. This secretion lasts longer (>30 min), continuing until blood glucose concentrations normalize. Impairment of this cellular cascade leads to type 2 diabetes, which is initially characterized by a defect in first-phase insulin secretion [1]. In response to hypoglycemia, pancreatic α-cells secrete glucagon, a counter-regulatory hormone that stimulates hepatic gluconeogenesis and glycogenolysis to elevate blood glucose levels. Pancreatic δ-cells secrete somatostatin which inhibits the secretion of glucagon, insulin, and other hormones.

5-HT was first reported to be present in pancreatic endocrine cells several decades ago [2, 3]. Autoradiography and electron microscopy have shown that 5-HT localizes inside β-cell granules. However, the mechanism underlying the finding, that 5-HT and HTRs are present in human β-cells, was unclear, due to the low expression of peripheral 5-HT synthesizing enzyme tryptophan hydroxylase-1 (TPH1) and the differences in STAT5 activities between humans and rodents [4, 5]. Recent bioinformatic analyses of human β-cells revealed that genes encoding 5-HT synthesizing enzymes (*TPH1*, *TPH2*, and *DDC*) and 15 HTRs are expressed in human islets [6, 7], with HTR2B among the most highly expressed HTR transcripts [6]. 5-HT and HTR2B protein were also shown to be present in β-cells [7–10].

1.1 The Role of 5-HT in β-Cell Function

5-HT synthesized in β-cells is involved in regulating β-cell function, both intracellularly and by binding to HTRs after secretion in an autocrine/paracrine manner. The role of 5-HT in regulating insulin secretion is unclear, as 5-HT [11, 12] and 5-hydroxytryptophan (5-HTP) [13] have been reported to stimulate insulin and/or glucagon secretion, to inhibit secretion [14, 15], or to have no effect [16]. As many of these studies were conducted in cell lines or isolated islets, interpretation of their results is limited, with the in vivo role of 5-HT and its derivatives remaining unclear.

Recent advances in mouse genetics have enabled the role of 5-HT in β-cell function to be studied using in vivo mouse models. Using *Tph1* knockout (KO) mice as an in vivo model showed that inhibiting peripheral 5-HT synthesis resulted in a defect in insulin secretion by β-cells [17]. 5-HT was shown to bind to the small GTPases RAB3A and RAB27A via 'serotonylation', thereby regulating the steps downstream of Ca^{2+} signaling in the insulin secretion cascade. Moreover, intracellular 5-HT was found to potentiate in vivo and in vitro insulin secretion by β-cells. Another study found that 5-HT synthesis was induced in β-cells during pregnancy and that inhibition of 5-HT synthesis by a tryptophan-free diet or by treatment with

the TPH inhibitor p-chlorophenylalanine (PCPA) resulted in gestational diabetes in mice [10]. Normally, glucose stimulated insulin secretion is enhanced during pregnancy due to the enhanced glucose responsiveness of β-cells; however, *Htr3a* KO mice showed impaired insulin secretion during pregnancy [18, 19]. Thus, 5-HT improves insulin secretion by enhancing glucose responsiveness through HTR3 in β-cells [18].

Administration of a high fat diet to β-cell-specific *Tph1* KO mice resulted in impaired glucose tolerance [19]. 5-HTP has been found to stimulate insulin secretion in vitro [13]. Because 5-HTP is rapidly converted to 5-HT upon entry into β-cells, intracellular 5-HT and/or tryptophan metabolites likely benefit β-cell function. These metabolites (i.e., 5-HT, 5-HTP, and melatonin) are indole derivatives that can potentially reduce intracellular oxidative stress [20]. Indeed, 5-HT and 5-HTP protect β-cells during lactation by scavenging reactive oxygen species, indicating that lactation improves long-term β-cell function [21].

To summarize, in vivo loss-of-function studies suggest that the production of 5-HT by β-cells benefits β-cell function [10, 19]. In addition, 5-HT production in β-cells was associated with a diabetes-free condition in humans [22]. The results of studies assessing the role of 5-HT in β-cells should be carefully interpreted in a context-dependent manner (i.e. pregnant vs. non-pregnant; intra- vs. extracellular).

1.2 The Role of HTR2B in β-Cell Function

5-HT, which is secreted by β-cells in response to glucose and/or other stimuli [2, 3, 23], binds to HTRs to exert its biological activities. Of the seven subfamilies of HTRs (HTR1 to 7), two members of HTR2 subfamily, HTR2A and HTR2B, are expressed in pancreatic islets, whereas the other, HTR2C, is expressed in the brain [10].

An in vitro study found that the HTR2B agonist α-methyl serotonin maleate (AMS) enhanced glucose-stimulated insulin secretion in human and mouse islets [24]. AMS modulated intracellular Ca^{2+} profiles, increasing peak duration and peak-to-peak distance and amplifying the response to glucose. This, in turn, activated mitochondrial enzymes, ultimately increasing the rate of mitochondrial oxygen consumption. Knock-down of *Htr2b* mRNA in the INS-1 (832/13) β-cell line was found to impair insulin secretion [24], with *Htr2b* KO mice showing glucose intolerance during pregnancy [10]. Treatment of pregnant mice with the HTR2B antagonist SB204741 reduced insulin secretion due to the reduced β-cell compensation in response to pregnancy. An ex-vivo experiment using islets isolated from pregnant *Htr2b* KO mice found that HTR2B did not affect insulin secretion, and a follow-up study showed that MIP-CreER-induced disruption of *Htr2b* gene in a β-cell-specific manner did not affect glucose tolerance in mice fed standard chow and a high fat diet [19]. HTR2B affected metabolic phenotypes only during pregnancy, possibly due to increased 5-HT availability, a condition inapplicable to male mice fed standard chow or a high fat diet. These findings, together with those of other in vivo and

in vitro studies, showed that the direct effect of HTR2B on insulin secretion was limited. Additional in vivo studies using β-cell-specific *Htr2b* KO mice are needed to confirm the in vivo effects of HTR2B on insulin secretion.

In contrast to its limited effects on insulin secretion, HTR2B plays an important role in regulating β-cell mass. The mass of pancreatic β-cells increases as metabolic demand increases, due, for example, to weight gain and insulin resistance. Physiological β-cell proliferation occurs during the perinatal period and pregnancy to achieve a proper β-cell mass. Interestingly, 5-HT synthesis in β-cells increases markedly during these two periods, suggesting that 5-HT may be involved in regulating β-cell masses [9, 10, 25]. During pregnancy, placental lactogen induces *Tph1* expression via the prolactin receptor (PRLR)-janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) cascade, with STAT5 phosphorylation partially mediated by phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase kinase (MEK)1/2 [10, 26]. Inhibition of 5-HT synthesis with a tryptophan-free diet or PCPA reduced β-cell proliferation and impaired glucose tolerance during pregnancy [10]. β-cell proliferation and mass were shown to be lower in *Htr2b* KO mice than in wild-type mice, as well as being lower in wild-type mice treated with the HTR2B antagonist SB204741. These findings indicate that 5-HT secreted by β-cells during pregnancy binds to HTR2B, activating the Gq signaling cascade and inducing β-cell proliferation (Fig. 15.1).

In contrast to pregnancy, neither prolactin nor placental lactogen induced 5-HT production in β-cells during the perinatal period, as shown by the persistent 5-HT production in β-cells of *Prlr* KO mice. Instead, growth hormone (GH) during the perinatal period binds to growth hormone receptor (GHR) and activates STAT5 signaling, inducing 5-HT production in β-cells [27]. Perinatal β-cell proliferation was markedly reduced in both β-cell-specific *Tph1* KO mice and β-cell-specific *Htr2b* KO mice, with adults of both types of mice exhibiting impaired glucose tolerance and decreased β-cell mass. Thus, 5-HT signaling through HTR2B plays a critical role in the determination of adult β-cell mass by regulating β-cell proliferation during the perinatal period.

Upregulation of 5-HT and induction of β-cell proliferation via HTR2B occur in response to physiologic stimuli, but do not compensate for increased metabolic demand. Feeding with a high fat diet does not induce the transcription of *Tph1* and *Htr2b* mRNA, nor aggravate glucose intolerance in β-cell-specific *Htr2b* KO mice [19]. Studies are needed to investigate the role of HTR2B in 5-HT producing conditions, such as pancreatic endocrine development and lactation, especially when β-cell demand is increased.

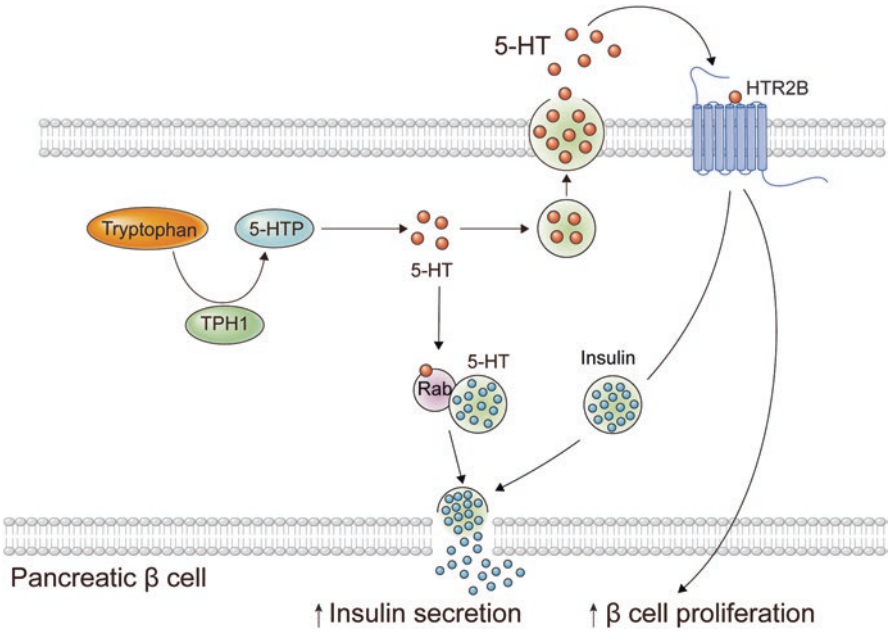

Fig. 15.1 Metabolic regulation of 5-HT and HTR2B in pancreatic β-cells. *5-HTP* 5-hydroxytryptophan, *TPH1* tryptophan hydroxylase-1

2 Adipocytes

2.1 The Role of 5-HT in Adipocyte Energy Metabolism

Adipose tissue is a dynamic metabolic organ that both stores and consumes energy. Until recently, adipose tissues were classified as white adipose tissue (WAT) and brown adipose tissue (BAT), which were functionally distinct [28]. More recently, a third functionally relevant subtype, beige adipocytes, was identified. These cells are located in WAT depots but function as brown adipocytes [29]. WAT mainly acts as an energy storage depot in the body. In the fed state, white adipocytes absorb excess energy and store it as triglycerides. In the fasting state, WAT catabolizes triglycerides supplying other organs with free fatty acids (FFAs) and glycerol [30]. In addition to their lipid storage function, brown and beige adipocytes consume energy to generate heat and maintain optimal body temperature [31].

Although 5-HT is present in adipose tissue [32], it was unclear whether adipocytes synthesize their own 5-HT. Recent evidence showed that adipocytes have a functional system for 5-HT synthesis and that both TPH1 expression and 5-HT content increase during adipocyte differentiation [33]. Inhibition of peripheral 5-HT synthesis by knocking out *Tph1* or by treatment with a TPH1 inhibitor resulted in the protection of high fat diet-induced obesity by mitochondrial uncoupling protein

1 (UCP1)-dependent thermogenic mechanisms in brown and beige adipose tissues [34, 35]. 5-HT inhibits brown adipocyte differentiation and β3-adrenergic induced thermogenic activation in a cell-autonomous manner [34, 36]. Similar to *Tph1* KO mice, adipocyte-specific *Tph1* KO mice showed resistance to high fat diet-induced obesity by increasing energy expenditure [35]. However, inhibiting 5-HT synthesis specifically in the gut, a major source of 5-HT in the periphery, did not protect against high fat diet-induced obesity [37]. Collectively, these findings indicate that regional 5-HT synthesis is a critical factor in regulating adaptive thermogenesis in adipose tissue.

2.2 The Role of HTR2B in Adipocyte Energy Metabolism

One of the main roles of 5-HT in mature adipocytes is inducing lipolysis through HTR2B signaling [38]. 5-HT treatment of epididymal and subcutaneous fat pads increased the release of glycerol and FFAs in a dose-dependent manner, suggesting that 5-HT directly promotes lipolysis. HTR2B is highly expressed in adipocytes, and adipocyte-specific *Htr2b* KO mice exhibited decreased levels of circulating glycerol, FFA, and β-hydroxybutyrate under fasting conditions. Furthermore, 5-HT induced the phosphorylation of hormone-sensitive lipase (HSL), a key enzyme in the lipolysis pathway, on serine residues 563 and 660 in an HTR2B-dependent manner. Taken together, these findings indicate that adipocyte HTR2B signaling maintains energy homeostasis by inducing lipolysis under fasting conditions (Fig. 15.2).

3 Hepatocytes

3.1 The Role of 5-HT in Hepatocyte Energy Metabolism

Hepatocytes regulate circulating glucose and lipids in the body. When nutrients are in excess, hepatocytes sequester glucose and fatty acids as glycogen and triglycerides, respectively. Under fasting conditions, hepatocytes maintain blood glucose levels by promoting glucose release, both by the breakdown of glycogen (glycogenolysis) and the de novo synthesis of glucose from glycerol and amino acids (gluconeogenesis).

Hepatocytes do not synthesize 5-HT, but have a functional serotonergic system. The 5-HT that acts on the liver is derived from the gut (free 5-HT) or platelets, depending on physiological conditions [38, 39]. 5-HT regulates lipid metabolism in hepatocytes. In vitro studies demonstrate that 5-HT has an additive effect on lipid accumulation in hepatocytes incubated with fatty acids [40, 41]. The lipogenic effects of 5-HT on primary hepatocytes were mediated by activation of mammalian target of rapamycin (mTOR). In contrast, the lipogenic effects of 5-HT on the

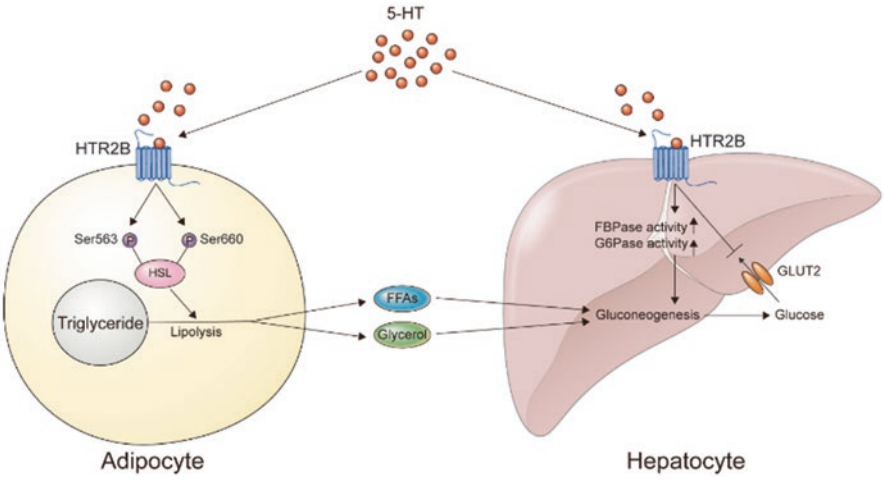

Fig. 15.2 Metabolic regulation of HTR2B in adipocytes and hepatocytes. *FFAs* Free fatty acids, *HSL* hormone-sensitive lipase, *FBPase* fructose 1,6-bisphosphatase, *G6Pase* glucose 6-phosphatase, *GLUT2* glucose transporter 2

hepatic cancer cell lines HepG2 and SK-Hep1 were mediated by activation of Notch signaling and induction of autophagy. In vivo studies showed that 5-HT had steatotic effects on the liver under conditions of diet-induced obesity. For example, inhibition of peripheral 5-HT synthesis in *Tph1* KO mice fed a high fat diet ameliorated hepatic steatosis indirectly through UCP1-dependent thermogenic mechanisms [34]. Hepatic steatosis in gut-specific *Tph1* KO mice fed a high fat diet was also prevented by downregulating the hepatic lipogenesis pathway, but did not affect systemic energy metabolism [37]. Taken together, these findings showed that gut-derived 5-HT acts directly on the liver (see also Chap. 14).

3.2 The Role of HTR2B in Hepatocyte Energy Metabolism

5-HT regulates hepatic carbohydrate metabolism through HTR2B signaling. HTR2B mediates the gluconeogenic activity of 5-HT in hepatocytes [38]. 5-HT treatment of primary hepatocytes stimulated the conversion of glycerol, lactate, and pyruvate to glucose in an HTR2B-dependent manner. Glucose levels were lower in hepatocyte-specific *Htr2b* KO mice than in control mice under fasting conditions after injection of glycerol or pyruvate. Moreover, the activities of two rate-limiting enzymes in gluconeogenesis, fructose 1,6-bisphosphatase (FBPase) and glucose 6-phosphatase (G6Pase), were lower in hepatocyte-specific *Htr2b* KO mice than in control mice. Hepatic HTR2B signaling regulates glucose metabolism not only by promoting gluconeogenesis but also by suppressing glucose uptake [38]. 5-HT treatment of primary hepatocytes reduced glucose uptake in an HTR2B-dependent

manner. Glucose tolerance tests showed that glucose clearance was lower in hepatocyte-specific *Htr2b* KO mice than in control mice. In addition, glucose transporter 2 (GLUT2) protein levels and glucokinase activity were higher in hepatocyte-specific *Htr2b* KO mice than in control mice. Taken together, these findings indicate that hepatocyte HTR2B signaling contributes to fasting adaptation by promoting gluconeogenesis and suppressing glucose uptake (Fig. 15.2).

4 Outlook and Prospects

HTR2B-dependent metabolic pathways in peripheral organs, insulin secretion and cell proliferation in pancreatic β-cells, lipolysis in adipocytes, gluconeogenesis and glucose uptake in hepatocytes, are important for the development of diabetes, obesity, and non-alcoholic fatty liver disease. Since the metabolic role of HTR2B signaling in adipocytes and hepatocytes under diet-induced obesity condition is missing, future studies should elucidate whether it mediates similar functions as well as fasting condition.

5-HT signaling is very complex because 5-HT is synthesized at multiple sites, it signals through auto-, para-, and endocrine actions, and it binds to at least 14 receptors. Many studies to date have tested the effects of TPH inhibitors and HTR2B agonists/antagonists or *Tph1* KO and *Htr2b* KO mice. However, these strategies have limitations in unraveling the tissue specific effects of HTR2B-dependent 5-HT signaling in in vivo models. Future studies should utilize in-vivo tissue-specific KO models to assess the tissue-specific roles of 5-HT and HTR2B.

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Part III
Brain Actions

Chapter 16
Drugs of Abuse Affecting 5-HT_{2B} Receptors

Dino Luethi and Matthias E. Liechti

Abbreviations

MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
CYP	Cytochrome P450
LSD	Lysergic acid diethylamide
NPS	New psychoactive substances
SERT	Serotonin transporter

1 Introduction

A variety of drugs of abuse affect monoaminergic neurotransmission including the serotonergic system. On the one hand, serotonergic stimulants target the plasmalemmal serotonin transporter (SERT), either as blockers such as cocaine or as substrates such as 3,4-methylenedioxymethamphetamine (MDMA) [1–5]; on the other hand, serotonergic psychedelics mediate their mind-altering effects mainly through activation of serotonergic 5-hydroxytryptamine (5-HT) 2A receptors [6–11]. Moreover, several stimulant-type substances interact with serotonergic receptors [2, 12–16] and some psychedelics inhibit transporter-mediated serotonin reuptake [17] in addition to their main action at the 5-HT_{2A} receptor. Besides stimulants and psychedelics, other drug classes such as synthetic cannabinoids and opioids have been shown to interact with serotonin transporers and receptors [18–20] in addition to their main effects at cannabinoid and opioid receptors, respectively. The 5-HT_{2B} receptor is one potential interaction site for serotonergic drugs of abuse. However, the 5-HT_{2B} receptor is not a primary target for serotonergic drugs as its main expression is in peripheral organs such as liver, kidneys, stomach, and gut, and there is

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only limited expression in the brain [21–25]. Nevertheless, it has been associated with pathways that modulate drug abuse and reinforcing effects of stimulants [26–28]. Furthermore, 5-HT_{2B} receptor interactions with drugs of abuse are of interest as receptor activation has been associated with cardiac valvulopathy, resulting in the market removal of several 5-HT_{2B} agonist prescription drugs, including the appetite suppressant fenfluramine [29–32]. The major metabolite of fenfluramine, norfenfluramine (3-trifluoromethylamphetamine), displays higher affinity and efficacy at the 5-HT_{2B} receptor in comparison to the parent compound [32, 33], indicating that it is mainly responsible for fenfluramine-induced cardiac valvulopathy. Even though fenfluramine has structural similarity to amphetamine, it does not share the potent stimulant effects and abuse is therefore rare [34, 35]. However, the chemical structures of fenfluramine and norfenfluramine suggest that drug-induced cardiac valvulopathy is a potentially severe complication to consider for any amphetamine-type drugs of abuse that stimulate serotonin 5-HT_{2B} receptors [36]. This chapter should give a basic overview over the involvement of 5-HT_{2B} receptors in recreational drug action and associated adverse effects such as cardiac valvulopathy. Different stimulant and psychedelic drugs for which activity at the 5-HT_{2B} receptors has been tested will be discussed.

2 Drugs Stimulating 5-HT_{2B} Receptors

Interference with monoaminergic signaling is the main mechanism of action for stimulants and psychedelics [1, 4, 37]. In addition, interactions with monoaminergic targets have been shown for other drug classes, such as opioids [17] or dissociative anesthetics [38, 39]. Compared to other monoaminergic targets such as 5-HT_{2A} or dopamine receptors, relatively little research has focused on the interactions of drugs of abuse with 5-HT_{2B} receptors. Nevertheless, 5-HT_{2B} receptor interactions have been assessed for various stimulant and psychedelic drugs of abuse, including many new psychoactive substances (NPS) [3, 14, 16, 17, 40–45], which are shown in Tables 16.1 and 16.2, respectively.

2.1 5-HT_{2B} Receptor-Mediated Effects of Stimulants

Despite its limited expression in the brain, the 5-HT_{2B} receptor has been shown to contribute to the mechanism of action of stimulants. For instance, it has been demonstrated that selective 5-HT_{2B} receptor antagonism and 5-HT_{2B} receptor knockout reversed MDMA-induced hyperactivity in mice [28]. Furthermore, it has been demonstrated that inhibition and knockout of the 5-HT_{2B} receptors abolished MDMA-induced efflux of serotonin in the nucleus accumbens and ventral tegmental area [28]. The authors of that study hypothesized that presynaptic 5-HT_{2B} receptors modulate MDMA-induced 5-HT release in serotoninergic raphe neurons. In

Table 16.1 5-HT$_{2B}$ receptor interactions of stimulant drugs of abuse

Drugs	5-HT$_{2B}$ receptor activity			Reference
	K_i [μM]	EC_{50} [μM]	E_{max} [%]	
Aminoindanes				
5-Iodo-2-aminoindane	0.07			Iversen et al. [43]
MDAI	>5			Iversen et al. [43]
MMAI		>10		Luethi et al. [16]
N-methyl-2-AI		>20		Luethi et al. [16]
Benzofurans				
5-APB		0.28 ± 0.12	61 ± 17	Rickli et al. [14]
	0.014	0.015 ± 0.001	92 ± 1	Iversen et al. [43]
5-APDB		1.2 ± 0.6	50 ± 21	Rickli et al. [14]
6-APB		0.14 ± 0.06	70 ± 9	Rickli et al. [14]
	0.004	0.0041 ± 0.003	93 ± 1	Iversen et al. [43]
6-APDB		0.12 ± 0.03	66 ± 17	Rickli et al. [14]
5-MAPDB		>20		Rickli et al. [14]
4-APB		1.0 ± 0.5	38 ± 16	Rickli et al. [14]
7-APB		0.28 ± 0.52	52 ± 17	Rickli et al. [14]
5-EAPB		>20		Rickli et al. [14]
Cathinones				
α–PVP		>20		Rickli et al. [15]
β-Keto-MDA		>20		Rickli et al. [15]
1-Naphyrone	0.4			Iversen et al. [43]
2,3-DMMC		>10		Luethi et al. [16]
2,4-DMMC		>10		Luethi et al. [16]
3-MMC		>10		Luethi et al. [16]
3,4-DMMC		>20		Luethi et al. [16]
BMDP	1.7			Iversen et al. [43]
4–Bromomethcathinone		>20		Rickli et al. [15]
4–Ethylmethcathinone		>20		Rickli et al. [15]
4–Fluoromethcathinone		>20		Rickli et al. [15]
4–Methylmethcathinone		>20		Rickli et al. [15]
Benzedrone	>5			Iversen et al. [43]
MDPPP		>20		Rickli et al. [15]
MDPBP		>20		Rickli et al. [15]
MDPV		>20		Rickli et al. [15]
Mephedrone		>10		Luethi et al. [16]
	0.74			Iversen et al. [43]
Methcathinone		>20		Rickli et al. [15]
Methylethcathinone	>5			Iversen et al. [43]
Methylone		>10		Luethi et al. [5]
Naphyrone	>5			Iversen et al. [43]
		>20		Rickli et al. [15]
Pyrovalerone		>20		Rickli et al. [15]

(continued)

Table 16.1 (continued)

Drugs	5-HT_{2B} receptor activity			Reference
	K_i [μM]	EC_{50} [μM]	E_{max} [%]	
Phenethylamines				
4-Fluoroephedrine		>20		Rickli et al. [15]
4–Fluoroamphetamine		11.4 ± 4.6	49 ± 15	Rickli et al. [15]
4–Fluoromethamphetamine		>20		Rickli et al. [15]
4-Methylamphetamine		0.86 ± 0.38	54 ± 8	Luethi et al. [16]
D-Amphetamine		9.4	8 ± 2	Rickli et al. [15]
D-Methamphetamine		>20		Rickli et al. [15]
Ephedrine		>20		Rickli et al. [15]
MDA		0.85 ± 0.11	52 ± 12	Rickli et al. [14]
MDMA		>20		Rickli et al. [14]
Piperidines				
4-Fluoromethylphenidate		>10		Luethi et al. [3]
4-Methylmethylphenidate		>10		Luethi et al. [3]
Ethylnaphthidate		>10		Luethi et al. [3]
Ethylphenidate		>20		Luethi et al. [3]
Methylphenidate		>10		Luethi et al. [3]
Propylphenidate		>10		Luethi et al. [3]
Other				
4,4′-DMAR		>10		Maier et al. [44]
5-IT		1.5 ± 0.6	36 ± 5	Luethi et al. [16]
Cocaine		>10		Luethi et al. [3]
Dimethylamylamine	>5			Iversen et al. [43]
Methiopropamine	3.9			Iversen et al. [43]
Methylmorphenate		>10		Luethi et al. [3]
Modafinil		>10		Luethi et al. [3]

addition, inhibition and knockout of the 5-HT_{2B} receptor led to an absence of dopamine efflux in the nucleus accumbens, which may have been the result of a lack of activation of postsynaptic serotonin receptors [28]. In a follow-up study, MDMA was shown to induce locomotor sensitization and conditioned place preference in wildtype but not in 5-HT_{2B} receptor knockout or 5-HT_{2B} receptor antagonized mice, underscoring the possible role of 5-HT_{2B} receptors in the reinforcing effects of serotonergic stimulants [27]. However, an increased dose of MDMA induced behavioral effects in all mouse models, potentially due to direct and therefore 5-HT_{2B} receptor independent interaction of MDMA with the dopamine transporter [27]. This assumption is supported by in vitro studies showing serotonin transporter inhibition at low and dopamine transporter inhibition by MDMA at high concentrations [5, 12].

Table 16.2 5-HT$_{2B}$ receptor interactions of psychedelic drugs of abuse

Drugs	5-HT$_{2B}$ receptor activity			Reference
	K_i [μM]	EC_{50} [μM]	E_{max} [%]	
Benzodifuran				
2C-B-FLY		0.040	56	Rickli et al. [14]
Ergoline				
LSD	0.00057	0.0031	23	Eshleman et al. [42]
		12	71	Rickli et al. [40]
Phenethylamines				
25B-NBOMe		0.01	19	Rickli et al. [40]
25C-NBOMe		0.10	16	Rickli et al. [40]
25D-NBOMe	0.0021	0.032	48	Eshleman et al. [42]
		0.10	22	Rickli et al. [40]
25E-NBOMe	0.0011	0.024	49	Eshleman et al. [42]
		0.06	26	Rickli et al. [40]
25H-NBOMe	0.063	0.46	38	Eshleman et al. [42]
		0.34	11	Rickli et al. [40]
25I-NBOMe	0.0019	0.11	21	Eshleman et al. [42]
		0.13	32	Rickli et al. [40]
25N-NBOMe	0.0087	0.047	58	Eshleman et al. [42]
		0.07	26	Rickli et al. [40]
25P-NBOMe		0.17	23	Rickli et al. [40]
25T2-NBOMe		0.04	31	Rickli et al. [40]
25T4-NBOMe		0.20	27	Rickli et al. [40]
25T7-NBOMe		0.31	14	Rickli et al. [40]
2C-B		0.13	89	Rickli et al. [40]
		0.075	52	Luethi et al. [41]
2C-BI-1		>10		Luethi et al. [45]
2C-BI-2		>10		Luethi et al. [45]
2C-BI-3		>10		Luethi et al. [45]
2C-BI-4		>10		Luethi et al. [45]
2C-BI-5		>10		Luethi et al. [45]
2C-BI-7		>10		Luethi et al. [45]
2C-BI-8		0.22		Luethi et al. [45]
2C-BI-10		>10		Luethi et al. [45]
2C-BI-11		>10		Luethi et al. [45]
2C-BI-12		0.20		Luethi et al. [45]
2C-C		0.28	81	Rickli et al. [40]
2C-D		0.23	77	Rickli et al. [40]
2C-E		0.19	66	Rickli et al. [40]
2C-H		6.2	46	Rickli et al. [40]
2C-I		0.15	70	Rickli et al. [40]
2C-N		0.73	74	Rickli et al. [40]
2C-P		0.13	72	Rickli et al. [40]

(continued)

Table 16.2 (continued)

Drugs	5-HT$_{2B}$ receptor activity			Reference
	K_i [μM]	EC_{50} [μM]	E_{max} [%]	
2C-T-1		0.057	58	Luethi et al. [41]
2C-T-2		0.13	75	Rickli et al. [40]
2C-T-3		0.044	28	Luethi et al. [41]
2C-T-4		0.16	68	Rickli et al. [40]
		0.063	75	Luethi et al. [41]
2C-T-7		0.35	45	Rickli et al. [40]
		0.052	46	Luethi et al. [41]
2C-T-16		0.047	36	Luethi et al. [41]
2C-T-19		0.369	40	Luethi et al. [41]
2C-T-21.5		0.182	40	Luethi et al. [41]
2C-T-22		0.11	35	Luethi et al. [41]
2C-T-25		0.108	32	Luethi et al. [41]
2C-T-27		>10		Luethi et al. [41]
2C-T-28		0.081	34	Luethi et al. [41]
2C-T-30		0.051	61	Luethi et al. [41]
2C-T-31		3.3	44	Luethi et al. [41]
2C-T-33		>10		Luethi et al. [41]
Biscaline		>10		Luethi et al. [45]
DMA	1			Nelson et al. [46]
DOAc	0.31			Nelson et al., [46]
DOB	0.027			Nelson et al. [46]
DOBz	0.035			Nelson et al. [46]
DOC	0.032			Nelson et al. [46]
DOCN	0.77			Nelson et al. [46]
DOF	0.23			Nelson et al. [46]
DOHx	0.03			Nelson et al. [46]
DOI	0.02			Nelson et al. [46]
DOM	0.041	0.15	96	Eshleman et al. [42]
DON	0.17			Nelson et al. [46]
DOPR	0.054			Nelson et al. [46]
DOTB	0.025			Nelson et al. [46]
MEM	0.76			Nelson et al. [46]
Mescaline		>20		Rickli et al. [40]
Mescaline-NBOMe		>20		Rickli et al. [40]
TMA	0.31			Nelson et al. [46]
Tryptamines				
4-OH-DiPT		0.460	55	Rickli et al. [17]
4-OH-MET		>20		Rickli et al. [17]
5-MeO-AMT		0.004	104	Rickli et al. [17]
5-MeO-MiPT		1.5	12	Rickli et al. [17]
DiPT		1.0	103	Rickli et al. [17]

(continued)

Table 16.2 (continued)

Drugs	5-HT$_{2B}$ receptor activity			Reference
	K_i [μM]	EC_{50} [μM]	E_{max} [%]	
DMT		3.4	19	Rickli et al. [17]
Psilocin		>20		Rickli et al. [17]

2.2 Stimulant-Induced Cardiac Valvulopathy

5-HT$_{2B}$ receptors are, among others, expressed in cardiovascular tissues [47] and their activation potentially leads to cardiac valvulopathy [29, 48–50]. Therefore, cardiac valvulopathy is a concern to consider for drugs that increase plasma 5-HT levels, directly activate the 5-HT$_{2B}$ receptor, or both. In fact, several prescription drugs have previously been removed from the market due to their potential to induce cardiac valvulopathy in patients [29–32]. However, serotonergic drugs of abuse are typically not associated with a high abuse liability [51–54] and are therefore mostly used sporadically and not on a regular basis. This raises the question of the relevance of 5-HT$_{2B}$-mediated cardiac valvulopathy in recreational drug use. The regular use of the serotonergic drug MDMA has been associated with mild to moderate valvular heart disease, based on a case control study [55]. In this study, 8 of 29 regular MDMA users displayed abnormal echocardiographic results compared with none of the control group. The average use of the MDMA users was very high and described to have consisted of 3.6 MDMA tablets per week with an average duration of drug use of 6.1 years [55]. This underscores the assumption that in particular heavy recreational use of serotonergic stimulants may induce cardiac valvulopathy. Besides these clinical findings from a case control study, 5-HT$_{2B}$ receptor-mediated proliferation of cardiac valvular interstitial cells induced by MDMA has also been demonstrated in vitro [56].

2.3 Stimulants Acting on 5-HT$_{2B}$ Receptors

Table 16.1 shows an overview of 5-HT$_{2B}$ receptor binding and activation potency values for various stimulants, assessed in different studies. Notably, in a study by Rickli and colleagues, MDMA did not activate the 5-HT$_{2B}$ receptor in the functional assay at investigated concentrations (EC_{50} > 20 μM); however, 3,4-methylenedioxyamphetamine (MDA), the main psychoactive *N*-demethylated phase I metabolite of MDMA, potently activated the receptor at submicromolar concentrations [14]. This suggests that the metabolite MDA rather than MDMA itself may lead to valvulopathy and that there could be a significant metabolic contribution to MDMA-induced effects and adverse effect. MDA formation is mainly mediated by cytochrome P450 (CYP) 2B6, with additional contributions from CYP1A2, CYP2C19, and CYP2D6 [57–60]. Therefore, genetic polymorphisms in the genes coding for these enzymes could potentially influence the 5-HT$_{2B}$ receptor-

mediated adverse effects in MDMA users. Notably, however, the sensitivity of the calcium mobilization assays used to determine the functional 5-HT_{2B} receptor activity and the inter-correlation of data obtained with different assays is not clearly understood. For example, only poor correlation between 5-HT_{2A} receptor activation and effects for psychedelics has been observed in several studies [61–63], whereas binding affinity at this receptor was a good predictor of the clinical potency of psychedelics [63]. Thus, the available in vitro 5-HT_{2B} receptor functional data may not be a good predictor of cardiac valvulopathy risk in vivo.

Besides MDA, several benzofuran NPS potently activated the 5-HT_{2B} receptor at submicromolar concentrations [14, 43]. Therefore, as shown for MDMA in vivo [27, 28], 5-HT_{2B} receptor activation may directly contribute to the effects of these novel drugs of abuse. Furthermore, regular and heavy use of benzofuran NPS may potentially result in heart damage; however, benzofurans have so far not been linked to any case of cardiac valvulopathy. Only a few other non-benzofuran stimulants displayed potent agonism at the 5-HT_{2B} receptor, such as 4-methylamphetamine (4-MA) or 5-(2-aminopropyl)indole (5-IT). The amphetamine derivative 4-MA was originally developed as an appetite suppressant but was never marketed [64]. Its recent reappearance on the illicit drug market has almost exclusively been limited to being a contaminant in street amphetamine samples [65]. The mixture of amphetamine and 4-MA has been linked to extreme hyperthermia and several fatalities, likely explained by the high difference in dopaminergic *vs.* serotonergic activity of the two substances [15, 16, 65]. The indole derivative 5-IT is a highly potent stimulant NPS that has been associated with various fatal intoxications in recent years [16, 66–69]. Furthermore, Iversen and colleagues reported submicromolar binding affinities at the 5-HT_{2B} receptor for the NPS 5-iodo-aminoindane, mephedrone, naphyrone, 1-naphyrone, and methylenedioxy-aminotetralin [43]. Mephedrone is not a potent agonist at the receptor [16] and no functional activity has been determined for the other substances. Therefore, it is not certain whether these substances act as agonists at the 5-HT_{2B} receptor.

2.4 5-HT_{2B} Receptor-Mediated Effects of Psychedelics

The subjective effects of psychedelics are primarily mediated by 5-HT_{2A} receptor activation [9–11, 70–72]. In addition, correlation between receptor activation and psychedelic effect potencies have been reported for the 5-HT_{2B} [46] and 5-HT_{2C} receptors [6, 7, 63], which is not surprising given that 5-HT_2 receptors share significant sequence homology [73]. However, there is currently no clear consensus on the importance of the 5-HT_{2B} and 5-HT_{2C} receptors in the mechanism of action of psychedelics.

2.5 *Psychedelics Acting on 5-HT$_{2B}$ Receptors*

5-HT$_{2B}$ receptor interactions for various psychedelics are listed in Table 16.2. For most of the substances, only receptor activation potency but no receptor affinity values have been reported. Most phenethylamine and tryptamine psychedelics activated the 5-HT$_{2B}$ receptor at submicromolar or low micromolar concentrations. As reported for the 5-HT$_{2A}$ receptor, no correlation between 5-HT$_{2B}$ receptor activation and clinical potency of psychedelic was observed in a study comparing receptor activation potencies of a considerable amount of psychedelics with their reported human doses [63]. Eshleman and colleagues reported 5-HT$_2$ receptor affinities as well as functional activity for six phenethylamine psychedelics and lysergic acid diethylamide (LSD) [42]. All compounds displayed highest binding affinity and activation potency for the 5-HT$_{2A}$ receptors; nevertheless, for several substances, high affinity and activation potency (K_i and EC_{50} < 100 nM) was observed at the 5-HT$_{2B}$ receptor [42]. A remarkable difference in receptor activation in two different functional assays has been reported for LSD. Whereas an EC_{50} of 12 μM has been measured with a calcium mobilization assay [40], an EC_{50} of 3 nM has been reported when a stimulation of inositol monophosphate (IP-1) formation assay was used [42]. To gain a clearer picture of the involvement of 5-HT$_{2B}$ receptors in the action of psychedelics, more in vitro and in vivo research is needed.

3 Conclusion

Several stimulant and psychedelic drugs of abuse activate the 5-HT$_{2B}$ receptor at pharmacologically relevant concentrations. Animal studies with MDMA suggest that the 5-HT$_{2B}$ receptor contributes to the effects of serotonergic stimulants, possibly by 5-HT-dependent regulation of dopamine release. Furthermore, stimulants that activate the 5-HT$_{2B}$ receptor may put regular and heavy users at risk of cardiac valvulopathy. The main classes of stimulant drugs of abuse that interact with 5-HT$_{2B}$ receptors are benzofurans and amphetamines with a distinct serotonergic *vs.* dopaminergic activity.

In addition to stimulants, various phenethylamine and tryptamine psychedelics activate the 5-HT$_{2B}$ receptor. However, the role of 5-HT$_{2B}$ receptor activation in the mechanism of action of psychedelic remains unclear. As psychedelics do not lead to dependence and are mostly not used on a regular basis, cardiac valvulopathy is likely not a risk to consider for users.

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Chapter 17
The Role of 5-HT_{2B} Receptor on Aggression and Drugs of Abuse

Janitza L. Montalvo-Ortiz and Emil F. Coccaro

1 The Relationship Between Serotonin and Aggression

Monoamines, particularly serotonin (5-hydroxytryptamine; 5-HT), have been extensively studied in the context of aggression. 5-HT is known to play a key role in the modulation of aggressive, impulsive, antisocial, and violent behavior. One of the early studies linking 5-HT with aggression-related traits investigated the relationship between cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5-HIAA), a 5-HT metabolite, with aggressive and impulsive behavior in a population of adult military men [1]. Lower 5-HIAA is associated with higher aggressive behavior, a finding that was replicated in a men cohort with borderline personality disorder [1], in violent offenders [2–4], and in subjects with a history of aggressive behavior [5]. However, non-replications of the negative correlation between CSF 5-HIAA have also been reported [6–9], as well as studies showing opposite effects: 5-HIAA positively correlated with aggressive behavior [9].

Nevertheless, 5-HT remains as a central modulator of aggressive and violent behavior. There are several hypotheses that intend to explain the underlying relationship between 5-HT and aggressive behavior. One of these states that 5-HT plays a modulatory role in the response of both internal and external stimuli [10]. Specifically, low levels of 5-HT are associated with impulsive behavior and stimulus response, whereas high levels of 5-HT are associated with behavioral rigidity. Another hypothesis, primarily based on 5-HIAA findings, is the "low serotonin syndrome", which is believed to be present in violent or aggressive individuals. Briefly,

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5-HT plays a role in constrain behavior, thus a decrease in 5-HT is related to increased impulsive behavior [11]. Another hypothesis is the "Irritable Aggression Model" proposed by Coccaro et al. [12], which consists on a net hyposerotonergic state associated with increased irritability, in other words, a lower threshold for noxious stimuli response. This theory is based on studies with pharmacological challenges, an approach that assess the effects of an acute administration of a serotonergic drug, *d,l-fenfluramine*, and the related hormonal response (i.e., prolactin response) *(PRL[d,l-FEN])*. These studies found an inverse correlation between *PRL[d,l-FEN]* and irritability and aggressive behavior [13, 14]. This relationship was also found in antisocial individuals [15, 16], and substance abusers [17]. This theory is further supported by studies showing noxious stimuli to be required for aggressive response in a net hyposerotonergic state [18, 19].

A meta-analysis of 171 studies (using 5-HIAA assay, acute tryptophan depletion (AID), pharmacologic challenge, and endocrine challenge methods) examining the inverse relationship between 5-HT and aggressive behavior found a smaller effect size than expected ($r = -0.12$) [20]. Specifically, pharmacologic challenge studies exhibited the largest effect size ($r = -0.21$), while studies using the 5-HIAA assay obtained a non-significant small effect size ($r = -0.06$). Factors including sex, age, psychopathology, history of aggression and type of drug, did not moderate the association between 5-HT and aggressive behavior. These conflicting and null results depict a more complex relationship between 5-HT and aggression.

Neuroimaging studies examining neurotransmitter functioning in living patients using methods such as positron emission tomography (PET) and pharmacologic challenges have allowed a more accurate assessment of the 5-HT system activity and functioning in the brain. These studies in subjects with personality disorder with impulsivity and aggression have implicated brain regions within the prefrontal cortex including orbitofrontal cortex and anterior cingulate cortex (areas known for their roles in inhibiting aggressive behavior) showing that deficits in these regions may underlie, in part, abnormal 5-HT function [21–23]. Similarly, other studies have found abnormal 5-HT synthesis and reuptake in impulsively aggressive individuals with personality disorders [24, 25]. Taken together, while normal levels of 5-HT have an inhibitory effect on brain regions involved in aggressive behavior, a reduction in 5-HT activity increases aggression [26]. Inhibitory effects of 5-HT on aggressive behavior are mediated by 5-HT receptors within the prefrontal cortex, which in turn mediates 5-HT signaling in subcortical regions [27].

Genetic factors also are involved in the relationship between 5-HT and aggressive behavior. It is known that genetic factors explain 50–63% of the variance in aggressive behavior and several genetic variants that influence aggressive behavior have been identified [28, 29]. Monoaminergic genes, including serotonergic genes, – identified via unbiased approaches or selected as biological candidates – have been extensively studied in relation to aggression-related traits [29, 30]. An apparently unique mutation at the monoamine oxidase A locus (*MAOA*), which encodes an enzyme that catabolizes monoamines (including 5-HT), was associated with aggressive behavior in a Dutch kindred [31] after first being localized by genetic linkage analysis [32]. Caspi et al. [33] subsequently reported that carriers of a

different low-activity *MAOA* variant exhibited violent behavior only after exposure to moderate or severe levels of child abuse [34]. Sequencing of 5-HT system genes in a Finnish population of impulsive individuals revealed association between a stop codon in the serotonin 2B receptor gene (*HTR2B* Q20*) and risk of committing violent acts [35]. In a subsequent study, *HTR2B* Q20* carriers showed aggressive behavior, alcohol-related impulsivity, and emotional dysregulation [36]. A more recent study integrating human and animal data showed an association between *HTR2B* and aggressive behavior under the influence of cannabis [37]. This link was found by using a hypothesis-free approach.

In summary, though some of the literature linking 5-HT and aggressive behavior shows conflicting or null results, this may be due to the complexity of the 5-HT system and function. This is supported by neuroimaging findings implicating well-known brain regions in modulating impulsive behavior, such as the prefrontal cortex, in the underlying connection between 5-HT and aggression. Further, it shows an impaired brain 5-HT functioning in impulsively aggressive individuals. Genetic studies also show evidence of a link between 5-HT and aggressive behavior by identifying 5-HT-related genetic variants in impulsive and aggressive individuals. 5-HT modulatory effects on behavior are complex and implicate multiple receptor subtypes and systems. It is known that 5-HT effects are mediated by at least 14 receptor subtypes grouped into seven 5-HT receptor families (5-HT$_1$–5-HT$_7$ receptor). In the brain, 5-HT receptors are distributed pre- and post-synaptically and it is believed that these different subtypes may exert opposing effects on aggressive behavior [38]. In the following, we will focus on the 5-HT$_{2B}$ receptor and will discuss its role in the modulation of aggressive and impulsive behaviors as well as related traits such as drug abuse.

2 Pharmacological and Genetic Aspects of 5-HT$_{2B}$ Receptor

5-HT$_{2B}$ receptor is a member of the G-protein-coupled receptor superfamily expressed in various peripheral tissues and the brain, particularly in the frontal cortex, however, at very low levels [39–41]. Though its specific function is unknown, its modulatory effect in the brain and peripheral 5-HT system is well-documented. *HTR2B,* a gene encoding for 5-HT$_{2B}$ receptor is located at chromosomal position 2q36.3-q37.1 and contains 4 exons spanning 17 kb [42]. According to the Genotype-Tissue Expression (GTEx) dataset (GTEx Portal, V8 release), a publicly available multi-tissue gene expression dataset comprising 17,382 samples from 948 individuals, *HTR2B* transcript is predominantly enriched in endocrine tissues (i.e., adrenal gland), muscle tissue (i.e., esophagus), and female tissues (i.e., uterus and cervix) (Fig. 17.1). Enrichment is also observed –to a lesser extent- in tissues from the gastrointestinal tract, male tissues, gastrointestinal tract, and brain tissue.

Genetic variants at the *HTR2B* gene have been associated with a variety of traits and disorders, implicating both the peripheral and the central nervous system. By using GWASATLAS [43], a phenome-wide association study (PheWAS) approach

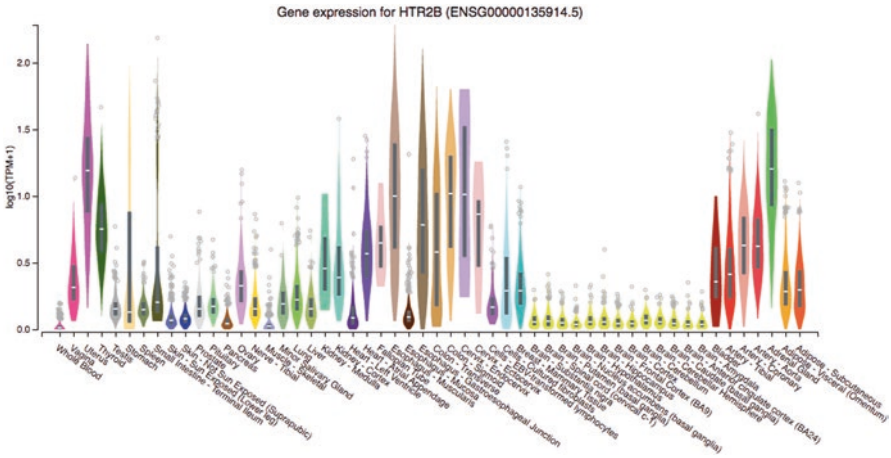

Fig. 17.1 Multi-tissue gene expression patterns of *HTR2B* in Humans. *HTR2B* gene expression patterns across multiple tissues from human data. Data is shown as RPKM (reads per kilobase transcript per million). The data used for the analyses described here were obtained from the GTEx Portal (http://www.gtexportal.org/home/) on 04/28/2020. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NIC, NHGRI, NIDA, NIMH, and NINDS

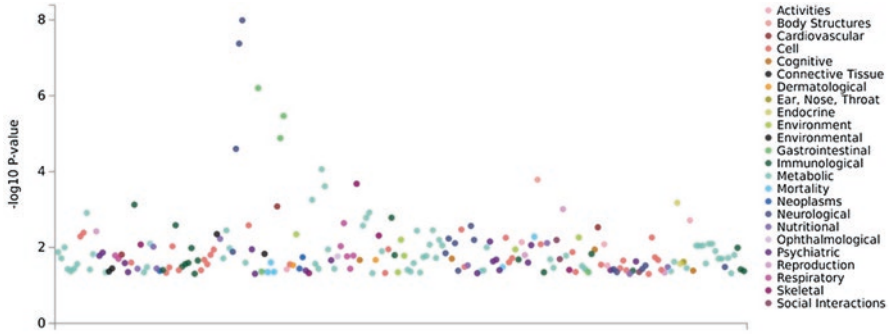

Fig. 17.2 Phenome-Wide Association Study Plot of *HTR2B* gene using GWASATLAS. Phenome-wide association study (PheWAS) plot is shown for *HTR2B* gene using data from genome-wide association studies (GWAS) 4756 studies. Bonferroni corrected *P*-value is set at 2.30×10^{-4}

was conducted including data from publicly available Genome-Wide Association Studies (GWAS) summary statistics and 600 GWAS based on UK Biobank release data (application IDL 16406). This method allows identifying associations between *HTR2B* genetic variants across multiple traits and disorders in a hypothesis-free manner. Five domains are significantly associated with *HTR2B* (Bonferroni corrected $p = 2.30 \times 10^{-4}$): neurological, gastrointestinal, metabolic, body structures, and skeletal (Fig. 17.2). This included traits or disorders such as cingulum

(hippocampus) radial/mean diusivities, diverticular disease, glomerular filtration rate, mouth/teeth dental problems, and femoral neck in females, respectively. Nominal associations are shown for psychiatric domain, including traits or disorders such as lithium response in Bipolar I patients ($p = 6.98 \times 10^{-3}$), miserableness ($p = 8.68 \times 10^{-3}$), depression ($p = 1.12 \times 10^{-2}$), and alcohol dependence ($p = 1.36 \times 10^{-2}$). This study does not include more recent GWAS, such as Montalvo-Ortiz et al. [37], that identified a *HTR2B* genetic variant associated with aggressive behavior while under the influence of cannabis and described later in the chapter.

Pharmacological activation of 5-HT$_{2B}$ receptors can increase extracellular 5-HT in neurons, showing an excitatory effect on 5-HT activity [44] and a positive regulatory role [45, 46]. Activation of 5-HT$_{2B}$ receptors by agonists is known to mimic antidepressant action [47] by increasing extracellular 5-HT levels in the dorsal raphe neurons. Further, acute and chronic administration of antidepressants can stimulate the 5-HT$_{2B}$ receptors in neurons astrocytes, also influencing 5-HT efflux [48]. Genetic ablation of these receptors or pharmacologically blocking its action can eliminate the acute and long-term effects of antidepressants on behavior and neurogenesis [44, 49]. Further, mice lacking the *Htr2b* gene display a hyposerotonergic phenotype [45]. Specifically, *Htr2b*$^{-/-}$ mice show reduced tonic firing frequency of dorsal raphe 5-HT neurons and an abolished efficacy of antidepressants. Similarly, decreased *Htr2b* expression in astrocytes is associated with the development of depressive-like behaviors in an animal model of Parkinson's disease [50]. Antidepressant administration reversed these effects by increasing *Htr2b* expression and decreasing depressive-like behaviors. These findings suggest that the therapeutic effects of antidepressants rely on long-term neuroadaptations within the 5-HT system [51].

3,4-methylene-dioxymethamphetamine (MDMA), commonly referred to as 'ecstasy', can also activate 5-HT$_{2B}$ receptors. Studies on mice have found that MDMA can selectively bind and activate 5-HT$_{2B}$ receptors to induce 5-HT efflux in the dorsal raphe nucleus [44]. This also leads to dopamine efflux in the nucleus accumbens and ventral tegmentum. Further, 5-HT$_{2B}$ receptor agonists can increase 5-HT transporter (SERT) phosphorylation [52], serving as a positive autoregulator of the serotonergic tone in dorsal raphe neurons. A study using 5-HT$_{2B}$ receptor antagonist RS127445 shows that 5-HT$_{2B}$ receptors are also located in GABAergic interneurons and exert a tonic inhibitory control on the activity of 5-HT neurons projecting to the medial prefrontal cortex [53]. This suggests that 5-HT$_{2B}$ receptor may play an important role in the modulation of the local, negative-feedback loop regulating 5-HT neuronal activity via GABA interneurons.

5-HT is known to also have an important modulatory role on inflammatory response. For example, 5-HT modulates the phenotypic and functional polarization of macrophages. Activation of 5-HT$_{2B}$ receptors, together with the 5-HT7 receptor, contributes to the maintenance of anti-inflammatory response by regulating macrophage and pro-inflammatory expression [54]. It inhibits TLR2, TLR3, and TLR7/8-induced proinflammatory cytokines and chemokines (TNK-α, IL-6, IL-8, IL-10, IL-12) and interferes with the polarization of CD1a^{+} human dendritic cells [55].

This suggests that 5-HT_{2B} not only acts as a neurotransmitter receptor, but also as an important modulator of both innate and adaptive immune responses.

Taken together, genetic studies, both in human and rodents, have allowed understanding better the role of *HTR2B* in the modulation of neuronal and peripheral systems. However, dissecting the specific actions and functionality of 5-HT_{2B} receptors has been challenging due, in part, to the lack of specificity of previous pharmacological agents targeting the 5-HT_{2B} receptor. The recent development of more selective 5-HT_{2B} receptor antagonists (i.e., SB206741) and agonists (i.e., BW723C86) can be used to further dissect the functionality 5-HT_{2B} receptor in both the neuronal and peripheral systems and its role in the modulation of health disorders.

3 The Role of 5-HT_{2B} Receptors in Psychiatric Disorders: A Focus on Aggression

Numerous studies have examined the association between psychiatric traits and 5-HT-related genes, including the *HTR2B*. The *HTR2B* gene has been implicated in impulsivity [35] and aggressive behavior [37] as well as other psychiatric traits including schizophrenia [35], substance abuse [56], personality traits [57], and autism spectrum disorders [58].

3.1 Human Studies

Substantial evidence has shown a crucial role of the 5-HT system in the pathogenesis of psychiatric disorders [59], where 5-HT_{2B} receptors seem to be an important contributor. For example, atypical antipsychotics, a common treatment for schizophrenia, are known to target 5-HT_{2B} receptors including clozapine, amilsulpride, asenapine, aripiprazole, or cariprazine [60–63], suggesting a role of 5-HT_{2B} receptors in the pathogenesis of psychotic disorder and related traits.

Bevilacqua et al. [35] is the first study implicating *HTR2B* in the risk of psychotic disorders, aggressive behavior, drug abuse, and related traits. Sequencing of 5-HT-system genes in a Finnish population of impulsive individuals revealed association between a stop codon in the *HTR2B* gene (*HTR2B* Q20*) and risk of committing violent acts [35]. These individuals exhibit higher prevalence psychosis, early-onset schizophrenia, substance abuse, suicide and depression [35, 64]. In a subsequent study, *HTR2B* Q20* carriers showed increased aggressive behavior, alcohol-related impulsivity, emotional dysregulation, and a passive-aggressive personality structure (i.e., low interest in exploratory activities, anxiety, fear of uncertainty, attachment, or dependence, and low persistence) [36]. Interestingly, homozygous for *HTR2B* Q20* -in males- are born prematurely with a low weight,

but with normal development and cognitive abilities. However, they suffer from alcohol dependence with an early onset and higher tendency to become physically aggressive while under the influence of alcohol [35].

Substance abuse has also been associated with *HTR2B* gene in a linkage scan study of a European American cohort with a history of illegal substance abuse and alcoholism ($n = 110$) [56]. Three single nucleotide polymorphisms (SNPs) mapping to the *HTR2B* region in chromosome 2, two of which result in a double-mutant of the 5-HT$_{2B}$ receptor protein, were associated with illicit substance abuse. Another linkage study in 105 families also identified several SNP markers around this region associated with alcoholism [65]. Further, a genome-scan study of polysubstance abuse in 1004 individuals identified SNP marker WIAF-1700 in the same region of chromosome 2 [66].

5-HT system-related genes, including *HTR2B*, have been implicated in the development of personality traits and novelty seeking [67], traits related to impulsivity and aggressive behavior. A previous study in a Chinese Han population ($n = 473$) examined six *HTR2B* polymorphisms and its association with behavioral inhibition, fun seeking, drive, and reward responsiveness [57]. Four *HTR2B* polymorphisms (rs6437000, rs10194776, rs16827801, and rs1549339) were significantly associated with fun seeking after Bonferroni correction. Another study examined the relationship between *HTR2B* polymorphisms rs10194776 and Q20* identified in previous studies [35, 57] and personality traits in healthy Japanese subjects ($n = 1334$) [68]. A nominal association was identified between *HTR2B* genotype and harm avoidance and self-directedness in females ($p = 0.037$ and $p = 0.043$, respectively), however, these did not survive multiple testing correction. The lack of association may be due to the cohort examined (i.e., healthy subjects) and the selected genetic variants. Studies on cohorts of personality and psychiatric disorders should be evaluated. Further, genome-wide analysis and fine mapping approaches should be considered in order to conduct a more comprehensive evaluation of genetic variants at *HTR2B* and other genes.

Another study evaluated the contribution of 80 genetic variants in 15 5-HT genes in traits related to autism spectrum disorders (ASD) in a Spanish cohort of children and young adults ($n = 141$) [58]. *HTR2B* polymorphism (rs10194776) was significantly associated with intelligence quotation (IQ). A second *HTR2B* polymorphism rs16827801 was also significantly associated with IQ, but also intellectual disability, and language onset delay. These findings suggest a potential role of *HTR2B* polymorphisms in specific traits within ASD, but its relationship with impulsivity- or aggression-related traits should be investigated next since these traits where not evaluated in this cohort, but are known to be somewhat prevalent in this disorder.

Individuals with Tourette syndrome (TS), manifested by motor and phonic ticks, often exhibit increased impulsivity. Dysfunction within the 5-HT system has been associated with TS [69]. A previous study examining mutations in the *HTR2B* gene in 132 individuals of European ancestry and 128 Chinese Han failed to identify genetic variants associated with TS [70]. Larger studies at the genome-wide level are needed to fully test this association.

3.2 Animal Studies

Mice lacking the *Htr2b* gene (*Htr2b*$^{-/-}$) exhibit hyperactivity to novelty environmental exposure, schizophrenia-like behaviors, including sensorimotor gating, selective attention, social interactions, and learning and memory processes [71]. These mice also show increased novelty seeking, hyperlocomotion, and high impulsivity in the delay discounting task [35, 44]. Further, they show decreased dopamine and glutamate neurotransmission in the dorsal striatum. After administration of psychostimulants dizocilpine and amphetamine, *Htr2b*$^{-/-}$ mice show hyperlocomotion and sleep alterations. These schizophrenia-like behaviors are phenocopied in *Htr2b*$^{+/+}$ mice treated with selective 5-HT_{2B} receptor antagonist, RS127445, and rescued after chronic haloperidol treatment.

An animal study mapping quantitative trait loci (QTLs) associated with intermale aggression examined 457 males from 55 strains in the resident-intruder paradigm following 10 days of isolation [72]. They identified a significant QTL on chromosome 1 that mapped a gene-sparse region between 82 and 88 Mb with the C57BL/6 J allele increasing aggression, measured as the latency to attack and number of total attacks. This association explained about 18% of the variance observed. In this region, *Hrt2b* gene that encodes the 5-HT_{2B} receptor, is the strongest candidate to drive the association with aggressive behavior given its a *priori* evidence. Mutations at the *Htr2b* gene are also linked to aggressive behavior in other species, including pigs [73] and zebrafish [74].

4 5-HT_{2B} Receptor as a Modulator of Aggression and Drug Abuse

Aggressive behavior is influenced by a combination of genetic and environmental factors, including substance use. A high proportion of all crimes are committed under the influence of substances of abuse [75, 76]. Studies in humans and animal models have established the relationship between 5-HT_{2B} receptor and aggressive behavior and drug abuse. These are observed in many types of drugs of abuse, including alcohol, cocaine, cannabis, and methamphetamine.

4.1 Alcohol

Antisocial alcoholic violent offenders show high novelty seeking, harm avoidance and reward dependence [76], thus showing that these personality features can predict risk-behavior under the influence of alcohol. The association between impulsivity or aggressive behavior and *HTR2B* is also mediated, in part, by alcohol use. Individuals with a *HTR2B* Q20* under the influence of alcohol exhibit increased

risk-taking behaviors; they show frequent aggressive out-bursts and increased impulsivity, are arrested for driving under the influence more often, and have a tendency to lose behavioral control [36]. They do not endorse alcohol dependence diagnosis, assessed by average alcohol consumption, but have a tendency to lose control while under the influence of alcohol [35]. Interestingly, homozygous for *HTR2B* Q20* show an early-onset alcohol dependence and alcohol-related aggressive behavior.

Epigenetic mechanisms also influence the 5-HT system by mediating gene regulation. DNA methylation, one of the most commonly studied epigenetic mechanisms, is altered in genes involved in the 5-HT system. DNA methylation site at *HTR2B* gene shows increased methylation in alcohol dependent individuals of European ancestry compared to controls ($p < 0.001$) [77]. Further, DNA methylation sites at the *HTR2B* gene are differentially methylated in whole blood cells from individuals with comorbid alcohol and nicotine dependence [78]. This effect was population specific: hypomethylation of one CpG site (cg27531267, $p = 7.2 \times 10^{-5}$) is observed in African Americans, whereas hypermethylation was observed in 16 CpG sites ($p_{range} = 10^{-9} – 10^{-5}$) in European Americans.

4.2 Cocaine

Cocaine abuse is commonly associated with aggressive behavior in both humans and animals. Cocaine addicts show increased frequency of violent acts [79]. These effects are partly mediated by the 5-HT system in the brain [80]. In mice, cocaine increases locomotor activity and defensive response to a predator [81]. Cocaine-induced locomotor activity is doubled in *Htr2b*$^{-/-}$ mice and after chronic 5-HT$_{2B}$ receptor inhibition [82]. In humans, imaging studies have shown that cocaine abusers exhibit a dampened neuronal activity and decreased dopamine efflux in the nucleus accumbens [83], part of the brain reward system. Similarly, *Htr2b*$^{-/-}$ mice show a decreased activity in dopaminergic neurons in the nucleus accumbens [84]. 5-HT$_{2B}$ receptor binding can modulate dopamine efflux in neurons, dampening 5-HT levels in the nucleus accumbens. Absence of 5-HT$_{2B}$ receptor can mimic the neuronal maladaptations observed in drug abusers, thus individuals that carry a genetic mutation that disrupts 5-HT$_{2B}$ receptor's functionality may be more vulnerable to becoming addicted.

Cocaine and/or crack users exhibit higher levels of impulsivity [85] and a high prevalence (20–25%) of comorbid attention deficit hyperactivity disorder (ADHD) [86]. These individuals also show a higher motor impulsivity when compared with those without ADHD [87]. A recent study in a small cohort of French Afro-Caribbean males ($n = 140$), a polymorphism at *HTR2B* (rs6736017) was associated with crack use disorder [88]. This association seems to be independent of ADHD or impulsivity; however, this may be due to lack of power given the small sample size of the cohort studied.

4.3 Cannabis

Cannabis, one of the most widely used drug of abuse worldwide, has been linked to increased impulsivity [89] and decreased behavioral inhibition [90, 91]. The relationship between cannabis use and aggression has been previously established [28, 92–94]. Studies have found that cannabis use is associated with a seven-fold risk of subsequent violent and aggressive behavior [28]. Further, a recent finding from the McArthur Risk Assessment study show that continuity of cannabis use is associated with increased risk of future violent behavior (n = 1136, OR = 2.44) [94].

A recent GWAS of aggressive behavior under the influence of cannabis identified a significant genetic variant mapping to the *HTR2B* (*HTR2B**rs177440378, $p = 2.16 \times 10^{-8}$) in an African American cohort ($n = 2587$) [37]. Figure 17.3a depicts a regional Manhattan plot showing the association between *HTR2B**rs17440378 and cannabis-related aggression. *HTR2B**rs17440378 also showed nominally significant association in the Grady Trauma Project cohort (p = 0.04, n = 89) with aggressive behavior in individuals with a lifetime prevalence of cannabis use. The risk effect of *HTR2B**rs17440378 is specific to cannabis-related aggression and not driven by cannabis or drug dependence alone, aggressive behavior, or aggression under the influence of other drugs. Further, *HTR2B**rs17440378 show important functional regulatory effects across several brain regions (e.g., medulla, substantia nigra, and putamen) and peripheral tissue.

These findings in humans were validated in an animal model (Fig. 17.3b). Using the resident intruder paradigm, a well-known behavioral assay for aggressive

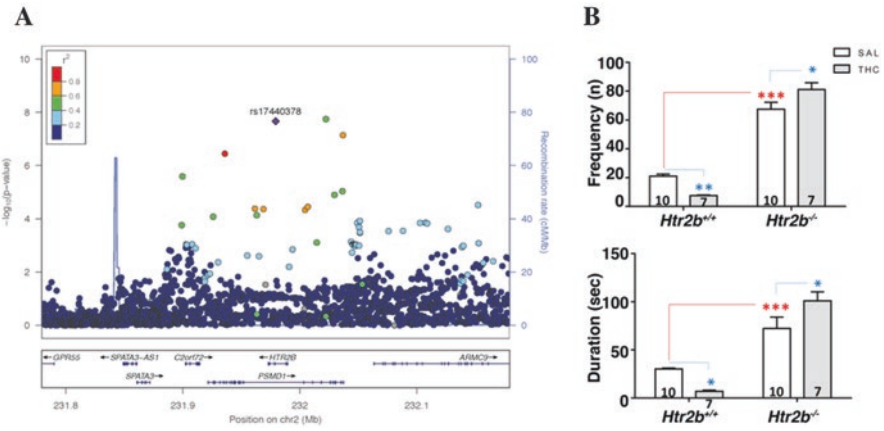

Fig. 17.3 Association of *HTR2B* and Cannabis-Related Aggression. (**a**) In humans, genetic variants mapping to *HTR2B* gene are associated with cannabis-related aggression. A regional Manhattan plot depicts the genome-wide significant SNPs mapping to *HTR2B* associated with cannabis-related aggression. (**b**) In mice, aggressive behavior is increased in $Htr2b^{-/-}$ group treated with THC, an active component of cannabis, as measured in the resident intruder paradigm. In contrast, THC decreases aggression in $Htr2b^{+/+}$ compared to saline-treated $Htr2b^{+/+}$ mice. * and *** represents $p < 0.05$ and 0.001, respectively. *Adapted from* [37]

behavior, *Htr2b*$^{-/-}$ mice exhibit increased aggressive responses and decreased social interaction [37]. After THC administration, an active component of cannabis, *Htr2b*$^{-/-}$ mice show a greater aggressive response than those receiving saline. Interestingly, *Htr2b*$^{+/+}$ showed decreased aggressive response after THC when compared to *Htr2b*$^{+/+}$ receiving saline. THC administration induces an opposite effect on aggressive response as a function of genotype, thus suggesting an important modulatory role of *HTR2B* in aggressive behavior and drug abuse.

Personality traits contribute to the relationship between aggressive behavior and cannabis use via genetic factors. By using polygenic risk score (PRS) analysis, a genetic overlap was identified between cannabis-related aggression and personality traits, specifically extraversion [37]. Higher extraversion is associated with greater risk for aggressive behavior under the influence of cannabis. Extraversion is positively associated with externalizing behavior [95], excitement- and attention-seeking behavior, as well as social and interpersonal dysfunction [96].

Additional evidence suggests that endocannabinoids and 5-HT functionally interact to modulate physiological and pathological functions in the brain, such as food intake, pain, drug addiction, depression, anxiety, and epilepsy [97–100]. Previous studies have also found a link between cannabinoid system and 5-HT_{2B} receptor. In mice, cannabinoid type 1 receptor (CB1 receptor) impairs 5-HT negative feedback and alters the expression and functionality of 5-HT_{2B} receptors in several brain regions [101]. Further, pharmacological co-activation of CB1 and 5-HT_{2B} receptors is essential for anti-epileptic effect, while single antagonism of either CB1 or 5-HT_{2B} receptor can block it [100]. It is possible that these two receptors may physically interact leading to a heteromer which, when activated, induces antiepileptic effects. The potential role of the interaction between the cannabis-related signaling and 5-HT needs to be further investigated in the context of aggressive behavior and drug abuse.

4.4 Other Drugs

MDMA, or 'ecstasy', has been associated with aggressive behavior, specifically with long-term exposure. In mice, MDMA inhibits social behavior in a dose-dependent manner (for Review, [102]). Further, in humans, chronic users of MDMA exhibit increased aggressive behavior, which may be mediated by MDMA pharmacological effects [103]. MDMA selectively binds to and activates 5-HT_{2B} receptors in mouse neuronal cells. This induces 5-HT in the raphe nuclei, thus leading to dopamine release in the nucleus accumbens and ventral tegmentum [44]. The 5-HT_{2B} receptor modulates the behavioral and molecular effects of MDMA. Genetic ablation or pharmacological inhibition of 5-HT_{2B} receptors in mice can eliminate MDMA-induced hyperlocomotion, sensitization, and conditioned place preference and 5-HT release [44, 104]. This effect may be mediated by blocking MDMA-induced 5-HT outflow in the nucleus accumbens and ventral tegmental area, brain regions involved in the brain reward system.

Psychoactive drugs, such as lysergic acid diethylamide (LSD) and many other hallucinogens, are known to act on 5-HT receptors. A recent study revealed a physical and functional interaction between LSD and 5-HT_{2B} receptors, suggesting a major role of this receptor in the modulation of LSD's psychoactive properties [105]. However, its potential role in aggressive behavior and drug abuse remains to be elucidated.

5 Conclusion

Evidence from human and animal studies implicate 5-HT in the etiology of psychiatric traits, including aggressive behavior and drug abuse. The 5-HT_{2B} receptor, both at the protein and gene levels, plays a critical role in the modulation of aggressive behavior and related traits. These findings suggest that therapeutic interventions targeting these receptors, such as 5-HT_{2B} receptor antagonists, could be useful in the treatment of psychiatric disorders and drug addiction. Future studies in animal models and human clinical studies should focus on the use of selective pharmacological agents of 5-HT_{2B} receptor. Human genetic studies using hypothesis-free approaches such as GWAS and fine mapping, should investigate the potential role of *HTR2B* genetic variants. Further, Mendelian randomization method could be used to dissect the potential causal effects of *HTR2B* genetic variants in the etiology of psychiatric traits and disorders, such as aggressive behavior and drug abuse. These studies will enhance our understanding of the mechanisms that underlie psychiatric disorders and further dissect the role of the 5-HT_{2B} receptor in the modulation of aggressive behavior and related traits.

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Chapter 18
Role of the Serotonin 2B Receptor in the Reinforcing Effects of Psychostimulants

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Abbreviations

MDA	3,4-methylenedioxyamphetamine
MDMA, Ecstasy	3,4-methylenedioxymethamphetamine
DA	Dopamine
DAT	Dopamine transporter
DRN	Dorsal raphe nucleus
GAD-67	Glutamate decarboxylase 67
MSN	Medium-size spiny neuron
NET	Noradrenaline transporter
NAC shell	Nucleus accumbens shell
PKC	Protein kinase C
PKG	Protein kinase G
SERT	Serotonin transporter
SSRIs	Selective serotonin reuptake inhibitors
TPH2	Tryptophan hydroxylase 2
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental area

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1 Introduction

Pioneer investigations have failed to detect the expression of the 5-HT_{2B} receptor in the rat brain [1–3], however, more recent studies have provided compelling evidence confirming, albeit at low levels, a broad expression of 5-HT_{2B} mRNA in various regions of the human, mouse, and rat brain. These brain regions include the dorsal raphe nucleus (DRN) [4–6], ventral tegmental area (VTA) [7], hippocampus, locus coeruleus, habenula, paraventricular nucleus of the hypothalamus [4], frontal and occipital cortices, and cerebellum [8, 9]. In the mouse VTA, expression of 5-HT_{2B} receptor mRNA was found in dopamine (DA) mesolimbic neurons projecting to the nucleus accumbens shell (NAC shell) [7]. In the DRN, the 5-HT_{2B} receptor mRNA was found in $PET1^{+}$/$TPH2^{+}$ 5-HT neurons in mice [9] and in glutamate decarboxylase 67 (GAD67)-positive neurons in rats [6] suggesting expression of the 5-HT_{2B} receptor in GABAergic neurons, although these neurons could be 5-HT neurons co-expressing a GABAergic phenotype [10–12]. At a protein level, the presence of the 5-HT_{2B} receptors was further confirmed in the frontal cortex, lateral septum, medial amygdala, dorsal hypothalamus and cerebellum in rats [13], and in the cerebellum, and hippocampus in mice [14]. Functional studies have finally revealed a pivotal role of 5-HT_{2B} receptors in the regulation of both 5-HT and DA neuron activity and the modulation of the reward system, including in the molecular and behavioral responses to psychostimulants such as amphetamine, the amphetamine- derivative MDMA, and cocaine [7, 15–20].

2 Role of 5-HT_{2B} Receptors in the Psychostimulant Effects of the Club Drug MDMA (Ecstasy)

The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) is a psychoactive drug that alters sensations and increases energy, empathy, and pleasure. As an amphetamine derivative, MDMA binds to, and is a substrate of, the membrane serotonin transporter (SERT), which causes an increase in extracellular 5-HT by potent inhibition of 5-HT uptake [21]. In addition, MDMA enters the synaptic vesicles and disrupts the activity of the vesicular monoamine transporter (VMAT) [22], which causes the depletion of 5-HT storage by reversing VMAT2 function [23]. This elevates the cytosolic concentration of 5-HT and reverses the activity of the SERT [24, 25], resulting in an exocytosis-independent release of 5-HT from the axon terminals [26]. As genetic ablation of the SERT or its blockade by selective serotonin reuptake inhibitors (SSRIs) has been shown to abolish or reduce the psychostimulant effects of MDMA in mice and rats, respectively [27–31], the SERT-mediated release of 5-HT and the subsequent activation of 5-HT receptors has been proposed to mediate the behavioral responses to MDMA (Fig. 18.1).

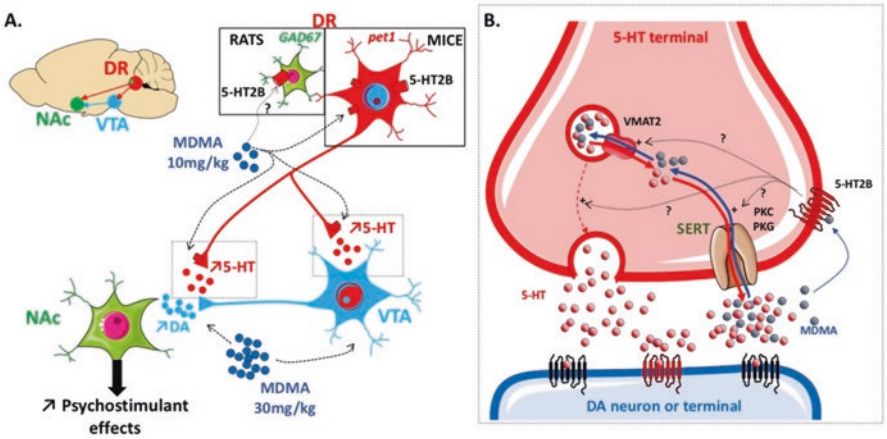

Fig. 18.1 Role of 5-HT_{2B} receptors in the effects of MDMA. (**a**) The 5-HT_{2B} receptors located in the DR 5-HT neurons (mice) positively modulate 5-HT release in the VTA and NAC in response to MDMA (10 mg/kg), resulting in a positive control of DA release in the NAC and the production of psychostimulant effects. The psychostimulant effects of higher doses of MDMA are independent of 5-HT_{2B} receptor signaling. In rats, 5-HT_{2B} receptors may be located in DR GAD67 interneurons, but their contribution to the psychostimulant effects of MDMA is uncertain. (**b**) MDMA binds to the SERT, is transported into the cytosol of 5-HT terminals and then transported into the synaptic vesicles through the VMAT2, where it chases 5-HT out of the vesicles and triggers a reverse transport mechanism by the SERT to release 5-HT in the synaptic cleft. The mechanism by which the 5-HT_{2B} receptor controls MDMA-induced 5-HT release is uncertain but could involve the regulation of VMAT2, SERT function or vesicular release, following direct (MDMA) or indirect (5-HT) stimulation

In serotonergic neuronal cells and primary neurons from the DRN, the transport of serotonin and the activity of the SERT are regulated by 5-HT_{2B} receptor-induced phosphorylations by the protein kinases C (PKC) and G (PKG) [32]. Since the mechanism by which amphetamines and amphetamine-derivatives like MDMA produce the reverse transport/efflux of 5-HT has been shown to be mediated, at least in part, by PKC-dependent phosphorylations of the SERT N-terminus [33–39], it has been hypothesized that 5-HT_{2B} receptor could also regulate MDMA-induced efflux of 5-HT. In addition, at recreational doses, MDMA and its metabolite 3,4-methylenedioxyamphetamine (MDA) preferentially bind to and activate the human 5-HT_{2B} receptor [40]. This suggests that the behavioral effect of MDMA may be mediated, at least in part, by the stimulation of 5-HT_{2B} receptors. Indeed, the essential contribution of the 5-HT_{2B} receptors to the psychostimulant effects of MDMA (10 mg/kg) was further confirmed with mice genetically ablated for the 5-HT_{2B} receptor (*Htr2b*$^{-/-}$) or WT mice pretreated with the selective 5-HT_{2B} antagonist RS127445 (0.5 mg/kg, i.p.) showing an absence of MDMA-induced hyperlocomotion [16], locomotor sensitization and conditioned place preference [15]. These findings are likely due to the abolition of MDMA-induced extracellular release of 5-HT in the VTA and NAC, and release of DA in the NAC [16], accompanied by an absence of MDMA-induced ERK1/2 phosphorylation in D1 receptor-containing

striatonigral medium-sized spiny neurons (MSNs) of the NAC shell [15]. Although these results suggest a pivotal role of the stimulation of 5-HT_{2B} receptors located on raphe 5-HT neurons in mediating MDMA-induced 5-HT (and DA) release and the subsequent psychostimulant and reinforcing effects of MDMA (10 mg/kg, i.p.) [15, 16], it is likely that a higher dose of MDMA (30 mg/kg, i.p.) acts independently of 5-HT_{2B} receptor activation to produce its effects (Fig. 18.1).

The detection of 5-HT_{2B} receptor mRNA expression in the raphe nuclei of WT mice supports the idea that the 5-HT_{2B} receptor may exert its control on 5-HT release at a presynaptic level. In line with this, the contribution of 5-HT_{2B} receptors to MDMA-induced 5-HT release was demonstrated in vitro in superfused midbrain synaptosome preparations [16]. In vivo, reverse microdialysis experiments in the raphe nuclei further revealed that the stimulation of the 5-HT_{2B} receptor by the preferential agonist BW723C86 produces an increase in 5-HT extracellular levels, which was blocked by the pretreatment with the selective 5-HT_{2B} receptor antagonist RS127445 [16]. Using a conditional knock-out mouse model in which 5-HT_{2B} receptors were genetically ablated from PET1-dependent 5-HT neurons, more recent work from our group not only confirmed the requirement of 5-HT_{2B} receptors located in 5-HT neurons to elicit MDMA-induced hyperlocomotion and locomotor sensitization, but further revealed that the 5-HT_{2B} receptor exerts a positive autoregulation of PET1-dependent 5-HT neuron firing activity, likely by counteracting 5-HT_{1A}-negative autoreceptor action [17, 41]. While there is evidence to suggest that 5-HT_{2B} receptors regulate the exocytic 5-HT release, as shown for the antidepressant and neurogenic responses to SSRIs [5, 17, 42], the exact mechanism by which 5-HT_{2B} receptors control MDMA-induced 5-HT release is not fully understood, and a concomitant modulation of MDMA-induced non-exocytotic/carrier-mediated 5-HT release by 5-HT_{2B} receptors cannot be ruled out in mice.

There are limited studies investigating the role of 5-HT_{2B} receptors in response to MDMA in rats. In synaptosomal preparations from whole brain minus caudate nucleus, MDMA-induced 5-HT release was unaffected by 5-HT_{2B} receptor activation by the preferential agonist BW783C86, or by its inhibition by the selective antagonist SB204741 [43]. Similarly, pretreatment with the 5-$HT_{2C/2B}$ antagonist SB-206553 (2 mg/kg) prior to MDMA (5 mg/kg) had no effect on MDMA-induced hyperlocomotion or increased striatal activity [44]. A possible explanation could be that in the rat DRN expression of the 5-HT_{2B} receptor has been observed in GAD-67-expressing neurons, where it exerts an inhibitory control on DR 5-HT neurons [6]. GAD-67 is the enzyme that produces GABA from glutamate, and therefore, its expression is attributed to GABAergic neurons, suggesting that the 5-HT_{2B} receptor is expressed in rat raphe GABAergic interneurons [6]. However, there is evidence to show that a subset of DR 5-HT neurons co-express tryptophan hydroxylase 2 (TPH2) and GAD-67, in mice [45] and rats [10, 46]. Therefore, it is possible that Cathala et al., [6] have detected the 5-HT_{2B} receptor mRNA in GAD-67^{+}/5-HT^{+} neurons rather than true GABA interneurons. Hence, whether this absence of effect of the manipulation of 5-HT_{2B} receptor activity on MDMA-induced 5-HT release in rats underlies true interspecies differences in 5-HT_{2B} receptor localization/function or rather relies on methodological variations needs to be further investigated (Fig. 18.1).

3 Role of 5-HT_{2B} Receptors in the Psychostimulant Effects of Amphetamine

Unlike MDMA that primarily modulates 5-HT neurotransmission by preferentially targeting the SERT, amphetamine preferentially acts on the DA transporter (DAT) to modulate DA neuron function in the dorsal and ventral striatum in a similar manner (for review, see [47]). Pioneer microdialysis studies in halothane- or chloral hydrate-anesthetized rats have revealed that blockade of the 5-HT_{2B}/$_{2C}$ receptors by the non-selective antagonist SB 206553 (5 mg/kg i.p.) potentiates basal DA outflow in the striatum and NAC [48–51] and this 5-HT_{2B}/$_{2C}$-dependent potentiation of basal DA outflow was confirmed in awake rats [52]. Amphetamine (2 mg/kg i.p.)-induced increase in DA release in the nucleus accumbens and striatum was however not affected [51].

Later, using more selective antagonists, the same group identified that the 5-HT_{2B} receptor modulates basal and amphetamine-stimulated DA outflow in ventral striatum (nucleus accumbens, NAC shell) but not in dorsal striatum of halothane-anesthetized rats [19]. In this study, the pharmacological blockade of 5-HT_{2B} receptor function by the selective antagonists LY266097 (0.63 mg/kg, i.p.) and RS127445 (0.16 mg/kg, i.p.) reduced the extracellular levels of DA in the NAc, up to 2 h after the injection, while stimulation of 5-HT_{2B} receptors by the preferential agonist BW723C86 (3 mg/kg, s.c.) had no effect [19]. This suggests that the 5-HT_{2B} receptor exerts a tonic facilitatory control on basal DA release. As a result, pretreatment with LY266097 (0.63 mg/kg, i.p.) prior to amphetamine significantly decreased DA release in the NAC induced by amphetamine (0.5 mg/kg, i.p.), and reduced the hyperlocomotion elicited by amphetamine (1 mg/kg, i.p.) [19], suggesting that 5-HT_{2B} receptor signaling *positively* modulates amphetamine-induced DA release in the NAC, as well as the subsequent amphetamine-induced hyperlocomotion (Fig. 18.2).

However, opposite effects on amphetamine-induced hyperlocomotion were found in mice genetically ablated for the 5-HT_{2B} receptor gene (*Htr2b*$^{-/-}$ mice), with an increased locomotor hyperactivity observed after amphetamine exposure (3 mg/kg, i.p.—unpublished data) and 10 mg/kg, i.p. [18], as compared to wildtype (WT) littermate controls. In addition, following re-exposure to amphetamine (3 mg/kg, i.p.), 7 days after the first exposure, *Htr2b*$^{-/-}$ mice did not show any locomotor sensitization (unpublished data). Unlike data in rats, these results rather support that 5-HT_{2B} receptor signaling exerts a negative modulation on amphetamine-induced hyperlocomotion in mice [18]. Since amphetamine-induced hyperlocomotion has been strongly correlated with the level of extracellular release of DA in the NAC shell [53], it is likely that 5-HT_{2B} receptors contribute in the negative regulation of DA signaling in the NAC shell. However, further investigations are needed to determine whether the negative modulatory role played by 5-HT_{2B} receptors on amphetamine-induced hyperlocomotion is accompanied by negative control of DA release in the NAC or the striatum in mice (Fig. 18.2). Although rats and mice studies have revealed fundamental discrepancies regarding the positive *vs.* negative

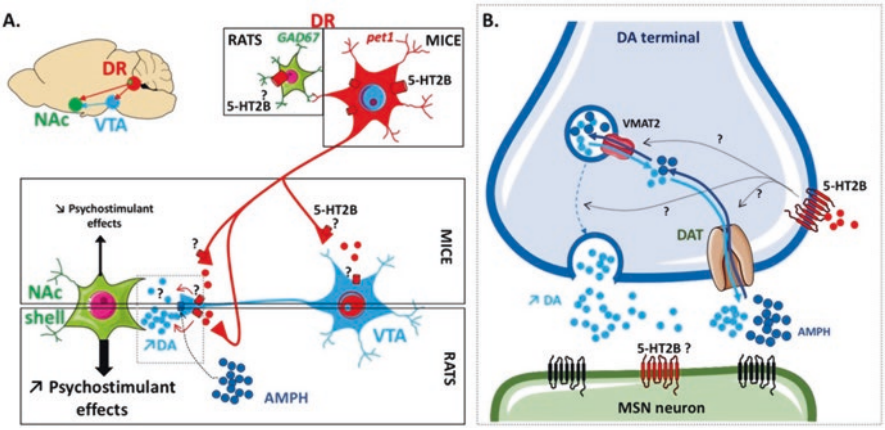

Fig. 18.2 Role of 5-HT_{2B} receptors in the effects of amphetamines. (**a**) Opposite effects have been observed in mice and rats. In mice, the 5-HT_{2B} receptor negatively modulates the psychostimulant effects of amphetamine. The cellular localization (5-HT and DA neuron or terminals) of the 5-HT_{2B} receptors involved in these effects as well as their role in the regulation of DA neurotransmission are unknown. In rats, 5-HT_{2B} receptors, likely located onto DA terminals in the NAC, positively modulate the psychostimulant effects of amphetamine and the concomitant release of DA in the NAC shell. (**b**) Similar to the 5-HT release produced by MDMA, amphetamine increased the release of DA onto the MSNs in the NAC shell via reverse transport of DA through the DAT. In both mice and rats, the exact localization and role of 5-HT_{2B} receptors in the modulation of amphetamine-induced DA release remain to be characterized

modulatory role potentially played by 5-HT_{2B} receptors on amphetamine-stimulated hyperlocomotion, it is likely that the 5-HT_{2B} receptors are somehow involved in the regulation of dopaminergic neurotransmission in both mouse and rat brain reward pathway [54].

4 Role of 5-HT_{2B} Receptors in the Psychostimulant and Rewarding Effects of Cocaine

Cocaine binds with similar affinity to the DAT, SERT and noradrenaline transporter (NET) and blocks the reuptake of these monoamines. While the contribution of the 5-HT_2 receptors, notably the 5-$HT_{2A/2C}$ receptors, has been well established (for review see [55]), there is a limited number of studies demonstrating the involvement of the 5-HT_{2B} receptors in the psychostimulant and rewarding effects of cocaine.

In rats, pioneer pharmacological studies have failed to identify the involvement of 5-HT_{2B} receptors in the discriminative stimulus effects of cocaine [56] but have suggested a role for 5-HT_{2B} receptors in cocaine-induced hyperlocomotion [57]. Pretreatment with lower doses of the 5-$HT_{2B/2C}$ receptor antagonist SB206553 (1 and 2 mg/kg, i.p.) reduced, while a higher dose (4 mg/kg, i.p.) increased cocaine-induced hyperactivity. The increased cocaine-induced hyperlocomotion following

higher doses of SB206553 pretreatment is likely due to the blockade of 5-HT_{2C} receptor activity [58–60] and the subsequent potentiation of cocaine-induced release of dopamine in the NAC and striatum [48, 61], however, the decrease in cocaine-induced hyperlocomotion produced by lower doses of SB206553 could be mediated by blockade of 5-HT_{2B} receptors. In line with this, pretreatment with the selective 5-HT_{2B} receptor antagonists RS127445 (0.16 mg/kg, i.p.) and LY266097 (0.63 mg/kg, i.p.), reduces cocaine (10 mg/kg, i.p.)-induced hyperlocomotion [20]. Although pharmacological blockade of 5-HT_{2B} receptor function reduces the basal extracellular release in the NAC shell, it appears that the reduction in hyperlocomotion was independent of an alteration in cocaine-induced DA release in the NAC shell, core or dorsal striatum [20]. Instead, 5-HT_{2B} receptor-mediated reduction of cocaine-induced hyperlocomotion seems to occur downstream to DA neurons. Indeed, 5-HT_{2B} blockade by RS127445 (0.16 mg/kg, i.p.) also reduces the late-onset hyperlocomotion induced by the dopamine D2 receptor agonist quinpirole (0.5 mg/kg, s.c.) [20], a dose known to elicit hyperlocomotion [62, 63], independently of changes in NAC DA outflow [64], supposedly via its action on postsynaptic D2 receptors [65]. These results therefore suggest that in rats, besides its positive regulation of basal and stimulated DA outflow in the NAC shell, the 5-HT_{2B} receptor might exert part of its positive modulatory role on cocaine-induced hyperlocomotion by modulation D2-containing postsynaptic neurons in the dorsal and/or ventral striatum [20]. This could however result from an indirect action involving the modulation of 5-HT neurotransmission/5-HT transport [32] by 5-HT_{2B} receptors located in the terminals of DR 5-HT neurons. Indeed, the blockade of the 5-HT_{2B} receptor-mediated positive autoregulation of 5-HT neuron activity would result in a decreased serotonergic tone [17], similarly to the stimulation of 5-HT_{1A} autoreceptors by low doses of the agonist 8-OH-DPAT (0.2–0.3 mg/kg, i.p.), which has been demonstrated to eliminate cocaine-induced hyperlocomotion in rats [66, 67] without affecting cocaine-induced DA turnover in the NAC. Further studies are however warranted to clarify the exact location and function of 5-HT_{2B} receptors in the modulation of the psychostimulant effects of cocaine in rats (Fig. 18.3).

Again, an opposite effect has been observed in mice, with 5-HT_{2B} receptors exerting a negative modulation of cocaine-induced hyperlocomotion. Mice genetically ablated for the 5-HT_{2B} receptors (*Htr2b*$^{-/-}$) hence display an increased hyperlocomotion and locomotor sensitization following cocaine (7.5, 15 and 20 mg/kg, i.p.) [7]. Interestingly, the increased hyperlocomotor effects of cocaine (15 mg/kg, i.p.) were not mimicked by acute pharmacological blockade with the 5-HT_{2B} antagonist RS127445 (0.5 mg/kg, i.p.) or the 5-$HT_{2B/2C}$ antagonist SB206553 (3 mg/kg, i.p.), but were reproduced by a chronic 4 week-treatment with RS127445 (1 mg/kg/day), suggesting that enhanced locomotor response and sensitization to cocaine is due to long-term neuroadaptations resulting from genetic/developmental (*Htr2b*$^{-/-}$) or sustained pharmacological (4 weeks) ablation of 5-HT_{2B} receptors [7]. Similar to the aforementioned findings in rats showing a dissociation between cocaine-induced locomotor activity and changes in DA release in the NAC shell, mice lacking the 5-HT_{2B} receptors display an increased cocaine-induced hyperlocomotion with abolished cocaine-induced DA-release but unaltered 5-HT-release, in the NAC shell [7].

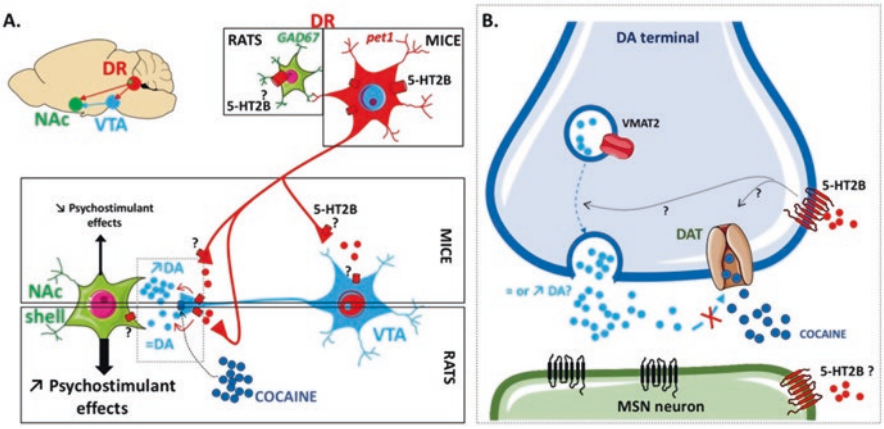

Fig. 18.3 Role of 5-HT_{2B} receptors in the effects of cocaine. (**a**) Distinct involvements of mouse and rat 5-HT_{2B} receptors in the psychostimulants effects of cocaine and the concomitant DA release in the NAC shell. In mice, 5-HT_{2B} receptors located in mesolimbic DA neurons projecting to the NAC shell, either in VTA cell bodies or axon terminals in the NAC, negatively regulate the psychostimulants effects of cocaine, but positively modulate cocaine induced-DA release in the NAC shell. In rats, the 5-HT_{2B} receptors, located on DA terminals or downstream to DA neurons, do not modulate cocaine-induced DA release in the NAC shell but positively modulate the psychostimulant effects of cocaine. (**b**) Cocaine produces an extracellular release of DA onto the MSNs of the NAC by blocking the reuptake function of the DAT. The 5-HT_{2B} receptor located in DA neurons positively modulates the release of DA in the NAC shell by a mechanism that remains to be described, but negatively modulates cocaine psychostimulant effects (mice). In rats, the 5-HT_{2B} receptor positively modulate the psychostimulant effects of cocaine independently of any modulation of DA release. Therefore, a contribution of 5-HT_{2B} receptors located downstream to DA neurotransmission in the psychostimulant effects of cocaine cannot be ruled out

As a result, cocaine-induced ERK1/2 phosphorylation within MSNs, a marker of DA-dependent D1-receptor stimulation and an essential component of signaling pathways underlying the long-term behavioral effects of drugs of abuse [68], was markedly reduced in the NAC shell of *Htr2b*$^{-/-}$ mice, although D1-dependent hyperlocomotion and locomotor sensitization are upregulated in these mice, with no change in D1 protein expression, receptor binding or G-protein coupling [7]. This result corroborates the idea that the locomotor and reinforcing effects of psychostimulants are not exclusively mediated by DA neurotransmission in the NAC shell. Conversely, the reduction of DA neurons sensitivity to substances of abuse following repeated exposures is the hallmark of addiction, and therefore suggests that *Htr2b*$^{-/-}$ mice display a phenotype similar to cocaine-exposed animals [7]. This was further evidenced by an enhanced inhibitory effect of cocaine on VTA DA neuron firing, accompanied by increased AMPA to NMDA receptor-mediated current ratio in these mice, which suggests that 5-HT_{2B} receptors act as an important factor for the prevention of drug-evoked synaptic plasticity [7] (Fig. 18.3).

Unlike the presynaptic 5-HT_{2B} receptor-dependent psychostimulant effect of low dose of MDMA (10 mg/kg) [17], the increased psychostimulant effects of cocaine

in *Htr2b*$^{-/-}$ mice is likely due to the absence of postsynaptic 5-HT_{2B} receptors located in mesolimbic DA neurons projecting to the NAC shell. Indeed, a similar increase in cocaine-induced hyperlocomotion and locomotor sensitization was observed in a conditional knockout mouse model where 5-HT_{2B} heteroreceptors receptors were selectively ablated in DAT-expressing DA neurons [7] (Fig. 18.3), which was unaffected by conditional ablation of 5-HT_{2B} autoreceptors in PET1^{+}-5-HT neurons (unpublished data).

Similar to the response to amphetamine, studies of the role of 5-HT_{2B} receptors in the psychostimulant effects of cocaine has yielded conflicting results between mice and rats. Although this could be due to the experimental design between studies, in which the 5-HT_{2B} receptor was targeted acutely (rats) or chronically/genetically (mice), further studies are needed to elucidate the role played by 5-HT_{2B} receptors in the psychostimulant effects of cocaine.

5 Conclusion

The role of brain 5-HT_{2B} receptors in the locomotor and reinforcing effects of psychostimulant remains controversial between mice and rats and therefore is still poorly understood. In mice, it appears that 5-HT_{2B} receptors are involved in the control of both 5-HT and DA ascending pathway neurotransmission. Studies from knockout and conditional knockout mice have demonstrated that presynaptic 5-HT_{2B} receptors located in DRN 5-HT neurons exert a facilitatory action on 5-HT and DA release, hyperlocomotion and locomotor sensitization in response to 5-HTergic psychostimulants such as MDMA, but postsynaptic 5-HT_{2B} receptors, likely located on DA mesolimbic neurons projecting to the NAC shell, exert an inhibitory action on DA release, hyperlocomotion and locomotor sensitization in response to DAergic psychostimulants such amphetamine and cocaine. In rats, however, it appears that 5-HT_{2B} receptors play only a minor role in the psychostimulant response to MDMA, but on the other hand facilitate the basal and amphetamine-stimulated (but not cocaine-stimulated) release of DA in the NAC shell and hyperlocomotion in response to both amphetamine and cocaine. Such opposite effects between mice and rats are striking and could underlie profound interspecies differences in the expression/function of the 5-HT_{2B} receptors, hence making it difficult to devise new pharmacotherapeutics to either inhibit or activate the 5-HT_{2B} receptors as treatment for psychostimulant addiction. Further studies are required to determine the role of 5-HT_{2B} receptors (facilitation or inhibition) in the reinforcing effects of psychostimulants in humans.

Impulsivity or lack of inhibitory control is a hallmark of addictive and relapsing behaviors, especially in subjects with a history of dependence to psychostimulants (for recent review see [69]). In both mice and humans, loss of 5-HT_{2B} receptor function produces impulsive behaviors [8, 9], suggesting that 5-HT_{2B} agonists might represent an effective treatment for psychostimulant abuse and relapse. However, drugs stimulating the 5-HT_{2B} receptor are known to elicit cardiac valvulopathy and

pulmonary hypertension [40, 70–75], which exclude their use as a therapeutic strategy.

Since the 5-HT_{2B} and 5-HT_{1A} receptors seem to play a bidirectional regulatory function, at least in mouse DRN 5-HT neurons, the aforementioned interspecies differences could be similar to those observed in the 5-HT_{1A} receptor localization and function between mice and rats. For instance, stimulation of 5-HT_{1A} receptor with agonists is known to elicit hypothermia in both mice and rats, however, this effect seems to be mediated by presynaptic 5-HT1A autoreceptors in mice and humans, and by postsynaptic 5-HT_{1A} heteroreceptors in rats, therefore supporting the idea interspecies differences may exist at a 5-HT receptors level. In line with this, the 5-HT_{2B} receptor has been localized in DRN 5-HT neurons in mice but proposed to be located on DRN GABA interneurons in rats. While this could explain some of the observed differences, further investigation is warranted to elucidate whether the observed differences in 5-HT_{2B} receptor-mediated regulation of 5-HT and DA neurotransmission results from methodological or interspecies variations. Unlike the studies in mice, microdialysis data in rats were principally collected in halothane-anesthetized animals, which has been shown to enhance DA metabolism [76], alter D2 autoreceptor signaling [77] and compete with cocaine binding sites [78], which ultimately leads to the potentiation of the effects of DAT blockers and amphetamine-derivatives on extracellular DA levels [79, 80]. This suggests that the observed differences might be the result of a combination of methodological and interspecies factors, and therefore highlights the need to perform more comprehensive comparative studies between mice and rats prior to proposing 5-HT_{2B}-targeting therapeutics for the treatment of psychostimulant addiction.

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Chapter 19
Serotonin 2B Receptor Interactions with Dopamine Network: Implications for Therapeutics in Schizophrenia

Adeline Cathala and Umberto Spampinato

Abbreviations

5-HT	Serotonin
5-HT_{2B}R	5-HT_{2B} receptor
APD	Antipsychotic drug
CNS	Central nervous system
DA	Dopamine
DRN	Dorsal raphe nucleus
EPS	Extrapyramidal side effects
FC	Frontal cortex
MDMA	3,4-methylendioxymethamphetamine
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
NOR	Novel object recognition
PCP	Phencyclidine
VTA	Ventral tegmental area

1 Introduction

The serotonin 2B receptor (5-HT_{2B}R) is the most recent addition to the 5-HT_2R family, which also comprises the 5-HT_{2A}R and the 5-HT_{2C}R subtypes [1]. Formerly called 5-HT_{2F}R, the 5-HT_{2B}R belongs to the seven transmembrane spanning receptor superfamily commonly referred to as G-protein-coupled receptors. It was first cloned and characterized in the rat stomach fundus [2, 3], then in mice [4] and in

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humans [5–7]. It has been shown to be present in various peripheral tissues in both rodents and humans, where it participates in the regulation of several physiological functions such as the gastrointestinal, the vascular, the pulmonary, the cardiac and the immune ones, for review, see [8]. In 1997, a few years after its cloning, the 5-HT_{2B}R was shown to be localized also in the mammalian brain. Immunohistochemistry studies assessing 5-HT_{2B}R protein expression in the rat brain demonstrated its presence in the frontal cortex (FC), the cerebellum, the lateral septum, the dorsal hypothalamus and the medial amygdala [9]. Subsequent investigations showed that 5-HT_{2B}R mRNA is expressed in additional rat brain regions such as the dorsal raphe nucleus (DRN), the locus coeruleus, the cerebellum, the habenula, the hippocampus and the hypothalamic paraventricular nucleus [10]. In humans, 5-HT_{2B}R mRNA was detected in the whole brain, and in particular in the cerebellum, the occipital cortex and the FC [5, 11]. Recent studies in mice have provided information about the cellular localization of 5-HT_{2B}Rs within the central nervous system (CNS), this issue remaining relatively unexplored in rats [8]. Thus, 5-HT_{2B}Rs have been shown to be expressed in primary astrocyte cultures from the neocortex [12], in 5-HT transporter-expressing primary neurons from embryonic raphe nuclei [13], in 5-HT neurons of raphe nuclei [14], in post-natal microglia [15], and in a subpopulation of ventral tegmental area (VTA) DA neurons innervating the nucleus accumbens (NAc) shell subregion [16].

With respect to the peripheral 5-HT_{2B}R, its functional role within the CNS has received much less attention until recently. Indeed, the first studies assessing the role of the central 5-HT_{2B}R on dopamine (DA) ascending pathway activity reported that the 5-HT_{2B}R agonist BW 723C86 and the 5-HT_{2B}R antagonist SB 204741 had no effect on DA neuron firing or on basal DA outflow in the FC, the NAc and the striatum [17, 18]. These negative findings, along with the risk of agonist-induced side effects related to heart-valve pathogenesis [19, 20], probably led to the discontinued use of 5-HT_{2B}R compounds in drug research and development when studying the central 5-HT system, and, in particular, the 5-HT/DA interaction within the CNS. Indeed, it was not until 2008 that the pivotal article by Maroteaux and coworkers showed that the central 5-HT_{2B}R participates in both the neurochemical and behavioral effect of 3,4-methylendioxymethamphetamine (MDMA) in mice [21]. They showed that selective pharmacological blockade with RS 127445 or genetic ablation of the 5-HT_{2B}R reverses MDMA-increased DA outflow in the NAc and 5-HT outflow in the NAc and the VTA, as well as MDMA-induced hyperlocomotion [21]. Subsequently, over the last decade and thanks to the development and availability of potent and high affinity 5-HT_{2B}R antagonists such as LY 266097 and RS 127445 [8, 22, 23], a growing number of studies have confirmed the key role of the central 5-HT_{2B}R in the control of DA and 5-HT neuron activity, and have highlighted its potential as a new pharmacological target for treating several neuropsychiatric disorders such as schizophrenia, depression and drug addiction [8, 14, 16, 24–30].

The present chapter provides an overview of the role of the 5-HT_{2B}R in the control of ascending DA pathway activity, covering neurochemical, electrophysiological and behavioral data mainly obtained from in vivo studies in the rat. After discussing

the role of 5-HT_{2B}Rs in controlling the release of DA in the medial prefrontal cortex (mPFC), the NAc and the striatum, we describe recent neurochemical and molecular findings providing the anatomo-functional basis underlying the effects of 5-HT_{2B}R antagonists on the activity of the mesocorticolimbic DA system. Finally, we present some behavioral data adding functional evidence for the therapeutic potential of 5-HT_{2B}R antagonists in the treatment of schizophrenia.

2 The Central 5-HT_{2B}R and DA Ascending Pathways

2.1 Regulation of DA Neuron Activity: In Vivo Neurochemical and Electrophysiological Data

Compelling in vivo biochemical and electrophysiological data demonstrate that, unlike 5-HT_{2B}R agonists [8, 17, 18, 24], 5-HT_{2B}R antagonists modulate DA ascending pathway activity in a differential manner. Thus, both the 5-HT_{2B}R antagonists RS 127445 and LY 266097 increase and decrease DA outflow in the mPFC and the shell subregion of the NAc, respectively, but do not modify DA outflow in the striatum or in the core subregion of the NAc [24–26]. In line with these results, electrophysiological findings have shown that selective blockade of 5-HT_{2B}Rs has no effect at the level of the substantia nigra pars compacta but decreases the firing rate of DA neurons in the VTA, presumably those projecting to the shell subregion of the NAc [26]. Based on these findings which provide additional support for the insensitivity of the nigrostriatal DA pathway to 5-HT_{2B}R modulation, it is tempting to hypothesize that 5-HT_{2B}R antagonism reduces accumbal DA outflow via an inhibitory modulation of mesoaccumbal DA neuronal firing. Nevertheless, as discussed elsewhere [26], in keeping with the cellular heterogeneity of the VTA [31–34], further studies are needed to identify DA neurons projecting to the NAc or to the mPFC. Altogether, these findings demonstrate that 5-HT_{2B}Rs independently control the activity of the three ascending DA pathways by specifically providing tonic excitatory and inhibitory controls on NAc and mPFC DA outflow, respectively, and no effect in the striatum (Fig. 19.1).

This conclusion contrasts with that offered by the first studies assessing the effect of this receptor on DA neuron activity and reporting that 5-HT_{2B}R blockade has no effect on DA ascending pathway activity [17, 18]. As discussed elsewhere [8], the use of high doses of non-selective 5-HT_{2B} compounds as well as some methodological drawbacks could be responsible for the discrepancies observed.

During recent years, much attention has been devoted to identifying the mechanisms and the anatomo-functional basis underlying the modulatory control exerted by 5-HT_{2B}Rs on the mesocorticolimbic DA system. Interestingly, it has been demonstrated that the opposite effect of 5-HT_{2B}R antagonists on mPFC and NAc shell DA outflow involves a functional interplay between 5-HT_{2B}Rs and 5-HT_{1A}Rs located in the DRN and in the mPFC, respectively (Fig. 19.2). By increasing cortical 5-HT

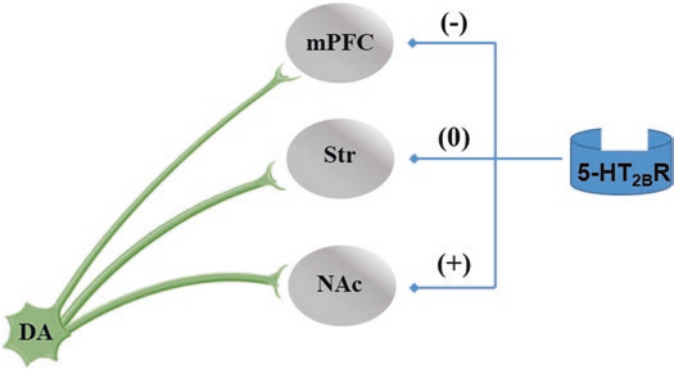

Fig. 19.1 Differential control exerted by central serotonin 2B receptors (5-HT_{2B}Rs) on the activity of ascending dopamine (DA) pathways. They exert a tonic inhibitory control on DA outflow in the medial prefrontal cortex (mPFC), a tonic excitatory control on DA outflow in the nucleus accumbens (NAc), but have no effect at the level of the striatum (Str), for details see [26]

outflow, intra-DRN 5-HT_{2B}R blockade triggers the stimulation of 5-HT_{1A}Rs located on mPFC GABAergic interneurons [35], thereby leading to the activation of pyramidal glutamatergic neurons [36] which drive opposite changes of mPFC and NAc DA outflow through direct or indirect interactions with VTA DA neurons [27, 37]. The involvement of these polysynaptic cortical-subcortical pathways is supported by the finding that the opposite change of mPFC and NAc DA outflow induced by the intra-DRN administration of RS 127445 is suppressed by the intra-mPFC perfusion of the selective 5-HT_{1A}R antagonist WAY 100635 [27]. These results provide the first evidence for a functional role of a specific 5-HT_{2B}R population in the regulatory control of DA neuron activity, and show that the DRN is a key brain region driving the 5-HT_{2B}R-DA system interaction.

Subsequent investigations exploring the mechanisms underlying the facilitatory effect of 5-HT_{2B}R antagonists on DRN 5-HT neurons innervating the mPFC demonstrated that 5-HT_{2B}Rs, in the rat DRN exert a GABA-mediated tonic inhibitory control on 5-HT neurons [38], (Fig. 19.2). This conclusion is supported by several compelling findings. First, it has been shown that intra-DRN perfusion of the GABAAR antagonist bicuculline prevents the increase in DRN and mPFC 5-HT outflow induced by intra-DRN administration of RS 127445 [38]. These results confirm and extend previous observations that peripheral administration of RS 127445 increases the firing rate of DRN 5-HT neurons and 5-HT outflow in the mPFC [27]. Second, the increase in DRN 5-HT outflow induced by the local administration of the selective 5-HT reuptake inhibitor citalopram is potentiated by the intra-DRN administration of RS 127445 only in the absence of bicuculline perfusion into the DRN [38]. Third, in agreement with the above-mentioned in vivo neurochemical findings, in vitro experiments coupling immunohistochemistry to reverse transcription-polymerase chain reaction revealed the presence of 5-HT_{2B}R mRNA on DRN GABAergic interneurons [38].While confirming the DRN as the main site of action of 5-HT_{2B}R antagonists, these results provide the first evidence for the

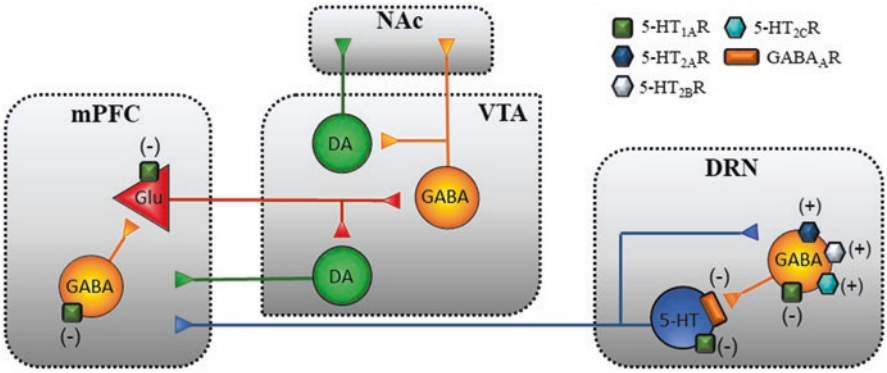

Fig. 19.2 Putative neuronal circuits involved in the opposite effect of serotonin 2B receptor (5-HT_{2B}R) antagonists on dopamine (DA) outflow in the medial prefrontal cortex (mPFC) and the nucleus accumbens (NAc). In the dorsal raphe nucleus (DRN), in addition to the autoinhibitory control exerted by somatodendritic 5-HT_{1A} autoreceptors, 5-HT neurons are regulated by a local negative-feedback circuit involving GABA interneurons. The 5-HT_{2B}R is expressed on these GABA interneurons, together with other post-synaptic 5-HTRs (5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R), and provides a tonic inhibitory control on 5-HT cells innervating the mPFC *via* $GABA_A$Rs. The 5-HT_{1A}R is expressed in the mPFC by GABA interneurons and pyramidal glutamatergic (Glu) neurons innervating the ventral tegmental area (VTA). In the VTA, Glu afferencies arising from mPFC Glu neurons provide a direct excitatory and GABA-mediated inhibitory control on the mesocortical and mesoaccumbal DA ascending pathways, respectively. Thus, by reducing GABA inhibitory tone, blockade of DRN 5-HT_{2B}Rs leads to increased activity of 5-HT neurons and consequently to increased 5-HT outflow in the DRN and the mPFC. Increased mPFC 5-HT outflow could trigger the stimulation of 5-HT_{1A}Rs expressed by local GABA interneurons. Subsequent disinhibition of mPFC Glu neurons innervating the VTA could respectively stimulate and inhibit the activity of the mesocortical and the mesoaccumbal DA pathways, thereby leading to increased and decreased DA outflow in the mPFC and the NAc, respectively, for details see [26, 27, 38]

location of the 5-HT_{2B}R in a specific cell population in the rat brain, and demonstrate its role in controlling the local negative-feedback loop regulating DRN 5-HT neuron activity via GABA interneurons (see Fig. 19.2), [38–41]. Of note, among the different 5-HTRs located on DRN GABA interneurons (5-HT_{2A}R, 5-HT_{2C}R and 5-HT_{1A}R) and participating in the local control of 5-HT neurons [39–41], the 5-HT_{2B}R is the only one providing a tonic control on 5-HT neurons [38]. From a functional point of view, these findings provide additional information on the mechanisms subsuming the effect of 5-HT_{2B}R antagonists on the mesocorticolimbic DA system, which has been shown to result from their ability to increase the activity of DRN 5-HT neurons projecting to the mPFC [27]. However, these data contrast with recent findings in mice showing that 5-HT_{2B}Rs are located on 5-HT neurons and exert a direct positive control on 5-HT neuron activity [42]. As discussed elsewhere [8, 38], these discrepant findings may result from species related anatomo-functional differences, so additional comparative studies between rats and mice are required to identify possible differences in the brain cellular distribution of the 5-HT_{2B}R.

2.2 5-HT$_{2B}$R Antagonists: Behavioral Data and Therapeutic Potential for the Treatment of Schizophrenia

Altogether, the neurochemical findings discussed above indicate that 5-HT$_{2B}$R antagonists may provide a useful pharmacological tool for treating neuropsychiatric disorders requiring the independent control of ascending DA pathways. In this context, schizophrenia is an emblematic mental illness that could benefit from 5-HT$_{2B}$R antagonist treatment. It is characterized by three main groups of symptoms: positive (i.e. hallucinations, delusions), negative (i.e. social interaction deficits, blunted affect) and cognitive (i.e. working and reference memory deficits, executive function impairments, decreased vigilance) [43–45]. This multimodal symptomatology is classically related to an imbalance in central DA neurotransmission: positive symptoms are thought to result from DA hyperfunction in the NAc, whereas negative and cognitive symptoms might involve DA hypofunction in the FC [45, 46]. The pharmacological treatment of schizophrenia is based on the use of DA-D$_2$ receptor antagonists classified as typical and atypical antipsychotic drugs (APDs) [43]. Although effective in controlling positive symptoms, typical APD such as haloperidol and chlorpromazine are responsible for the occurrence of extrapyramidal side effects (EPS) due to altered striatal DA activity [43, 47]. On the other hand, atypical APDs, of which clozapine is the prototype, display a wider therapeutic spectrum covering positive, negative and cognitive symptoms with a limited propensity to induce EPS [43, 44].

Thus, given their unique DAergic profile of effects, 5-HT$_{2B}$R antagonists should be able to improve all the symptoms of schizophrenia without inducing EPS by restoring normal DA function. This hypothesis has been demonstrated by recent studies in rats assessing their effectiveness in different DA-dependent behavioral models classically used to predict the ability of APDs to alleviate positive [hyperlocomotion induced by the non-competitive N-methyl-D-aspartate receptor antagonist phencyclidine (PCP)] and cognitive [PCP-induced deficit in novel object recognition (NOR) test] symptoms of schizophrenia, as well as their propensity to induce EPS (catalepsy test), [45]. These behavioral tests are known to be related to increased, reduced and altered DA function in the NAc, the mPFC and the striatum, respectively [47–49]. Thus, in line with their differential effects on DA outflow in these brain regions, the 5-HT$_{2B}$R antagonists RS 127445 and LY 266097 have been shown to reduce the hyperlocomotion induced by PCP [26]. This result is consistent with previous findings showing that 5-HT$_{2B}$R blockade reduces amphetamine-induced hyperlocomotion [24], another behavioral model used to investigate the potential of APDs to restore normal accumbal DA function [45]. Furthermore, both 5-HT$_{2B}$R antagonists were able to reverse PCP-induced NOR deficit to a similar extent as clozapine [26]. Finally, unlike haloperidol, neither RS 127445 nor LY 266097 produced a cataleptic state [26].

These findings providing additional support for the therapeutic relevance of 5-HT$_{2B}$R antagonists for treating schizophrenia suggest that these compounds could represent a new class of atypical APDs, given their ideal profile of effects expected

to alleviate cognitive and positive symptoms, without eliciting EPS [8, 26]. However, as discussed elsewhere [8, 26], this proposal has to be confirmed, so additional investigations are required to profile the acute or chronic effects of 5-HT_{2B}R antagonists in a palette of other experimental conditions predictive of therapeutic efficacy or side effects [45, 50]. Their involvement in metabolism, body mass and diabetic disorders, commonly referred to as "metabolic syndrome" [43, 45], as well as their ability to alleviate the negative symptoms of schizophrenia deserve dedicated studies.

In addition to the therapeutic potential of 5-HT_{2B}R antagonists per se, 5-HT_{2B}Rs could contribute to the therapeutic benefit of atypical APDs, many of which (clozapine, amisulpride, asenapine, aripiprazole, cariprazine) display antagonist properties at the 5-HT_{2B}R [51–55] and the DA-D_2R, together with partial agonist properties towards the 5-HT_{1A}R [45, 56]. This hypothesis is supported by the ability of 5-HT_{2B}R blockade to potentiate and decrease haloperidol-induced DA outflow in the mPFC and the NAc, respectively [24, 26], together with the functional role of 5-HT_{1A}R stimulation in the 5-HT_{2B}R-mediated control of DA outflow [27].

Importantly, these conclusions pointing to the potential of 5-HT_{2B}R antagonists for treating schizophrenia diverge from those offered by studies in mice showing that genetic ablation of 5-HT_{2B}Rs generate an antipsychotic-sensitive schizophrenic-like phenotype [29]. As discussed elsewhere [8, 26], in keeping with the role of 5-HT_{2B}Rs in brain maturation [15], developmental neural adaptations triggered by the permanent suppression of this receptor as well as species-related anatomo-functional differences may account for these divergences. Nonetheless, although additional investigations are warranted to clarify this issue, these findings support the role of 5-HT_{2B}Rs in the neurobiology and/or improved treatment of schizophrenia.

3 Conclusions and Perspectives

In conclusion, this chapter provides an updated overview of the important advances in the understanding of the physiological role of the central 5-HT_{2B}R in the control of DA ascending pathways and the anatomo-functional basis underlying this interaction. Specifically, the findings reported herein identify the DRN as a major site of action for the 5-HT_{2B}R-dependent control of DA and 5-HT neuron activity. First, the differential control exerted by 5-HT_{2B}R antagonists on the mesocorticolimbic DA system takes place in the DRN and involves complex polysynaptic cortico-subcortical pathways driven by a functional interplay between DRN 5-HT_{2B}Rs and mPFC 5-HT_{1A}Rs [27]. Second, in the DRN, 5-HT_{2B}Rs are located on GABA interneurons and exert a tonic inhibitory control on 5-HT neurons projecting to the mPFC by participating in the control of the local negative-feedback loop regulating 5-HT neuron activity [38].

From a clinical point of view and in keeping with their unique profile of effects on DA network, the data reported here highlight the therapeutic potential of 5-HT_{2B}R antagonists for the treatment of schizophrenia, a major neuropsychiatric disorder

whose optimal treatment requires the independent control of ascending DA pathways [8, 44, 45].

Additional experiments are warranted to obtain a deeper insight into the pathophysiological role of the 5-HT_{2B}R in the mammalian brain, and to verify the extent to which the contrasting findings observed between rats and mice are related to anatomo-physiological differences between species and/or to brain development-related factors. In addition, further investigations in a larger palette of experimental conditions including long-term treatments are mandatory to confirm the therapeutic potential of 5-HT_{2B}R antagonists for treating schizophrenia [8, 45]. In this context, investigations in advanced genetic models such as conditional 5-HT_{2B}R knock-out animals should be pursued. Finally, these data reported in this chapter provide additional knowledge about the regulation of ascending DA pathways by the central 5-HT system, and highlight the legitimacy of 5-HT_{2B}Rs as key modulators of the activity of the central DA network.

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Chapter 20
A Role for the 5-HT$_{2B}$ Receptor in the Neurobiology of Schizophrenia

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Abbreviations

ADHD	Attention-deficit hyperactivity disorder
CSF	Cerebrospinal fluid
MK-801	Dizocilpine
D2	Dopamine 2 receptor
DA	Dopamine
EEG	Electroencephalography
EMG	Electromyography
EPS	Extrapyramidal symptoms
HPLC	High performance liquid chromatography
NMDAR	N-methyl-D-aspartate receptor
NREM	Non-rapid-eye-movement sleep
NOR	Novel object recognition
NSF	Novelty suppressed feeding
NAc	Nucleus accumbens
PCP	Phencyclidine
PFC	Prefrontal cortex
PPI	Prepulse inhibition
qPCR	Quantitative polymerase chain reaction
REM	Rapid-eye-movement
5-HT	Serotonin; 5-hydroxytryptamine

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VTA Ventral tegmental area
WT Wild type

1 Atypical Antipsychotic Drugs and the 5-HT_{2B} Receptor

The therapeutic effects of antipsychotic drugs have long been attributed to their ability to modulate the dopaminergic system, most notably their antagonistic properties at dopamine 2 (D2) receptors [1]. However, the atypical antipsychotics have a complex pharmacology, with many displaying a high affinity for serotonin (5-hydroxytryptamine; 5-HT) receptors, as well as dopamine (DA) receptors [2]. While DA receptor antagonism is still considered important for the therapeutic properties of atypical antipsychotics, antagonistic (Ant), partial agonistic (PA), and even inverse agonistic (IA) properties of these drugs at 5-HT receptors has been proposed as an additional mechanism for antipsychotic drug action. Further, these actions on the serotonergic system and reduced D2 receptor antagonism most likely contribute to the reduced potential for atypical antipsychotics to induce adverse extrapyramidal symptoms [3]. However, the complex pharmacology of these drugs is also associated with increased incidence of metabolic side effects, such as weight gain and cardiovascular disease [4]. The 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptor subtypes have primarily been associated with antipsychotic action, however many of these drugs also display high affinity for 5-HT_{2B} receptors. Thus, a potential role of 5-HT_{2B} receptor modulation in the therapeutic action of atypical antipsychotic drugs has been proposed. Atypical antipsychotics currently in clinical use that exhibit affinity (either antagonistic or inverse agonistic) at 5-HT_{2B} receptors, include: clozapine, aripiprazole, cariprazine, amisulpride, brexpiprazole and asenapine. The binding affinity and action of these drugs on clinically relevant receptors has been recently reviewed [4, 5] (Table 20.1).

Clozapine is considered to be the prototypical atypical antipsychotic and is often used as a benchmark for new drugs due to its therapeutic efficacy and absence of motor side effects [6]. Indeed, clozapine exhibits a high antagonistic affinity for the 5-HT_{2B} receptor, in fact more so than for DA receptors. Amisulpride and asenapine

Table 20.1 Affinity (K_i, nM) and action of atypical antipsychotics on schizophrenia-relevant serotonin receptors, as reported in [7, 29–35]. Ant: antagonist; Ag: agonist; PA partial agonist, IA inverse agonist

	5-HT_{1A}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}
Asenapine	2.5 (Ag)	0.07 (Ant)	0.16 (Ant)	0.03 (Ant)
Aripiprazole	5.6 (Ag)	22 (Ag)	0.4 (IA)	76 (Ag)
Cariprazine	3 (Ag)	19 (Ant)	0.58 (Ant)	134 (Ant)
Brexpiprazole	0.12 (PA)	0.47 (Ant)	1.9 (Ant)	N/A
Clozapine	124 (Ag)	22 (Ant)	10 (Ant)	13 (Ant)
Amisulpride	>10,000	8304	13 (Ant)	>10,000

are also antagonistic to both DA receptors and the 5-HT$_{2B}$ receptor, with asenapine having greater 5-HT$_{2B}$ receptor affinity than amisulpride [4, 5, 7]. Finally, aripiprazole, brexpiprazole, and cariprazine all display unique drug profiles compared to other antipsychotic drugs [8–10]. These drugs display antagonistic actions on 5-HT$_{2B}$ receptors, although they possess agonistic properties at DA receptors. In conclusion, atypical antipsychotics bind to many targets, including the 5-HT$_{2B}$ receptor in many cases. Due to the complex pharmacology of these drugs, it is difficult to elucidate exactly what actions are at the core of their antipsychotic action. However, given that many of these drugs display affinity for the 5-HT$_{2B}$ receptor, it can be theorized that this receptor subtype may play a unique role in both their antipsychotic drug action and in the neurobiology of schizophrenia.

2 Genetic Ablation of the 5-HT$_{2B}$ Receptor Results in a Schizophrenic-Like Phenotype in Mice

Psychomotor agitation in response to psychostimulants is linked to the positive symptoms of schizophrenia, as it is characterized by hyperactivity and stereotypic movements [11]. In rodent models of schizophrenia, hyperactivity is assessed with behavioral tests that evaluate spontaneous and drug-induced locomotor activity. Indeed, spontaneous locomotor hyperactivity in response to a novel environment is observed in many genetic and neurodevelopmental rodent models of schizophrenia [12]. Further, this locomotor hyperactivity is often enhanced upon administration of psychostimulant drugs that target the dopaminergic and the glutamatergic systems, such as amphetamines, dizocilpine (MK-801), ketamine, and phencyclidine (PCP). Indeed, these drugs are known to induce psychosis in schizophrenic patients and to induce schizophrenic-like symptoms in healthy individuals [13]. However, it is important to note that locomotor hyperactivity is not unique to schizophrenia, but rather implicated in a wide variety of neuropsychiatric disorders such as bipolar disorder and attention-deficit hyperactivity disorder (ADHD).

The prepusle inhibition (PPI) of the startle reflex is typically considered an operational measure of sensorimotor gating, and its deficit has been reported in schizophrenic patients and in relevant animal models. Our studies have shown that constitutive deletion of the 5-HT$_{2B}$ receptor in mice (5-HT$_{2B}$ receptor KO) results in a global deficit in sensorimotor gating, as reflected by a decreased PPI of the startle reflex [14]. Moreover, 5-HT$_{2B}$ receptor KO mice display prominent hyperactivity when placed into a novel environment [14, 15]. Specifically, we have reported that genetic ablation of 5-HT$_{2B}$ receptor results in spontaneous locomotor hyperactivity, as assessed in a circular corridor with infrared beams placed at every 90°; furthermore, 5-HT$_{2B}$ receptor KO mice presented enhanced locomotor response to psychostimulant drugs, namely the D1 agonist SKF 81297 [15], amphetamine, and the NMDA receptor antagonist dizocilpine [14].

Asociality is a prominent negative symptom of schizophrenia. Further, social behavior in rodent models of this disorder is directly relatable to that seen in human patients. Indeed, it has been shown that both genetic and psychostimulant-based pharmacological models of schizophrenia exhibit impaired social function in a variety of behavioral paradigms [16]. Most of these tests quantify social behavior as number of contacts or time spent interacting with a juvenile or adult conspecific. We recently assessed social behavior in 5-HT_{2B} receptor KO mice by using a 3-compartmental sociability test that assesses preference for the interaction with a novel social stimulus *versus* an asocial stimulus (i.e., empty compartment) [14]. In this study, it was reported that the 5-HT_{2B} receptor KO mice showed no preference for the social stimulus, as they spent equal time interacting with the empty compartment as with an adult conspecific. Further, when a novel mouse was placed in the previously empty compartment, the 5-HT_{2B} receptor KO mice had no preference for the novel social stimulus, indicating impaired social memory [14]. Together, these findings support the notion that genetic ablation of 5-HT_{2B} receptor is associated not only with positive, but also with negative symptoms of schizophrenia.

Cognitive symptoms, including learning and memory deficits, represent the third widely recognized cluster of schizophrenic symptoms. Indeed, rodent models of schizophrenia recapitulate cognitive deficits observed in the human disorder. Interestingly, 5-HT_{2B} receptor KO mice were shown to exhibit cognitive deficits in the novel object recognition (NOR) and the fear conditioning tasks. The NOR task is a widely used learning and memory test in mice; it is conducted in an open field arena, in which mice are allowed to explore two identical objects during a learning (i.e., acquisition) session. Next, in the retention session, a novel object is presented to the mouse along with the familiar object, and time spent interacting with each object is recorded. Our studies showed that 5-HT_{2B} receptor KO mice exhibited lower preference for the novel object at both 1 h and 24 h after the learning session, indicating impaired short- and long-term object recognition memory [14]. Further, 5-HT_{2B} receptor KO mice exhibited an impairment in fear learning assessed in the fear conditioning task. In this test, mice are typically placed in a novel environment and exposed to an auditory tone (i.e., cue), accompanied by a painful foot shock. After this learning session, the mouse is placed back into the environment, and freezing response to the environment (i.e., context) and the cue were recorded. Indeed, 5-HT_{2B} receptor KO mice exhibited a decreased fear response to both the context and the cue, indicating an impairment in fear learning and memory [14]. An extension of this fear conditioning is the latent inhibition paradigm. In this assessment, mice are first pre-exposed to the environment without a foot shock in order to learn that the environment is safe. Following this initial exposure, mice undergo the same fear conditioning procedure as mentioned before. In the latent inhibition assessment, 5-HT_{2B} receptor KO mice were unable to remember that the environment was previously safe, evidenced by no difference in freezing time between the pre-exposed and non-pre-exposed groups [14]. These data suggest that constitutive loss of 5-HT_{2B} receptor function in mice impairs learning and memory processes, echoing the cognitive symptoms observed in human patients suffering from schizophrenia.

Impulsivity is defined as action without foresight and is heavily associated with a variety of neuropsychiatric disorders, including schizophrenia. Indeed, impulsive

behaviors are observed in rodent models for this disorder. Bevilacqua et al. [15] reported increased novelty seeking and impulsivity in the 5-HT$_{2B}$ receptor KO mice in three behavioral paradigms, namely: exposure to a novel object, novelty suppressed feeding (NSF) test, and the delay discounting test. During exposure to a novel object, an unfamiliar object was placed in the home cage of each mouse. The number of contacts with this object is a direct indicator of the novelty seeking activity of the mouse; 5-HT$_{2B}$ receptor KO mice initiated contacts with the novel object more frequently than their WT counterparts, suggesting that these mice are novelty seekers. In the NSF paradigm, mice are deprived of food for 12h, and then placed into a novel environment with a single food pellet in the center. The latency to feed is then recorded; a higher latency to feed indicates increased anxiety, whereas a decreased latency to feed indicates anxiolysis and increased novelty seeking behavior. Bevilacqua et al. [15] reported decreased latency to feed in 5-HT$_{2B}$ receptor KO mice, an indication that these mice display lower levels of anxiety accompanied by increased novelty seeking. Lastly, the delay discounting test was used to assess impulsivity in response to a rewarding stimulus. This was conducted in a computer-controlled operant chamber with two holes with infrared beams that respond to a nose poke from the mouse. One hole presents a small food reward, while the other presents a large food reward. After the mice are habituated to this system and learn to use it (~10 days), the testing phase (~7 days) is commenced. In this phase, both rewards start with no delay from nose poke to reward. Naturally, the mouse will exhibit a preference to the large reward. However, during each day of the testing phase the delay for the large reward is increased. As the delay for the large reward increases, the mouse will lose preference for it, eventually showing a preference for the short-delay reward over the long-delay, large reward. Impulsivity is indicated when a mouse develops a preference for the short delay reward sooner. Indeed, in this assessment, 5-HT$_{2B}$ receptor KO mice developed a preference for the short-delay reward more quickly than did WTs, indicating that global loss of 5-HT$_{2B}$ receptor function is associated with impulsive behavior [15].

Taken together, findings from these studies support a critical neurodevelopmental role for the 5-HT$_{2B}$ receptor. Specifically, genetic ablation of this receptor results in many behavioral alterations observed in other animal models of schizophrenia. These include spontaneous and psychostimulant-induced locomotor hyperactivity, asociality, impaired short-term and long-term memory, impaired social and fear learning, and increased novelty seeking and impulsivity. These behavioral symptoms represent a schizophrenia-like phenotype comprised of positive, negative, and cognitive symptoms clinically observed in patients suffering from this disorder.

3 Pharmacological Ablation of 5-HT$_{2B}$ Receptor in Rats

Devroye et al. [17] have also conducted psychopharmacological studies using the 5-HT$_{2B}$ receptor antagonists RS127445 and LY266097 to further investigate how impaired function of this receptor may be associated with psychotic states in rats. Using chronic PCP administration to induce psychosis in rats, the therapeutic

potential of RS127445 and LY266097 was assessed in three behavioral paradigms: locomotor hyperactivity in response to PCP, the NOR test, and the catalepsy test. These behaviors represent the positive and cognitive symptoms of schizophrenia, and the potential for antipsychotics to induce adverse effects, respectively. Interestingly, neither RS127445 nor LY266097 affected distance travelled when administered alone, however both were able to abolish PCP-induced hyperactivity. This finding suggests that RS127445 and LY266097 have therapeutic potential for the positive symptoms of schizophrenia. Next, the potential of RS127445 and LY266097 to ameliorate short-term memory deficits induced by PCP administration was assessed in the NOR test. Indeed, rats that had received chronic PCP treatment were unable to discriminate the novel object from the familiar one. However, PCP-treated rats administered a single dose of RS127445, LY266097, or the atypical antipsychotic clozapine, exhibited a near-complete rescue of short-term object recognition memory. This indicated that both RS127445 and LY266097 have the potential to ameliorate learning and memory deficits seen in schizophrenia patients. Finally, the ability of RS127445 and LY266097 to induce extrapyramidal symptoms (EPS; e.g., irregular, jerky movements and muscle spasms and rigidity) was assessed. To assess the potential of RS127445 and LY266097 to produce these adverse effects, the catalepsy test was performed. In this paradigm, rats were positioned with their forelimbs on a 9-cm tall wooden block and hindlimbs on the floor of the testing arena, and the time elapsed before the animal dismounted from this block was recorded. A large time elapsed before dismount indicates that the drug in question has the tendency to induce EPS-like adverse effects. Indeed, in rats treated with the typical antipsychotic drug haloperidol a much larger time elapsed before dismount in the test, as compared to vehicle (VEH)-treated animals. Interestingly, neither RS127445 nor LY266097 significantly affected the time before dismount as compared to vehicle. This would suggest that 5-HT_{2B} receptor antagonism induces less adverse effects than typical antipsychotics and could be a safer alternative to medications currently in use. The behavioral studies conducted by Devroye et al. [17] show some therapeutic promise in 5-HT_{2B} receptor antagonists in the treatment of schizophrenia. Indeed, such drugs could have the potential to ameliorate positive symptoms of psychosis and cognitive deficits, without the ability to produce adverse symptoms such as EPS. However, additional neurobehavioral assessments are needed to fully understand their therapeutic and adverse effects.

4 A Role for the 5-HT_{2B} Receptor in Sleep Architecture

Disruption of circadian rhythmicity is a symptom shared by many neuropsychiatric disorders, including schizophrenia as well as bipolar disorder and major depressive disorders [18]. Sleep architecture in rodents is typically assessed with electroencephalography (EEG)-based polysomnography. Specifically, EEG distinguishes sleep states by measuring electrical oscillatory activity of neurons in broad cortical areas. From these oscillations and muscle electrical activity (electromyography,

EMG), three major distinct vigilance states can be identified: wakefulness, non-rapid-eye-movement sleep (NREM), and rapid-eye-movement (REM) sleep. Indeed, schizophrenia patients may exhibit insomnia and fragmented sleep [19]. Further, as sleep has been shown to be critical to memory consolidation these sleep disturbances may likely contribute to the cognitive deficits seen in schizophrenia [20]. Considering the behavioral and neurochemical schizophrenia-like phenotypes observed in 5-HT$_{2B}$ receptor KO mice and the role of sleep in schizophrenia, EEG-based polysomnography was used to investigate disturbances in the sleep architecture due to genetic ablation of the 5-HT$_{2B}$ receptor in mice. Duration of wakefulness, NREM, and REM were all assessed over a 48h period. Interestingly, 5-HT$_{2B}$ receptor KO mice had reduced total sleep duration and reduced NREM duration as compared to wild type (WT; control) mice, however REM sleep duration was unaffected [14]. These differences were most pronounced at the end of the dark cycle and the onset of the light period. Interestingly, the sleep disturbances observed were antipsychotic drug-sensitive as these were abolished by 4-week chronic oral haloperidol administration. Notably, reduced latency to REM sleep has been reported in patients suffering from schizophrenia and mood disorders [21]; 5-HT$_{2B}$ receptor KO mice also exhibited reduced latency to REM sleep, defined as the time elapsed from the beginning of a bout of sleep after the animal had been awakened, to the first incident of REM sleep [14]. Overall, genetic ablation of the 5-HT$_{2B}$ receptor in mice results in antipsychotic-sensitive schizophrenia-like sleep disturbances. This further supports a potential neurodevelopmental role for the 5-HT$_{2B}$ receptor in schizophrenia pathogenesis.

5 5-HT$_{2B}$ Receptor and Schizophrenia: Clinical Evidence

In addition to their behavioral studies in mice, Bevilacqua et al. [15] have also shown that the 5-HT$_{2B}$ receptor gene is implicated in severe impulsivity and related neuropsychiatric disorders in humans, including schizophrenia. In this study, several candidate genes related to the serotonergic and dopaminergic neurochemical systems were screened in two Finnish populations of violent offenders and healthy subjects free of psychiatric diagnoses. A stop codon mutation in the 5-HT$_{2B}$ receptor gene (*HTR2B* Q20*), which renders the receptor nonfunctional, was found to co-segregate with impulsivity-related disorders, such as antisocial personality disorder and alcohol use disorder. This clinical finding was supported by behavioral data in the 5-HT$_{2B}$ receptor KO mice [15]. Moreover, individuals with at least one immediate family member diagnosed with schizophrenia were found to be numerically more likely to be heterozygote carriers of this mutation. These findings further suggest that 5-HT$_{2B}$ receptor genetic impairment contributes to schizophrenia and impulsivity-related neuropsychiatric disorders. Lastly, this study employed the digit span test to assess working memory in the same Finnish population. This is a simple test in which participants see or hear a sequence of digits and are asked to recall them in forward or reverse order. Recall of longer sequences requires superior

working memory capacity. Interestingly, male carriers of this mutation scored lower on this test as compared to non-carriers, however there was no difference between female carriers and non-carriers. This supports previously mentioned behavioral data in mice suggesting that genetic disruption of the 5-HT_{2B} receptor impairs memory function [14]. Interestingly, testosterone levels in the cerebrospinal fluid (CSF) were found to be elevated in Q20* mutation carriers as compared to non-carriers, and plasma testosterone was found to be elevated in 5-HT_{2B} receptor KO mice. This suggests a role of 5-HT_{2B} receptor function in stress axis and testosterone production as a mechanism for the observed impulsive phenotype, although more research is needed to confirm this.

Interestingly, a recent study has also identified the 5-HT_{2B} receptor gene as a potential neurodevelopmental risk gene in schizophrenia [22]. Using RNA-Seq, transcriptomic analysis was undertaken on cultured neural progenitor cells derived from the olfactory epithelium (CNON cells) in adult patients suffering from schizophrenia and healthy control subjects. Interestingly, CNON cells are actively proliferating and differentiating and have a gene expression profile most similar to the second trimester fetal brain, a critical period in the development of schizophrenia. Thus, these CNON cells represent a practical way to study cellular and molecular abnormalities associated with schizophrenia in the developing human central nervous system. In this analysis, 53 genes were found to be differentially expressed in CNON cells between schizophrenia patients and healthy control subjects. Among these genes is the *HTR2B* gene, which was found to be expressed slightly higher in individuals with schizophrenia. Further, this difference was found to be driven by African American individuals; when separating the African American cohort, there was no significant difference in non-African American schizophrenic patients *versus* healthy control groups. Taken together, these studies provide evidence that altered function of the 5-HT_{2B} receptor in humans may play a role the development of schizophrenia or related neuropsychiatric disorders, although more research is needed to elucidate this.

6 Role of the 5-HT_{2B} Receptor in Regulating the Brain's Neurochemistry: Ex Vivo and In Vivo Neurochemical Studies

In addition to the behavioral abnormalities observed in 5-HT_{2B} receptor KO mice, we have reported several neurochemical alterations that accompany the genetic ablation of the 5-HT_{2B} receptor [14]. Currently, two major neurochemical hypotheses exist to explain how symptoms arise from brain dysfunction in schizophrenia; these hypotheses revolve around the brain's dopaminergic and glutamatergic systems. The dopamine hypothesis suggests that two dopamine pathways are disrupted in schizophrenia, both originating in the ventral tegmental area (VTA) of the brain. The mesolimbic pathway is comprised of dopaminergic VTA neurons that innervate

the nucleus accumbens (NAc), located in the ventral striatum (STR), while the mesocortical pathway is comprised of dopaminergic VTA neurons that project to the prefrontal cortex (PFC). In schizophrenia, excess dopaminergic activity in the mesolimbic pathway is thought to underlie positive symptoms, whereas deficient dopaminergic activity in the mesocortical pathway is believed to underlie negative and cognitive symptoms [23]. Indeed, dopaminergic dysfunction is well characterized in established animal models of the disorder [24]. We have implemented high performance liquid chromatography (HPLC) to assess total neurotransmitter content in schizophrenia-relevant brain regions in 5-HT$_{2B}$ receptor KO mice *ex vivo*. Specifically, decreased DA levels were observed in the dorsal STR of 5-HT$_{2B}$ receptor KO mice, as opposed to WT mice, but no differences were observed in the PFC. Interestingly, chronic haloperidol treatment for 4 weeks with the typical antipsychotic and D2 receptor antagonist haloperidol, reversed this neurochemical striatal deficit. In addition, decreased striatal levels of DA were accompanied by decreased expression of the D2 receptor mRNA in this brain region using quantitative polymerase chain reaction (qPCR); this molecular alteration was also rescued by chronic haloperidol treatment [14]. The other prevailing neurochemical hypothesis for schizophrenia suggests that symptoms arise from dysfunction of the glutamatergic system. Specifically, N-methyl-D-aspartate receptor (NMDAR) hypofunction has been proposed as a central mechanism for this disorder. Theoretically, impaired NMDAR function in cortical glutamatergic neurons results in an impaired sensory filter and dysregulation of the mesolimbic and mesocortical dopamine pathways, resulting in schizophrenia pathology [25]. This is supported by observations of psychoactive NMDAR antagonist drugs such as PCP and ketamine to induce psychotic states when administered to humans [26]. Further, chronic PCP and ketamine administration is a widely used model of schizophrenia in rodents. Thus, altered glutamatergic neurotransmission is viewed as central to the development of schizophrenic symptoms. Interestingly, in our study, we reported reduced glutamate in the striatum of 5-HT$_{2B}$ receptor KO mice, which is abolished by chronic administration of haloperidol [14]. Taken together, these ex vivo neurochemical findings suggest that constitutive loss of 5-HT$_{2B}$ receptor function results in dopaminergic and glutamatergic dysfunction, presumably contributing to the observed schizophrenia-like behavioral phenotype.

In addition to these ex vivo neurochemical studies, Auclair et al. [27] and Devroye et al. [17] have conducted in vivo microdialysis studies in rats to investigate the role of the 5-HT$_{2B}$ receptor in schizophrenia-relevant dopaminergic pathways. As symptoms of schizophrenia are hypothesized to arise due to dysregulated DA pathways in the brain, possible control over these pathways by 5-HT$_{2B}$ receptor modulation is of particular interest. In the in vivo brain microdialysis technique, a probe containing a semi-permeable membrane connected to a two-way fluidics system is implanted into a specific brain region of interest in the rodent. Artificial cerebrospinal fluid (CSF) is pumped through the system and into the probe, where small molecule neurotransmitters will diffuse through the probe and out of the fluidics system to be collected and analyzed. In this set-up, drugs can be administered through peripheral administration (intraperitoneally, i.p., or subcutaneously, s.c.) or

through the microdialysis probe into a specific brain region, in the case of reverse dialysis. Auclair et al. [27] have conducted microdialysis studies in rats on 5-HT_{2B} receptor control over the dopaminergic system. In this study, the 5-HT_{2B} receptor antagonists RS127445 and LY266097, along with the agonist BW723C86 and inverse agonist SB206553, were used to assess 5-HT_{2B} receptor control over DA outflow in the NAc and STR. In agreement with Devroye et al. [28], this study found that peripheral administration of RS127445 and LY266097 (0.16 mg/kg and 0.63 mg/kg, i.p., respectively) decreased DA outflow in the NAc, but have no effect in the dorsal STR. Interestingly, the 5-HT_{2B} receptor agonist BW723C86 (3 mg/kg, s.c.) had no effect on either of these brain regions. Next, the interplay of antagonist LY266097 and inverse agonist SB206553 was assessed. When given alone, SB206553 (5 mg/kg, i.p.) significantly increased both striatal and accumbal DA outflow. Interestingly, when LY266097 and SB206553 were administered in concert, a significant reduction of SB-induced DA outflow was observed in the NAc, but not the STR. A similar effect was observed upon co-administration of LY266097 and the typical antipsychotic haloperidol. Indeed, LY266097 was able to rescue haloperidol-induced DA outflow in the NAc, but not in the STR. Further, when 5-HT_{2B} receptor agonist BW723C86 and haloperidol were administered in concert, BW723C86 had no effect on DA outflow in the STR nor the NAc in either vehicle or haloperidol-treated rats. Finally, the effect on LY266097 to reduce amphetamine-induced DA outflow in both the STR and NAc was assessed. Indeed, LY266097 again partially rescued amphetamine induced DA outflow in the NAc, but not the STR. In order to better understand this neurochemical finding, Auclair et al. [27] also assessed the effect of locomotor activity in rats. Indeed, it was also found that LY266097 partially rescued amphetamine-induced hyperactivity.

Devroye et al. [17] further investigated 5-HT_{2B} receptor control over the dopaminergic system, specifically in the mPFC in addition to the STR and NAc. Antagonists RS127445 and LY266097 were used to assess the effects of pharmacologic ablation of this receptor in rats. Microdialysis probes were placed in the NAc, STR, and mPFC and dialysates were collected and analyzed upon i.p. administration of saline, RS 127445 (0.08 and 0.16 mg/kg) and LY266097 (0.16 and 0.63 mg/kg) and analyzed with HPLC. In that study, it was reported that the high dose (0.16 mg/kg) of RS127445 decreased DA outflow in the NAc at 30 min post-injection, lasting until 120 min. Further, an increase of DA was observed in the mPFC upon i.p. administration of high doses of both RS127445 (0.16 mg/kg) and LY266097 (0.63 mg/kg) at 30–120 min. RS127445 was also found to potentiate haloperidol-induced dopaminergic transmission in the mPFC when these drugs were co-administered. Finally, no effect of RS127445 on DA outflow in the STR was reported. These data support a potentially therapeutic role for 5-HT_{2B} receptor antagonists, as hyperactive mesolimbic dopaminergic neurotransmission and a hypoactive mesocortical dopaminergic tone are hypothesized to underlie positive and negative schizophrenia symptoms, respectively. Further, these drugs could have reduced extrapyramidal side-effects than typical antipsychotics, as striatal DA release was unaltered. In addition to in vivo microdialysis, the firing rate of VTA and substantia nigra (SNc) dopaminergic neurons was also assessed in rats *via* in vivo extracellular recordings [17]. Rats

were anesthetized and a recording electrode was implanted in either the VTA or SNc. Individual neural recordings were obtained under three conditions: baseline firing (1–2 min), saline injection (5 min), and RS127445 injection (15 min). Interestingly, RS127445 administration reduced VTA neuron firing rate as compared to baseline. However, RS127445 did not affect the firing rates of neurons in the SNc. Overall, these neurophysiological data lend support to the microdialysis studies, suggesting that acute pharmacological brain-wide 5-HT$_{2B}$ receptor blockade may inhibit the mesolimbic dopaminergic pathway, while having no major effect over the nigrostriatal dopaminergic pathway.

7 Conclusions

A battery of preclinical experimental evidence shows that global genetic ablation of the 5-HT$_{2B}$ receptor results in schizophrenia-relevant behavioral, neurochemical, and neurophysiological alterations in mice [14]. Specifically, 5-HT$_{2B}$ receptor KO mice exhibit hyperactivity, increased novelty seeking, asociality, impulsivity, and impaired memory; these behavioral phenotypes closely mirror clinical symptoms of schizophrenia. Constitutive deletion of the 5-HT$_{2B}$ receptor also resulted in striatal dopaminergic and glutamatergic neurochemical alterations. Further, 5-HT$_{2B}$ receptor KO mice display disrupted sleep rhythms. Interestingly, behavioral and neurochemical deficits were rescued in some capacity upon antipsychotic drug administration, further supporting a role of 5-HT$_{2B}$ receptor dysfunction in the pathophysiology of schizophrenia. Notably, subsequent psychopharmacological studies have provided evidence pointing to the therapeutic potential of 5-HT$_{2B}$ receptor antagonists in schizophrenia, while providing a potential neurochemical mechanism for these effects [17, 28]. Further, pharmacological blockade of the 5-HT$_{2B}$ receptor seems to ameliorate locomotor hyperactivity, as well as learning and memory deficits observed upon PCP administration; increases in mesocortical DA outflow induced by pharmacological ablation of the 5-HT$_{2B}$ receptor could underlie this response. It is important to note that there are conceptual inconsistencies in the studies assessing the effects of the genetic *vs* the pharmacological ablation of the 5-HT$_{2B}$ receptor. While our studies suggest that genetic ablation of the 5-HT$_{2B}$ receptor in mice results in a schizophrenic-like behavioral phenotype [14], data from Devroye et al. [17] in rats show that loss of function of this receptor *via* pharmacological blockade results in antipsychotic behavioral effects with accompanying dopaminergic alterations. This can be attributed to a number of experimental factors; it is critical to keep in mind that global genetic ablation of the 5-HT$_{2B}$ receptor mirrors the developmental impact of permanent loss of this receptor, while pharmacological ablation following RS127445 or LY266097 treatment probes acute blockade of 5-HT$_{2B}$ receptor in otherwise normal rodents. For instance, Doly et al. [28] showed that 5-HT$_{2B}$ receptor KO mice exhibit increased locomotor response to cocaine, that was not observed upon acute pharmacological inhibition of the 5-HT$_{2B}$ receptor. Furthermore, discrepancies could also be due to the different

rodent species used (i.e., mice *versus* rats), or the experimental methods implemented (i.e., ex vivo *versus* in vivo neurochemical analyses). Notably, increasing experimental evidence also supports an important role for the 5-HT_{2B} receptor in the neurobiology of schizophrenia and responsiveness to antipsychotic drug treatments. In the clinical arena, data show that individuals who carry a non-functional 5-HT_{2B} receptor mutation exhibit impulsivity and impaired working memory, two symptoms closely related to schizophrenia [15].

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Chapter 21
5-HT_{2B} Receptors and Antidepressants

Silvina L. Diaz

Abbreviations

5-Hydroxytryptamine, 5-HT	Serotonin
DRN	Dorsal raphe nucleus
FST	Forced swimming test
GIRK	G protein-coupled inwardly-rectifying potassium channels
KO	Knock-out
NSF	novelty suppressed feeding
SERT	Serotonin transporter
SNP	Single-nucleotide polymorphism
SSRIs	Selective serotonin reuptake inhibitors
TPH2	Tryptophan hydroxylase
UCMS	Unpredictable chronic mild stress
VMAT2	Vesicular monoamine transporter

1 Introduction

Serotonin (5-Hydroxytryptamine, 5-HT) is a neurotransmitter involved in many psychiatric diseases including depression. The serotonergic neurons that innervate forebrain originate predominantly from the rostral cell group of neurons in the dorsal raphe nucleus (DRN) [1, 2]. These neurons express the serotonergic markers tryptophan hydroxylase (TPH2), and serotonin transporter (SERT), and also the negative autoreceptors, 5-HT_{1A} and 5-HT_{1B}, whose expression is restricted to

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somatodendritic compartments of serotonergic neurons, and to axonal terminals, respectively [3]. The 5-HT_{1A} autoreceptor activation elicits an outward current carried through G protein-coupled inwardly-rectifying potassium channels (GIRK) of the Kir3 family leading to membrane hyperpolarization and inhibition of serotonergic neuron firing [4]. The presence of synaptic vesicles in dendrites of serotonergic neurons led to the suggestion that autoinhibition is mediated via dendritic release of 5-HT, for review see [5]. However, activity of serotonin DRN neurons can also be positively modulated by 5-$HT_{2A/2B/2C}$ receptors triggering directly or indirectly inward currents [6–10]. Upon electrical stimulation of leech serotonergic neurons, transmembrane Ca^{2+} entry through L-type channels first evokes an early dendritic exocytosis; subsequently, the released serotonin activates dendritic 5-HT_2 autoreceptors coupled to Gq and phospholipase C, resulting in a positive feedforward loop that maintains sustained exocytosis [11]. It has thus been proposed that DRN neurons can display responses ranging from inhibition to excitation depending on a balance of functional 5-HT_{1A} and 5-HT_2 receptors [12].

A single-nucleotide polymorphism (SNP) introducing a stop codon at the beginning of the human 5-HT_{2B} receptor (Q20*) is associated with psychiatric diseases [13]. Interestingly, 70% of the Q*20 male cases displayed impulsive suicidal behavior, and 66% had at least one life-threatening suicide attempt by age 33.5 [13] supporting a possible action of 5-HT_{2B} receptors on serotonergic neurons. Genetic (KO) or pharmacologic (antagonist) manipulation of 5-HT_{2B} receptors in mice interferes similarly with effects of molecules that target serotonergic neurons including amphetamine-derivatives, and serotonin releasers MDMA [14–16]. Furthermore, local infusion of the 5-HT_{2B} receptor preferential agonist BW723C86 [16] in DRN by microdialysis increased extracellular serotonin that was blocked by RS127445, supporting a functional role of this receptor within the raphe and the selectivity of BW723C86 for this effect [15].

The precise localization and way of action of 5-HT_{2B} receptors are still poorly identified. Main difficulties include a lack of specific antibody as well as low level of expression of 5-HT_{2B} receptors in the mouse [17] or in human brain [18–20]. Nevertheless, the expression of 5-HT_{2B} receptor mRNA was confirmed in several brain nuclei including DRN [13, 21]. Besides, previous study using single cell RT-PCR [22] established a 5-HT_{2B} receptor expression not only in the raphe but more specifically in serotonergic neurons. Together, these data suggested that 5-HT_{2B} receptors could be implicated in 5-HT-dependant behavior by acting directly onto serotonergic neurons.

2 Serotonin Syndrome

The serotonin syndrome is a serious disorder reported in humans that most commonly appears after serotonergic antidepressant overdose or after combining several psychotropic medications acting at serotonin levels [23]. The exaggerated serotonergic function induced by these conditions can be simulated in experimental

animals by administration of serotonin-enhanced drugs like serotonin precursors, serotonin transporter inhibitors, or serotonin receptors agonists [23, 24]. Thus, signs of serotonin syndrome are possibly due to an excess of extracellular serotonin activating both, central and peripheral serotonin receptors. Indeed, specific reversible inhibitors of the rate-limiting enzyme TPH are being studied as tool for the treatment of definite signs of the serotonin syndrome [25].

2.1 Mouse Model of Serotonin Syndrome

The acute serotonin toxicity has been characterized in mouse model by expression of certain behavioral and physiological responses as hind limb abduction, forepaw treading, backward movement, Straub tail, head weaving, tremor and low flat posture [26–29]. Autonomic responses also observed in rodents include temperature dysregulation, piloerection, and defecation [30]. These behavioral and autonomic responses are usually scored during a defined period of time to quantify or asses the intensity of a serotonin syndrome in mice models. Rodent models of serotonin syndrome are induced by administration of serotonergic antidepressants, serotonin agonists, or serotonin precursors. Indeed, the precursor 5-HTP is able to cross the blood brain barrier and, at high doses, this drug is able to induce marked behavioral and autonomic responses [30]. The most widely used antidepressants, the selective serotonin reuptake inhibitors (SSRIs), induce an acute increase in extracellular serotonin concentrations by blocking SERT [31, 32]. SERT regulates the extracellular serotonin concentration by removing serotonin from the synaptic cleft [33], and various molecules modulate this activity, including the A_3 adenosine receptor [34] and kinases such as PKG/p38 MAPK [35]. Serotonin syndrome has been also observed in mice treated with high dose of SSRIs, Fluoxetine or paroxetine in different mice strains [36, 37]. The forced swimming test (FST) is the behavioral paradigm most employed for screening new molecules potentially efficacious as antidepressants. A highly heterogeneous sensitivity to antidepressants effects and particularly to SSRIs, among several strains of mice was clearly demonstrated in this paradigm [36, 38]. This fact suggested a fine genetic-dependent regulation in components of serotonin neurotransmission systems involved in antidepressant effects. Many subtypes of serotonin receptors have been proposed as regulating the response in FST either acting at the presynaptic membrane [39, 40] or at a postsynaptical levels [9, 41–43]. One sign of serotonin syndrome, hind limb abduction was expressed specifically by 129S2 mice in FST at doses of SSRIs that are efficacious for other strains of mice [36]. 5-HT$_{1A}$, 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors participate in development of acute serotonin toxicity [41, 44, 45].

The interesting U-shaped dose-response curve observed in SSRI-treated 129S2 mice (Fig. 21.1 a, b) could be explained by a higher extracellular serotonin concentration attained at a single high dose, thus activating additional serotonin receptors [46].

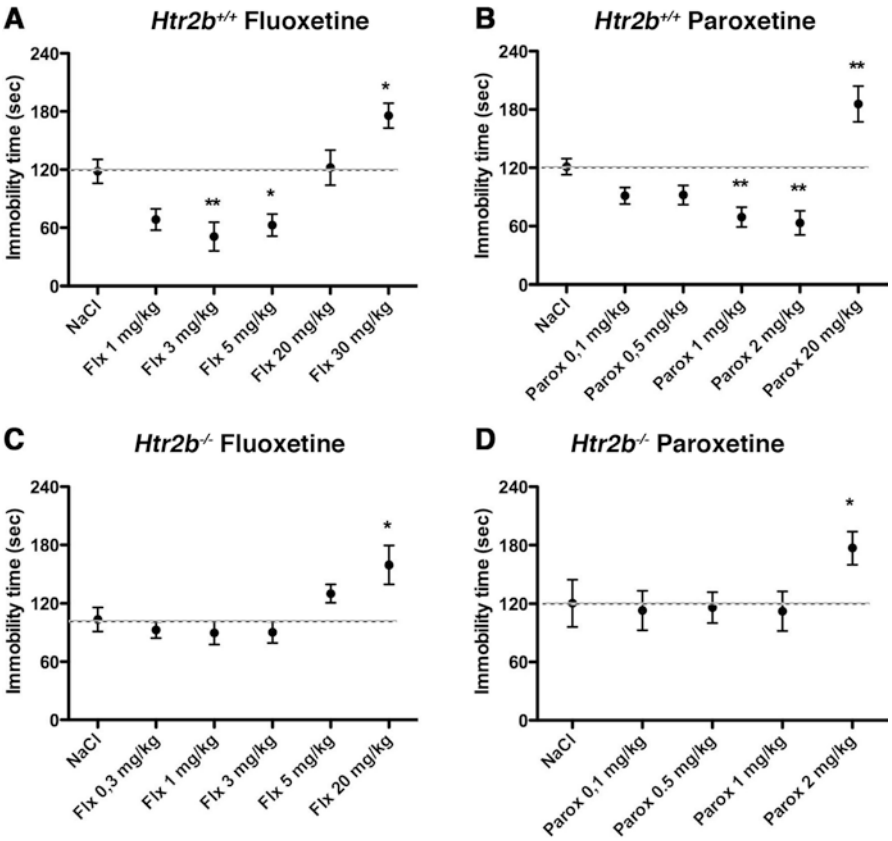

Fig. 21.1 Dose-response curve to SSRIs in the Forced Swimming Test. Immobility time was measured 30 min after ip injection of various doses of SSRIs. The time spent immobile in FST was determined in *Htr2b*$^{+/+}$ after Fluoxetine (Flx -**a**) and Paroxetine (Parox -**b**) and in *Htr2b*$^{-/-}$ mice after Flx (**c**) and Parox (**d**). One-way ANOVA, followed by Dunnet's post-hoc test; **$p < 0.01$; *$p < 0.05$. Data are expressed as mean ± SEM (n = 8–14 mice for each group). Adapted from Diaz et al. [46]

The serotonin syndrome signs observed in mice treated with high doses of SSRIs were unexpected, although 129S2 mice are frequently described as a non- or weakly- responsive strain in these behavioral tests [47, 48]. Nevertheless, 129S2 mice are able to develop classical responses to antidepressants when administered at appropriated doses as it was confirmed by the dose-response curve. Concerning the serotonin syndrome, the importance of mice genetic background in influencing behavioral and neurochemical phenotypes has been previously noticed [23]. Accordingly, a study on sensitivity to antidepressants in different mice strains showed that an increase in immobility time was surprisingly observed only in 129S2 mice receiving Fluoxetine over 20 mg/kg, but not other strains like C57Bl/6J [36]. Thus, it was argued that the dose of SSRIs administered could apparently interfere

with hind limb movements of mice inducing an opposite effect. Likewise, 129S2 mice receiving fluoxetine 30 mg/kg experienced hind limb abduction and Straub tail, both characteristic signs of a serotonin syndrome.

2.2 5-HT$_{2B}$ Receptor in Serotonin Syndrome

Mice knocked-out (KO) for the 5-HT$_{2B}$ receptor gene (*Htr2b*$^{-/-}$) did not respond to the doses of fluoxetine or of paroxetine that were efficacious in reducing immobility time in WT mice (Fig. 21.1 c, d). Moreover, *Htr2b*$^{-/-}$ mice developed characteristic signs of 5-HT syndrome when receiving fluoxetine 20 mg/kg, paroxetine 2 mg/kg, or 5-HTP 50 mg/kg, doses that did not induce a syndrome in WT mice (Fig. 21.1 c, d). Previous studies demonstrated that the absence of 5-HT$_{2B}$ receptors impairs the response to drugs targeting the serotonin system. In particular, 5-HT$_{2B}$ receptors are necessary for the serotonin releasing effect of SERT-targeting drugs, like MDMA (the club-drug ecstasy) and dexfenfluramine [14–16]. In line with these results, acute paroxetine administration to *Htr2b*$^{-/-}$ mice reduced (1/4) the increase in hippocampal serotonin levels compared to WT mice. Pharmacological experiments indicated that the 5-HT$_{2B}$ receptor agonist BW723C86 [16] mimicked SSRI action in the FST, which was abolished by injection of RS127445, a 5-HT$_{2B}$ receptor antagonist, or in *Htr2b*$^{-/-}$ mice [15, 46]. Another receptor that has been involved in acute antidepressant effects of SSRI is the 5-HT$_{1B}$ receptor. Agonist stimulation of 5-HT$_{1B}$ receptors induced classical acute response of antidepressants in the FST in WT as well as in *Htr2b*$^{-/-}$ mice. This effect is clearly independent of 5-HT$_{2B}$ receptors, consistent with previous results demonstrating that 5-HT$_{1B}$ receptor agonists mimicked SSRI effects in FST in rats by acting at postsynaptic receptors [42, 49]. Stimulation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors have an effect opposed to that induced by antidepressants in FST and that is independent of 5-HT$_{2B}$ receptors. Other results suggest that functions of both 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors are opposite in FST. This hypothesis is supported by the fact that 5-HT$_{2C}$ receptors are present on GABAergic interneurons with a constitutive inhibitory activity on raphe neurons [50]. Therefore, and taking into account results with 5-HT$_{2C}$, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors agonists, it seems that at high doses of SSRIs , the increased extracellular serotonin activates these receptors, which are involved in expression of serotonin syndrome, having hindering or facilitating properties in serotonin syndrome.

Although different receptors are mediating serotonin syndrome, the 5-HT$_{2B}$ receptors seem to have a protective role in this syndrome. Indeed, the absence or pharmacological blockade of 5-HT$_{2B}$ receptors increases the risk to develop a serotonin syndrome in response to SSRI administration. Surprisingly, *Htr2b*$^{-/-}$ mice responded similarly to WT mice to agonists of 5-HT$_{1A}$ receptor 8-OH-DPAT, 5-HT$_{2C}$ receptor WAY161503 , or 5-HT$_2$ receptor DOI, suggesting that these post-synaptic pathways are intact in *Htr2b*$^{-/-}$ mice. In addition, plasma serotonin concentrations determined after administration of high doses of SSRIs were similar in either genotype. Therefore, differences in systemic serotonin levels induced by these com-

pounds could not be invocated to explain the differential reactivity. It is conceivable, thus, that the increased sensitivity to develop a serotonin syndrome, when 5-HT_{2B} receptors are not functional, is related to events inherent to 5-HT_{2B}-mediated actions at serotonergic neurons.

3 Chronic SSRIs Responses

Previous studies have suggested a functional interaction between SERT and 5-HT_{2B} receptors. Ex vivo studies have indicated that 5-HT_{2B} receptors might participate in the control of SERT in raphe neurons [51], while in vivo studies further confirmed that 5-HT_{2B} receptors contribute to the behavioral and physiological effects of the SERT-targeting serotonin releasers, MDMA (the club-drug ecstasy) and dexfenfluramine [14–16]. Whereas the increase in serotonin levels is immediately attained after SSRI administration, therapeutic antidepressant effects are only observed after weeks of treatment. The delay before the onset of clinical effects in depressive individuals appears to rely on the time required for stabilization of monoamine levels and other neuroadaptations, including neurogenesis [52]. As well, regulation of serotonin receptors appears to be required for either the acute or chronic effects of SSRIs, as it was largely demonstrated for 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors [41, 52–54]. Importantly, fewer than 50% of all patients with depression show remission with optimized available antidepressant treatments [31]. Despite extensive research, the neurobiological mechanisms underlying antidepressant effects or the resistance to antidepressants are not yet well understood [55, 56].

3.1 5-HT_{2B} Receptors in Chronic SSRIs Responses

Putative positive regulation of dorsal raphe by 5-HT_{2B} receptors has been proposed [57]. Strikingly, long-term effects of SSRIs both in behavior and neurogenesis were eliminated after genetic ablation of 5-HT_{2B} receptors or upon selective chronic antagonist treatment [22]. Conversely, pharmacological experiments indicated that chronic agonist stimulation of 5-HT_{2B} receptors mimicked chronic SSRI action on behavior and neurogenesis, which were abolished in *Htr2b*$^{-/-}$ mice, confirming that effects seen in mutant mice are not a consequence of developmental compensation [22]. Notably, the baseline response in the novelty suppressed feeding (NSF) test is altered in both groups of mice and might result from specific settings established as a consequence of chronically inactive 5-HT_{2B} receptors. The SSRI-reduced latency to feed in the NSF is associated with anxiolytic-like effects, seen after chronic administration. The ability of *Htr2b*$^{-/-}$ mice to show anxiolysis clearly persists, as evidenced by their response to diazepam. The activation of 5-HT_{2B} receptors is necessary for chronic SSRI actions, and chronic stimulation with a 5-HT_{2B} receptor

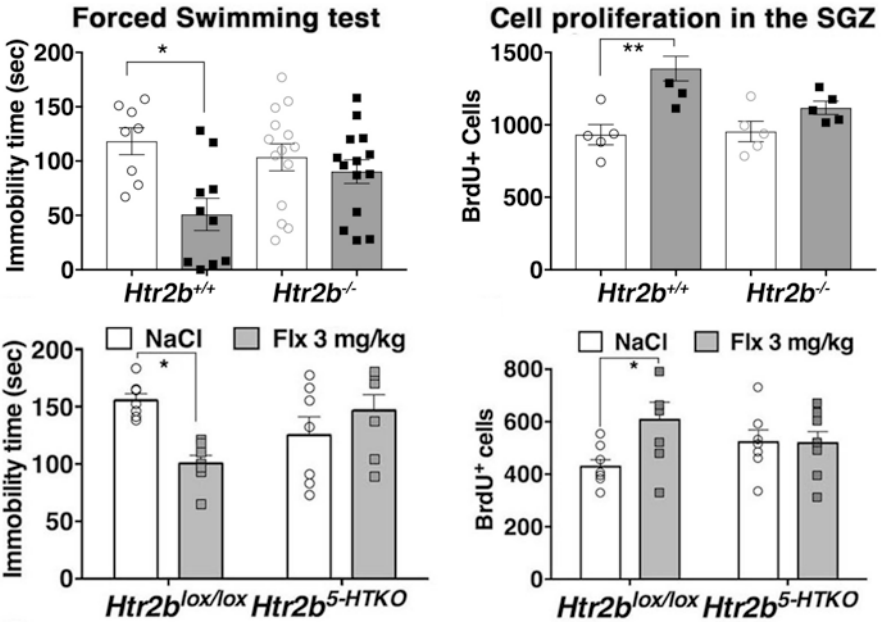

Fig. 21.2 Effects of SSRIs are impaired in the absence of 5-HT$_{2B}$ receptors. (Left) Behavioral effects of SSRIs are impaired in the absence of 5-HT$_{2B}$ receptors. The SSRIs Flx (3 mg/kg/day), decreased significantly the immobility time in the FST of WT mice, but no effect was observed in either *Htr2b*$^{-/-}$ or *Htr2b*$^{5\text{-}HTKO}$ mice. (Right) Hippocampal neurogenic effects of SSRIs are impaired in the absence of 5-HT$_{2B}$ receptors. The SSRIs Flx (3 mg/kg/day), administered daily for 4 weeks, induced a significant increase in cell proliferation in the SGZ of WT mice, but no effect was observed in either *Htr2b*$^{-/-}$ or *Htr2b*$^{5\text{-}HTKO}$ mice. Adapted from Diaz et al. [22, 46] and Belmer et al. [58]

agonist is sufficient to mimic SSRI effects in WT mice. The fact that both 5-HT$_{2B}$ receptor agonist and antagonist induced a similar decrease in the latency to feed appears paradoxical. Moreover, the fact that basal neurogenesis is unaltered in either WT mice chronically treated with RS127445 or in *Htr2b*$^{-/-}$ mice is more consistent with an effect of impulsivity in the NSF in mice with non-functional 5-HT$_{2B}$ receptors than an antidepressant-like action (Fig. 21.2. see also Fig. 21.1 [22]). Furthermore, the reduced increase in extracellular serotonin levels induced by SSRIs in the absence of functional 5-HT$_{2B}$ receptors might be insufficient to trigger behavioral and neurogenic actions [22].

The expression of 5-HT$_{2B}$ receptors by a subset of serotonergic neurons of the raphe nuclei is consistent with a positive regulatory role for these receptors in synaptic serotonin homeostasis. The inhibition of SSRI-induced extracellular serotonin accumulation in mice with no functional 5-HT$_{2B}$ receptors likely reflects a lack of positive control exerted by serotonin signaling in raphe neurons. Indeed, in vivo microdialysis experiments performed with BW723C86 confirm that local 5-HT$_{2B}$ receptor activation in the raphe nuclei is sufficient to induce extracellular

serotonin accumulation [15]. Moreover, stimulation of 5-HT_{2B} receptors appears to be presynaptic since BW723C86-induced acute behavioral effects are abolished in *Pet1*$^{-/-}$ mice (a genetic model of serotonin depletion) or in *Sert*$^{-/-}$ mice. Together, these data identified 5-HT_{2B} receptors as possible positive serotonin autoreceptors, acting in an opposing manner to the 5-HT_{1A} and 5-HT_{1B} receptors, which are well established as negative autoreceptors [59]. This concept is consistent with previous electrophysiology studies showing reduced or abolished 5-HT-dependent depolarization of serotonergic neurons by 5-HT_2 receptor antagonists (ketanserin or mesulergine) in the presence of the 5-HT_{1A} receptor antagonist (WAY100635) [6]. This last point could have important physiological consequences in the mode of action of the receptor.

3.2 5-HT_{2B} Receptors Positively Regulate Serotonergic Neurons

There is a growing consensus that serotonergic neurons are non-homogeneous as supported by anatomical, biochemical and electrophysiological studies [12, 60–62]. Sub-populations of serotonergic neurons, either within the DRN or between various raphe nuclei, are interconnected, and form complex circuits [63–65]. The activity of serotonergic neurons can be modulated by both 5-HT_{1A} and 5-$HT_{2A/2B/2C}$ receptors [6, 8]. Identified serotonergic neurons are known to respond to 5-HT_{1A} receptor agonists by a 5-HT-induced outward current [6–8, 66]. A significant proportion of TPH2-positive neurons (about 50%) also respond to 5-HT_2-receptor activation by an inward current [12].

Tonic spiking of serotonergic neurons establishes synaptic serotonin levels. Cell-attached recordings of identified wildtype raphe Pet1-positive neurons, revealed that stimulation of 5-HT_{2B} receptors by BW723C86 can increase their firing frequency [58]. Independent electrophysiological current-clamp recordings showed that overexpression of 5-HT_{2B} receptors in Pet1-positive serotonergic neurons was sufficient to increase their excitability [58]. These results indicate that 5-HT_{2B} receptors can positively control the firing of serotonergic neurons. This was confirmed in-vivo by extracellular recordings in *Htr2b*$^{5\text{-}HTKO}$ mice of putative serotonergic neurons that showed a significant shift to low firing rate [58]. Together, these results revealed a need for 5-HT_{2B} receptors in serotonergic neurons to positively regulate their activity.

3.3 Serotonergic Tone Results from an Opposite Control Exerted by 5-HT$_{1A}$ and 5-HT$_{2B}$ Receptors

The lack of effects of MDMA and fluoxetine in the absence of 5-HT$_{2B}$ receptors in Pet1-positive serotonergic neurons [58], previously observed in *Htr2b*$^{-/-}$ mice associated to reduced extracellular serotonin accumulation as assessed by microdialysis [15, 22], raised the possibility of an interaction of 5-HT$_{2B}$ receptors with SERT. However, the absence of modification in SERT uptake and expression lowers this possibility. The unique control of dendritic serotonin release has important implications for DRN physiology and actions of SERT-targeting drugs, SSRIs and MDMA. Packaging by the vesicular monoamine transporter (VMAT2) is essential for serotonin transmission; glutamate receptor activation in dorsal raphe brain slice can evoke somatodendritic release by vesicle exocytosis [67]. SSRI antidepressants markedly increase extracellular serotonin in DRN that involves both somatic and dendritic release [67]. The serotonin released within DRN induces feedback inhibition of serotonergic neurons firing activity by stimulation of somatodendritic 5-HT$_{1A}$ negative autoreceptors, which results from local release rather than extended diffusion of serotonin throughout the extracellular space [68].

The hypothermic response to 8-OHDPAT, known to be mediated by 5-HT$_{1A}$ auto- but not hetero-receptors in mice [69], is attenuated by pretreatment with the 5-HT$_{2B}$ receptor agonist BW723C86 [58]. Richardson-Jones et al. [69] generated a mouse strain differing in 5-HT$_{1A}$ autoreceptor expression by approximately 30–40% below the wildtype level (1A-Low). These 1A-Low mice showed reduced 8-OHDPAT-induced hypothermia and their neurons exhibit a shift toward higher firing rates. To the opposite, in the absence of 5-HT$_{2B}$ receptors in Pet1-neurons (in *Htr2b*$^{5\text{-}HTKO}$ mice), a significant increase in hypothermic response to 8-OHDPAT and a significant shift toward lower frequency firing neurons were observed [58]. These findings support that the lack of 5-HT$_{2B}$ receptor in Pet1-positive serotonergic neurons is associated with a higher 5-HT$_{1A}$-autoreceptor reactivity and thus a lower activity of serotonergic neurons.

The lower serotonergic tone observed in the absence of 5-HT$_{2B}$ receptors in Pet1-positive neurons would thus result from the opposite control exerted by 5-HT$_{1A}$ and 5-HT$_{2B}$ receptors on DRN neurons. This may explain the lack of actions of SERT-targeting drugs, SSRIs and MDMA, although the detailed mechanism remains to be identified. An interaction between 5-HT$_{1A}$ and 5-HT$_{2B}$ receptors directly or via trans regulation could be involved as previously reported cross-talks between 5-HT$_{1B}$ and 5-HT$_{2B}$ receptors [70].

3.4 5-HT$_{2B}$ Receptors Contribute to SSRI Therapeutic Effects

The excess of inhibitory control exerted by 5-HT$_{1A}$ receptors in the absence of 5-HT$_{2B}$ receptors in Pet1-positive serotonergic neurons may also explain the lack of response to chronic SERT blockers (fluoxetine) in *Htr2b*$^{5\text{-}HTKO}$ mice. Chronic SSRI antidepressant responses are at least partially ascribed to desensitization of somatodendritic 5-HT$_{1A}$ receptors [69]. Recent works using chemogenetic approaches (i.e., Designer Receptors Exclusively Activated by Designer Drugs-DREADDs) showed that CNO activation of SERT- or Pet1-positive neurons expressing the Gq-coupled M3Gq DREADD induced an increase in serotonergic neuron firing rate and a reduction in immobility in FST [71, 72]. Activation of 5-HT$_{2B}$ Gq-coupled receptors with BW723C86 mimicked both acute and chronic behavioral and neurogenic effects of SSRI antidepressants and led to extracellular serotonin accumulation, which were eliminated in *Htr2b*$^{-/-}$ mice or by RS127445 [15, 22]. Knocking-out the 5-HT$_{2B}$ receptors exclusively from Pet1-positive neurons (*Htr2b*$^{5\text{-}HTKO}$) mice is sufficient to eliminate behavioral effects in FST and neurogenic effects of fluoxetine (Fig. 21.2) and 5-HT$_{2B}$-receptor overexpression increases Pet1-positive neuron excitability [58]. It appears thus that 5-HT$_{2B}$ receptors contribute to SSRI therapeutic effects by their positive Gq-dependent signaling on adult raphe serotonergic neurons, which may be revealed upon somatodendritic 5-HT$_{1A}$-receptor desensitization.

3.5 The Serotonergic Neuron Firing Relies on a Balance of Functional 5-HT$_{1A}$ and 5-HT$_{2B}$ Receptors

The reason why positive 5-HT$_{2B}$ receptors acting in an opposite manner to negative 5-HT$_{1A}$ autoreceptors has not been previously identified could have several explanations. Recently, the role for 5-HT$_{1A}$ receptor-mediated autoinhibition of the DRN in homeostatic control of firing rate has been questioned. As discussed by Andrade et al. [5], 5-HT$_{1A}$ autoinhibition may participate in regulating glutamate signaling to serotonergic neurons [73] or in mediating inputs from distal serotonergic cell groups [63]. De Kock et al. [74] first showed that following calcium influx through NMDA receptors, serotonin could be released from DRN neuron dendrites in the absence of postsynaptic firing. Colgan et al. [73] reported that serotonin release from dendrites is secondary to calcium influx through L-type calcium channels that open in response to the local dendritic depolarization elicited by synaptically released glutamate. A contribution of dendritic serotonin release to 5-HT$_{1A}$-autoreceptor activation would thus result from excitatory glutamatergic inputs to DRN via locally triggered calcium influx rather than by neuronal firing. Independently, it has been reported that upon electrical stimulation of leech serotonergic neurons, transmembrane Ca^{2+} entry through L-type channels can first evoke an early dendritic exocytosis; subsequently, the released serotonin activates 5-HT$_2$ autoreceptors coupled to Gq and phospholipase C, resulting in a positive feedforward loop that maintains

sustained exocytosis [11]. In frog motoneurons, a potentiation of NMDA-induced depolarization has been shown to depend on the activation of 5-HT$_{2B}$ receptors causing an influx of extracellular Ca^{2+} through L-type Ca^{2+} channels and a reduction of the open-channel block of NMDA receptors [75]. Since serotonergic DRN neurons can respond to serotonin with responses ranging from inhibition to excitation with the net effect of serotonin relying on a balance of functional 5-HT$_{1A}$ and 5-HT$_{2A/2B/2C}$ receptors [12], combined expression levels of these receptors in various serotonin subpopulations may set-up DRN firing levels. Since DRN receives serotonergic inputs from caudal raphe nuclei [63], serotonin released in DRN may also originate from extrinsic serotonergic afferents. In conclusion, 5-HT$_{2B}$ receptors can positively modulate serotonergic neuron activity, and counteract 5-HT$_{1A}$ negative autoreceptor actions.

4 Stress-Induced Depressive Like State

Depression is one of the diseases that has been exhaustively studied although several questions remain opened around its ethiopathology and treatment [76]. Antidepressant effects evaluated in "normal" mice may engage different neurobiological mechanisms than those involved in the response of "depressed" individuals [77]. Depressive-like symptoms in animals are not easy to model since many clinical signs of depression are difficult or even impossible to evaluate in animals, such as guiltiness or suicidal ideation [78]. In this respect, measures of anhedonic behaviors like decreased preference for sucrose consumption or reduced interest for hygienic habits are preferred outcomes as they might be indicative of depressive-like behaviors. Additionally, when describing a depressive-like phenotype in animals, the evaluation of several signs rather than single behavioral parameters adds consistency to conclusions. Several paradigms and models have been developed to simulate "depressed" condition in mice like olfactory bulbectomy, learned helplessness, or unpredictable chronic mild stress (UCMS) [78]. The use of chronic social isolation appears as a milder and more appropriate stress paradigm for 129S2 strains [79].

4.1 5-HT$_{2B}$ Receptor in Stress-Induced Depressive Like State

Two behavioral parameters (i.e. coat score and splash test) plus an histologic outcome (DG cell proliferation) were used to characterize the depressive-like state induced by chronic stress and found almost similar outcomes in both *Htr2b*$^{-/-}$ and *Htr2b*$^{+/+}$ mice subjected to chronic social isolation [80]. The lack of 5-HT$_{2B}$ receptors does not modify the vulnerability to develop a depressive-like state following chronic stress. In other words, the 5-HT$_{2B}$ receptor does not appear to participate in the establishment of stress-induced depressive state, whereas it has a key role in the

effects of serotonergic antidepressant. Similar dichotomies have been suggested for other aspect linked to antidepressants. For example, while neurogenesis appears necessary for antidepressant effects [81], it is not clear if defects in neurogenesis play a role in depression [82]. This supports the idea that antidepressants do not necessarily target the causative factors triggering depression.

Following chronic stress, acute antidepressant effects in FST are retained in both *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice for desipramine targeting the norepinephrine system; however, fluoxetine was only efficient in *Htr2b*$^{+/+}$ mice, further supporting the specific alteration of the serotonergic system in these mice [80]. In addition, parameters altered after chronic isolation can be reversed by fluoxetine only in *Htr2b*$^{+/+}$ mice but not in *Htr2b*$^{-/-}$ mice, including the increase in time of grooming in the splash test, and the decrease in latency to feed in the NSF test [80]. Cell proliferation in the DG cell layer is a correlate of chronic treatment with antidepressants originally described in rats [81] and later extended to mice [55]. A significant decrease in SGZ proliferation after chronic isolation was observed in *Htr2b*$^{+/+}$ mice, and a similar trend was detected in *Htr2b*$^{-/-}$ mice. However, treatment with SSRI only reversed this condition in *Htr2b*$^{+/+}$ mice, but not in *Htr2b*$^{-/-}$ mice after chronic isolation. These findings correlate with previous observations made in non-stressed *Htr2b*$^{-/-}$ mice (Fig. 21.2), see also Fig. 3.c and 5.c–d [80], [22, 46]. From a work on astrocytes, it has been suggested that fluoxetine and other SSRIs could be acting as direct 5-HT$_{2B}$ receptor agonists independently of SERT [83, 84]. Previous data [22] do not support this hypothesis due to the absence of antidepressant effects of fluoxetine in mice lacking either the serotonin transporter (*Sert*$^{-/-}$) or differentiated serotonergic neurons (*Pet1*$^{-/-}$). This rules out that the antidepressant effects of fluoxetine could be independent of SERT, and indicates that serotonergic neurons expressing SERT (and 5-HT$_{2B}$ receptors) are necessary for the 5-HT$_{2B}$ receptor effects independently of other cell types. This also 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 two mutant mice (*Sert*$^{-/-}$ and *Pet1*$^{-/-}$) [84] (See also Chap. 1, Fig. 1.2).

4.2 BDNF in Stress-Induced Depressive Like State

Increased BDNF levels observed in the hippocampus of *Htr2b*$^{-/-}$ mice do not dampen the stress response to chronic social isolation [80]. Additionally, when expression of BDNF transcripts were analyzed by qPCR, total BDNF expression was increased in the hippocampus of *Htr2b*$^{-/-}$. Mice heterozygous for *Bdnf* have been proposed as a mouse model of genetic resistance to antidepressants, since *Bdnf*$^{+/-}$ mice do not respond to antidepressant neither in the FST [85, 86] nor in the cell proliferation assay [87]. In *Bdnf*$^{+/-}$ mice, hippocampal extracellular serotonin levels do not increase after acute paroxetine administration [88] as it is the case for *Htr2b*$^{-/-}$ mice [22, 46]. These results suggest that altered levels of BDNF impair the actions of antidepressants. After a report showing an increase of BDNF expression in the hippocampus of rats chronically treated with antidepressants [89], several studies con-

firmed these results in rodents [81, 90, 91], suggesting that increased levels of this neurotrophin could protect neurons from the noxious effects of stress. Further, antidepressant effects were reproduced in rats by infusing BDNF in the midbrain [92] or the hippocampus [93]. In contrast, the role of this neurotrophin in the ethiopathogeny of depression is less studied. The increased BDNF in the *Htr2b*$^{-/-}$ mice could be at least partially responsible for the lack of antidepressant effect of SSRI in these mutant mice [22, 46]. These results are in agreement with a study conducted in *Bdnf*$^{+/-}$ mice in which the altered levels of BDNF attenuate the effect of antidepressants in the resident/intruder test and the tail suspension test but do not affect vulnerability to UCMS-induced stress [94].

In addition to the decreased latency to feed in NSF test, new specific behavioral and neurochemical parameters including a basal increase in hippocampal BDNF levels, with normal TrkB and p75 protein levels, and an increased preference for sucrose consumption were identified. A combination of three independent informations, (a) increased hippocampal BDNF levels and normal TrkB and p75 expression, (b) a significant preference for sucrose consumption, and (c) a decreased latency to feed in the NSF test, supports that *Htr2b*$^{-/-}$ mice display a basal phenotype comparable to animals chronically treated with antidepressants or "antidepressant-like phenotype".

5 Outlook and Prospects

As a high proportion of clinical patients do not respond to classical pharmacotherapies, animal models of resistance to antidepressants are required to more thoroughly study the underlying neurobiological causes of this process and to develop new pharmacological targets. These experiments describe *Htr2b*$^{-/-}$ mice as a useful tool to explore neurochemical and molecular basis of resistance to SSRI antidepressant, one major unsolved problem in clinical treatment of depression. A remaining question is why both positive and negative autoreceptors are needed to regulate serotonergic neuron activity. These findings established that Gq-coupled 5-HT$_{2B}$ receptors expressed by Pet1-positive serotonergic neurons act in an opposite manner as to 5-HT$_{1A}$ autoreceptors. The 5-HT$_{2B}$ receptor can thus be considered as a positive modulator of serotonergic tone that acts at serotonergic neuron excitability. This positive modulation has to be taken into account in the studies of the regulatory mechanisms of serotonergic neurons including those of antidepressants.

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Chapter 22
Serotonin and the 5-HT_{2B} Receptor in Amyotrophic Lateral Sclerosis

Alizée Arnoux and Luc Dupuis

Abbreviation

ALS	Amyotrophic lateral sclerosis
CTs	Clinical trials
DPR	Di-peptide repeat proteins
FTD	Fronto-temporal dementia
GPCR	G-protein coupled receptor
MAO-B	Monoamine oxidase B
PD	Parkinson's Disease
POMC	Pro-opiomelanocortin
ROS	Reactive oxygen species
SERT	Serotonin transporter
SOD1	Superoxide dismutase 1
TDP43	TAR DNA-binding protein 43

1 Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig or Charcot's disease, was described by the French Neurologist Jean-Martin Charcot, in the nineteenth century. This progressive paralysis, usually fatal within a few years after

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onset of motor symptoms, was initially thought to be restricted to the motor system. In recent years however, it appeared that ALS is a much more widespread disease than initially considered, and involves, among other neuronal types, degeneration of brainstem serotonin neurons. In this chapter, we will introduce ALS pathogenesis and mouse models, describe serotonergic alterations, and current knowledge on the involvement of the 5-HT_{2B} receptor in this disease.

2 ALS: Definition and Clinical Presentation

ALS is the major adult onset motor neuron disease, with onset of motor symptoms generally in the fifth of sixth decade of life and progression of disease leading to death, usually from respiratory insufficiency, within the 3 to 5 years after motor onset. ALS epidemiology varies depending on the region and population observed. In Europe, the incidence rate is 3 new cases per year and per 100.000 inhabitants, similar to multiple sclerosis, with a mean age of onset of 65 years of age. ALS is characterized by the simultaneous degeneration of upper motor neurons, in the motor cortex, and of lower motoneurons, in the brainstem and spinal cord, underlying the progressive motor symptoms including paralysis and spasticity. ALS can initially manifest in various sites of onset, leading to three major initial clinical presentations : spinal, bulbar and respiratory [1]. A number of ALS cases are associated with a family history and are called familial ALS (fALS). The remaining 90% cases, without family history are called sporadic ALS cases. More than 30 genes have been linked to fALS, which is thus a genetically heterogenous disease, and five major genes are currently associated with ALS (*C9ORF72*, *SOD1*, *TARDBP*, *FUS* and *TBK1*) and detailed below [2, 3]. Importantly, most of these genes are also associated with another neurodegenerative disease, fronto-temporal dementia (FTD). In particular, the ALS-associated *C9ORF72* mutation, responsible for more than 30% of ALS cases, is also responsible for 25% of FTD cases [2, 4]. Consistent with a substantial overlap between both diseases, a large subset of patients with ALS develop FTD-like cognitive impairment, whose with bulbar onset being more susceptible [5]. Both ALS and FTD patients generally develop cytoplasmic aggregates of the TDP-43 protein. It is thus generally considered that ALS and FTD constitute two extremes of a clinical continuum.

3 Familial ALS Highlights Converging Pathophysiological Mechanisms

3.1 A Subset of ALS Cases Are Caused by Monogenic Mutations

Despite the heterogeneity of ALS, approximately 70% of all European familial cases are caused by monogenic mutations in five genes : *C9ORF72*, *SOD1*, *TARDBP*, *FUS and TBK1* [3, 6]. *SOD1* encodes for the antioxidant enzyme superoxide dismutase 1 and mutations in the *SOD1* gene represent 20% of all familial cases and 1–3% of sporadic cases [7, 8]. Superoxide dismutase 1, or Cu/Zn superoxide dismutase, is a ubiquitously expressed protein, and its mutations lead to ALS through a gain of toxic function, that remains incompletely understood more than 25 years after its discovery [9]. Mutations in *TARDBP* (coding for TAR DNA-binding protein 43; TDP43) were discovered in 2008 [10, 11] following the initial observation that TDP-43 was a major protein component of ALS and FTD protein aggregates [12]. Physiologically located in the nuclei, TDP-43 is an RNA-binding protein involved in multiple aspect of RNA metabolism such as RNA splicing, transport and translation [13]. Importantly, TDP-43 proteinopathy is found in neurons and glial cells in 95–97% ALS patients (sporadic and familial) [14] even in the absence of germline *TARDBP* mutation. Mutations in *FUS,* a RNA-binding protein functionally related to TDP-43, are found in 5% of familial ALS cases, and cause the most severe forms of ALS known to date. As TDP-43, FUS proteinopathy can also be observed in a subset of FTD patients, in the absence of *FUS* mutations and FUS mislocalization is frequent in many ALS cases including sporadic [15]. The most frequent mutation observed in ALS patients is an intronic expansion in the *C9ORF72* gene and was discovered in 2011 [16, 17]. Mutations in *C9ORF72* accounts for more than one third of familial ALS cases and 5–10% of sporadic cases in Europe [18]. The mutation consists of an expansion of the repetition of the hexanucleotide GGGGCC in the first intron of the *C9ORF72* gene. C9ORF72 is involved in vesicular trafficking, especially in autophagy, and the ALS/FTD *C9ORF72* mutation is thought to trigger its toxic effects through (a) haploinsufficiency, due to decreased expression of the mutant allele, (b) RNA-mediated toxicity and (c) translation of the mutant RNA into toxic di-peptide repeat proteins (DPR) [19–21]. TBK1 is the most recently discovered prevalent mutation, with a frequency in patient of 4% [3, 22]. Half of patients carrying TBK1 mutations will also develops cognitive impairment or FTD. TBK1 is involved in the regulation of autophagy [23].

3.2 Impairment of Proteostasis and Autophagy

Ubiquinated protein aggregates are a pathological hallmark of ALS, indicating a disruption in proteostasis. Consistent with this, a large number of genes associated to ALS appears to encode for proteins involved in autophagy [14]. Indeed, VCP, UBQLN2, OPTN and SQSTM1 coding respectively for VCP, ubiquilin 2, optineurin and sequestosome 1 [24, 25] are all autophagy adaptors that allow the targeting of specifically labelled proteins to the autophagy pathway. C9ORF72 is involved in vesicular trafficking and several studies have shown its importance in autophagosome lifecycle [19, 20]. Last, TBK1 is encoding for a major NF kappa B kinase, also phosphorylating optineurin, and critical for induction of autophagy in multiple cell types. Importantly, mutations in TBK1 impair this function of TBK1 in controlling autophagy [3]. Besides, it should be noted that a number of ALS-related mutations, in particular in SOD1 or FUS, increase the aggregative potential of the mutant protein, and thereby could overload the autophagic machinery by clumped protein aggregates.

3.3 Impairment of RNA Metabolism

TDP-43 and FUS are both RNA-binding proteins, and found mutated in a subset of fALS cases, while most patients develop cytoplasmic TDP-43 aggregates. Importantly, these proteins are involved at all steps of RNA metabolism, from transcription, to splicing, transport, translation and degradation. Therefore, their cytoplasmic aggregation is able to lead to loss of their critical nuclear functions, hence multiple RNA metabolism defects. This has been documented for both FUS and TDP43 [13, 26–30].

3.4 Impairment of Cytoskeleton and Vesicular Trafficking

With an axonal length up to 1m and a dissymmetric morphology, motor neurons greatly rely on their cytoskeleton to transport vesicle from the body cell through the axon, to the synapse. A number of cases of familial and sporadic ALS are linked to mutations leading to cytoskeleton disorganization [7]. Indeed, mutations in genes involved in cytoskeleton and vesicular trafficking can alter neurons formation and function by inhibiting axon outgrowth (PFN1; [31]), destabilizing the microtubule's network (TUBA4A; [32]), slowing vesicle transport (KIF5A, DCTN1; [33, 34]) and preventing vesicle formation (ANXA11; [35]). Importantly these different mechanisms are not mutually exclusive as impairment of proteostasis, or of RNA metabolism might also interfere, directly or indirectly with cytoskeleton and vesicular trafficking.

4 Current Treatment Options for ALS

Development of drugs that modify ALS progression has been shown to be quite challenging. There are many reasons for this failure, including high heterogeneity of patients, poorly understood etiology and difficult recruitment for clinical trials. In a study published in early 2017, Petrov et al [36] reviewed a total of 51 studies on 23 different potential treatments in advanced-stage clinical trials. Out of these 51 studies, only 2 reported positive results. We present here the two FDA-approved treatment currently on the market and the primary promising drugs in development.

4.1 FDA-Approved Treatments

The involvement of glutamate excitotoxicity in the pathogenesis of ALS was one of the first hypothesis proposed in the 90s subsequent to observations of abnormalities in the metabolism and transport of glutamate in ALS patients [9, 37]. Riluzole is an antagonist of glutamate, however the exact mechanism of its protective effect in ALS remains unclear, as it has also several other, glutamate independent, effects such as inhibition of sodium persistent inward currents [38]. Riluzole has been shown to slow down disease progression and increase survival in ALS patients in two consecutive clinical trials [37, 39], and this effect seems to occur mostly in the latest phase of the disease [40]. This modest, yet significant, effect led to FDA approval in 1995 and until recently it was the only treatment approved for ALS. At the time of its development there was no mouse model of ALS available, and it was thus directly tested in patients [37]. Now that ALS mouse models are widely available, researchers are trying to replicate these findings on various models. In a study by Hogg et al [41], no effect of riluzole was observed despite the use of 3 different mouse models (SOD1 G93A, TDP-43 A315T and FUS (1-359)). Importantly, riluzole remains the only anti-excitotoxic drug that showed efficacy in ALS, although many other drugs targeting the same pathway failed in clinical trials [36].

Edaravone was approved by the FDA to treat ALS at the end of 2017. As a radical scavenger, edaravone targets the abnormal production of reactive oxygen species (ROS), a long-standing potential culprit in ALS. Indeed, oxidative stress is a shared feature across all form of ALS (familial/sporadic), no matter the mutation involved [4]. In the SOD1 G93A mouse model of ALS, intraperitoneal injection of edaravone had a protective effect on motoneurons, and decreased SOD1 deposits [42]. In humans it appears to be effective in patients in an early stage and specific inclusion criteria [43]. It remains unknown why edaravone seems protective in a subset of ALS patients while several other antioxidant drugs failed in clinical trials. We currently do not have any retrospective study documenting the efficacy of edaravone in a larger ALS population. It is important to notice that the protective effect of edaravone in ALS is highly controversial [44–46], especially as the current mode of administration of this drug might have blurred the results of the clinical trial by

exacerbating the placebo arm of the trial [45]. As such, further studies are warranted to ascertain the efficacy of edaravone.

4.2 Clinical Trials (CTs)

Considering the necessity of developing an effective treatment in ALS and the number of potential targets involved, the number of CTs is unsurprisingly high, with 47 currently active phase 2 or 3 CTs worldwide (from clinicaltrials.gov).

Rasagiline is a MAO-B selective irreversible inhibitor used as a treatment for Parkinson's Disease (PD) and currently being investigated for the treatment of ALS [47, 48]. Monoamine oxidase B (MAO-B) is an enzyme present in the serotonergic neurons of the raphe nuclei and in glial cells. It is mainly responsible for the oxidative catabolism of dopamine [49]. The first neuroprotective effect of rasagiline comes from its antioxidant property as a MAO-B inhibitor. Structurally, rasagiline contains a propargyl group, which is responsible of the anti-apoptotic effect of both rasagiline and its metabolite [50]. In ALS, the drug was found to prolong survival by 20% in the SOD1 G93A mouse model [51]. The results of three phase 2 CTs are currently available. In all three studies, the primary outcome criteria (improvement of the rate of decline in the ALS functional rating scale—revised for two out of three studies, and survival for the third one) was not met [52–54]. However, some results suggest either a target engagement [53], or a potential efficacy in a large subset of patients [54]. All three studies discuss the methodology of patient inclusion and conclude on the necessity to further investigate the potential effect of rasagiline in ALS.

Masitinib is a highly selective kinase inhibitor. Its efficacy has been demonstrated in other diseases such as Alzheimer's disease [55] or multiple sclerosis [56]. Out of the 24 compounds reviewed by Petrov et al. [36] it was the only one to show only positive results. Masitinib has been shown to have a positive effect on survival, microgliosis, neuroinflammation and to control apparition of aberrant glial cells in the SOD1 G93A rat model and in microglial culture [57]. It distinguishes itself from other drugs in development by its capacity to prolong survival of rats after disease onset. A preliminary phase 2/3 trial was recently published suggesting efficacy of masitinib [58]. A phase 3 trial should confirm these promising results.

Other therapeutic opportunities include modification of nutrition. Specifically, increased caloric intake was found protective in mouse models of ALS, and BMI and adiposity are positively correlated with survival of ALS patients [59–63]. Importantly, a small randomized CT including patients with gastrostomy indicated that hypercaloric diet could improve survival of patients [64]. Several randomized CTs are ongoing investigating the efficacy of increasing caloric intake in ALS.

4.3 Mouse Models of ALS

As SOD1 was the first gene that have been linked to ALS, it also was the first transgenic mouse developed as an ALS model. The SOD1 G93A is the most widely used mouse model today, although high expression of the mutant protein leads to artifactual mitochondrial vacuolization that might confound the ALS phenotype [65]. Other mutations include human G37R and G85R mutations, or the mouse G86R mutation, corresponding to the hG85R mutation [66–68]. These models are based on a toxic gain of function of the protein [69]. They share features like a relatively early disease onset and progression, and reproduce the cardinal features of SOD1-ALS, including loss of spinal and cortical motoneurons as well as pathological hallmarks such as astrocytosis and microgliosis or presence of ubiquitinated SOD1 inclusions [70]. Interestingly, disease characteristics are potently altered by alteration of genetic background, suggesting the existence of genetic modifiers, at least in mice [71, 72]. Considering the shared features between the human disease and these mice, these models are of interest for preclinical research. It is important to note however that mutant SOD1 mice also have a number of limitations. First, as a model of *SOD1*-ALS, they reproduce the pathology of these patients, which appears atypical in the absence of TDP43 aggregates. Second, artifactual phenotypes, in particular mitochondrial vacuolization, is possibly confounding preclinical research in this model, and might underlie the failure of clinical trials based on results on SOD1 mice. Other mouse model of ALS/FTD express TDP43 [26, 73], C9ORF72 [74] and FUS [27–29] mutations, and might be useful to confirm and extend results obtained in SOD1 mice.

5 The Serotonergic System in ALS

5.1 Early Studies

Involvement of the serotonergic system in ALS has been studied since the 80s, but most early studies are severely limited by small sample size and use of post-mortem tissues that could heavily confound biochemical results. These early studies mainly focused on serotonin metabolism and do not allow definitive conclusions. Most studies did not find altered levels of serotonin in the spinal cord [75–77], but not all [78], while results on 5-HT metabolite 5-HIAA levels are conflicting [75–78]. Only one study examined the activity of MAO-A in the spinal cord, and activity levels were not detectable, nor in patients or in control [79].

5.2 Peripheral Serotonin in ALS

Peripheral and central pools of serotonin are distinct, and peripheral serotonin is produced in the gut and accumulates in platelets. Indeed, platelets and central serotonergic neurons are equipped with similar serotonin related enzymes and receptor [80, 81]. Dupuis et al. [82] hypothesized that potential alterations in central serotonin could be reflected in platelet serotonin levels. Indeed, these authors showed that the level of platelet, but not plasma-unconjugated, serotonin is decreased in 31% of patients. In this study, plasma 5-HIAA levels were unchanged between patients and controls. Platelet serotonin level was also positively correlated with survival [82].

5.3 Central Serotonin in ALS

As serotonin does not cross the blood brain barrier and ALS is affecting the central nervous system, central serotonin is more likely involved in ALS. Consistently, Dentel et al showed that central serotonergic neurons degenerate in ALS patients and SOD1 mice, and that this leads to decreased serotonin levels in several brain and spinal cord area at disease onset of SOD1 mice [83]. In mice, loss of central serotonin precedes the onset of motoneurons degeneration.

To characterize downstream effects of serotonin loss, it would be critical to target central serotonin itself. However, 5-HT does not cross the blood brain barrier, and this rescue can only be achieved by administering 5-HTP, the precursor of 5-HT. Interestingly 5-HTP treatment appears to improve both survival and locomotor function of SOD1 G93A mouse even if the basal level of 5-HT was not modified in these mice [84]. An alternative strategy would be to decrease serotonin catabolism. To this aim, fluoxetine, as inhibitor of the serotonin transporter (SERT), appears promising. However, fluoxetine treatment appears to exacerbate disease progression in a dose dependent manner when $SOD1^{G93A}$ mice were treated perinatally [85]. Such deleterious effect was not present when fluoxetine was provided to adult mice. Arguably, fluoxetine administration at neonatal stages have been shown to negatively regulate development of the serotonergic system [86]. Therefore, the deleterious effect of neonatal fluoxetine could result from altered development of serotonergic neurons [87, 88]. In rats, MAO-A activity was decreased by 15% after chronic treatment with rasagiline [89]. Several studies have shown that at high concentration or as chronic treatment in vitro, rasagiline, a current treatment candidate, also inhibit partially MAO-A, paradoxically increasing its activity, thus potentially modifying 5-HT brain levels [90, 91]. The encouraging results of several clinical trials for rasagiline in ALS could thus be caused by modulation of serotonin.

5.4 What Could be the Pathophysiological Consequences of Decreased Central Serotonin in ALS?

First, serotonin is a direct modulator of motor neuron excitability. Indeed, serotonin potentiates excitatory transmission, thereby possibly exacerbating excitotoxic mechanisms downstream of glutamate. Interestingly, in the brainstem, groups of motoneurons are either densely innervated by serotonin, such as facial motoneurons, and others receive less 5-HT input [92]. In ALS, those receiving dense 5-HT innervation are more subject to denervation. On the contrary, motoneurons less subject to denervation receives little 5-HT input [92]. In regard of the limbs being the onset site in 70% of patients, it is interesting to note that motoneurons innervating the trunk and limbs are densely innervated by 5-HT [92]. Moreover, a study has recently shown that mice invalidated for TPH2 have altered swallowing function [93]. The fact that most ALS cases begin in the limbs, and that early symptoms includes difficulty swallowing or speaking would thus be consistent with a pathogenic role of serotonergic denervation of motor neurons [9].

Importantly, the alteration of motor neuron excitability caused by serotonergic denervation of motor neurons could significantly contribute to spasticity, a major ALS symptom. Indeed, such a role for serotonin deprivation in spasticity has been previously characterized in spinal cord injury [94], and Dentel et al. [83] demonstrated that 5-HT$_{2B/C}$ inverse agonists could alleviate spasticity in ALS mice. More recently, El Oussini et al. [95] genetically rescued serotonin neurons in a conditional mouse model of ALS, and observed that this rescue prevented the development of spastic like muscle contractions. However, this genetic manipulation, while preventing spasticity, also worsened motor function and accelerated disease onset [95]. This is consistent with the observation that spasticity in patients allows to maintain a basal level of motor function, and thereby prevents the decline of motor functions.

Besides motor neuron excitability, serotonin is involved in other mechanisms of importance in ALS. Weight loss is a critical negative prognostic factor in ALS, and several studies point to dysregulation of hypothalamic networks controlling food intake and energy expenditure as a cause of weight loss [96–98]. Pro-opiomelanocortin (POMC) and AgRP neurons, collectively termed melanocortin system, in the hypothalamic arcuate nucleus are critical modulators of energy metabolism [99] and highly regulated by serotonin [100]. Interestingly, Vercruysse et al. [96] observed defects in the melanocortin pathway, indirectly in patients and more directly in SOD1 mice. Loss of POMC expression could be rescued fluoxetine treatment in mutant SOD1 mice, along with the observed compensatory hyperphagia. Thus, defects in the melanocortin pathway appears downstream and could be subsequent of the loss of serotonin neurons [96].

Roos et al. [101] showed that patients have a higher risk of depression before diagnosis of ALS, and the year after. Since serotonin is critically involved in depression, and degeneration of serotonergic neurons appears before onset, at least in mice, it would be reasonable to hypothesize a serotonergic contribution to ALS-related depression, and the use of serotonin reuptake inhibitors, the first-line

treatment of depression, was suggested to be safe for patients [85]. Serotonin could also be involved in other behavioural alterations in ALS patients, such as inappropriate sexual behavior. Indeed, in a patient exhibiting inappropriate sexual behavior and aggressivity, Sertraline, a selective serotonin reuptake inhibitor, alleviated these symptoms [102]. Further research is needed to evaluate whether the many drugs targeting the serotonin pathway could prove useful in treating symptomatically ALS-related symptoms.

6 Serotonin Receptors in ALS

An alternative manner to determine whether serotonin might be an appropriate target in ALS is to pharmacologically intervene on serotonin signaling. Serotonin acts by binding to its receptors classified in seven families (5-HT_1 to 5-HT_7). All these receptors are G-protein coupled receptors (GPCR) except 5-HT_3 which is an ion channel. Modification of expression of several of these receptors have been described in ALS. Two families of receptors are of particular importance in ALS: 5-HT_1 and 5-HT_2. Other serotonin receptor such as 5-HT_3, 5-HT_5, 5-HT_6 and 5-HT_7 are present on motoneurons but their expression and functions have not yet been studied in ALS.

6.1 5-HT_1 Receptors

5-HT_{1A} and 5-HT_{1B} receptors are the most described regarding their modulation in ALS. They are found in the nervous system, both central and peripheral, and are coupled to a Gi/o effector. Interestingly, 5-HT_{1A} and 5-HT_{1B} receptors are found on motoneurons and serotonergic neurons, respectively on cell bodies and dendrite, and on preterminal axons [103, 104]. 5-HT_{1A} polymorphisms are linked to pathologies like schizophrenia or Tourette's syndrome and to an altered response to 5-HT stimuli [105–107]. Knockout mice for this receptor have an anxious phenotype compared to wild type littermates, without modification of 5-HT levels [108, 109]. Regarding 5-HT_{1B} receptor, polymorphisms have been linked to numerous disorders such as attention deficit hyperactivity disorder, depression and substance abuse [110–112]. Genetic invalidation of this receptor in mice induces aggressive behavior and abolished the hyperlocomotor effect of an agonist targeting both 5-HT_{1A} and 5-HT_{1B} [113].

5-HT_{1A} expression and binding are decreased in the brain and spinal cord of ALS patients by more than 50% and has been shown both by binding assays in post-mortem tissues and PET-Scan in sporadic ALS as well as in presymptomatic SOD1 mutation gene carriers [77, 114]. This receptor being present on both motoneurons and serotonergic neurons, these diminutions could however be secondary to the loss

of these particular neurons. The pathological significance of this loss of 5-HT_1 receptor in ALS remains unknown.

6.2 5-HT_2 Receptors

There are three 5-HT_2 receptors (A, B and C), that are all expressed in the nervous system: 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} [104]. 5-HT_2 receptors are typically coupled to a Gq/11 protein. Early binding studies did not distinguish between 5-HT_2 subtypes and suggested decreased 5-HT_2 receptors binding in the motor cortex of ALS patients by 20% [77], while globally unchanged in the spinal cord. However, these binding sites are grouped in "hot spots" at the post synaptic level [77].

Among 5-HT_2 receptors, 5-HT_{2A} receptor is present on pyramidal cells, which includes motoneurons and is responsible for the motoneuron's increased sensitivity to glutamate-mediated excitation [115, 116]. Its function in ALS has been investigated using drugs with limited specificity. Turner and al. [84] used clozapine, an antipsychotic agent antagonizing the 5-HT_{2A} receptor, but also showing other pharmacological properties including partially 5-HT_{1A} antagonism. In $SOD1^{G93A}$ mice, a low dose of clozapine (2.5 mg/kg PO) rescued motor performance and increased survival [84]. However, high dose of clozapine (7.5 mg/kg PO) yielded opposite effects are observed, with an increased mortality and worsening of motor impairment. The relevance of this drug treatment to ALS, as well as target engagement of 5-HT_{2A} remains unknown.

5-HT_{2B} receptor is also expressed in the central nervous system and several recent observations have suggested its relevance to ALS. 5-HT_{2B} receptor is expressed on serotonergic neurons and microglial cells, where it regulates microglial processes mediated by serotonin [117, 118]. Dentel and al., initially observed an upregulation of 5-HT_{2B} mRNA levels in the spinal cord of $SOD1^{G86R}$ mice at onset motor symptoms, that are coincident with the appearance of spasticity that can be objectively quantified with measurement of tail muscle spasms [83]. Consistent with a potential role of 5-$HT_{2B/C}$ receptors in spastic-like phenotypes, 5-$HT_{2B/C}$ inverse agonists were able to potently prevent their appearance (Fig. 22.1).

To determine whether 5-HT_{2B} or 5-HT_{2C} receptor was involved in this phenotype, El Oussini et al. crossed $SOD1^{G86R}$ mice with mice lacking 5-HT_{2B} receptor (*Htr2b*$^{-/-}$). Disease associated muscle spasms were unaltered in SOD1 mice without 5-HT_{2B} receptor suggesting that this phenotype is more likely due to the 5-HT_{2C} receptor. However, ablation of 5-HT_{2B} receptor led to a decreased survival of 30%, an atrophy of motoneurons cell bodies and an overall exacerbation of the disease. Indeed, the upregulation of the 5-HT_{2B} receptor is intrinsically microglial [119]. Consistent with this hypothesis, expression levels of genes involved in microglial homeostasis were decreased in *$SOD1^{G86R}$; Htr2b*$^{-/-}$ mice and survival of primary microglia was dependent upon tonus of the 5-HT_{2B} receptor. Last, El Oussini et al. [119] identified a polymorphism in the human *HTR2B* gene that appeared associated with higher mRNA levels in spinal cord and decreased signs of microglial degeneration.

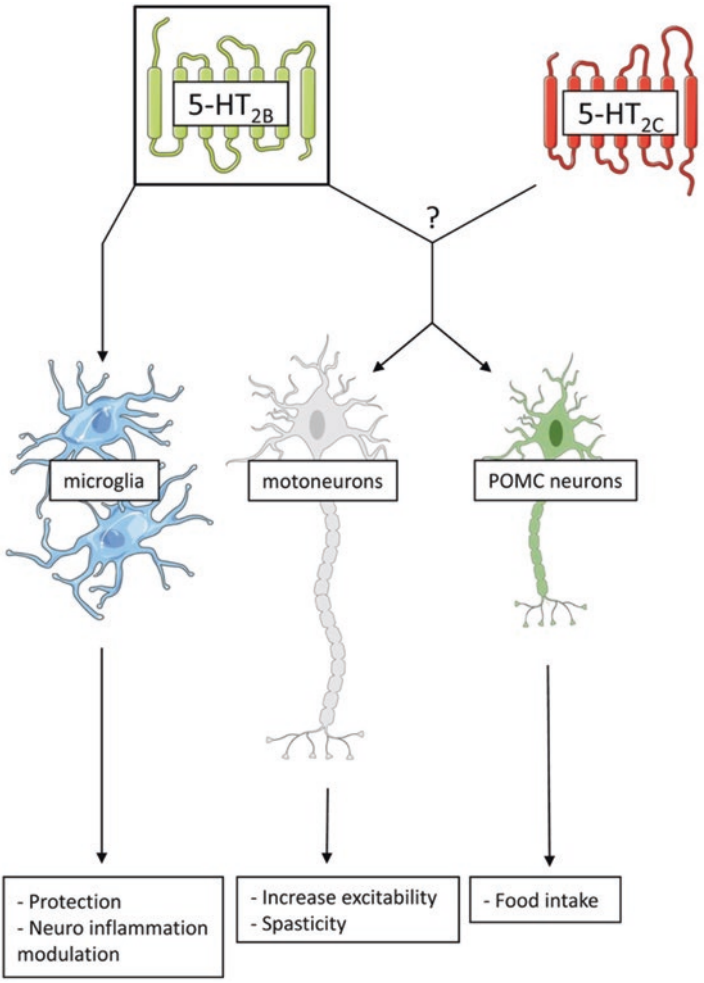

Fig. 22.1 5-HT2B/2C contribution to ALS pathomechanisms. This figure presents 5-HT_{2B}-mediated effects on microglia, as well as 5-HT_{2B}- and/or 5-HT_{2C}-mediated effects on POMC neurons and motoneurons

Interestingly, patients carrying this polymorphism showed slightly increased survival as compared with those not carrying the polymorphism (Fig. 22.2).

Altogether, these data suggest that loss of the 5-HT_{2B} receptor could prove detrimental in ALS. It remains to be tested whether stimulation of the 5-HT_{2B} receptor could be protective.

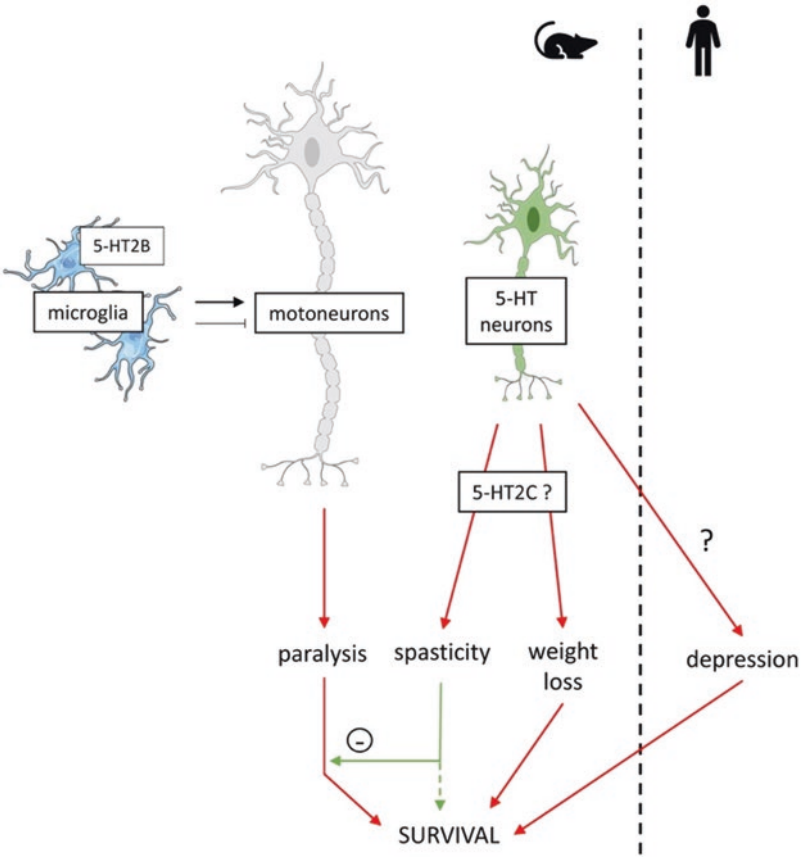

Fig. 22.2 Involvement of the serotonergic system in survival modulation. Left part of the figure has been demonstrated in mouse models, in regard to serotonergic-mediated behavior modulation studied in patient (right part of the figure). Red arrows indicates a negative effect on survival, and green arrows indicate a beneficial effect

7 Outlook and Prospects

In all, involvement of the serotonergic pathway in ALS has been demonstrated, both in the development of the pathology, and in the development of symptoms linked to the disease such as depression or behavior modifications. Whether or not the modulation of this pathway could improve ALS pathogenesis or disease outcome need to be determined by further studies. In particular, receptors 5-HT$_{2B}$ and 5-HT$_{2C}$ appear to be linked to critical features like the development of spasticity. Their presence on numerous cell types in the central nervous system make them potentially good targets for neuroprotection.

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