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Serotonin

A Chemical Messenger Between All Types of Living Cells

Edited by Kaneez Fatima Shad

SEROTONIN - A CHEMICAL MESSENGER BETWEEN ALL TYPES OF LIVING CELLS

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Meet the editor

Professor Kaneez Fatima Shad is an Australian neuroscientist, and she did her PhD degree studies in 1994 from the School of Physiology and Pharmacology, Faculty of Medicine, UNSW. She is an academic, with more than 35 years of experience in teaching Medical and Health Sciences in various universities including Australia, the USA, UAE, Bahrain, Pakistan, and Brunei. She wrote 50 peer-reviewed papers, 3 text book chapters, and 2 invited reviews along with more than 90 international conference abstracts mostly as a result of her 22 successful research grants and 35 postgraduate students. She also edited 3 books. Her research passion is to identify peripheral markers for cerebro and cardiovascular disorders by using skills such as patch clamping, tissue culture, MRI, platelet electro-pharmacology, as well as molecular biological and biochemical techniques.

Contents

Preface

In this book, we are fortunate to have chapters written by the experts in their field covering from phylogenic distribution, types of receptors, to metabolism of serotonin and its effect on different systems ranging from neurological, immunological, to cardiovascular system.

We divided this book into four sections according to the contents of the chapters included. Under *Phylogeny*, we have three very interesting chapters encompassing from the origin of serotonin to its function in bivalves, followed by the three chapters in the *Types* section, in which two very upright papers by different authors focusing on 5-HT1A receptor, which is the most widely spread serotonin receptor controlling almost all systems including endocrine and neuromodulation, and one paper exquisitely describe the influence of ligand-gated serotonin receptor 5-HT3R in carving brain. In *Metabolism* section, three decent papers discuss the outcomes of different serotonergic metabolic products on both brain and body. In the *System* section, we selected four brilliant papers on the effects of serotonin on neurological, immunological and cardiovascular systems.

This book is for a wide range of audience and we tried to include a variety of interesting chapters to cater all types of scientific taste buds.

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Section 1

Phylogeny

Chapter 1

Introductory Chapter: Serotonin - The Most Ancient Neurotransmitter, Hormone and Trophic Factor

Kaneez Fatima Shad

Additional information is available at the end of the chapter

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1. Introduction

This book, as its title indicates, is about "Serotonin: a chemical messenger for all living cells", which is not only present in every tissue of human body but also detected in all aerobic organism including plant and bacteria. In humans, serotonin acts as a trophic factor starts soon after conception and is directly related to the production of serotonin by mother's enterochromaffin cells in the gut and its transfer into the platelets in mother's blood. At the same time from a very early stage of gestation, fetus also starts synthesizing its own serotonin in a special group of nuclei of the midbrain. Soon after serotonergic neurons distribute it throughout the brain and body of the fetus in turn increases division, migration and maturation of both central and peripheral tissues.

Serotonin, which is also known as 5-hydroxytryptamine (5-HT), acts both as neurotransmitter and hormone and is mainly found in the brain, bowels and blood platelets. In 1948, Rapport identified a serum agent that affected vascular tone and called it serotonin. Later, in 1950, he identified chemical composition of serotonin and named it 5-hydroxytryptamine. Serotonin, a biogenic amine is produced by the conversion of amino acid tryptophan in the presence of an enzyme tryptophan hydroxylase (TPH) that exists both in brain and bowel. Till now this enzyme is of two types: TPH1 (found in peripheral organs and CNS) and TPH2 (present only in the brain).

Enteroendocrine cells derived 5-HT acts also as a hormone, which perform multiple functions including inhibition of osteoblast proliferation and promotion of hepatic regeneration. The chemical formula of 5-HT is $N_2OC_{10}H_{12}$, such that 15.8970% nitrogen, 9.0793% oxygen, 68.1598% carbon and 6.8638% hydrogen.

2. Effect of serotonin on brain and body

In the central nervous system, serotonin is synthesized by the neurons of the Raphe nuclei from the amino acid tryptophan through a short metabolic pathway that contains two enzymes: tryptophan hydroxylase and amino acid decarboxylase, and is distributed along the length of the brainstem. Serotonin is released from the varicosities along the axon into the extra-neuronal space this provides a larger area for serotonin to activate its receptors that exist on the dendrites, the cell bodies and the presynaptic terminals of the adjacent neurons. Thus serotonin not only stimulates postsynaptic serotonin receptors but also present on the extrasynaptic neurons.

Thus, it is not a surprise to find significant role of serotonin in the modulation of many behavioral and psychotic disorders such as mood, sleep, appetite, vomiting, sexuality, memory, learning, temperature, cardiovascular and endocrine regulation.

Both high and low levels of serotonin have harmful effect. High serotonin levels cause severe toxicity termed as "serotonin syndrome", which can be fatal in some cases, whereas low levels of serotonin have been associated with migraines, bipolar disorders, apathy, fear, feelings of worthlessness, insomnia, fatigue, anxiety and depression. These pathologies may be explained by the fact that 10% of large dorsal raphe nuclei (largest source of serotonin synthesis) are projected to amygdala and other medium raphe nuclei project to caudate, putamen and olfactory bulb. It is important to mention here that serotonin is required for the metabolism of stress hormone.

Scientific evidence confirms that genetic polymorphisms in the enzyme tryptophan hydroxylase in both TPH1 and TPH2 forms can affect the susceptibility to depression and anxiety. Furthermore, ovarian hormones can affect the expression of tryptophan hydroxylase, triggering postnatal depression and premenstrual stress syndrome, and expression of abnormal serotonergic neurons in infants may lead to high possibility of having sudden infant death syndrome (SIDS). Serotonin is also involved in the regeneration of organs such as liver and bone and induces cell division throughout the body.

Large diversity of serotonin receptors creates its pharmacological complexity. There are at least seven types and eight subtypes of serotonin receptors that have been identified in different areas of the body, and they all have diverse effects. Serotonin receptors are activated by psychoactive drugs such as ecstasy (MDMA), LSD, DMT and psilocybin (a substance found in psychedelic mushrooms). A small dose of ecstasy, for example, stimulates a big release of serotonin in the body causing feelings of well-being and comfort but with many side effects.

5-HT receptors (1–7) are mainly second messenger-gated receptors of which only serotonin type-3 receptor is Ligand-gated ion channel and is involved in nausea and emesis as well as a therapeutic target for depression and other mental conditions. 5-HT1 receptor has five subtypes (5-HT1A–1F, no 1E subtype) which are potential site for anxiolytics and antidepressants as these receptors are mainly responsible for regulating emotions and proprioception. Activation of 5-HT1B and 1D receptors cause vasoconstriction and their antagonists are used for the treatment of schizophrenia and migraines and their partial agonist acts as therapeutic targets for anxiety and depression.

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Similarly, 5-HT2 receptor has three subtypes (5-HT2A–2C) and responsible for sleep, pain and motor regulation and are targeted for conditions such as anxiety, migraine and eating disorders. There are certain psychiatric medications that modulate the levels of serotonin in the human body. These drugs are classified into four general categories: (1) monoamine oxidase inhibitors (MAO), (2) tricyclic (TCA) antidepressants, (3) atypical antipsychotics and (4) selective serotonin reuptake inhibitors (SSRIs). SSRIs are prescribed for the treatment of social phobia, anxiety disorders, panic disorders, obsessive-compulsive disorders (OCD), major depression, irritable bowel syndrome (IBS) and eating disorders.

It is vital to know the mechanism of neuronal communication to understand the mode of action of SSRIs. Briefly, two neurons talk to each other by releasing their neurotransmitters in a space (known as synapse) between them. These neurotransmitters travel from presynaptic neurons via synapse to their specific receptors present on the postsynaptic neurons and stimulate them. Once the postsynaptic neurons receive this signal and get activated, these neurotransmitters go back through the transporters present on the presynaptic neurons. SSRIs are the class of drugs that inhibit these serotonin transporter's activities and let serotonin to halt in synapse for a longer period of time. Whereas TCA will let both serotonin and norepinephrine to stay in the gap for extended period of time than normal so both types of postsynaptic neurons can stimulate completely.

As mentioned earlier, all neurotransmitters, especially biogenic amines (serotonin, norepinephrine and dopamine) regulate each other, for example, stimulating 5-HT2 and 5-HT3 receptors by using SSRIs and result in the decrease in levels of dopamine released from the Substantia Nigra, leading to serious mental health problems. Patients on SSRIs or TCA for a longer period of time or in combination with MAOs become very agitated, having tremor and involuntary muscle contraction leading to impaired respiration, increased carbon dioxide pressure and hypoxia.

The role of 5-HT receptors is a topic of intense research, so more therapeutic applications may be discovered in the future. Concisely, role of serotonin in central nervous system is to control appetite, vomiting, sleep, mood, hallucinations and pain perception, and peripherally, responsible for the contraction of vascular and non-vascular smooth, platelet aggregation, increased capillary permeability and modulation of the release of other neurotransmitters.

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Chapter 2

Pharmacology and Molecular Identity of Serotonin Receptor in Bivalve Mollusks

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Additional information is available at the end of the chapter

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Abstract

It is now known that 5-HT regulates several neurobehavioral systems such as mood, appetite, sleep, learning, and memory. It also plays critical roles in the physiological functions of peripheral organs involved in stress, growth, and reproduction in the animal kingdom. 5-HT content has seen to be higher in the nervous system of bivalves than those of other examined invertebrates and vertebrates. Thus, bivalves have been considered as an excellent model to investigate 5-HT functions in neurological and peripheral systems. The present study reviews knowledge on 5-HT signaling mediated through 5-HT receptor and its physiological contribution to regulate reproduction in bivalves. Two G-protein-coupled 5-HT_1-like receptors have been cloned in bivalve species. However, binding affinities of the 5-HT agonists and antagonists to the isolated plasma membrane proteins and their effects on spawning in bivalves suggest the presence of a single or mixed 5-HT_1-, 5-HT_2-, and 5-HT_3-like receptors. It has suggested that the 5-HT-like receptors in bivalves are distinct from those of mammalian 5-HT receptors due to pharmacological properties. The present review pays a special attention to future research perspectives to better understand 5-HT regulation of reproduction in bivalves, which can provide us with satisfactory knowledge to elucidate reproductive disorders associated with dysfunctions of the neurotransmitter system.

Keywords: gonad, nervous system, oocyte, serotonin biosynthesis, serotonin metabolism and reuptake, serotonin receptor, sperm

1. Introduction

5-hydroxytryptamine called serotonin (5-HT) is a transmitter substance of the nervous system in animal kingdom. 5-HT has also been identified in bivalves from the period of its first discovery and earlier studies on these animals have led to convince the neurobiologist that it acts as a neurotransmitter.

A brief bibliography of discovery for 5-HT receptor and its physiological functions is provided in **Table 1**. Gaddum and Picarelli [6] were the first who demonstrated that 5-HT acts through a receptor-mediated pathway. Further studies have then directed toward pharmacological characterization of the 5-HT receptors in the nervous system and peripheral organs using radiolabelled ligands [7, 8] until the first molecular identity of the 5-HT receptor [9]. In 1960–1980s, 5-HT neurons have localized in the nervous system and peripheral organs (including gonad) of bivalves. Then, Sugamori et al. [10] and Tanabe et al. [11] cloned the 5-HT receptors in the nervous system and reproductive system of pond snail (*Lymnaea stagnalis*) and Yesso scallop (*Patinopecten yessoensis*), respectively. Taken together, bivalves and mammals become model organisms to investigate receptor-mediated mechanism of 5-HT physiological function because of small size, a simple nervous system and a high content of 5-HT in the nervous system.

Year	Scientists	Contribution to discovery of identification, localization, and characterization of 5-HT	References
1957	Gaddum and Picarelli	Suggestion of two types of 5-HT receptors (5-HT_M and 5-HT_D) in the guinea-pig ileum	[6]
1978	Fillion et al.	Identification of 5-HT receptors in the bovine brain using radiolabelled ligands: [^{3}H]-5-hydroxytryptamine and [^{3}H]-lysergic acid diethylamide	[7]
1979	Peroutka and Snyder	Evidence for the presence of two distinct 5-HT (5-HT_1 and 5-HT_2) in the rat brain derived from their selective recognition by radiolabelled ligands	[8]
1982	Matsutani and Nomura	Serotonin stimulates spawning in Yesso scallop (Bivalvia, Mollusca)	[18]
1984	Hirai and Koide	5-HT stimulates oocyte maturation in surf clam	[27]
1985	Osanai	5-HT regulation of the oocyte signaling required to undergo germinal vesicle breakdown	[28]
1988	Fargin et al.	Molecular identity of 5-HT_{1A} receptor	[9]
1991	Bandivdekar and Koide	Pharmacological identification of serotonin receptor in surf clam	[29]
1993	Sugamori and Van Tol	Molecular identity of 5-HT receptor in pond snail (Gastropoda, Mollusca)	[10]
2010	Tanabe and Osada	Molecular identity of 5-HT receptor in Yesso scallop	[11]

Species: pond snail, *Lymnaea stagnalis*; surf clam, *Spisula solidissima*; Yesso scallop, *Patinopecten yessoensis*.

Table 1. Bibliography of 5-hydroxytryptamine (serotonin, 5-HT) receptor: from discovery to physiological characterization.

Serotonin regulates various neurobehavioral systems (such as mood, appetite, sleep, learning, and memory). However, studies have revealed that it also plays critical roles in physiological functions of peripheral organs such as stress and growth [1–3]. One of the major system that 5-HT contributes to its regulation is reproduction. In both mammals and bivalves, it has observed that 5-HT regulates reproductive endocrine system, oocyte maturation, and sperm motility [12–23].

Although 5-HT biosynthesis and its receptor structure have been reviewed in bivalves [24–26], however, there is a gap of review on physiological signaling of 5-HT in these animals. The present study reviews the biology of 5-HT in bivalves, particularly, its contribution to reproduction. Particular attention has then paid to pharmacological characteristics of the 5-HT receptor and 5-HT-stimulated spawning through a receptor-mediated mechanism. This study provides future perspectives that await investigation to better understand 5-HT network and signaling in bivalve reproduction.

2. Molecular identity and pharmacological characteristics of the 5-HT receptors

Since the time Gaddum and Picarelli [6] suggested the presence of two kinds of tryptamine receptor, further studies have been conducted to identify and localize the 5-HT receptors to elucidate serotonergic signaling in biological systems. Fargin et al. [9] were the first who reported that the protein product of an orphan receptor (G21) encoding a G-protein-coupled receptor (GPCR) transiently expressed in monkey kidney cells possesses all the typical ligand-binding characteristics of the 5-HT_{1A} receptor. Molecular identity of 5-HT receptors has revealed that there are, so far, a total of 14 structurally and pharmacologically distinct mammalian 5-HT receptors which are classified into seven groups. Except of the 5-HT_3 receptor that is a ligand-gated ion channel [35, 36], the 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 belong to GPCR superfamily [4, 5, 37–41]. In invertebrates, pharmacological properties of the 5-HT receptors do not allow us to classify them in mammalian categories, although some signal transduction characteristics are similar [26].

2.1. Pharmacological characteristics of 5-HT receptors in bivalves

In bivalves, primary studies have used pharmacological 5-HT agonists and antagonists to investigate their binding affinities onto isolated membrane proteins of the oocytes and sperm using radiolabelled [^{3}H]5-HT [29, 42–45]. The results showed that only 5-HT and its analogs are capable of inhibiting [^{3}H]5-HT-specific binding to the isolated plasma membrane proteins of the oocytes in surf clam, whereas other monoamines (such as acetylcholine, haloperidol, carbachol, pyrilamine, and so on) are without effects [43, 44].

In surf clam, 1 μM ICS 205930, 5-HT, 5-CT, mianserin, methysergide, 8-OH-DPAT, 2-methyl-5-HT, BMY 7378, α-methyl-5-HT, ketanserin, quipazine, and PBG inhibit [^{3}H]5-HT binding to the isolated proteins of the oocyte plasma membrane by 49, 46, 40, 40, 37, 35, 33, 28, 26, 25, 22, and 11%, respectively [29]. The authors suggested that 5-HT receptors in the oocyte of

surf clam possess sites that interact with the 5-HT_1 and 5-HT_3 receptor analogs, because of the binding affinity of the 5-HT_1 receptor (5-CT, mianserin, methysergide, and 8-OH-DPAT) and the 5-HT_3 receptor (ICS 205930 and 2-methyl-5-HT) analogs. However, current pharmacological characterization of 5-HT receptor analogs reveals that 5-CT is a non-selective agonist, and mianserin and methysergide are particularly selective antagonists of the 5-HT_2 receptor (**Table 2**). These may suggest that the 5-HT_2 receptor also exist on the membrane of the oocytes in surf clam, in addition to the 5-HT_1 and 5-HT_3 receptors [29, 46].

Krantic et al. [43, 44] studied dose-dependent effects of the 5-HT analogs and observed that 5-HT, 8-OH-DPAT, metoclopramide, MDL 72222, mianserin, ICS 205930, ritanserin, imipramine, propranolol, and TFMPP inhibit specific [^{3}H]5-HT binding to the isolated membrane

Receptor	Agonists	Reference	Antagonist	Reference
5-HT_1	8-OH-DPAT (5-HT_{1A})	[47]	Propranolol (5-HT_{1B})	[49, 50]
	TFMPP (5-$HT_{1A, 1B, 1D}$)	[48]	NAN-190 (5-HT_{1A})	
			BMY 7378	[61, 62]
5-HT_2	TFMPP (5-$HT_{2A, 2C}$)	[49]	Ketanserin (5-HT_{2A})	[50]
	mCPP (5-$HT_{2B, 2C}$)	[50]	Spiperone (5-HT_{2A})	[50]
	PBG	[51]	1-NP (5-$HT_{2A, 2B, 2C}$)	[63, 64]
			Cyproheptadine (5-$HT_{2A, 2B}$)	
			Mianserin (5-$HT_{2A, 2B, 2C}$)	[50]
			Ritanserin (5-$HT_{2A, 2B, 2C}$)	[65]
			Methysergide (5-$HT_{2B, 2C}$)	[66]
5HT_3	1-m-c-b (mCPBG)	[52]	Metoclopramide	[67, 68]
	2-methyl-5-HT	[53]	ICS 205-930 (Tropisetron)	[53, 69–71]
	Quipazine	[54]	LY-278584	[72, 73]
			MDL-72222 (Bemesetron)	[69, 74]
			Ondansetron	
Non-selective	α-Methyl-5-HT (5-$HT_{1, 2}$)	[55]	Methiothepin (5-$HT_{1A, 1B, 1D, 5A}$)	[75]
	5-CT (5-$HT_{1A, 1B, 1D, 5A, 7}$)	[56–60]		

α-methyl-5-HT, α-methyl-5-hydroxytryptamine; 1-m-c-b, 1-methyl-chlorophenyl biguanide; 2-methyl-5-HT, 2-methyl-5-hydroxytryptamine; 1-NP, 1-(1-naphthyl)piperazine; 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 7-(dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol; mCPP, *meta*-chlorophenylpiperazine; MDL-72222 (Bemesetron) PBG, 1-phenylbiguanide; and TFMPP, 3-trifluoromethylphenylpiperazine.

8-OH-DPAT also acts as a 5-HT_7 receptor agonist [76] and possesses serotonin reuptake blocking property [77]. TFMPP binds to SERT and evokes 5-HT release [78]. *mCPP acts as 5-HT reuptake inhibitor/releasing agent* [79]. Unlike mCPP, TFMPP has insignificant affinity for the 5-HT_3 receptor [80]. BMY-7378 is a weak partial 5-HTlA agonist compared to 8-OH-DPAT that is a full 5-HT1A agonist [81, 82] and is a selective antagonist of α_{1D}-adrenoceptors [83]. PBG and mCPBG have dopamine releasing properties [84]. Methysergide also acts as a 5-$HT_{1A, 1B, 1D}$ receptors' partial agonist. 5-HT and methysergide appear not to compete for the same site, whereas ketanserin and methysergide do appear to compete for the same site [56, 66, 85]. Quipazine also acts via 5-HT_2 receptor as an agonist [86, 87] or antagonist of 5-HT_3 receptor [88, 89]. Metoclopramide acts as antagonist of dopamine D_2 receptors [90] and as a 5-HT_4 receptor agonist [91].

Table 2. Pharmacological agonists and antagonists of the 5-hydroxytryptamine (serotonin, 5-HT) receptors.

proteins of the oocytes in surf clam by 100, 67, 63, 61, 57, 57, 55, 49, 47, and 12% with IC_{50} of 0.52, 0.05, 0.06, 0.13, 0.45, 3.05, 0.42, 4.2, 1.32, and >100 μM, respectively. Hence, these results showing affinities of the 5-HT analogs to the 5-HT_1, 5-HT_2, and 5-HT_3 receptors in the oocyte of surf clam; however, the receptor possesses distinct 5-HT binding sites from 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 receptors in mammals and *Drosophila*. For instance, the 5-HT_{1A} receptor is more sensitive to 8-OH-DPAT than 5-HT, insensitive to ritanserin, and relatively sensitive to TFMPP in mammals. 8-OH-DPAT is a weak agonist on the *Drosophila* 5-HT receptors. Ritanserin, but not TFMPP, inhibits [^{3}H]5-HT binding to the isolated membrane protein of the oocyte in surf clam, although isolated 5-HT receptor is highly sensitive to 8-OH-DPAT more than that of 5-HT. The 5-HT receptor in the oocyte of surf clam does not possess pharmacological 5-HT_2 receptor characteristics in mammals, as it is not equally sensitive to TFMPP and 8-OH-DPAT. The pharmacological characteristics of the isolated 5-HT receptor also differ from the 5-HT_3 receptor. In mammals, the 5-HT_3 receptor is at least 100-fold more sensitive to 8-OH-DPAT than to metoclopramide; however, 8-OH-DPAT and metoclopramide are equipotent in inhibition of [^{3}H]5-HT binding to the 5-HT receptor in the surf clam. Based on these different responses of the isolated membrane protein of the surf clam oocytes to the 5-HT analogs, the authors suggested the presence of a novel 5-HT receptor in the plasma membrane of the surf clam oocytes.

In Yesso scallop, Osada et al. [45] observed that [^{3}H]5-HT binding to the oocyte plasma membrane is inhibited to 93, 83, 70, 44, 41, and 36% in the presence of 100 μM metoclopramide, 8-OH-DPAT, 5-HT, ritanserin, α-methyl-5-HT, and methiothepin, respectively. In the Pacific oyster, [^{3}H]5-HT binding to the oocyte plasma membrane is inhibited to 96, 83, 58, 49, 21, and 16% in the presence of 100 μM metoclopramide, 8-OH-DPAT, 5-HT, α-methyl-5-HT, ritanserin, and methiothepin respectively [45]. Ritanserin-, α-methyl-5-HT-, and methiothepin-inhibited [^{3}H]5-HT binding to the 5-HT receptor isolated from the oocyte of Yesso scallop suggest that mixed 5-HT_1 and 5-HT_2 receptors function in this species. However, the authors suggested that a single 5-HT_1 receptor functions in the Pacific oyster as methiothepin acts mainly as a 5-HT_1 antagonist (**Table 2**). In addition, this study shows that metoclopramide does not influence [^{3}H]5-HT binding to 5-HT receptor isolated from the oocyte of Yesso scallop and the Pacific oyster and 8-OH-DPAT is also a weak agonist, suggesting that 5-HT signaling is not mediated by 5-HT_3 receptor and is distinct from mammalian 5-HT_{1A} receptors in these species.

Pharmacological characteristics of the 5-HT receptor in sperm have only studied in surf clam [42]. The results have shown that 1 μM ICS 205930, 2-methyl-5-HT, 8-OH-DPAT, BMY 7378, 5-HT, 5-CT, mianserin, methysergide, α-methyl-5-HT, PBG, and ketanserin inhibit 45, 43, 37, 32, 31, 31, 30, 26, 13, 4, and 1% of [^{3}H]5-HT binding to the sperm plasma membrane, respectively. Considering current pharmacological characterization of 5-HT receptors, analogs of 5-HT_3, 5-HT_1, and 5-HT_2 receptors are more potent to compete with 5-HT to inhibit [^{3}H]5-HT binding to the sperm plasma membrane.

2.2. Molecular identity and cellular localization of 5-HT receptors in bivalves

In mollusks, the 5-HT_{Lym} and 5-HT_{2Lym} are first identified in the central nervous system of the pond snail (*L. stagnalis*). They display some pharmacological characteristics of the 5-HT_1 and 5-HT_2 receptors in mammals, and thus are currently considered as the 5-HT_1-like receptor and the 5-HT_2-like receptor, respectively [10, 92]. The Ap5-HT_{B1} and Ap5-HT_{B2} [93], 5-HT_{1AP}[94],

and 5-HT_{2AP} [95] are identified in California sea slug (*Aplysia californica*). The Ap5-HT_{B1} and Ap5-HT_{B2} (79.5% homologous to each other) are expressed in the reproductive system and the nervous system, respectively; however, they are not classified into any 5-HT receptor subtypes in mammals due to differences in their amino acid sequences [93]. The 5-HT_{1AP} is distributed in most organs, including the nervous system, kidney, gills, and heart, and its amino acid sequence and pharmacological profiles suggest that it is a 5-HT_1 receptor subfamily [94]. The 5-HT_{2AP} shares 68 and 34% of its amino acid sequence identity with the 5-HT_{Lym} and 5-HT_1 receptor in mammals, its pharmacological characteristics is very similar to those of the 5-HT_{Lym} receptor, and it is only expressed in the nervous system [95].

In bivalves, the 5-HT receptors are cloned in the ovary of the Yesso Scallop [11], and Pearl oyster, *Pinctada fucata* [96] (**Figure 1**). Molecular identity of the 5-HT receptor is also predicted for the Pacific oyster (5-HT_{cg}) [97]. In the Yesso scallop, an 1818 bp cDNA encodes a putative 5-HT_{py} receptor that includes a 232-bp 5′-untranslated region (UTR), a 1362-bp open reading frame (ORF) encoding a putative protein of 454 amino acids, and a 224-bp 3′-UTR. In the Pearl oyster, a 2541 bp cDNA encodes a putative 5-HT_{pf} receptor that includes a 296-bp 5′-UTR, a 1416-bp ORF encoding a putative protein of 471 amino acids, and an 829-bp 3′-UTR. The 5-HT_{pf} is calculated to have a molecular weight of 53.55 kDa. The hydrophobicity analysis of the deduced amino acid sequence revealed seven putative transmembrane domains, which are highly conserved between 5-HT_{py}, 5-HT_{pf}, and other 5-HT_1 receptors coupled with $G_{i/o}$. The 5-HT_{py} contains two potential sites for N-linked glycosylation in the extracellular N-terminal region and the third intracellular domain. The 5-HT_{pf} receptor contains five potential sites for N-linked glycosylation in the extracellular N-terminal region. There are 12 and 8 sites for phosphorylation by protein kinase A or C in the Yesso scallop and Pearl oyster, respectively, among which 7 sites are located in the third cytoplasmic loop. A relatively long third cytoplasmic loop and a short fourth inner terminal domain (C-terminal tail) are present in the 5-HT_{py} and 5-HT_{pf} sequence.

An amino acid sequence alignment of 5-HT receptor homologs from different species reveals that a relatively high level of amino acid sequence identity exists between 5-HT_{py} and 5-HT_{pf} (52%) and between 5-HT_{py} and 5-HT_{cg} (48%). The amino acid sequence identity is between 5-HT_{pf} and 5-HT_{cg} (71%). There are conserved amino acid regions when the 5-HT_{py} and 5-HT_{pf} are aligned to 5-HT_1 subtypes in human (**Figure 1**). The 5-HT_{py} amino acid sequence is 40, 40, 37, 38, and 38% identical to the human 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptor, respectively. The 5-HT_{pf} amino acid sequence is 42, 39, 39, 40, and 40% identical to the human 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} receptor, respectively. The 5-HT_{cg} was not considered in alignment analysis as it is a predicted sequence. The amino acid sequence identity is higher within the transmembrane domains, compared to those of the intracellular and extracellular region. However, lower amino acid sequence identity exists between the 5-HT receptors in bivalves with the other 5-HT receptors (5-HT_2, 5-HT_5, 5-HT_6, and 5-HT_7) in vertebrates. The phylogenetic analysis of the 5-HT receptors in invertebrates suggests that the 5-HT receptors of bivalves resemble the 5-HT receptors in the California sea slug (*A. californica*), pond snail (*L. stagnalis*), and air-breathing snail (*Planorbella trivolvis*), which are known to be as 5-HT_1-like receptor (**Figure 2**). These known 5-HT receptors are differentiated into a major branch, compared to the other known invertebrate 5-HT receptors. Four 5-HT receptors

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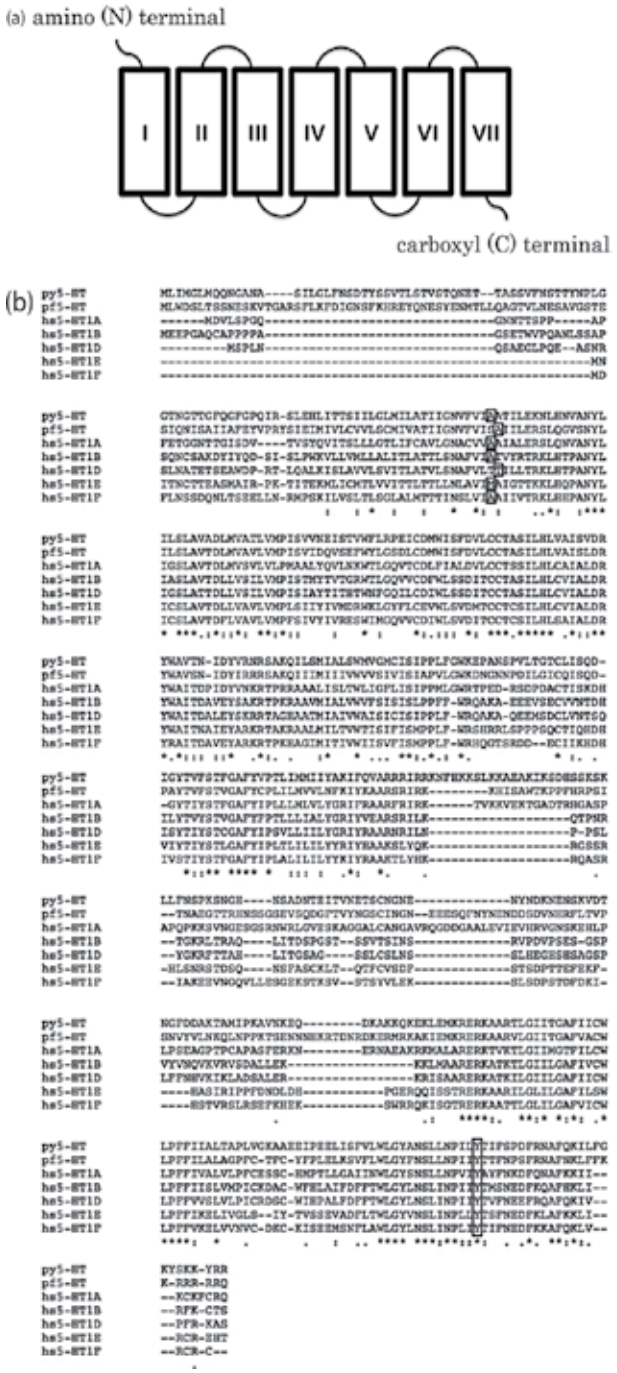

Figure 1. A schematic representation of the G-protein-coupled 5-hydroxytryptamine (serotonin, 5-HT) receptor showing seven transmembrane domains (A). (B) Multiple alignment of deduced amino acid sequence of 5-HT receptors of the Yesso scallop (*Patinopecten yessoensis*, py5-HT) and pearl oyster (*Pinctada fucata*, pf5-HT) with the 5-HT$_{1A\text{-}F}$ receptors in human. The marked amino acids indicate seven transmembrane regions. Sequences are aligned with MUSCLE configured for highest accuracy (www.phylogeny.fr).

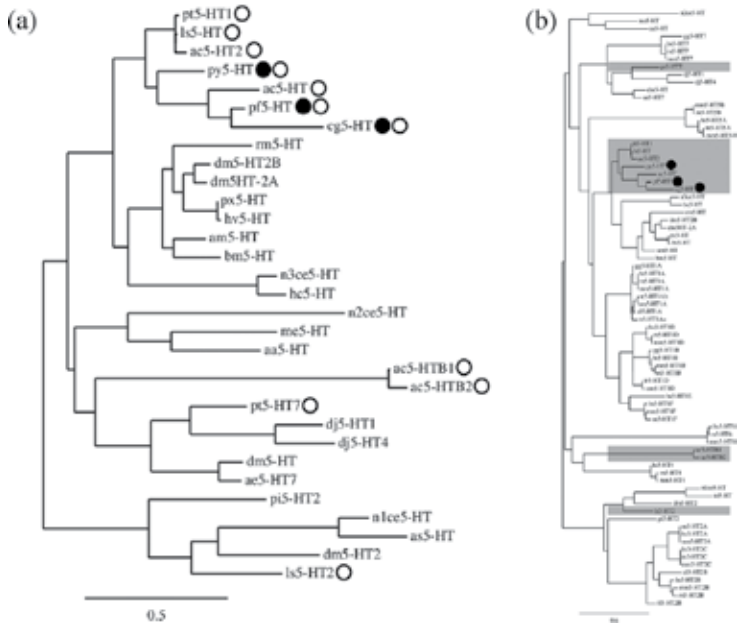

Figure 2. Phylogenetic analysis of the 5-hydroxytryptamine (serotonin, 5-HT) receptor known from invertebrates (A) and from invertebrates and vertebrates (B). Filled circles indicate bivalve species. Open circles or dark background indicate mollusk species. Note that the 5-HT_3 receptors are excluded in this analysis, as they are ligand-gated ion channel. Phylogeny trees are constructed using the maximum likelihood method implemented in the PhyML program. The amino acid sequences of the 5-HT receptors are aligned with MUSCLE configured for highest accuracy (MUSCLE with default settings). After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) are removed (www.phylogeny.fr). Accession numbers of applied 5-HT receptors are as follows: **invertebrates** dm5-HT (AAA28305, 5HT-dro), dm5-HT2 (CAA57429, 5-HT2-dro), dm5HT-2A (CAA77570, 5HT-dro2A), dm5-HT2B (CAA77571, 5HT-dro2B), ae5-HT7 (AAG49292), am5-HT (NP-001164579), px5-HT (BAD72868), rm5-HT (AAQ89933), bm5-HT (CAA64862), hv5-HT (CAA64863), dj5-HT1 (BAA22404), dj5-HT4 (BAA22403), 1ce5-HT (AAC15827), 2ce5-HT (NP-491954), 3ce5-HT (NP-497452), as5-HT (AAC78396), hc5-HT (AAO45883), ac5-HTB1 (Q16950, Ap5HTB1), ac5-HTB2 (Q16951, Ap5HTB2), ac5-HT2 (AAM46088, Ap5-HT2), ac5-HT (AAC28786, Ap5-HT), ls5-HT2 (AAC16969, Lym5-HT2), ls5-HT (AAA29290, Lym5-HT), pt5-HT1 (AAQ95277), pt5-HT7 (AAQ84306), py5-HT (BAE72141), pf5-HT (AIW04132), cg5-HT (EKC38511), pi5-HT2 (AAS57919, 5-HT type 2), me5-HT (AAS05316), aa5-HT (BAA12013), and **vertebrates** tr5-HT1Aa (CAA65175, 5-HT1Aalpha), tr5-HT1Ab (CAA65176, 5-HT1Abeta), om5-HT1A (AAP83427), xl5-HT1A (CAA69208), gg5-HT1A (NP-001163999), rn5-HT1A (NP-036717), mm5-HT1A (NP-032334), hs5-HT1A (NP-000515), gg5-HT1B (NP-001166252), rn5-HT1B (NP-071561), mm5-HT1B (NP-034612), hs5-HT1B (AAH69065), tr5-HT1D (CAA58745), om5-HT1D (AAP83428), rn5-HT1D (NP-036984), mm5-HT1D (NP-032335), hs5-HT1D (NP-000855), hs5-HT1E (NP-000856), rn5-HT1F (NP-068629), mm5-HT1F (NP-032336), hs5-HT1F (NP-000857), rn5-HT2A (NP-058950), mm5-HT2A (NP-766400), hs5-HT2A (NP-000612), tf5-HT2B (CAC85912), xl5-HT2B (CAD71264), rn5-HT2B (NP-058946), mm5-HT2B (NP-032337), hs5-HT2B (NP-000858), rn5-HT2C (NP-036897), mm5-HT2C (NP-032338), hs5-HT2C (NP-000859), rn5-HT4 (NP-036985), mm5-HT4 (CAA70775), hs5-HT4 (CAC22248), rn5-HT5A (NP-037280), mm5-HT5A (NP-032340), hs5-HT5A (NP-076917), rn5-HT5B (NP-077371), mm5-HT5B (NP-034613), rn5-HT6 (NP-077341), mm5-HT6 (NP-067333), hs5-HT6 (NP-000862), gg5-HT7 (NP-001165240), rn5-HT7 (NP-075227), mm5-HT7 (NP-032341), hs5-HT7 (NP-000863). First letters of the genus and species are used to construct the phylogenetic analysis; fruit fly (*Drosophila melanogaster*, dm); mosquito (*Aedes aegypti*, ae); honey bee (*Apis mellifera*, am); butterfly (*Papilio xuthus*, px); tick (*Rhipicephalus microplus*, rm); silkworm (*Bombyx mori*, bm); moth (*Heliothis virescens*, hv); planarian flatworm (*Dugesia japonica*, dj); nematode roundworm (*Caenorhabditis elegans*, ce); nematode roundworm (*Ascaris suum*, as); nematode (*Haemonchus contortus*, hc); California sea slug (*Aplysia californica*, ac); pond snail (*Lymnaea stagnalis*, ls); air-breathing snail (*Planorbella trivolvis*, pt); scallop (*Mizuhopecten yessoensis*, py); Pearl oyster (*Pinctada fucata*, pf); Pacific oyster (*Crassostrea gigas*, cg); lobster (*Panulirus interruptus*, pi); shrimp (*Metapenaeus ensis*, me); barnacle (*Amphibalanus amphitrite*, aa); pufferfish (*Takifugu rubripes*, tr); pufferfish (*Tetraodon fluviatilis*, tf); Tilapia (*Oreochromis mossambicus*, om); frog (*Xenopus laevis*, xl); chicken (*Gallus gallus*, gg); rat (*Rattus norvegicus*, rn); mouse (*Mus musculus*, mm); and human (*Homo sapiens*, hs).

of mollusks (5-HT_2 in pond snail, 5-HT_7 in the air-breathing snail, 5-HT_{B1} and 5-HT_{B2} in the California sea slug) are differentiated into different branch. Except of two latter case which display difficulties to be classified in terms of 5-HT receptors in vertebrates [26], the 5-HT_2 in pond snail and the 5-HT_7 in the air-breathing snail are considered as the 5-HT_2-like and the 5-HT_7-like receptors, respectively [92, 98].

The 5-HT_{py} and 5-HT_{pf} are expressed in most of the organs, including the ovary, testis, mantle, adductor muscle, gill, the nervous system (cerebral-pedal ganglia and VG), digestive gland, or kidney [11, 96]. *In situ* hybridization has shown that the 5-HT_{py} mRNA is localized in the oocytes and epithelium of the gonoducts in the ovary and in the spermatids and epithelium of the gonoduct in the testis [11]. It has histologically observed that, at spawning, mature oocyte and sperm are collected and evacuated from the acini into the surrounding aquatic environment via gonoducts in the great scallop [99]. Real-time PCR analyses of the 5-HT_{pf} mRNA transcription reveals that the order of decreasing is as follows: mature ovary > mature testis, VG, and digestive gland > mantle, gills, and adductor muscle. In addition, the testicular and ovary 5-HT_{pf} mRNA transcription does not differ among resting, developmental, and mature stages, however, increases in the ovary at spawning stage [96].

3. Receptor-mediated 5-HT stimulation of spawning in bivalves

Matsutani and Nomura [18] observed that injection of homogenates of CG, PG, or VG into the gonad of Yesso scallop induces spawning in 100% of males; however, they are without effects on females. In another experiment, they observed that 5-HT induces spawning in 100% of males and 73.3–80% of females. No other neurotransmitters, including adrenaline, noradrenaline (NA), and ϒ-aminobutyric acid, induced spawning [100–103]. Acetylcholine and dopamine (DA) induce spawning in males (40%), however they are without effects on females. Similarly, further studies have shown that neurotransmitters except of 5-HT are not potent to induce spawning in the surf clam [40], Zebra mussel [104], and Peruvian scallop [33, 105]. It is worth to note that DA at high dose (2×10^{-3} M) is capable of inducing spawning in males of Peruvian scallop [105] and in both males and females of Lion's paw scallop (*Nodipecten nodosus*) and Nucleus scallop (*Argopecten nucleus*) [106]. Omitting these exceptions, it has been accepted that 5-HT is the most potent neurotransmitter that induce spawning in bivalves at physiological concentration (**Table 3**). Other studies also show that injection of 0.4 mM 2–20 $\times 10^{-4}$ M 5-HT induces spawning in bivalve species, including the Atlantic deep-sea scallop, butter clam (*Saxidomus gigantea*), Gaper clam (*Tresus capax*), Manila clam (*Ruditapes philippinarum*), Pacific geoduck (*Panopea generosa*), Pacific littleneck clam (*Protothaca staminea*), Pacific oyster, Pacific razor clam (*Siliqua patula*), Pink scallop (*Chlamys rubida*), Rock scallop (*Hinnites multirugosus*), Weathervane scallop (*Patinopecten caurinus*), and Yesso scallop [107, 108]. It has also observed that 10^{-4} to 10^{-6} M 5-HT stimulates the release of the oocytes from the ovary tissues and sperm from the testicular tissues following a 90-min incubation, *in vitro* [109–112]. These are in agreement with identification of 5-HT and localization of nerve fibers transferring 5-HT from nervous system to gonad, which are observed around acini or gamete collective tubules. Both males and females response to exogenous 5-HT in a dose-dependent

manner. However, it seems that females usually require higher amount of 5-HT than that of a male to release the oocytes. The observed sex-specificity might be related to inter-sex differences in the concentration of 5-HT, which are shown to be higher in males than in females [32, 34]. Moreover, studies show that 5-HT fully stimulates spawning in ripe individuals.

As 5-HT fibers are localized in the gonad of bivalves, these observations pioneered further research to elucidate mechanism through which 5-HT induces spawning. In Zebra mussel, methiothepin, a non-selective 5-HT_1 receptor antagonist (**Table 2**), decreases 5-HT-induced spawning when it is added into the aquarium 5 min after addition of 5-HT. However, it is without effects on 5-HT-induced spawning when it is added into the aquarium 10 min after addition [120]. A 2 h pre-treatment of the Zebra mussel with 10^{-4} M methiothepin decreases parturition from 65 to 8% and from 82 to 1% in the individuals treated with 10^{-4} and 10^{-3} M 5-HT, respectively. These suggest that 5-HT-induced spawning requires a certain period of time and that 5-HT-induced spawning is irreversible.

To better understand which type of 5-HT receptor is involved in 5-HT-induced spawning, further experiments have conducted using 5-HT receptor analogs. It has observed that 10^{-4} M 8-OH-DPAT, 5-HT, and TFMPP induce 80, 70, and 56% spawning in Zebra mussel; however,

Species	Notes	Spawning of female (%)		Spawning of male (%)		References
		Control	5-HT (mM)	Control	5-HT (mM)	
Yesso scallop *Patinopecten yessoensis*	T: 6.7–10.5M: Injection to gonadD: 0.4 ml of 5-HT solution C: FSW	011.1	2: 73.3, 800.2: 1000.02: 200.002: 0	0	2: 1000.2: 800.02: 1000.002: 800.0002: 400.00002: 0	[18]
Yesso scallop *Patinopecten yessoensis*	T: 17–19M: Injection to gonadD: 0.4 ml of 0.1 mM 5-HT C: ASW	12.5	T_e: 87.5T_e: 91.7T_e: 100	–	100	[30]
American oyster *Crassostrea virginica*	T: 25M: Injection to gonadD: 0.4 ml of 2 mM 5-HT C: FSW	0	0	0	100	[2][113]
Bay scallop *Argopecten irradians*	T: 20–21M: Injection to gonadD: 0.4 ml of 2 mM 5-HT C: FSW	33.3	3.5	66.7	96.6	[2][113]
Hard clam *Mercenaria mercenaria*	T: 28–29M: Injection to muscleD: 0.4 ml of 2 mM 5-HT C: FSW	0	15.3	0	84.7	[2][113]
Hard clam *Mercenaria mercenaria*	T: 20M: Injection to muscleD: 0.4 ml of 5-HT solution C: FSW	20: 02: 00.2: 00.02: 0	20: 02: 1.10.2: 12.20.02: 2.2	20: 02: 00.2: 00.02: 0	20:23.32: 40.00.2: 36.60.02: 14.4	[114]

Species	Notes	Spawning of female (%)		Spawning of male (%)		References
Ocean quahog *Arctica islandica*	T: 15–16M: Injection to muscleD: 0.4 ml of 2 mM 5-HTC: FSW	0	T_{e1}: 16.7T_{e2}: 22.2T_{e3}: 23.1T_{e4}: 9.1	0	T_{e1}: 83.3T_{e2}: 77.8T_{e3}: 76.9T_{e4}: 90.9	[3][115]
Ocean quahog *Arctica islandica*	T: 15–16M: Injection to muscleD: 0.4 ml of 2 mM 5-HTC: FSW	0	21.1	0	79.0	[2][113]
Ribbed mussel *Geukensia demissa*	T: 28M: Injection to muscleD: 0.4 ml of 2 mM 5-HTC: FSW	0	11.1	100	88.9	[2][113]
Surf clam *Spisula solidissima*	T: 19M: Injection to gonadD: 0.4 ml of 2 mM 5-HTC: FSW	100	33.3	0	66.7	[2][113]
Surf clam *Spisula solidissima*	T: NDM: Injection to gonadD: 0.5 ml of 5-HT solutionC: ASW	0	2: 1000.2: 66.70.02: 66.70.002: 250.0002: 0	0	2: 1000.2: 85.70.02: 400.002: 00.0002: 25	[19]
Japanese baking scallop *Pecten albicans*	T: 12–16M: Injection to gonadD: 0.5–1 ml of 5-HT solutionC: FSW			0	2.5: 900.25: 87.50.025: 93.8	[4][116]
Giant clam *Tridacna gigas*	T: 27.8–30.5M: Injection to gonadD: 1–7 ml of 2 mM 5-HTC: FSW	0	2.6	0	66.7	[1][117]
Southern giant clam *Tridacna derasa*	T: 27.8–30.5M: Injection to gonadD: 1.5–4.5 ml of 2 mM 5-HTC: FSW	0	4.3	0	47.8	[1][117]
Maxima clam *Tridacna maxima*	T: 27.8–30.5M: Injection to gonadD: 0.5–2 ml of 2 mM 5-HTC: FSW	0	18.8	0	93.8	[1][117]
Crocus clam *Tridacna crocea*	T: 27.8–30.5M: Injection to gonadD: 0.5–1 ml of 2 mM 5-HTC: FSW	0	0	0	73.3	[1][117]
Scaly clam *Tridacna squamosal*	T: 27.8–30.5M: Injection to gonadD: 1.5–3 ml of 2 mM 5-HTC: FSW	0	0	0	67	[1][117]

Species	Notes	Spawning of female (%)		Spawning of male (%)		References
Bear paw clam *Hippopus hippopus*	T: 27.8–30.5M: Injection to gonadD: 1–5 ml of 2 mM 5-HT C: FSW	0	52.5	0	100	[1][117]
Zigzag scallop *Pecten ziczac*	T: 20M: Injection to muscle and gonadD: 0.4 ml of 2 mM 5-HT C: FSW	Feb.: 0Mar.: 0Apr.: 0	Feb.: 0Mar.: 0Apr.: 0	Feb.: 0Mar.: 0Apr.: 0	Feb.: 55Mar.: 100Apr.: 90	[118]
Doughboy scallop *Mimachlamys asperrima*	T: 15M: Injection to gonadD: 0.05 ml of 5-HT solutionC: Saline solution (Instant Ocean, Sarrebourg, France)	0	0.001: 00.01: 1000.1: 1001: 10010: 100	20	0.001: 200.01: 600.1: 601: 10010:100	[119]
Zebra mussel *Dreissena polymorpha*	T: 12M: 5-HT has added into aquarium, *in vivo*	0	1: 1000.1: 48.7	0	1: 1000.1: 65.4	[120]
Fingernail clam *Musculium transversum*	T: 23M: 5-HT has added into aquarium, *in vivo*	0	1 M: 1000.1: 560.01:0			[5][121]
Peruvian scallop *Argopecten purpuratus*	T: NDM: Injection to gonadD: 0.4 ml of 0.02–2 mM 5-HT C: FSW	0	0–20	0	100	[105]
Japanese clam *Mactra chinensis*	T: NDM: Injection to footD: 0.4 ml of 0.001–2 mM 5-HT C: FSW	0	2: 1001: 1000.1: 93.30.05: 1000.02: 1000.01: 26.70.001: 0	0	2: 1001: 1000.1: 93.30.05: 1000.02: 1000.01: 26.70.001: 0	[122]
Catarina scallop *Argopecten ventricosus*	T: 23M: Injection to gonadD: 0.025–2.5 mM 5-HTC: ND	0	0	0	100	[123]
Manila clam *Ruditapes philippinarum*	T: NDM: Injection to footD: 0.2 ml of 5-HT solution C: FSW	0	8.8	0	10: 801: 600.1: 86.70.01: 1000.001: 500.0001: 0	[124]
Nucleus scallop *Argopecten nucleus*	T: 22M: Injection to gonadD: 0.2 ml of 1 mM 5-HT solution C: FSW	40	67	20	90	[106]

Species	Notes	Spawning of female (%)		Spawning of male (%)		References
Lion's paw scallop *Nodipecten nodosus*	T: 22M: Injection to gonadD: 0.2 ml of 1 mM 5-HT solution C: FSW	6	48	24	93	[106]
Atlantic deep-sea scallop *Placopecten magellanicus*	T: 5 and 10M: Injection to gonadD: 0.4 ml of 2 mM 5-HT C: FSW			0	100	[125]

Abbreviation: ASW, artificial seawater; C, injection to control; D, dose; FSW, filtered seawater; M, method; ND, not determined; T, temperature (°C), T_e, experimental trial.

[1]Values for control are 0% as no individual injected with filtered seawater exhibited spawning behavior [117].

[2]Numbers of female and male injected with 5-HT are not determined. Values show percentage of spawned females and males from total number of individuals that spawned following injection of 5-HT. Total percentage of spawning are 27.1% (Ocean quahog), 82.9% (Bay scallop), 70% (American oyster), 45.0% (Ribbed mussel), 41.6% (Hard clam), and 60.0% (Surf clam). In the control group of Bay scallop, Ribbed mussel, and Surf clam, 8.6, 5.0, and 2.2% spawned, respectively. Individual in the control group of American oyster, Hard clam, and Ocean quahog did not spawn.

[3]Numbers of female and male injected with 5-HT are not determined. Values show percentage of spawned females and males from total number of individuals that spawned following injection of 5-HT. Total percentage of spawning are 17.1, 22.5, 37.1, and 35.5% in individual spawning trail 1 (T_{e1}), individual spawning trial 2 (T_{e1}), mass spawning trial 1 (T_{e3}), and mass spawning trial 2 (T_{e4}), respectively. Individual spawning represents spawning of a specimen placed in a glass dish (1 l FSW). Mass spawning represents placing of all individuals in troughs (140 l FSW). Individual in any control group did not spawn.

[4]Induction of spawning in the male phase of hermaphrodite scallop.

[5]Animals are exposed, and the percentage of parturition is evaluated based on the number of the release of juveniles.

Table 3. 5-hydroxytryptamine (serotonin, 5-HT) stimulates spawning in various species of bivalve mollusks.

2-methyl-5-HT and α-methyl-5-HT are without effects (4.1 and 0%) [104]. None of these 5-HT receptor agonists induce spawning at 10^{-5} M. A 2 h pre-treatment of Zebra mussel with 10^{-4} M cyproheptadine and mianserin results in 50 and 30% inhibition of 10^{-3} M 5-HT-induced spawning, respectively, whereas propranolol, 1-NP, NAN-190, and ketanserin are without effects. In addition, cyproheptadine is the only effective analog that totally inhibits 10^{-4} M 5-HT-induced spawning. A 2 h pre-treatment of Zebra mussel with 10^{-4} M cyproheptadine or mianserin totally suppress spawning at 10^{-4} or 10^{-3} M 8-OH-DPAT-induced Zebra mussel. In addition, 10^{-4} and 10^{-3} M 8-OH-DPAT-induced spawning are inhibited by 30 and 60% in the presence of 10^{-4} M NAN-190, respectively. These results may suggest that 5-HT_1 receptor agonists are potent to induce spawning. Antagonists of 5-HT_2 receptor are strongly potent to interfere with spawning induced by 5-HT_1 receptor agonist; however, they are capable of partially inhibiting 5-HT-induced spawning. The latter note, itself, represents interaction between 5-HT binding sites [104] or suggests the presence of more than one type 5-HT receptor to regulate 5-HT-induced spawning.

In Japanese clam [122], 1, 10, 20, 50, 100, and 1000 μM α-methyl-5-HT injected into the foot induces spawning in 0, 25, 31, 63, 75, and 100% of specimens, respectively, compared to 0% in control and 100% in ≥20 μM 5-HT. In addition, Japanese clams injected with 10, 100, and

1000 μM 8-OH-DPAT into the foot spawns 15, 33, and 100%, respectively. In this species, neither TFMPP nor mCPBG induces spawning in Japanese clam. Injection of mianserin into the foot of Japanese clam decreases spawning to 25 and 0% at 100 and ≥500 μM, respectively. The mianserin-inhibited spawning can be partially overcome by the second injection of 20 μM 5-HT, resulting in 60 and 50% spawning at 100 and 500 μM, respectively. Based on the rank order of potency of the 5-HT agonists, the authors suggested that a mixed $5\text{-HT}_1/5\text{-HT}_2$ receptor mediates 5-HT-induced spawning in this species. However, spawning of the individual pre-treated with mianserin may also suggest that 5-HT binding sites to induce spawning are different from those of mianserin. On the other hand, there might be more than one 5-HT receptor in the Japanese clam; however, 5-HT signaling seems to be mediated via a 5-HT_1 receptor.

4. Conclusion and future research perspectives

A few studies exist that investigate the characteristics of 5-HT binding site in the plasma membrane of the oocyte and sperm. Pharmacological profiles of binding sites in competition experiments suggest the presence of a single or mixed 5-HT_1, 5-HT_2, and 5-HT_3 receptors in bivalves. The phylogenetic analysis of 5-HT receptor suggests that classification of the bivalve 5-HT receptors based on available mammalian 5-HT receptor classification is not successful. It might be due to sensitivity and insensitivity of 5-HT binding sites to 5-HT analogs. On the other hand, the 5-HT receptor(s) in bivalves is distinct from those of other organisms. However, molecular identity of 5-HT receptor shows that the 5-HT receptor in bivalve seems to be a homolog of 5-HT_1 receptors in mammals.

Tissue distribution of the 5-HT receptor has shown that it is widely expressed in various organs, although its mRNA transcription is relatively high in the ovary and testis. This suggests multifunctional characteristics of 5-HT in bivalves. In addition, transcription of the 5-HT receptor undergoes seasonal variation. Studying 5-HT content and expression of 5-HT receptor in the nervous system and the gonad of bivalves will help us to better understand 5-HT signaling in reproduction.

To better understand receptor-mediated 5-HT signaling, it requires to produce genetic models of bivalves that do not express 5-HT receptor(s). Another valuable biological tool is to use bivalves that show natural alternations in 5-HT biosynthesis or natural disruption of reproduction. Bivalves host some parasites that particularly infect the reproductive system. For instance, Garnerot et al. [31] observed histopathological changes in the gonad of soft-shell clam infected with a trematode *Prosorhynchus squamatus*. In infected individual, the follicles and genital follicles are not surrounded by 5-HT-IR fibers around, and 5-HT staining is clearly visible inside the parasite. Another example is protozoan *Marteilioides chungmuensis* that become mature in the oocyte of the pacific oyster [126]. The parasites affect the reproductive follicles causing irregular enlargement of the infected gonadal tissues [127]. Although infected female oysters produced oocytes continuously and spawned repeatedly, however the parasites cause nutritional wasting and mortality, and affect the reproductive output of

infected female oyster [127, 128]. Ngo et al. [129] also reported that *M. chungmuensis* delays spawning and cause damages to ripe oocytes. These biological examples of parasite-infected bivalves can provide us with model organisms to study 5-HT regulation of gonadal development and gamete maturation.

Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

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Chapter 3

Structure-Function of Serotonin in Bivalve Molluscs

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Additional information is available at the end of the chapter

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Abstract

It has been observed that 5-HT excites the heart nerves in hard clam and regulates contraction and relaxation of the anterior byssus retractor muscle in the blue mussel. It is now known that 5-HT regulates several neurobehavioral systems such as mood, appetite, sleep, learning, and memory. It also plays critical roles in the physiological functions of peripheral organs involved in stress, growth, and reproduction in the animal kingdom. The present study reviews conserved 5-HT biosynthesis and its localization in the nervous system, and its physiological contribution to regulate reproduction in bivalves. In the cytosol of neurons, tryptophan hydroxylase catalyzes hydroxylation of L-tryptophan to 5-hydroxytryptophan, which is converted to 5-HT by aromatic L-amino acid decarboxylase. A 5-HT transporter and a monoamine oxidase reuptakes and metabolizes 5-HT to control the amount of released 5-HT in the nervous system and peripheral organs. Perikarya and fibers of 5-HT neurons are mostly located in the cortices and neuropil of ganglia, respectively, and innervate the gonad. However, distribution and 5-HT content differ among species and sexes and undergo seasonal variations associated with gonadal development. The present review pays a special attention to future research perspectives to better understand 5-HT regulation of reproduction in bivalves.

Keywords: gonad, nervous system, oocyte, serotonin biosynthesis, serotonin metabolism, reuptake, sperm

1. Introduction

5-Hydroxytryptamine called serotonin (5-HT) is a transmitter substance of the nervous system in animal kingdom. From its first discovery in the 1940s, many laboratories have been directing their studies toward understanding the biology of 5-HT and its physiological functions on various biological systems especially on mammals as model organism [1–15]. However, 5-HT has been also identified in bivalves from the period of its first discovery and earlier studies on these animals have led to convince the neurobiologist that it acts as a neurotransmitter.

A brief bibliography of 5-HT discovery and its physiological functions is provided in **Table 1**. Rapport et al. [16] was the first who isolated a vasoconstrictor substance from the blood serum in a crystalline form and tentatively identified it as 5-HT in a creatinine sulfate complex [17].

Year	Scientists	Contribution to discovery of identification, localization, and characterization of 5-HT	References
1947	Rapport et al.	Isolation of a substance from the blood serum that constricts blood vessels and contracts isolated intestinal strips	[16]
1948	Rapport et al.	The substance contains an indole ring	[42]
1949	Rapport	Identification of chemical structure of 5-HT as a creatinine sulfate complex	[17]
1951	Hamlin and Fisher	Synthesis of 5-HT	[18]
1953	Twarog and Page	Identification of 5-HT in the extract of the brain of mammals (dog, rat, and rabbit)	[19]
1953	Gaddum	Assigning a role for 5-HT in normal cerebral function in mammals	[43]
1953	Welsh	5-HT, in contrast to acetylcholine, excites the heart nerves in hard clam (Bivalvia, Mollusca) originating from visceral ganglion	[21]
1954	Amin et al.	Localization of 5-HT in the central nervous system (brain) of mammals (dog)	[20]
1954	Wooley and Shaw	Human schizophrenia might be due to 5-HT deficiency	[44]
1954	Twarog	5-HT-mediated relaxation of byssus retractor muscle in the blue mussel (Bivalvia, Mollusca) is antagonist of acetylcholine contracting the muscle	[22]
1956	Hoyle and Lowy	5-HT is a putative neurotransmitter controlling contraction and relaxation of the anterior byssus retractor muscle in the blue mussel	[23]
1957	Brodie and Shore	Assigning 5-HT function as a neurotransmitter	[24]
1957	Welsh	Identification of 5-HT in the extract of nervous system of various bivalve mollusks	[25]
1962	Falck et al.	Development of Falck-Hillarp method to visualize monoamine-containing cells as intense yellow-green fluorescence	[45]
1964	Dahlström and Fuxe	Identification of 5-HT cell bodies in the pons and midbrain, from where they project with their axons to the forebrain, medulla, and spinal cord	[46]
1968	Sweeney	Identification and localization of 5-HT in whole body extract and in the nervous system of blue mussel using Falck-Hillarp's method	[47]

Year	Scientists	Contribution to discovery of identification, localization, and characterization of 5-HT	References
1982	Matsutani and Nomura	Serotonin stimulates spawning in Yesso scallop (Bivalvia, Mollusca)	[33]
1984	Hirai and Koide	5-HT stimulates oocyte maturation in surf clam	[48]
1985	Osanai	5-HT regulation of the oocyte signaling required to undergo germinal vesicle breakdown	[49]

Species: Blue mussel, *Mytilus edulis*; hard clam, *Mercenaria mercenaria*; surf clam, *Spisula solidissima*; Yesso scallop, *Patinopecten yessoensis*.

Table 1. Bibliography of 5-hydroxytryptamine (serotonin, 5-HT): from discovery to physiological characterization.

Within next 5 years, 5-HT has been synthesized [18], identified in the extract of mammalian brain [19], and localized in the brain of mammals [20]. Along with these studies on mammals, Welsh [21], Twarog [22], and Hoyle and Lowy [23] demonstrated that 5-HT excites the heart nerves in hard clam (*Mercenaria mercenaria*), and regulates contraction and relaxation of the anterior byssus retractor muscle in the blue mussel (*Mytilus edulis*) that both belong to Bivalvia, Mollusca. These observations resulted in identification of 5-HT as a neurotransmitter in the nervous system of mammals [24]. In the same year, Welsh [25] identified 5-HT in the nervous system of bivalves and demonstrated that 5-HT content in these animals is higher than other invertebrates and vertebrates [26]. Moreover, bivalves have served some advantages to be used as experimental model: (A) they are small which is a great opportunity to conduct serial examinations on the whole organism, (B) they have a simple nervous system, (C) the nervous system is relatively large in size and easy to dissect ganglia and connectives, and (D) the nervous system contains high amount of 5-HT.

Serotonin regulates various neurobehavioral systems (such as mood, appetite, sleep, learning, and memory). However, studies have revealed that it also plays critical roles in physiological functions of peripheral organs such as stress and growth [3–5]. One of the major systems that 5-HT contributes to is the regulation of reproduction. In both mammals and bivalves, it has been observed that 5-HT regulates reproductive endocrine system, oocyte maturation, and sperm motility [27–38].

Although 5-HT biosynthesis and its receptor structure have been reviewed in bivalves [39–41], there is a gap of review on physiological signaling of 5-HT in these animals. The present study reviews the biology of 5-HT in bivalves; particularly its contribution to reproduction. Biosynthesis pathway of 5-HT in the nervous system and cellular localization of 5-HT neurons in the nervous system are studied. Particular attention has then paid to 5-HT content and distribution of 5-HT neurons in the gonad. This study provides future perspectives that await investigation to better understand 5-HT network and signaling in bivalve reproduction.

2. Biosynthesis, metabolism, and reuptake of 5-HT in the nervous system

Hamlin and Fisher [18] were the first who synthesized 5-HT from tryptophan. A year later, Blaschko [50] suggested that 5-hydroxytryptophan (5-HTP) is the substrate for 5-HT. This

suggestion led to the discovery of an enzyme in mammalian kidney [51], later called aromatic L-amino acid decarboxylase (AADC) [52] that mainly decarboxylates 5-HTP to 5-HT [53]. In parallel, studies have shown that the extract of mammalian brain contains 5-HT [19], and administration of exogenous 5-HTP or tryptophan increases 5-HT level in the brain and peripheral organs [54, 55]. A year later, Welsh and Moorhead [56] observed that homogenates of ganglia of hard clam are capable of synthesizing 5-HT from 5-HTP, *in vitro*. Further studies using the blue mussel (*Mytilus edulis*) demonstrated presence of precursors of 5-HT (either tryptophan or 5-HTP) [57–59], and decarboxylation of 5-HTP to 5-HT [60, 61]. Thus, 5-HT biosynthesis in bivalves is similar to those of higher vertebrates. Although aforementioned studies have shown biosynthesis pathway of 5-HT and demonstrated that both nervous system and peripheral organs contain 5-HT; however, it was still unknown where the 5-HT biosynthesis takes place and how it gets transferred to other organs.

In 1960s, Bertaccini [62] and Gal et al. [63] demonstrated that the brain contains 5-HT even after partial or complete removal of 5-HT in the gastro-intestinal tissues and the brain produces 5-HT after intracerebral injection of radioactive labeled tryptophan. It is worth noting that it has previously been shown that the intestine contains large amount of 5-HT [64]. These studies provided the scientists with very important information that the brain independently synthesizes 5-HT from L-tryptophan, and suggested that exogenous 5-HT administration incorporates to 5-HT contents in the nervous system. Next studies resulted in molecular identity of two major enzymes in 5-HT biosynthesis pathway: tryptophan hydroxylase (TPH) and AADC [6, 14, 65, 66] (**Figure 1**). In the cytosol of the nerve cells, TPH catalyzes hydroxylation of L-tryptophan to produce 5-HTP by incorporation of an atom of atmospheric oxygen into L-tryptophan and the other is reduced to water, in the presence of the cofactor agent, tetrahydrobiopterin. The pathway is rate-limiting step meaning that suppression of TPH activity results in stopping 5-HT biosynthesis. The AADC catalyzes conversion of 5-HTP to 5-HT which is not rate-limiting step. It has also been shown that the rate at which 5-HT is produced in the central nervous system highly depends on availability of tryptophan, tryptophan uptake into the brain, and dietary contents of tryptophan and other amino acids (such as tyrosine and phenylalanine) that compete with tryptophan uptake or transport carrier into the brain [8, 14, 67].

In the snail, it has been observed that certain nerves are capable of accumulating radioactive labeled 5-HT [68]. Using bivalves, Stefano and Aiello [69] observed that fluorescence intensity of 5-HT-immunoreactive (5-HT-IR) neurons increases in the blue mussel after administration of exogenous 5-HT. Thus, as in mammals, 5-HT biosynthesis in bivalve mollusks also takes place in the nervous system.

Further studies have shown that there are biological systems through which external amounts of the released 5-HT is regulated, as its rise may cause abnormal physiological functions or might be lethal for cells. Reuptake and metabolism of 5-HT are key determinants to remove and/or inactivate significant amount of released 5-HT, respectively. Metabolism of 5-HT is mediated by monoamine oxidase (MOA) located in the outer membrane of mitochondria, and catalyzes the oxidative deaminative of 5-HT to 5-hydroxy-3-indolacetaldehyde (5-HIAL), which is further metabolized into 5-hydroxy-3-indolacetic acid (5-HIAA) by an

NAD^+-dependent aldehyde dehydrogenase. In addition, an NADH-dependent aldehyde reductase or an NADPH-dependent alcohol-dehydrogenase converts 5-HIAL to 5-hydroxytryptophol (5-HTOL) [6, 70] (**Figure 1**). In mollusks, small amount of MOA has been reported [71]. Boutet et al. [72] cloned MOA molecular structure in the Pacific oyster. Administration of MAO inhibitor leads to increase in the number and intensity of 5-HT-IR neurons in the blue mussel [69]. Thus, metabolism of 5-HT is active in bivalve mollusks. However, studies have demonstrated that 5-HT action at the synapse is mostly terminated by its reuptake across the presynaptic membrane [73–77].

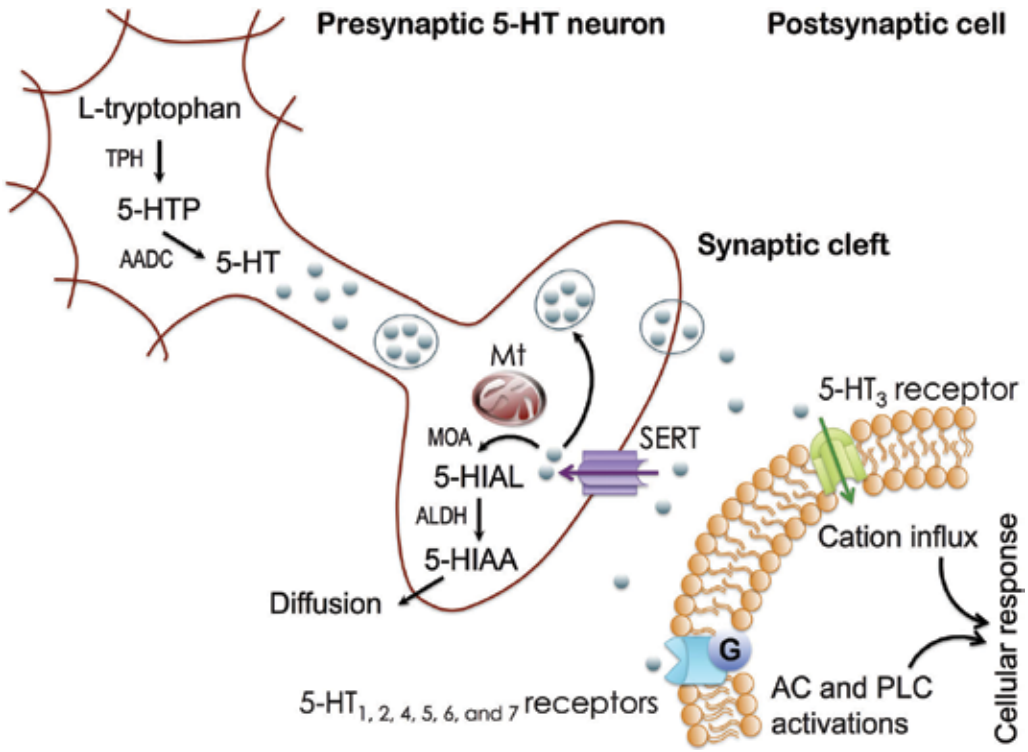

Figure 1. Biosynthesis, metabolism and reuptake of 5-hydroxytryptamine (serotonin, 5-HT) in bivalves. In the cytosol of the 5-HT neurons, tryptophan hydroxylase (TPH) catalyzes hydroxylation of L-tryptophan to produce 5-hydroxytryptophan (5-HTP) that becomes converted to 5-HT by aromatic L-amino acid decarboxylase (AADC). Conversion of L-tryptophan to 5-HTP is rate-limiting step meaning that suppression of TPH activity results in stopping 5-HT biosynthesis, however AADC-catalyzed conversion of 5-HTP to 5-HT is not rate-limiting pathway. The 5-HT vesicles are transferred to axon terminal and released to synaptic cleft. Reuptake and metabolism of 5-HT are key determinants to inactivate significant amount of the released 5-HT. In mollusks including bivalves, 5-HT reuptake from synaptic cleft is more than the enzymatic destruction. It is an ionic-coupled system and mediated by a serotonin transporter (SERT) that transports 5-HT from synaptic cleft to the presynaptic 5-HT neuron. However, enzymatic destruction of 5-HT also exists which is mediated by monoamine oxidase (MOA) located in the outer membrane of mitochondria (Mt). The MOA catalyzes the oxidative deaminative of 5-HT to 5-hydroxy-3-indolacetaldehyde (5-HIAL) that is metabolized into 5-hydroxy-3-indolacetic acid (5-HIAA) by aldehyde dehydrogenase (ALDH). Released 5-HT binds to its receptor(s) on the surface of a postsynaptic cell or postsynaptic neuron (not shown in the figure) to trigger intracellular signaling required for a cellular response, e.g., stimulation of oocyte and sperm maturation. The 5-HT receptors are mainly G-protein coupled receptor ($5\text{-HT}_{1, 2, 4, 6, 5, \text{and } 7}$ receptors), which induce adenylate cyclase (AC) or phospholipase C signaling (PLC). However, the 5-HT_3 receptor is a ligand-gated ion channel and regulates ionic influx.

The 5-HT reuptake is also similar between mollusks and mammals. It is an ionic-coupled pathway mediated by a serotonin transporter (SERT) that transport 5-HT from synaptic cleft to the presynaptic neuron [9, 12, 78]. SERT first binds a Na^+ ion, followed by 5-HT, and then a Cl^- ion in the synaptic cleft and transport to presynaptic neuron. After releasing 5-HT, K^+ efflux is involved in the translocation mechanism of SERT. This is an energy dependent process and a Na^+/K^+ ATPase maintains the extracellular Na^+ concentration as well as the intracellular K^+ concentration [79]. This mechanism results in the inactivation of 5-HT by removing it from the synaptic cleft. Studies have also shown that a 5-HT reuptake inhibitor (SRI) interferes with SERT function to inhibit or suppress 5-HT reuptake [80, 81].

3. Anatomy of the nervous system in bivalves

3.1. Nervous system

In bivalves, the nervous system is bilaterally symmetrical, decentralized, and consists of cerebral ganglia (CG), pedal ganglia (PG), and visceral ganglia (VG). The ganglia are joined by a cerebral commissure, a visceral commissure, and cerebral-pedal, cerebral-visceral, and cerebral-visceral-pedal connectives [82–86] (**Figure 2**). Each ganglion is surrounded by a perineurium.

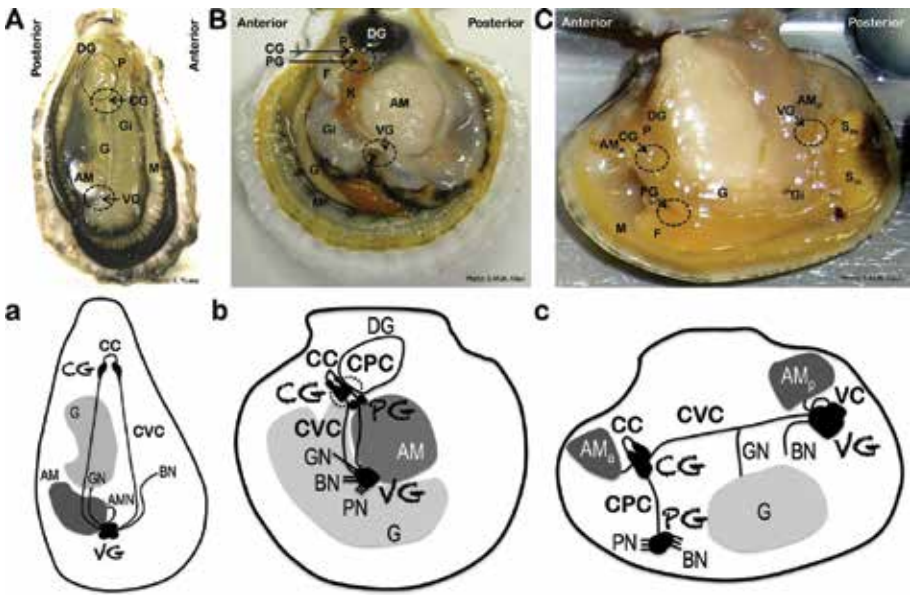

Figure 2. Anatomy of the nervous system in bivalves. It is decentralized and consists of bilaterally symmetrical cerebral ganglia (CG), pedal ganglia (PG), and visceral ganglia (VG). The locations of ganglia highly differ among species; however, they are connected by nerve connectives. The PG are absent in oysters (e.g., Pacific oyster, *Crassostrea gigas*, A). All parts of the nervous system exist in scallops (e.g., Yesso scallop, *Patinopecten yessoensis*, B) and clam species (e.g., Manila clam, *Ruditapes philippinarum*, C). Panels a, b, and c are representative schematics of intercommunicating ganglia in Pacific oyster [83], Yesso scallop (the authors), and soft-shell clam, *Mya arenaria* [89], respectively. In most bivalves, VG innervates the gonad. $AM_{a \text{ and } p}$, anterior and posterior adductor muscle; AMN, adductor muscle nerves; BN, bronchial nerves; CC, cerebral commissure; CPC, cerebral-pedal connective; CVC, cerebral-visceral connective; DG, digestive gland; F, foot; G, gonad; GN, gonad nerves; Gi, gills; K, kidney; M, mantle; P, labial palp; PN, pallial nerve; S_{in}, incurrent siphon; S_{ex}, excurrent siphon.

The neuronal cell bodies "perikarya" are located at the cortices and the axonal processes lie at central core called "neuropil".

The pairs of CG lie on the sides of esophagus and are connected by a cerebral commissure in bivalves. In oyster species, CG are less developed and positioned at the sharp angle anterior to the labial palp, gills, and digestive gland [83]. In mussel and clam species, CG are located anterior to the digestive gland, and beneath the anterior adductor muscle [82, 84]. In freshwater pearl mussel (*Hyriopsis bialata*), CG are fused [87]. In scallop species, the foot is positioned anterior to CG, and adductor muscle and digestive gland are located posterior to CG [82, 86]. Each CG consists of an anterior lobe and a posterior lobe [88]. The CG innervate the palps, anterior adductor muscle, and parts of mantle [83, 84, 86].

In most bivalves, the pairs of PG lie on the foot and are connected by a pedal commissure [84–86]. However, PG are absent in oyster species [83]. In soft-shell clam (*Mya arenaria*), the PG are fused [89]. In freshwater pearl mussel, PG are positioned in the visceral mass [87]. The PG innervate the foot [84, 86].

The paired VG are located on the ventral side of the adductor muscle, usually posterior to foot. In most bivalves, ganglia of VG are fused into a single organ [83, 89–91]. In scallop species, VG consist of five lobes; two anterior lobes, a posterior lobe, and two lateral lobes [88, 90]. There is an accessory ganglion that locates at the point of the lateral lobes. The CG and VG are joined by a pair of cerebral-visceral connective that pass through the digestive gland or gonad. The VG innervate various organs, including gonads, gills, hearts, sensory organs, posterior adductor muscle, and parts of mantle [83, 84, 86].

3.2. Anatomy and annual cycle of neurosecretory cells in bivalves

Rawitz [92] seems to be first who isolated pear- or club-shaped neurons from the European flat oyster (*Ostrea edulis*). The neurons are classified into unipolar, bipolar, and multipolar neurons (**Figure 3**) [93]. Illanes-Bucher [94] classified the neurosecretory cells into A1, A2, A3, and A4 in the blue mussel. The A1-type neurons are small (6–15 μm), unipolar, and nucleus is located opposite to the axonal cone. The A2-type nerve cells are large (20–30 μm), multipolar, and nucleus is eccentric. The A3-type nerve cells are large (20–25 μm), unipolar, and nucleus is eccentric. The A4-type nerve cells are medium in size (12–15 μm), apparently unipolar, and contain numerous vacuoles surrounded by neurosecretory granules. Blake [95] observed that the neurosecretory cycle of neurons in the CG of the Bay scallop (*Argopecten irradians*) appeared identical to that of the VG. The neurosecretory cells also undergo distinct annual cycle [96–99]. Seasonal changes in the activity of neurosecretory cells are also associated with gonadal development, and the cells release their products at maturity stage [96]. Moreover, number of active neurosecretory cells positively correlates with progress of the gonad development in the Bay scallop [95], clam (*Katelysia opima*) [100], blue mussel [101], and green-lipped mussel (*Perna canaliculus*) [102].

3.3. Identification and cellular localization of 5-HT

Cellular localization of 5-HT neurons and its quantitative bioassay in the nervous system and gonads provide us with highly satisfactory knowledge to elucidate ontogeny and developmental

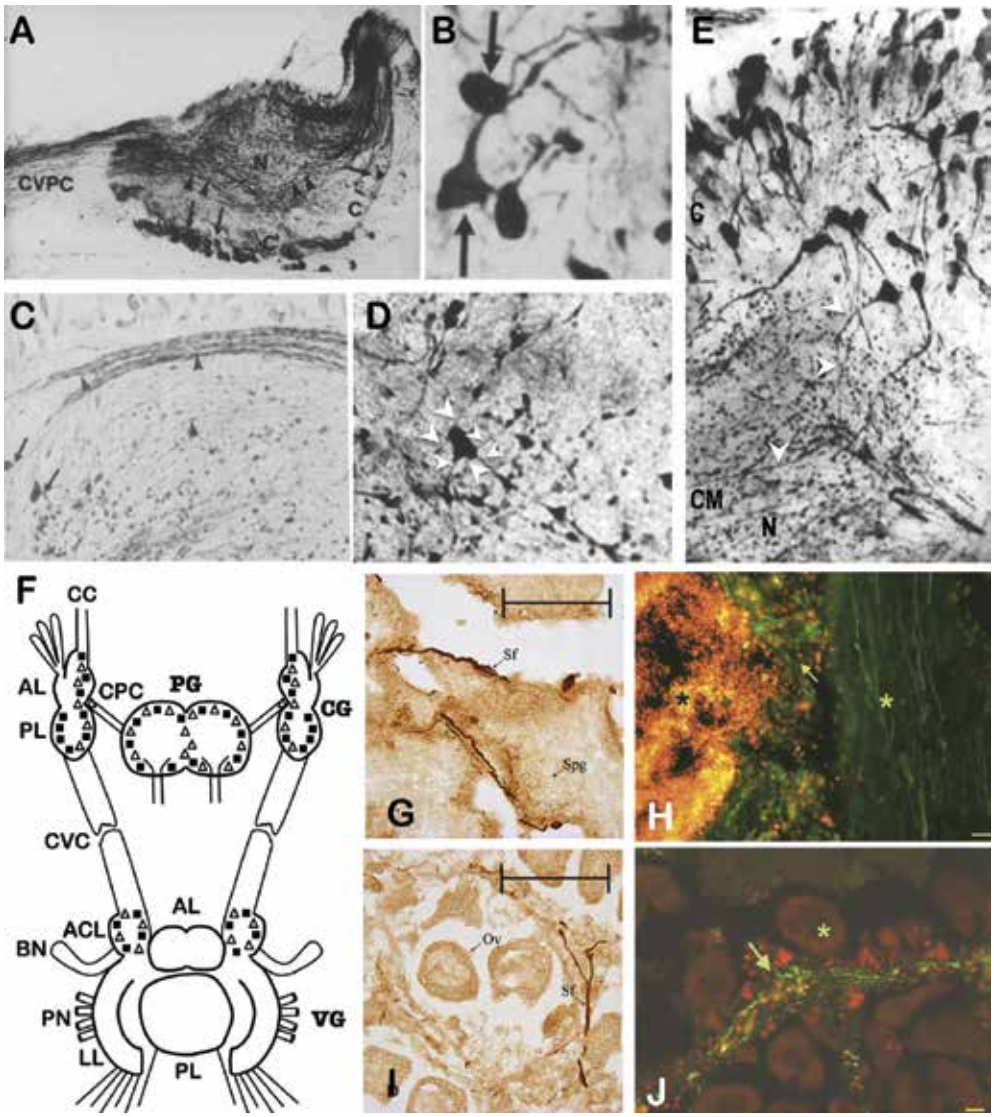

Figure 3. Cellular localization of 5-hydroxytryptamine (serotonin, 5-HT) in the nervous system (A–F) and gonad (G–J) of bivalves. (A) The 5-HT immunoreactive (5-HT-IR) cell bodies (arrows) and fibers (arrowheads) in the cortex (C) and neuropil (N) of cerebral ganglia (CG) (135×). (B) A few 5-HT-IR unipolar neurons with cell bodies (arrows) and their process in the CG (360×). (C) 5-HT-IR neurons (arrows) and fibers (arrowheads) in the visceral ganglion (380×). (D) a 5-HT-IR multipolar neuron with its processes (arrows) in pedal ganglion (PG) (800×). (E) Pear-shaped unipolar 5-HT-IR neurons and fibers in cortex (C) and neuropil (N) of PG. The arrowheads show long process of (the axon) of a 5-HT-IR neuron that runs toward commissure (CM) (315×). CVPC is cerebral-visceral-pedal connective. (A)–(C) [103], (D) and (E) [104] show localization of the 5-HT neurons in *Mytilus galloprovincialis*. (F) A schematic of localization of the 5-HT neurons in Yesso scallop, *Patinopecten yessoensis* (■) [105] and great scallop, *Pecten maximus* (Δ) [90]. (G) and (H) the 5-HT-IR fibers in the testis of *Mya arenaria* and *Venus verrucosa*, respectively. The 5-HT-IR fibers (Sf) are seen in the testis containing spermatogonia (Spg) at early stage II of the development (I). The 5-HT-IR fibers (arrows) originated from cerebral-visceral connective (yellow asterisk) surround acini full of sperm (black asterisk) (H). (I) and (J) The 5-HT-IR fibers in the ovary of *M. arenaria* and *V. verrucosa*, respectively. The 5-HT-IR fibers (Sf) surround the ovary containing post-vitellogenic oocytes (Ov) (I). The 5-HT-IR fibers (arrow) surround the wall of the follicles filled with mature oocytes (asterisk) (J). Scale bar G and I = 100 μm [106] and H and J = 20 μm [91].

biology of 5-HT biosynthesis, release, and reuptake, and to understand 5-HT regulation of reproduction in bivalves.

3.3.1. 5-HT in the nervous system of bivalves

Welsh [25] was the first who identified 5-HT in the nervous system of the hard clam using a paper chromatography method. Then, Welsh and Moorhead [26, 56, 107] used a spectrofluorometric method to measure 5-HT in over 60 species from 11 different phyla that includes 7 bivalve species (**Table 2**) [108]. They reported that (A) the nervous system of bivalves contains much higher 5-HT than that of other invertebrates. In the phylum Annelida, 5-HT is measured 0.1–10.4 μg/g wet in the nerve cords. In the phylum of Arthropoda, 5-HT is measured from <1.0 μg/g wet in the nerve cords, ventral ganglia, and green ganglia. In vertebrates, 5-HT is measured 0.3–2.6 μg/g wet in different parts of cat brain [109]. (B) Content of 5-HT is higher in the nervous system than the peripheral organs. (C) Content of 5-HT differs among various parts of the nervous system. It is higher in the ganglia than the connective nerves. In addition, they observed that 5-HT content is slightly lower in VG than those of CG and PG (10 vs. 15 μg/g wet) in the blue mussel. (D) The blood does not contain 5-HT. The authors suggested that 5-HT is produced in the nervous system: in cell bodies or synaptic region of neurons.

Species	Notes	Nervous system	Gonad	Reference
Brown mussel *Perna perna*	M: HPLC-ED V: pg/mg wet (mean ± SEM) Jul.: Resting stage Sep.: Developmental stage I–II Mar.: Maturation stage IIIA Apr.: Egg-laying stage	5-HT 74 ± 16 (PG), 51 ± 7 (CG) (Jul.) 115 ± 20 (PG), 61 ± 6 (CG) (Sep.) 293 ± 54 (PG), 63 ± 7 (CG) (Mar.) 302 ± 47 (PG), 150 ± 9 (CG) (Apr.) 5-HIAA 79 ± 22 (PG), 56 ± 30 (CG) (Jul.) 122 ± 30 (PG), 11 ± 1 (CG) (Sep.) 166 ± 46 (PG), 46 ± 12 (CG) (Mar.) 56 ± 16 (PG), 83 ± 40 (CG) (Apr.)	5-HT 8.7 ± 0.6 (Jul.) 31 ± 5.7 (Sep.) 142 ± 49.6 (Mar.) 142 ± 14.3 (Apr.) 5-HIAA 188 ± 36 (Jul.) 443 ± 70 (Sep.) 29 ± 6 (Mar.) 51 ± 5 (Apr.)	[110]
Pacific lion's paw scallop *Nodipecten subnodosus*	M: HPLC V: ng/mg dry (mean ± SD) I: Resting stage II: Initial development stage III: Maturing stage IV: Mature stage V: Partially spent stage VI: Fully spent stage		5-HT I: ND (O), 0.35 ± 0.63 (T) II: ND (O), 0.87 ± 0.94 (T) III: 0.04 ± 0.07 (O), 0.65 ± 0.72 (T) IV: 0.12 ± 0.19 (O), 2.04 ± 2.18 (T) V: ND (O), 0.42 ± 0.56 (T) VI: ND (O), ND (T)	[111]

Species	Notes	Nervous system	Gonad	Reference
Surf clam *Spisula solidissima*	M: HPLC V: ng/g wet (mean ± SEM) I. Active stage II. Ripe stage III. Spawning stage IV: Spent stage * shows $P < 0.05$ compared to stages I and IV		5-HT I: 625 ± 100 (O), 550 ± 100 (T) II: 175 ± 50* (O), 225 ± 65 (T) III: 350 ± 95* (O), 500 ± 150 (T) IV: 1050 ± 250 (O), 575 ± 400 (T)	[112]
Peruvian scallop *Argopecten purpuratus*	M: Spectrofluorometer V: ng/mg wet (mean ± SEM) It is a hermaphroditic species VG innervates mainly the female portion of the gonad CG and PG innervate mainly the male portion of the gonad * shows $P < 0.05$ compared to before spawning	5-HT CG + PG + VG 29.4 ± 4.3 (before spawning) 17.9* ± 0.6 (after sperm release) 22.5 ± 0.5 (after oocyte release) 21.3* ± 2.3 (24 h after spawning) CG + PG 107.3 ± 12.9 (**b**efore spawning) 63.6 ± 2.1* (spawned) 100.0 ± 16.3 (unspawned) VG 50.7 ± 4.3 (before spawning) 51.8 ± 5.1 (spawned) 53.3 ± 12.4 (unspawned)	5-HT Ovary portion of gonad 1.0 ± 0.03 (before spawning) 0.6* ± 0.02 (after sperm release) 0.5* ± 0.05 (after oocyte release) 0.7 ± 0.15 (24 h after spawning) Testis portion of gonad 1.7 ± 0.15 (before spawning) 0.8* ± 0.05 (after sperm release) 0.7* ± 0.09 (after oocyte release) 1.2 ± 0.05 (24 h after spawning)	[113]
Atlantic deep-sea scallop *Placopecten magellanicus*	M: HPLC-ED V: pg/mg wet (mean ± N.D.) Samples of March	CG + PG + VG 5-HTP: 1650 ± 715 5-HT: 1150 ± 525 5-HIAA: 180 ± 90	5-HTP: 2035 ± 520 5-HT: 1000 ± 180 5-HIAA: 90 ± 15	[114]
Atlantic deep-sea scallop *Placopecten magellanicus*	M: HPLC-ED V: pg/mg wet (mean ± N.D.) Samples of March–May	CG + PG + VG 5-HT: 1483 ± 828	5-HT: 791 ± 408	[115]
Peruvian scallop *Argopecten purpuratus*	M: Spectrofluorometer V: ng/mg wet (mean ± SEM)	5-HT CG + PG + VG 48.3 ± 7.2 (0 d) 46.2 ± 9.7 (0.5 d) 40.0 ± 5.6 (1 d) 37.9 ± 3.5 (7 d) 44.5 ± 5.7 (14 d) 39.0 ± 6.0 (21 d) 47.2 ± 6.2 (28 d) 63.3 ± 12.6 (35 d)	5-HT Gonad ovary (O) or testis (T) portion 1.3 ± 0.02 O, 6.8 ± 0.5 T (0 d) 0.7 ± 0.03 O, 2.2 ± 0.7 T (0.5 d) 0.7 ± 0.02 O, 2.5 ± 0.5 T (1 d) 1.5 ± 0.34 O, 3.0 ± 0.5 T (7 d) 1.6 ± 0.02 O, 4.8 ± 0.4 T (14 d) 1.4 ± 0.03 O, 4.6 ± 1.3 T (21 d) 1.0 ± 0.04 O, 4.4 ± 0.4 T (28 d) 1.1 ± 0.01 O, 4.9 ± 0.9 T (35 d)	[116]

Species	Notes	Nervous system	Gonad	Reference
Great scallop *Pecten maximus*	M: HPLC-ED V: ng/g.p. (mean ± SEM) Samples of mature individuals (3-year old)	CG + PG 330 (Jul., 1991) 405 (Aug., 1991) 510 (Nov., 1991) 510 (Dec., 1991) 180 (Jan., 1992) 270 (Feb. 1992) 240 (beginning of Mar., 1992) 210 (middle of Mar., 1992) 180 (end of Mar., 1992) 225 (Apr., 1992) 300 (May, 1992) 300 (June, 1992) VG 350 (Jul., 1991) 410 (Aug., 1991) 550 (Nov., 1991) 405 (Dec., 1991) 290 (Jan., 1992) 350 (Feb. 1992) 290 (beginning of Mar., 1992) 200 (middle of Mar., 1992) 315 (end of Mar., 1992) 350 (beginning of Apr., 1992) 450 (middle of Apr., 1992) 350 (beginning of May, 1992) 425 (end of May, 1992) 425 (June, 1992)		[90]
California mussel *Mytilus californianus*	M: HPLC-ED V: nM/ganglia pair (mean ± SEM) Samples of mature individuals in March–May	0.09 ± 0.02 (CG) 0.22 ± 0.05 (PG) 0.41 ± 0.07 (VG)		[117]
Blue mussel *Mytilus edulis*	M: HPLC-ED V: nM/g.p. (mean ± SEM) Samples of mature individuals in March–May	0.04 ± 0.01 (CG) 0.06 ± 0.003 (PG)		[117]
Gaper clam *Tresus capax*	M: HPLC-ED V: nM/g.p. (mean ± SEM) Samples of mature individuals in March–May	0.70 ± 0.11 (CG) 0.39 ± 0.06 (PG) 0.48 ± 0.06 (VG)		[117]
Cockle clam *Clinocardium nuttallii*	M: HPLC-ED V: nM/g.p. (mean ± SEM) Samples of mature individuals in March–May	0.22 ± 0.01 (PG) 0.24 ± 0.04 (VG)		[117]

Species	Notes	Nervous system	Gonad	Reference
Bent-nose clam *Macoma nasuta*	M: HPLC-ED V: nM/g.p. (mean ± SEM) Samples of mature individuals in March–May	0.20 ± 0.06 (CG) 0.15 ± 0.004 (VG)		[117]
Blue mussel *Mytilus edulis*	M: Spectrofluorometer V: μg/g wet (mean ± SEM) *, **, and *** show $P < 0.005$, $P < 0.001$, and $P < 0.05$ compared to Jan, respectively	2 CG + 2 PG + 2 VG 25.10 ± 2.71 (Jan.) 26.96 ± 2.11 (Feb.) 32.17 ± 3.85 (Mar.) 41.98 ± 1.22* (Apr.) 48.15 ± 1.02** (May) 53.13 ± 1.71** (Jun.) 51.74 ± 3.14** (Jul.) 57.28 ± 2.49** (Aug.) 48.90 ± 1.13* (Sep.) 44.80 ± 1.51* (Oct.) 35.71 ± 2.70*** (Nov.) 28.97 ± 2.64 (Dec.)		[118]
Blue mussel *Mytilus edulis*	M: Spectrofluorometer V: ng/ganglion pair (mean ± SD)	5-HT 123 ± 12 – 252 ± 34 (PG)		[119]
Blue mussel *Mytilus edulis*	M: Spectrofluorometer V: μg/g wet (mean ± N.D.)	5-HT 5.4–8.6 (PG, Mar.) 26.2-42 (PG, Apr.)		[120]
Fingernail clam *Sphaerium sulcatum*	M: Spectrofluorometer V: ng/individual (mean ± N.D.)	13.4 ± 2.5 (whole body extracts)		[47]
Ocean quahog *Arctica islandica*	M: Spectrofluorometer V: μg/g wet	5-HT CG + PG + VG 20		[26]
Atlantic jackknife clam *Ensis directus*	M: Spectrofluorometer V: μg/g wet	5-HT CG + PG + VG 21-39		[26]
Soft-shell clam *Mya arenaria*	M: Spectrofluorometer V: μg/g wet	5-HT CG + PG + VG 22		[26]
Hard clam *Venus mercenaria*	M: Spectrofluorometer V: μg/g wet 26 assays during 16 months	5-HT CG + PG + VG 30–40		[26, 107]
Atlantic surf clam *Spisula solidissima*	M: Spectrofluorometer V: μg/g wet	5-HT CG + PG + VG 8.0–14.3 Ganglia connectives 2.2		[26]
Atlantic deep-sea scallop *Placopecten magellanicus*	M: Spectrofluorometer V: μg/g wet	5-HT 36 (VG)		[26]

Species	Notes	Nervous system	Gonad	Reference
Blue mussel *Mytilus edulis*	M: Spectrofluorometer V: μg/g wet	5-HT 15 (CG) 15 (PG) 10 (VG)		[26]

Abbreviation: CG, cerebral, cerebral-pleural or cerebroid ganglion; d, day; g.p., ganglia pair; HPLC-ED, high-performance liquid chromatography coupled with electrochemical detection; M, methods; N.D., not determined; O, ovary; PG, pedal ganglion; T, testis; SD, standard deviation; SEM, standard error of mean; V, values; VG, visceral ganglion.

Table 2. Identification of 5-hydroxytryptophan, (5-HTP), serotonin (5-hydroxytryptamine, 5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in the nervous system and gonad of bivalve mollusks.

Following development of cellular and molecular methods, 5-HT has been localized in the nervous system and gonad of several bivalve species (**Table 3**). Firstly, Falck-Hillarp's method has been used to localize 5-HT in fingernail clam (*Sphaerium sulcatum*) [47], blue mussel [69], and Yesso scallop [88]. In this method, histological sections are exposed to gaseous formaldehyde or glyoxylic acid to visualize monoamine containing neurons [45, 121, 122]. In all examined bivalve species, 5-HT-IR neurons are observed in CG, PG, and VG (**Table 3**). However, the Falck-Hillarp's method is not always useful as 5-HT fluorescence tends to faint rapidly. In addition, catecholamines neurons show similar intensity to that of 5-HT neurons at high concentrations [123]. In 1978, Steinbusch et al. [124] developed a rat monoclonal antibody against a 5-HT-bovine serum albumin conjugate to localize 5-HT in nervous system. Further studies have used monoclonal or polyclonal antibody against 5-HT to localize 5-HT-IR neurons in the nervous system and peripheral organs of bivalves (**Table 3**). The advantage of immunohistochemistry method using antibodies against 5-HT is to describe morphology of 5-HT neurons, and to localize 5-HT distribution within different parts of nervous system, precisely. The 5-HT-containing neurons are mostly unipolar, although their sizes may differ among species (**Table 3**). Using an electron microscopy, it has been observed that 5-HT-IR neurons are often in close connection with each other, but without indication of gap junctions or other specialized junctions. The neurons possess numbers of granular vesicles (100–180 nm in Mediterranean mussel) containing 5-HT that concentrated at the cell periphery [104, 125]. It has confirmed that 5-HT-IR fibers are the axon or axon terminals of 5-HT containing neurons that transport 5-HT to peripheral organs. Within the nervous system, 5-HT-IR fibers seem to be synaptic region, an area where release and reuptake of 5-HT occur.

In general, studies on bivalves show that 5-HT-IR neurons are mostly located in the cortices, and 5-HT-IR fibers are located in the neuropil of CG, PG, and VG (**Table 3**). In Yesso scallop, 5-HT-IR neurons are located in the cortices of the right side of the left lobe and in the left side of the right lobe in anterior lobe (AL) of CG, while they are located throughout their cortices in PG and the posterior lobe (PL) of CG [105] (**Figure 3**). In the great scallop [90], distribution of 5-HT-IR neurons in the posterior lobe of CG slightly differs compared to Yesso scallop. In VG, 5-HT-IR neurons are restrictively scattered in the accessory lobe of scallop species [90, 105, 115] or at the roots of branchial nerves in clams [89]. Large numbers of 5-HT-IR fibers have also been observed in the cerebral-pedal, and cerebral-visceral-pedal connectives [90, 103], suggesting that 5-HT transports from CG to VG [69, 89, 90, 105]. Comprehensive overview of

Species	Methods	Cerebral ganglia	Visceral ganglia	Pedal ganglia	Gonad	Reference
Fingernail clam *Sphaerium sulcatum*	Histochemistry using a paraformaldehyde-induced fluorescence method	5-HT-IR unipolar cells (μm length) are located in the cortices at the dorsal and anteriomedial surfaces of the ganglion. 5-HT-IR fibers are located in the anterior pallial nerve, the CVC, CC, and CPC	No traces of 5-HT-IR neurons are observed in the VG. 5-HT-IR fibers are observed	5-HT-IR fluorescences are uniformly distributed in the cytoplasm of unipolar neurons (10–25 μm length). Green-yellow fibers extend throughout neuropil and across the PC		[47]
Blue mussel *Mytilus edulis*	Histochemistry using a paraformaldehyde-induced fluorescence method	5-HT-IR neurons (9–14 μm d.) are only located in the cortex. Fluorescence is observed in the perikarya	A few 5-HT-IR neurons (11–14 μm d.) are located in the cortex and neuropil. 5-HT-IR fibers are observed in the CVC			[69]
Yesso scallop *Patinopecten yessoensis*	Histochemistry using a glyoxylic acid-induced fluorescence method	Fluorohistochemical reaction is detected in the neuropil, and its tendency is higher than PG and VG	Fluorohistochemical tendency is high in the accessory ganglia	Fluorohistochemical reaction is detected in the neuropil close to CPC	Muscles of the gonoduct stretched under the epithelium in the gonad	[88]
Yesso scallop *Patinopecten yessoensis*	Immunohistochemistry using a rat monoclonal 5-HT antibody against a 5-HT-bovine serum albumin conjugate (coded YC5/45 HL, Sera-Lab, UK)	5-HT-IR neurons are distributed in the AL (right side of the left lobe and left side of the right lobe), and throughout the cortex in PL	ND	5-HT-IR neurons are distributed throughout the cortex		[105]
Mediterranean mussel *Mytilus galloprovincialis*	Immunogold labeling of nerve cells using an anti-5-HT raised in rabbits against formaldehyde cross-linked 5-HT-bovine serum albumin (Immunonuclear, Incstar Co, Stillwater, MN)	5-HT-IR unipolar neurons are mostly located in the cortex with a few numbers in the neuropil. 5-HT-IR fibers are seen in the CC and CVPC	5-HT-IR neurons are unipolar and located in the cortex. Number of 5-HT-IR neurons is lower than CG.5-HT-IR fibers are seen in the visceral commissure and CVC	Large numbers of 5-HT-IR unipolar neurons and a few bipolar or multipolar are clustered in the cortex. 5-HT-IR fibers are observed in neuropil		[103, 104, 125, 126]

Species	Methods	Cerebral ganglia	Visceral ganglia	Pedal ganglia	Gonad	Reference
Great scallop *Pecten maximus*	Immunohistochemistry using an anti-5-HT polyclonal antibody (coded, PS10, TEBU)	5-HT-IR neurons are mostly located in the cortex: 10 or 20–25 μm d. 5-HT-IR fibers are seen in the CVC	A small number of 5-HT-IR neurons are seen in VG, restricted to ACL at the base of CVC	5-HT-IR neurons are mostly located in the cortex with size of 10 μm d. (small cells) or 20–25 μm d. (large cells)	5-HT-IR fibers surround periphery of gonadal lobules (acini) and in the subepithelial layer of the gonoducts	[90]
Atlantic deep-sea scallop *Placopecten magellanicus*	Immunohistochemistry using a rabbit anti-5-HT antibody (Incstar Co., Stillwater, MN)	5-HT-IR neurons are widely distributed over the anterior surface and only sparsely over the posterior surface. 5-HT-IR fibers are located in neuropil	5-HT-IR neurons are mainly distributed in the accessory ganglia. 5-HT-IR neurons and fibers are far fewer than CG and PG	5-HT-IR neurons are unipolar (5–15 μm d.) and located along the medial, dorsal, and ventral margins, of the anterior surface of each PG. 5-HT-IR fibers are located in neuropil	5-HT-IR fibers occasionally surround periphery of acini at early gametogenesis. After spawning, 5-HT-IR fibers abundantly surround the empty germinal acini	[115]
Surf clam *Spisula solidissima*	Immunohistochemistry using a rabbit anti-5-HT antibody (Incstar Co., Stillwater, MN)				5-HT-IR fibers surrounds periphery of gonadal lobules (acini) in males and females throughout reproductive cycle. The 5-HT-IR fibers are interrupted or expelled from each acinus after spawning	[112]
Warty venus *Venus verrucosa*	Immunohistochemistry using a rabbit anti-5-HT antibody (Biogenesis, UK)	5-HT-IR oval perikarya are clustered at the roots of the branchial nerves in the cortex. They are unipolar (15–25 μm d.). 5-HT-IR fibers are located in the neuropil			5-HT-IR fibers are observed at the periphery of the follicle and seminiferous acini filled with mature oocytes and sperm, respectively	[91]
Soft-shell clam *Mya arenaria*	Immunohistochemistry using a rabbit polyclonal anti-5-HT antibody (Sigma-Aldrich Co. LLC.)	Largest number of 5-HT-IR cells scattered throughout the cortex	5-HT-IR cells are symmetrically restricted to clustered population called "glomeruli"	5-HT-IR cells are symmetrically distributed in the cortex	Early spermatogenesis stage in males and post-vitellogenic stage in females	[89, 106]

Species	Methods	Cerebral ganglia	Visceral ganglia	Pedal ganglia	Gonad	Reference
Freshwater pearl mussel *Hyriopsis bialata*	Immunohistochemistry using a rabbit polyclonal anti-5-HT IgG (Zymed Laboratories, San Francisco, CA or Sigma-Aldrich Co. LLC.)	5-HT-IR neurons are large (10 × 30 μm d.) and located at the periphery of CG. 5-HT-IR fibers are occasionally detected	5-HT-IR perikarya are large (10 × 30 μm d.) and located in the cortex of VG. 5-HT-IR fibers are mostly observed in the neuropil. Expression of 5-HT-IR fibers or neurons is higher in females than males	5-HT-IR neurons are large (10 × 30 μm d.) and located at the periphery of PG		[87, 127]

Abbreviations: 5-HT-IR, serotonin-immunoreacted; ACL, accessory lobe; AL, anterior lobe; CC, cerebral commissure; CPC, cerebral-pedal connective; CVC, cerebral-visceral connective; CVPC, cerebral-visceral-pedal connective; PC, pedal commissure; d., diameter; ND, no 5-HT-IR neurons or fibers are detected; PL, posterior lobe.

Table 3. Cellular localization of 5-hydroxytryptamine (serotonin 5-HT) in the nervous system and gonad of bivalve mollusks.

cellular localization of 5-HT indicates that localization and distribution of 5-HT-IR neurons may differ among subclasses of bivalve, for instance between Heterodonta (genus *Mya, Ruditapes*, and *Venus*) and Pteriomorphia (genus *Pecten, Patinopecten*, and *Mytilus*) (**Table 3**). It might be due to differences in location of various parts of nervous system in the body to innervate peripheral organs.

Using histochemistry or immunohistochemistry methods, studies have shown that a few 5-HT-IR neurons are located in the cortex and neuropil of VG compared to those of the CG or PG, for instances in the blue mussel [47, 69, 128], Mediterranean mussel (*Mytilus galloprovincialis*) [103], great scallop [90], Atlantic deep-sea scallop (*Placopecten magellanicus*) [115], and soft-shell clam [89]. Matsutani and Nomura [105] reported no 5-HT-IR neurons in the VG of the Yesso scallop. Although VG contain a few 5-HT-IR neurons, they are usually rich in 5-HT-IR fibers. These studies confirm the Welsh and Moorhead's observation that 5-HT content differs among various parts of the nervous system.

Studies used spectrofluorometric method [26, 47, 56, 118–120] or electrochemical detection coupled with a high-performance liquid chromatography (HPLC-EC) to study 5-HT content in the nervous system of bivalves [90, 110, 114, 115, 117] (**Table 2**). Results confirm aforementioned differences in 5-HT content among various parts of the nervous system, for instance it is higher in the CG than the VG of gaper clam (*Tresus capax*) and bent-nose clam (*Macoma nasuta*) [117]. In addition, the metabolite of 5-HT (5-HIAA) is detected in the nervous system of the brown mussel (*Perna perna*) [110] and Atlantic deep-sea scallop [114], suggesting that metabolism of 5-HT takes place in the nervous system.

Welsh and Moorhead [56] observed that *in vitro* 5-HT synthesis by the nerve tissues undergoes a seasonal variation and suggested seasonal variation of amine oxidase. Further studies have shown that 5-HT content in the nervous system undergoes seasonal variation along with gonadal development in bivalves (**Table 2**). Content of 5-HT increases in the nervous system from early gonadal development to maturity stage in the brown mussel [110] and decreases following spawning in Peruvian scallop (*Argopecten purpuratus*) [113]. York and Twarog [120] reported that 5-HT in the PG of blue mussel is higher in April than March. It has also observed that 5-HT content in the whole nervous system of the blue mussel increases from April to October [118]. As the blue mussel spawns from late spring to late summer [129, 130], these data suggest that 5-HT content increases during spawning. 5-HT content also correlates with the content of its metabolite (5-HIAA), suggesting that metabolism of 5-HT is in parallel to its biosynthesis in the nervous system [110].

3.3.2. 5-HT in the gonad of bivalves

Localization of 5-HT in the gonad has studied in a few species of bivalves (**Table 3**). Using method of Falck-Hillarp, Sweeney [47] and Matsutani and Nomura [88, 105] observed the 5-HT-IR fibers in the gonoduct and epithelium around gonad in the Fingernail clam and Yesso scallop, respectively, and suggested that the 5-HT-IR fibers originate from CVC to innervate the gonad. Further studies using antibodies against 5-HT confirmed existence of 5-HT-IR fibers in the gonad of Yesso scallop [105], great scallop [90], Atlantic deep-sea scallop [115], surf clam [112], warty venus [91], and soft-shell clam [106]. These studies clearly indicated

that the nervous system innervation of the gonads is mostly emerged from VG or derived from CVC. The 5-HT-IR fibers surround periphery of collecting tubes and of gonadal lobules (acini) in males and females filled with sperm and oocytes, respectively (**Figure 3**).

As seasonal-dependent 5-HT content in the nervous system, distribution of 5-HT fibers also changes in the gonad throughout reproductive cycle [91, 106, 112, 115] (**Figure 3**; **Tables 2** and **3**). Generally, the 5-HT-IR fibers are occasionally observed around the germinal acini, and extensively distributed around the collective tubes at early developmental stage. However, the 5-HT-IR fibers around the acini are more frequent at maturity stage [112]. After spawning, the 5-HT-IR fibers still exist around collecting tubes, and are abundant around gamete empty acini.

Using spectrofluorometric or HPLC-EC method, 5-HT content has been measured in the gonad of the Atlantic deep-sea scallop [114, 115], surf clam [112], Pacific lion's paw scallop (*Nodipecten subnodosus*) [111], and brown mussel [110]. Matsutani [131] reported a tendency toward an increase and a decrease of 5-HT content in the testis and ovary of Japanese scallop (*Chlamys farreri nipponensis*) during spawning, respectively. It has shown that 5-HT content increases from early developmental stage of the gonad to maturity stage in males and females [110, 111]. In surf clam, Masseau et al. [112] reported that changes in 5-HT content are uncertain in males during testicular development and after spawning. However, in females, 5-HT is high at early development stage, decreases at maturity stage and spawning, and then increases after spawning. They also reported that 5-HT content does not differ between males and females when they are compared at similar gonadal development stage. Klouche et al. [110] pooled the data of males and females in brown mussel, as there are no differences between sexes, and observed that 5-HT content increases toward maturation of gonad. In Peruvian scallop, 5-HT content decreases in the male and female portions of gonad following spawning [113, 116]. Observed differences in 5-HT content among studies may represent inter-species differences associated with 5-HT regulation of reproduction that might also be different between sexes. Klouche et al. [110] reported that the gonadal content of 5-HT metabolite (5-HIAA) in brown mussel is high at early development and become decreased at maturity stage. As 5-HT content is high at maturity, these suggest that 5-HT-dependent reproduction associates with decreasing 5-HT inactivation mediated by its metabolism.

A few studies show 5-HT content in both nervous system and gonad, for instance in the Peruvian scallop [113, 116] and brown mussel [110]. Results show higher 5-HT content in the nervous system than gonadal tissue as 5-HT content is lower in connective nerves than 5-HT neurons [26, 56].

Croll et al. [115] observed that distribution of 5-HT-IR neurons and fibers is similar between juvenile and adult in the Atlantic deep-sea scallop or between sexes in the surf clam [112]. However, abundance or distribution of 5-HT neurons and 5-HT content may differ between sexes. Martínez and Rivera [116] observed that 5-HT content is higher in the male portion than female portion of the gonad of the Peruvian scallop. Expression of 5-HT-IR fibers or neurons has been seen to be higher in the VG of females than that of males [127]. These studies may suggest inter-sex difference in 5-HT biosynthesis or inter-sex difference in 5-HT regulatory function of reproduction.

4. Conclusion and future research perspectives

The essential components of 5-HT biosynthetic pathway are highly conserved in the animal kingdom. The 5-HT biosynthesis from the essential amino acid L-tryptophan is catalyzed by TPH, which convert L-tryptophan to 5-HTP, and by AADC, which convert 5-HTP to 5-HT. All precursors of 5-HT are identified in the nervous system of bivalves. In mammals, there are two isoforms of TPH (TPH1 and TPH2), which are predominantly expressed in the peripheral organs and in the nervous system, respectively. However, TPH1 is the primary form and expresses earlier in neural development [132, 133]. Molecular sequence of the gene encoding AADC has also been identified and localized in mammals [134, 135]. It has a non-specific tissue distribution and is expressed in wide range of cell types [66]. In bivalves, molecular identity, localization, and characterization of TPH and AADC are unknown. These studies will provide us with satisfactory information to better understand ontogeny of 5-HT neurons in the nervous system and to elucidate developmental biology of 5-HT regulation of reproduction.

It has been seen that the first 5-HT-IR neurons appearing within the nervous system correspond to the location of the CG and apical ganglion (AG) during the late trochophore stage: 30–32 h postfertilization in blue mussel [136], 24 h postfertilization in surf clam [137], and 27 h postfertilization in the Bay mussel (*Mytilus trossulus*) [138]. Kreiling et al. [137] reported that the 5-HT-IR neurons appear in VG of surf clam at 48 h postfertilization. Following 72 h postfertilization, the 5-HT-IR neurons emerging from the CG and AG extend their processes to the VG, through which connections of the 5-HT-IR neurons between CG/AG and VG are formed at 96 h postfertilization. During the embryonic development, the size of the 5-HT area in the CG/AG and VG increases from 24 h to 96 h postfertilization, which is associated with an increase in 5-HT content. Cann-Moisan et al. [139] reported that 5-HT content undergoes variation throughout the larval and postlarval stages. It rises from 2 d to 27 d postfertilization (15–50 pg/μg of protein, respectively); however, it decreases to less than 1 pg/μg of protein after 55 d postfertilization. These indicate that 5-HT neurons form at the embryonic stage, and 5-HT content increases from embryonic development to metamorphosis, and decreases after metamorphosis. Voronezhskaya et al. [138] observed that 5-HT-IR neurons innervate the peripheral organs in the postmetamorphic stage, suggesting that 5-HT biosynthesis undergoes developmental variation. This might be related to the availability of the 5-HT precursors or inactivation mechanisms of 5-HT. However, further studies are required to investigate development of 5-HT fibers in the gonad through developmental stage.

As animals lost the ability to synthesize tryptophan, there possess developed biological mechanisms through which animals obtain tryptophan from their diets. Thus, 5-HT biosynthesis highly depends on dietary factors including availability of tryptophan and competitive uptake or transport of tryptophan with other amino acids (such as tyrosine and phenylalanine) into the 5-HT neurons. Studying nutritional effects on 5-HT biosynthesis will lead to better understanding of physiological relationships between seasonal variation in 5-HT content and gonadal development. In addition, it can help us to investigate the impacts of parental nutrition on gamete maturation and fertility in bivalves. These studies can provide us with

knowledge to better understand 5-HT controls of feeding behaviors such as appetite and satiety, which have been demonstrated in mammals [140].

Mechanisms of 5-HT inactivation in the nervous system and peripheral organs of bivalves are poorly understood. It requires molecular identity, localization, and characterization of SERT and MOA. In this regard, several types of SERT and MOA inhibitors are available [80, 114, 141] that provide us with useful tools to elucidate molecular signaling that control 5-HT reuptake and metabolism. A few studies show that selective 5-HT reuptake inhibitors modulate 5-HT-induced spawning in bivalves. Fong [142] and Fong et al. [143, 144] reported spawning of Zebra mussel treated with selective 5-HT reuptake inhibitors (fluvoxamine, fluoxetine, zimelidine, and paroxetine). Both males and females are capable of releasing their gametes after treatment with fluvoxamine at 10^{-7} and 10^{-6} M, respectively. Following treatment with fluoxetine, 100% of males have spawned at 10^{-4} to 10^{-5} M, however spawning has induced in 50–60% of females at 10^{-5} M. Zimelidine induces spawning in 100 and 60–70% of males and females at 10^{-4} M. Paroxetine induces spawning in 50 and 20% of males and females at 10^{-6} and 10^{-5} M, respectively. Considering spawning of males and females at 10^{-3} M 5-HT, these results indicate that selective 5-HT reuptake inhibitors stimulate spawning in Zebra mussel at concentrations lower than that of 5-HT. Further examinations have revealed that mianserin and cyproheptadine interfere with fluvoxamine-, fluoxetine-, and zimelidine-induced spawning [144] suggesting that antagonists of 5-HT_2 receptor block stimulatory function of selective 5-HT reuptake inhibitors in spawning. Inhibition of 5-HT reuptake may increase the synaptic 5-HT concentrations, which in turn activate postsynaptic 5-HT receptor to induce spawning. It is also possible that selective 5-HT reuptake inhibitors act as ligands at postsynaptic receptor rather than inhibition of SERT. Overall, these studies suggest that 5-HT transport plays a key role in reproduction; however, the mechanisms of action are largely unknown.

So far, histochemistry and immunohistochemistry methods have been employed to localize the 5-HT neurons and fibers, and spectrofluorometric and HPLC-EC methods have been used to identify 5-HT content in the nervous system and gonad of various bivalve species. Successful implication of various mammalian monoclonal or polyclonal antibodies indicates that 5-HT structure is highly conserved through evolution across the animal kingdom. However, mechanisms through which 5-HT acts on a biological system may differ. The present review shows that 5-HT content highly differs in the nervous system and gonad of bivalve species. The inter-species differences in 5-HT content might be related to capability of nervous system to synthesize 5-HT, differences in 5-HT inactivation or 5-HT transport from nervous system to the gonad. In the latter case, 5-HT content in the gonad may correspond to 5-HT concentration that requires to stimulate spawning. The present review shows that 5-HT concentration to induce spawning highly differs between sexes, and among species. It is worth to note that tissue sampling, extraction procedure, and analytical method affect the results of 5-HT content. In addition, 5-HT content undergoes seasonal variation and change following spawning.

Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

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Types

Chapter 4

4WD to Travel Inside the 5-HT_{1A} Receptor World

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Additional information is available at the end of the chapter

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Abstract

5-HT_{1A} receptor is one of the most important members of the numerous families of serotoninergic receptors. Though it was the first 5-HT receptor to be identified and cloned, the knowledge of its activation/transduction mechanisms, mediated effects, and connection with other systems is still uncompleted. For this reason, relevant is the study of the four Ws of the title: first of all "who" this receptor is, then "why" it continues to be a so attractive target after several years after its identification, then "where" is 5-HT_{1A} receptor expressed within the body, and, finally, "what" effects this receptor can elicit under physiological and pathological conditions. Obviously, more and more potent, safe, and selective "drugs" might be discovered once the responses to these questions are given.

Keywords: 5-HT_{1A} receptor, 5-HT_{1A} transduction mechanisms, central nervous system diseases, 5-HT_{1A} ligands, structure-activity relationship studies

1. Introduction

The rational research of novel efficacious and safe drugs is mainly based on the knowledge of biological systems, whose dysfunctions cause several pathological conditions. Receptors and enzymes are the most common targets to which the so-called charmed bullets by Paul Ehrlich (1854–1915), Nobel Prize in Physiology and Medicine in 1908, should be addressed to mean the selectivity of interaction and, therefore, the reduced occurrence of unwanted side effects. Serotonin receptors (5-HTRs) are the most widespread targets of drugs because of the numerous

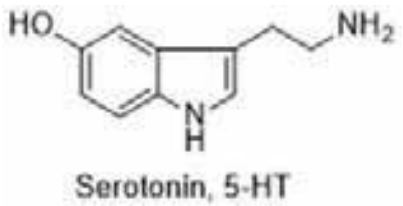

Figure 1. Chemical structure of 5-HT.

biological effects of the endogenous ligand serotonin (5-HT; **Figure 1**) and the wide presence of different 5-HTR subtypes in both the central and peripheral nervous systems (CNS and PNS) [1].

5-HT is biosynthesized at the periphery into the gut by intestinal enterochromaffin cells and in the CNS in the raphe nucleus from the essential amino acid L-tryptophan. A 5-HT reuptake protein (SERT) is responsible for carrying the neurotransmitter from the synaptic cleft to its target nerve and acts as a regulator of 5-HT levels. In the CNS, SERT is a key target for various antidepressant drugs such as tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs). 5-HT is mainly deaminated by monoamine oxidase A (MAO A) to the corresponding aldehyde in the liver. The physiological effects of 5-HT are mediated by several 5-HTRs, whose heterogeneity was hypothesized from pharmacological characterization in the 1950s. From radioligand experiments, the first evidences of 5-HT subtypes were reported in 1979 [2]. To date molecular cloning techniques, amino acid sequence determination, evaluation of its pharmacological properties, second messenger coupling, and signal transduction characterization have allowed the identification of at least seven subfamilies (5-$HT_{1–7}$), some of which are further subdivided into different subtypes (**Figure 2**).

Figure 2. Classification of 5-HT_{1A}Rs.

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While 5-HT$_3$Rs are cation-permeable ion channels, all the others are G-protein-coupled receptors (GPCRs) and are classified as rhodopsine-like receptors (class A). Among the 5-HTRs, the 5-HT$_{1A}$ subtype was the first to be cloned [3] and pharmacologically characterized, and it is one of the most studied. For this reason, it is often ironically called "old target" [4]. The human 5-HT$_{1A}$R consists of 422 amino acid residues with a molecular weight of about 46,000 Da. Though its structure is still unknown, mutagenesis studies have allowed the identification of amino acid residues responsible for ligand binding and G-protein coupling [1].

2. Localization

5-HT$_{1A}$Rs are widely expressed in the brain of mammals, including humans [5]. The main expressions are in limbic areas, such as the hippocampus, lateral septum, cortical brain regions, as well as dorsal and medial raphe nuclei (DRN and MRN) (**Figure 3**).

5-HT$_{1A}$Rs are located within the brain both pre- and postsynaptically. Presynaptic 5-HT$_{1A}$Rs are expressed in all 5-HT neurons (autoreceptors) and in a lot of non-5-HT neurons (heteroreceptors). The latter modulate the release of several neurotransmitters, including glutamate and dopamine, and hormones (adrenocorticotropin (ACHT), oxytocin, prolactin, growth hormone, β-endorphin). In the brainstem, presynaptic autoreceptors are expressed in serotonergic neurons in DRN and MRN, where their activation inhibits cell firing rate. These neurons send ascending 5-HT fibers to the forebrain attenuating 5-HT synthesis, turnover, and release in projection areas from axon terminals, working on a basis of a negative feedback. Presynaptic

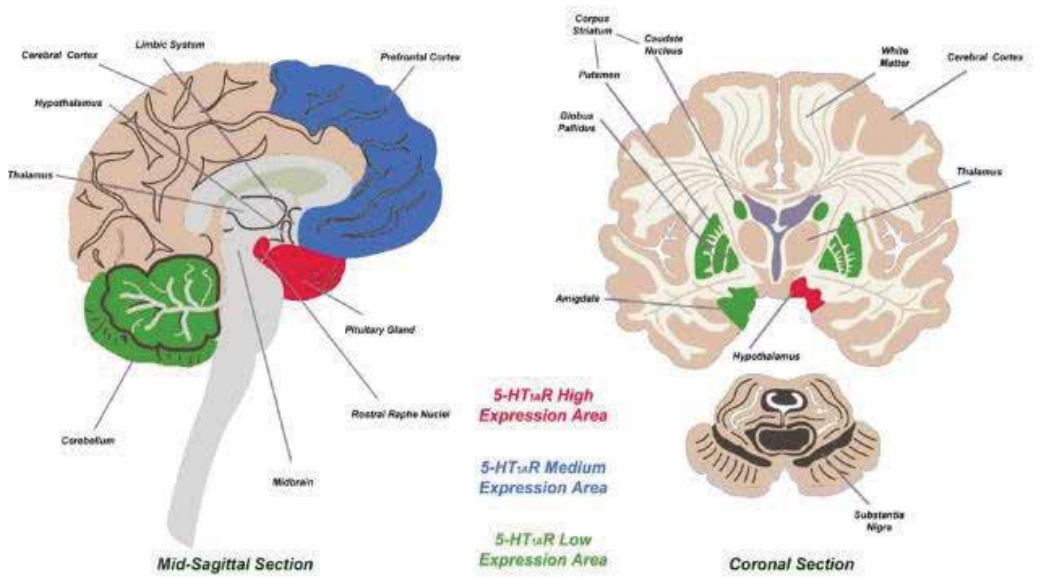

Figure 3. Central localization of 5-HT$_{1A}$Rs (Adapted from CNSforum image bank, Lundbeck Institute "Distribution of 5-HT$_{1A}$ receptors" http://www.cnsforum.com/imagebank/item/hrl_Rcpt_sys_SN1A_dist/default.aspx).

5-HT_{1A}Rs expressed in DRN, through coupling to G$\alpha_{i/o}$ proteins, decrease rate of cell firing by the activation of inwardly rectifying potassium channels. Postsynaptic 5-HT_{1A}Rs are found at high density in limbic regions, such as the hippocampus and septum, and in the frontal and entorhinal cortices. Lower 5-HT_{1A}R levels are observed in the amygdala. As in the case of presynaptic receptors, the activation of postsynaptic 5-HT_{1A}Rs generally decreases the firing rate of postsynaptic cells. Electrophysiological, pharmacological, and biochemical evidences have demonstrated that 5-HT_{1A}Rs are localized in primary afferent neurons [4]. They are also present in the gut, in the enteric nervous system, as well as in smooth muscle, where their activation inhibits relaxation or contraction.

3. Signal transduction pathways of 5-HT_{1A}Rs

The primary transduction pathway of 5-HT_{1A}Rs is the inhibition of adenylate cyclase (AC). Nevertheless, various other pathways are coupled to this receptor depending on the target cell. Indeed, 5-HT_{1A}R stimulation activates or inhibits different enzymes, channels, and kinases, as well as modulates the production of several second messengers (**Figure 4**) [6, 7].

Whatever is the activated second messenger, the signals initiated by the stimulation of 5-HT_{1A}Rs implicate the involvement of $G_{i/o}$ protein. Moreover, a G-protein-independent pathway of 5-HT_{1A}R coupling to a smooth inward current has also been suggested.

3.1. AC inhibition

The activation of 5-HT_{1A}Rs inhibits AC and reduces the production of cAMP with a consequent decrease of protein kinase A (PKA) activity. The Gα_i-induced inhibition of AC is coupled to 5-HT_{1A} heteroreceptors, whereas the situation is still unclear for 5-HT_{1A} autoreceptors. Indeed, some results reveal that 5-HT_{1A}R partial agonists negatively regulate presynaptic AC activity in raphe nuclei. On the other hand, a lot of evidences highlight that 5-HT_{1A}R agonists do not inhibit forskolin-stimulated AC activity in homogenates of the raphe region, suggesting that these autoreceptors do not couple to AC. 5-HT_{1A}R agonists also reduce PKA activity in the hippocampus, determining increased protein phosphatase-1 activity and reduction of Calcium/calmodulin-dependent protein kinase II phosphorylation. This signaling effect is joined to cognitive deficits. Therefore, cognitive behaviors can be mediated by the inhibition of AC/PKA activity induced by 5-HT_{1A}Rs.

3.2. GIRK and Ca^{2+} channel opening

Through coupling to G$\alpha_{i/o}$ proteins, 5-HT_{1A}Rs activate inwardly rectifying potassium channels (GIRKs) in the hippocampus and DRN. Such an action hyperpolarizes neurons and decreases firing. Moreover, Ca^{2+} entry is reduced by the inhibition of voltage-gated Ca^{2+} channel following 5-HT_{1A}R activation.

3.3. ERK/MAPK pathway activation

The stimulation of 5-HT_{1A}Rs induces the release of $\beta\gamma$-complex that participates in the activation of phosphatidylinositol-3 kinase (PI3K). It triggers the activation of extracellular signal-regulated

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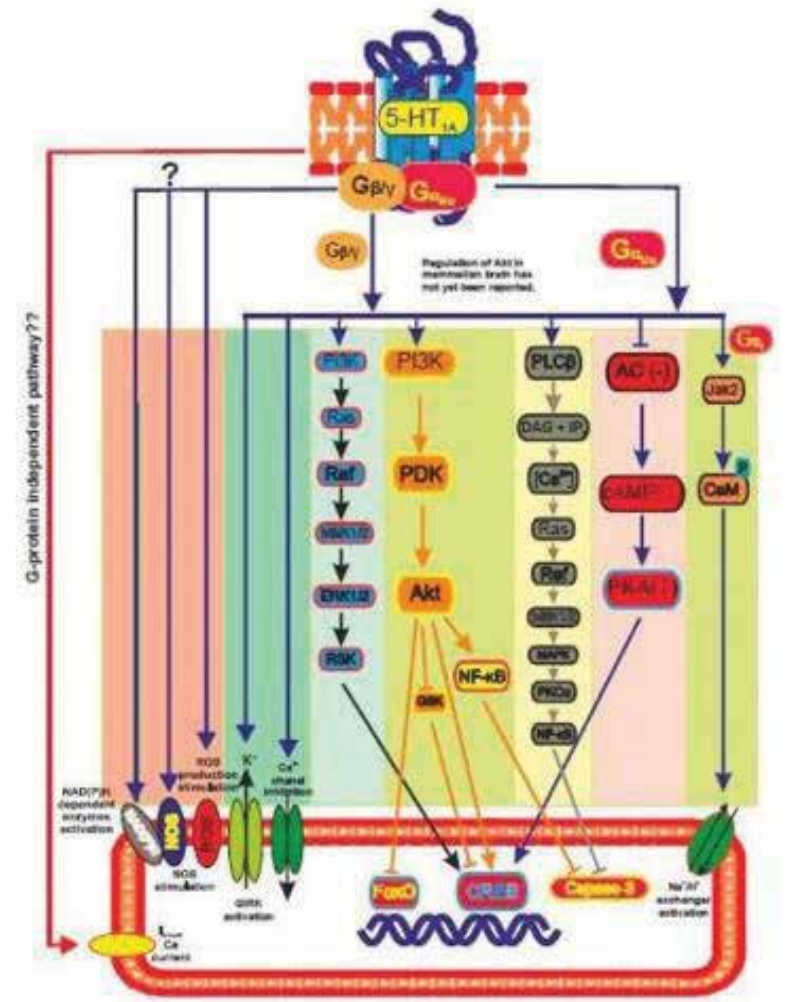

Figure 4. Main transduction pathways of 5-HT$_{1A}$Rs (Reprinted with permission from Ref. [6]).

protein kinase (ERK) (or MAPK), implicated in cell proliferation and differentiation through two pathways involving Ras-Raf-MEK proteins. In addition, 5-HT$_{1A}$-induced ERK activation in nonneuronal cells can be mediated by phosphatidylcholine-specific phospholipase C (PC-PLC) in a G-protein-dependent manner. In neuronal cells, the effects on ERK activity produced by 5-HT$_{1A}$Rs can be different. Indeed, in the hypothalamus a rapid but transient increase of ERK phosphorylation is observed, and this effect might be an intermediate step for the 5-HT$_{1A}$R-mediated increase of oxytocin, ACTH, and prolactin. In HN2-5 hippocampal-derived cell lines, 5-HT$_{1A}$R activation favors ERK phosphorylation and activity. This effect does not occur in the primary culture of hippocampal or fetal rhombencephalic neurons. On the contrary, in the rat hippocampus, 5-HT$_{1A}$R activation decreases ERK phosphorylation. Analogously it reduces MEK activity and ERK phosphorylation in differentiated raphe neurons. Different ERK-related effectors can be modulated by 5-HT$_{1A}$Rs: activation of the ribosomal S6 kinase (RSK), stimulation of

nuclear factor κB (NF-κB), and inhibition of caspase 3. This pathway seems to be involved in neuroprotective mechanisms. ERK also activates cAMP response element binding (CREB), a transcription factor that plays fundamental roles in stress, anxiety, and depression. Finally, the activation of MAPK/ERK transduction pathway may inhibit apoptosis by phosphorylation of the proapoptotic protein Bad and by increasing the expression of antiapoptotic Bcl-2.

3.4. PI3K and Akt pathway activation

5-HT_{1A}R stimulation can also regulate the activation of the PI3K/Akt signaling pathway through βγ-complex. The Akt protein kinase plays a key role in several cellular processes, such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. In the mammalian brain, the PI3K/Akt pathway is also implicated in synaptic plasticity, learning, and memory. Consequently, Akt dysfunction can be associated with metabolic diseases (e.g., diabetes and obesity), central disorders (e.g., depression, schizophrenia, and drug abuse), and the most frequent alterations observed in human cancer and tumor cells. Akt phosphorylates and inactivates the protein glycogen synthase kinase 3 (GSK3), whose inhibition produces antidepressant and antimanic effects. Active Akt also phosphorylates and inactivates Forkhead box O (FoxO) transcription factors, whose deficiency in mice develops antidepressive and anxiolytic behavioral phenotypes.

3.5. Na^+/H^+ exchanger activation

Another complex pathway following 5-HT_{1A}R stimulation and involving G(i2)α and/or G(i3)α induces Janus kinase 2 (Jak2) activation, which leads to tyrosine phosphorylation of calmodulin (CaM). The consequent increase of CaM binding to Na^+/H^+ exchangers (NHEs) induces a conformational modification that activates NHEs, unmasking an obscured proton-sensing and/or proton-transporting region. NHEs, expressed on the surface of all mammalian cells, regulate cell volume, intracellular pH, and transepithelial transport of Na^+ and acid-base equivalents.

3.6. NO production

5-HT_{1A}Rs can also regulate the production of nitric oxide (NO) that plays an important role in the brain. In rat ventral prostate cells, 5-HT_{1A}Rs can stimulate NO synthase (NOS) activity, whereas in the adult rat hippocampus and in human neocortical slices, they inhibit NMDA-induced NO production. Therefore, the regulation of NO synthesis by 5-HT_{1A}Rs is complex and appears to be cell specific.

4. Biological interest of 5-HT_{1A}Rs

5-HT_{1A}R is one of the most important among the 5-HTRs because of its high affinity for 5-HT and involvement in nearly all 5-HT-mediated effects. The main behavioral and physiological functions mediated by this receptor are summarized in **Figure 5**.

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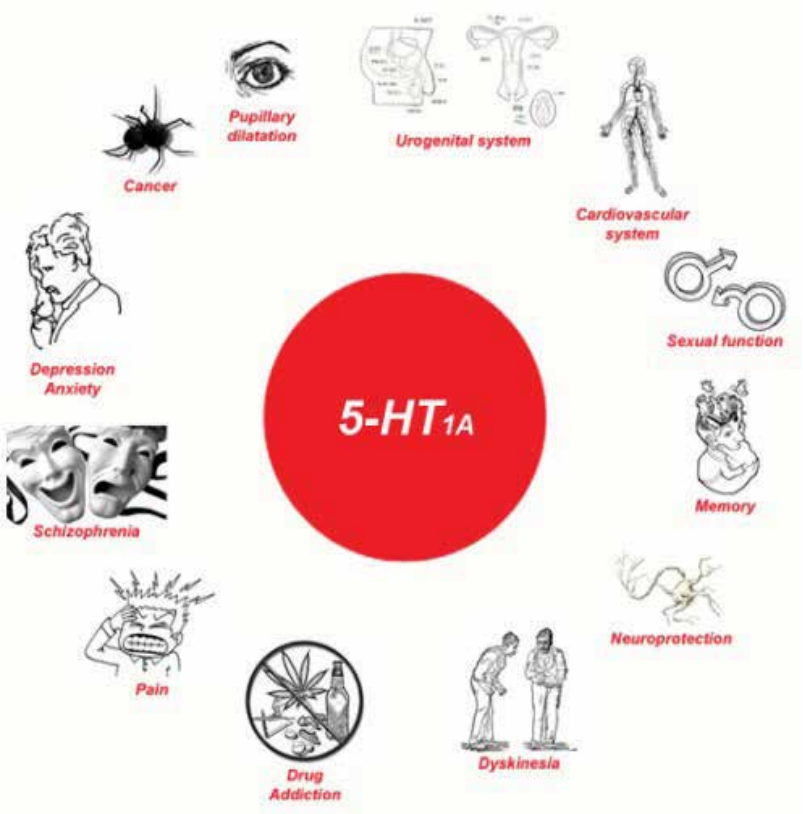

Figure 5. Main behavioral and physiological functions mediated by 5-HT$_{1A}$Rs.

4.1. Depression

The dysfunction of 5-HT$_{1A}$ autoreceptors has been proven to be associated with the major depressive disorders. This correlation is confirmed by the observation that significant antidepressant activity is elicited by 5-HT$_{1A}$R agonists [4]. Though the mechanism responsible for their antidepressant action is still unclear, desensitization or downregulation of presynaptic 5-HT$_{1A}$Rs appears to be implicated in this pharmacological effect. Indeed, in DRN and MRN, prolonged treatment with 5-HT$_{1A}$R agonists desensitizes presynaptic 5-HT$_{1A}$Rs inducing a reduction of autoreceptor-mediated inhibition of 5-HT release.

SSRIs represent the first-line treatment of depression. However, the inhibition of the reuptake of 5-HT increases 5-HT concentration in the synaptic cleft and simultaneously activates 5-HT$_{1A}$ autoreceptors, with a consequent suppression of 5-HT release from presynaptic terminals [8]. Therefore, only prolonged treatment with SSRIs allows the desensitization of 5-HT$_{1A}$ autoreceptors, leading to the recovery of neurotransmission in 5-HT neurons. Beneficial effects on depression are also produced by the combination of SSRIs with 5-HT$_{1A}$R agonists or antagonists, leading to faster onset of antidepressant action and greater antidepressant efficacy.

In particular, 5-HT_{1A}R antagonists can improve the efficacy of SSRIs by blocking inhibitory 5-HT_{1A} autoreceptors, while 5-HT_{1A}R agonists exert antidepressant-like effect through the activation of postsynaptic 5-HT_{1A}Rs and/or faster desensitization of 5-HT_{1A} autoreceptors. Finally, antidepressant-like effect can also be produced by 5-HT_{1A} partial agonism combined with 5-HT reuptake inhibition [4].

4.2. Anxiety

Several studies have been performed to demonstrate the possible role of 5-HT_{1A}Rs in anxiety [1]. Interestingly, mice with genetically inactivated 5-HT_{1A}R gene develop an anxiety-like phenotype, probably resulting from impaired autoinhibitory control of midbrain 5-HT neurons. On the contrary, mice with overexpressed 5-HT_{1A}Rs display diminished anxiety when compared to wild-type animals. These findings support the crucial role of the stimulation of 5-HT_{1A}Rs in the control of anxiety-like behavior. Therefore, 5-HT_{1A}R agonists and partial agonists have been developed as novel anxiolytic agents, devoid of dependence and side effect profile of other anxiolytics and antipsychotics.

4.3. Schizophrenia

Several studies performed in postmortem schizophrenia patients report an overexpression of 5-HT_{1A}Rs in the prefrontal cortex, indicating that these receptors are not adequately stimulated by 5-HT [1]. Therefore, 5-HT_{1A}R agonists might be useful to contrast this apparent deficit. Two mechanisms are advantageously activated by 5-HT_{1A}R stimulation in the treatment of schizophrenia. The first one involves the attenuation of parkinsonian symptoms, such as catalepsy, caused by the antagonism at dopamine D_2 receptor (D_2R) produced by antipsychotics. Since atypical antipsychotic drugs, such as clozapine, quetiapine, and ziprasidone, also behave as potent 5-HT_{1A}R agonists, it has been suggested that the reduced incidence of motor side effects observed with these drugs might be due to their inherent 5-HT_{1A}R agonism. The second mechanism involves the ability of 5-HT_{1A}R agonists to increase dopamine release in the prefrontal cortex, consequently reducing the negative symptoms of schizophrenia. Based on these observations, a novel approach in the treatment of schizophrenia concerns the development of novel atypical antipsychotic agents characterized by a mixed D_2R antagonist/5-HT_{1A}R agonist profile.

4.4. Pain

Full and partial 5-HT_{1A}R agonists are beneficial in pain treatments, including efficacy in neuropathic pain models, arousing great interest as future therapeutic agents. In knockout mice, 5-HT_{1A}Rs have also been demonstrated to mediate an endogenous inhibitory control of nociception evoked by thermal noxious stimuli [4].

4.5. Drug addiction

A critical role in the effects of psychostimulants, including addiction, is played by 5-HT_{1A}Rs. Some psychostimulant drugs, including cocaine, amphetamine, methamphetamine,

and 3,4-methylenedioxymethamphetamine (MDMA), increase not only dopamine but also 5-HT that can hyperactivate 5-HT_{1A}Rs. Interestingly, the contribution of pre- and postsynaptic 5-HT_{1A}Rs can be dissociated and frequently is responsible for opposite effects. In fact, 5-HT_{1A} autoreceptors indirectly facilitate psychostimulant addiction-related behaviors by reducing 5-HT response in projection terminal areas, while postsynaptic 5-HT_{1A}Rs directly contrast the expression of various addiction-related behaviors [9]. Several studies have also demonstrated that 5-HT_{1A}R agonists alleviate opioid-induced respiratory depression in rodent models. The mechanisms involved in this effect are still unclear. However, concomitant decreases in opioid-induced analgesia, as well as altered baseline ventilation and behavior, have also been observed.

4.6. Dyskinesia

5-HT_{1A}Rs are involved in the regulation of locomotor activity. In particular, the stimulation of 5-HT_{1A}Rs facilitates the establishment of locomotor sensitization [10]. Parkinsonian patients in therapy with L-3,4-dihydroxyphenylalanine (L-DOPA) may develop motor complications, such as dyskinesia. The development of this side effect involves several pathways, including an abnormal 5-HT-mediated neurotransmission [4]. It has been highlighted that parkinsonian animals chronically treated with L-DOPA have increased levels of 5-HT_{1A}Rs in the striatal matrix. Accordingly, treatment with 5-HT_{1A}R agonists attenuates dyskinesia but, in some cases, also reduces the antiparkinsonian benefit of L-DOPA. Some evidences suggest that a lot of 5-HT_{1A}R agonists are also endowed with D_2R antagonism, which alleviates dyskinesia, though at the expense of worsening parkinsonism. The challenge is to obtain compounds able to selectively stimulate 5-HT_{1A}Rs in striatus and/or in middle layers of the cortex, avoiding the involvement of 5-HT_{1A}Rs in external cortical layers.

4.7. Neuroprotection

The activation of 5-HT_{1A}Rs exerts a neuroprotective effect in different animal models of ischemia, interfering with excitotoxic and apoptotic cell death processes in the postischemic brain [1]. Though the cellular mechanisms underlying such a neuroprotective effect are still unclear, the hyperpolarization of pyramidal neurons inhibits the glutamate-induced excitotoxicity consequent to cerebral ischemia. 5-HT_{1A}Rs may mediate brain protective mechanisms, by contrasting the effects of glutamatergic NMDA receptor overstimulation and the consequent NMDA-induced Ca^{2+} influx. Moreover, the inhibition of 5-HT_{1A}R-induced cyclases might produce neuroprotective effects due to the reduction of adenylyl cyclase excess following reperfusion after ischemic attack. 5-HT_{1A}R agonists can also be useful for the treatment of traumatic brain injury (TBI) [11].

4.8. Memory

Several experimental evidences highlight that the activation of postsynaptic 5-HT_{1A}Rs, attenuating the neuronal activity, impairs emotional memory. On the contrary, presynaptic 5-HT_{1A}R activation reduces 5-HT release and exerts pro-cognitive effects. 5-HT_{1A}R antagonism facilitates memory retention, probably by the activation of 5-HT_7Rs, and evidence is provided that 5-HT_7Rs can facilitate emotional memory upon reduced 5-HT_{1A}R

transmission [12]. Moreover, tonic and phasic 5-HT release can exert different and potentially opposite effects on emotional memory, depending on the states of 5-HT_{1A}Rs and 5-HT_7Rs and their interaction. Consequently, individual differences due to genetic and/or epigenetic mechanisms play an essential role in the responsiveness to drug treatment [13].

4.9. Sexual function

5-HT_{1A}Rs and 5-HT_{2C}Rs produce two distinct and opposite effects on sexual function: the activation of 5-HT_{1A}Rs decreases ejaculatory latency and erection, directly promoting the sympathetic emission, while the activation of 5-HT_{2C}Rs increases them, directly favoring parasympathetic expulsion and erection [4]. Therefore, 5-HT_{1A}R antagonists are under investigation for the treatment of primary premature ejaculation.

4.10. Cardiovascular system

Several studies have demonstrated that 5-HT_{1A}Rs in the medullary raphe mediate protective responses to stress [4]. Indeed, the activation of 5-HT_{1A}Rs induces bradycardia and blood pressure decrease, suggesting that 5-HT_{1A}Rs can reduce the sympathetic outflow. Moreover, 5-HT_{1A}R agonists reduce the cutaneous vasoconstriction evoked by physical and psychological stressors. 5-HT_{1A}Rs located in limbic regions can also reduce stress-evoked cardiovascular responses. However, this action does not occur via a direct effect on brainstem cardiovascular neurons, but is consequent to the anxiolytic effect. Psychological stress, cold exposure, or fever might elicit cardiovascular responses also mediated by neurons within the dorsomedial hypothalamus. Therefore, 5-HT_{1A}R agonists might be useful therapeutic agents to reduce the sympathetic responses occurring in some forms of hypertension and heart failure. The cardiovascular responses of 5-HT_{1A}R agonists could also be useful to reduce side effects in the treatment of hyperphagia and obesity with noradrenaline (NA) uptake inhibitors. Such inhibitors are able to reduce food intake due to increased noradrenergic activity that also causes an increased cardiovascular activity. When 5-HT_{1A}R agonists are combined with NA uptake inhibitors, side effects, such as hypertension and tachycardia, are mitigated. Postsynaptic 5-HT_{1A}R activation may contribute to hypophagia efficacy. Moreover, presynaptic 5-HT_{1A}Rs may reduce food intake by inhibiting spontaneous noradrenergic cell firing.

4.11. Urogenital system

5-HT_{1A}Rs mediate effects in the lower urinary tract function [4]. Indeed, their stimulation activates the micturition reflex, inducing an increase in the frequency of isovolumic bladder contractions. Conversely, 5-HT_{1A}R agonists elicit periodic external urethral sphincter relaxation, inducing an increase in micturition volume, a decrease in bladder capacity, and an increase in voiding efficiency.

4.12. Pupillary dilation

Pupillary response to 5-HT_{1A}R agonists is species dependent [14]. Indeed, 5-HT_{1A}R activation produces miosis in humans and rabbits and mydriasis in mice. In humans, 5-HT_{1A}Rs induce

miosis solely by inhibiting sympathetic mechanisms. However, evidences suggest that the parasympathetic nerve is also involved. Indeed, the activation of central 5-HT_{1A}Rs induces NA release, which in turn reduces parasympathetic neuronal tone to the iris sphincter muscle by the stimulation of postsynaptic α_2-adrenoceptors (α_2-ARs) within the Edinger-Westphal nucleus.

4.13. Cancer

5-HT_{1A}Rs are known to be involved in the proliferation of human tumor cells, but their function still remains poorly understood [4]. 5-HT_{1A}R antagonists inhibit the growth of different prostatic tumor cell lines, such as PC-3, DU-145, and LNCaP, as well as the proliferation of PC-3 xenografted subcutaneously in athymic nude mice. Multitarget ligands, acting as α_{1A}/α_{1D}-AR and 5-HT_{1A}R antagonists, in which a synergic effect occurs, have proved to be useful in the management of benign prostatic hyperplasia. 5-HT_{1A}Rs are also reported to be involved in the mitogenic effect of 5-HT in human small cell lung carcinoma cells.

5. Ligands

Several structurally different ligands, such as aryloxyalkylamines, arylpiperazines, aminotetralins, indolyl-alkylamines, ergolines, and aporphines, are known to bind 5-HT_{1A}Rs [15]. Recently, new classes of ligands, including 2-imidazoline and 1,4-dioxane derivatives, have also shown high 5-HT_{1A}R affinity. Due to the high homology among 5-HT_{1A}Rs and other receptor systems, in binding studies several molecules show nanomolar and subnanomolar affinity not only for 5-HT_{1A}Rs but also for other receptors (5-HT_{2A}Rs, 5-HT_{2C}Rs, 5-HT_7Rs, α_1- and α_2-ARs, as well as D_1Rs and D_2Rs).

5.1. Aryloxyalkylamines

The sequence analysis of the 5-HT_{1A}R genomic clone indicates 43% amino acid homology with the β_2-AR in the transmembrane domain. Therefore, some compounds show good affinity for both systems. The first examples of dualistic interaction are offered by pindolol (**1**) and propranolol (**2**) (**Figure 6**) [16].

In several studies, an Asn amino acid residue in the putative helix VII of 5-HT_{1A}Rs has been demonstrated to play a crucial role in the binding of aryloxypropanolamines. Indeed, for example, propranolol **2** shows significantly reduced affinity for human 5-HT_{1A}Rs, in which

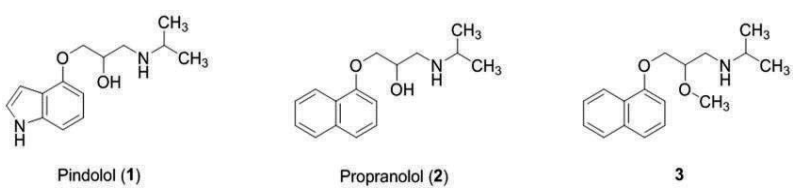

Figure 6. Chemical structures of **1–3**.

the Asn386 is replaced by valine, while the affinity of the neurotransmitter 5-HT is hardly affected. It was initially hypothesized that the formation of two hydrogen bonds occurs between the oxypropanol moiety and the amide group of Asn386. Moreover, since the (*S*)-enantiomer of propranolol is 13-fold more potent than the (*R*)-enantiomer at wild type (pK_i 5-HT_{1A}R = 6.8 and 5.7, respectively) and the enantioselectivity is significantly reduced (three-fold) in Asn386Val mutant human 5-HT_{1A}Rs (pK_i 5-HT_{1A}R = 5.4 and 5.0, respectively), Asn386 proves to behave as a chiral discriminator. Moreover, the observation that the replacement of the hydroxyl substituent of **2** with a methoxy group does not affect the high affinity for the wild-type receptor suggests that one or both ether oxygen atoms of (*S*)-**3** may act as hydrogen bond acceptors. (*S*)-**3** (pK_i 5-HT_{1A}R = 6.8) also shows high affinity for the Asn386Val mutant receptor because of a favorable lipophilic contact of its methoxy group with Val386.

5.2. Arylpiperazines

Arylpiperazines are one of the most important classes of 5-HT_{1A}R ligands from which a second generation of anxiolytics, including buspirone (**4**), the antipsychotics ziprasidone (**5**), perospirone (**6**), and aripiprazole (**7**), and several pharmacological tools originated (**Figure 7**) [8].

These ligands bind with high affinity to different GPCRs; the two multitarget drugs **5** and **6**, for example, acting as D_2R antagonists and 5-HT_{1A}R agonists, were marketed in 2001 and 2002, respectively, for the management of schizophrenia [4]. Compound **4** is the most known member of long-chain arylpiperazines (LCPAs) [17]. It was initially investigated as a putative antipsychotic agent devoid of the typical side effects of this class of drugs but was launched in the market as an anxiolytic in the USA in the 1980s. It behaves as a

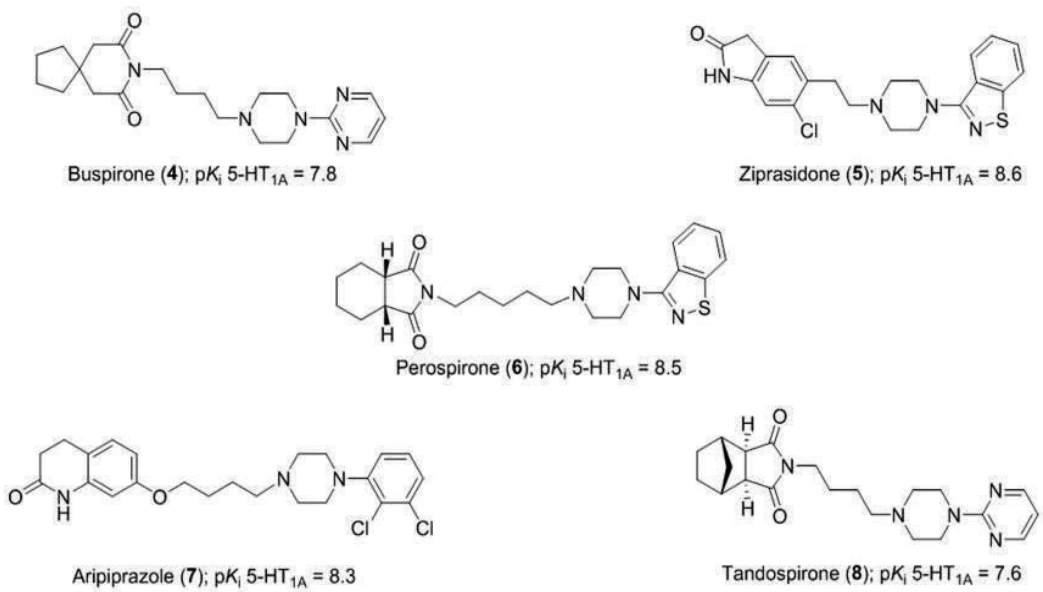

Figure 7. Chemical structures of **4**–**8**.

potent but nonselective partial 5-HT$_{1A}$R agonist and D$_2$R antagonist. Since its launch, several *N*4-(2-pyrimidinyl)piperazines containing an *N*1-imidobutyl substituent have originated as the third generation of anxiolytic agents, including the partial agonist tandospirone (**8**) (**Figure 7**).

The general structure of arylpiperazines consists of a terminal fragment containing an amide, imide, alkyl, arylalkyl, heteroarylalkyl, or tetralin function linked through a flexible aliphatic chain of variable length to the *N*1-arylpiperazine moiety [8]. The search for new derivatives has been focused on the modification of one or more portions of such a pharmacophore. Some of the main changes are schematically reported in **Figure 8**.

5.2.1. Modification of the aryl group

The replacement of the 2-pyrimidinyl moiety of **4** with a 2-methoxyphenyl group leads to the antidepressant BMY 8227 (**9**), from which BMY 7378 (**10**) originates by shortening its butyl

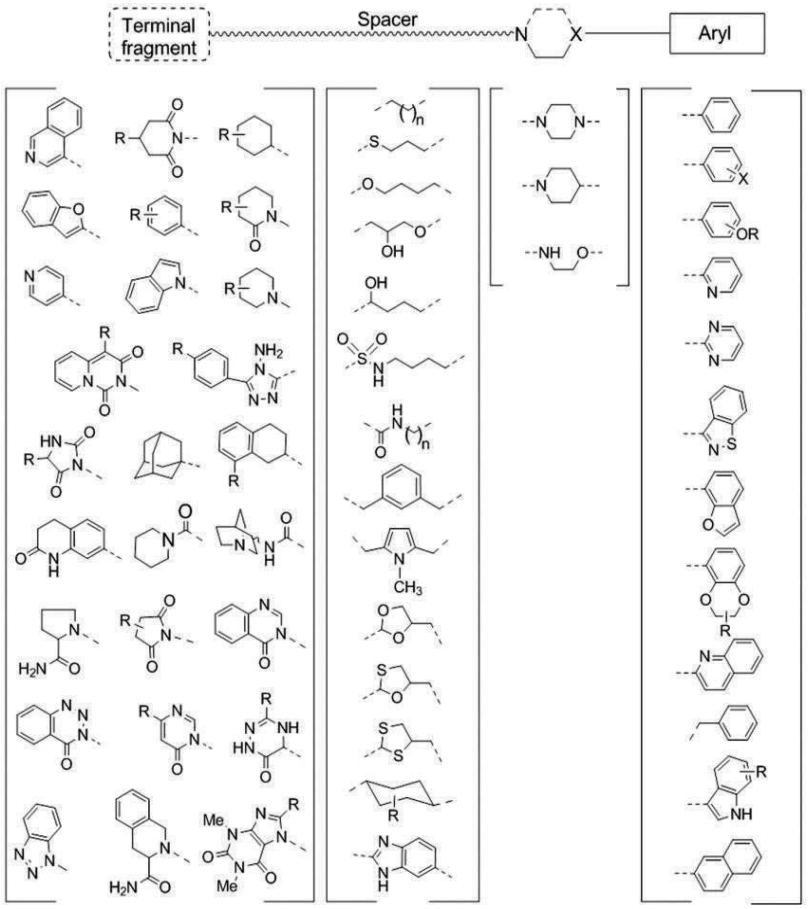

Figure 8. Pharmacophore of arylpiperazines.

to ethyl chain (**Figure 9**) [15]. Compounds **9** and **10** belong to a generation of postsynaptic 5-HT_{1A}R antagonists, which also behave as low efficacy partial agonists [4].

The 2-methoxyphenyl group is also present in the WAY series, including WAY 100135 (**11**) and WAY 100635 (**12**) (**Figure 9**). These compounds, also called "silent" 5-HT_{1A}R antagonists, behave as antagonists at both pre- and postsynaptic 5-HT_{1A}Rs. In the case of **11**, the (*S*)-enantiomer is 28-fold more potent than its (*R*)-antipode.

The incorporation of the *o*-methoxy group into an annulated benzodioxane or benzofurane ring, affording two series of heterobicyclic arylpiperazines, is consistent with the maintenance of high 5-HT_{1A}R affinity [15]. The benzodioxane fragment is present in the structure of flesinoxan (**13**) (**Figure 10**), a potent agonist at both pre- and postsynaptic 5-HT_{1A}Rs [15]. An example of benzofuran derivative showing high 5-HT_{1A}R affinity is compound **14** (**Figure 10**).

Moderate to high affinity for 5-HT_{1A}Rs and SERT and low affinity for 5-HT_{2A}R are recorded by ligands, whose four-carbon chain bears a quinoline moiety (**Figure 11**) [8].

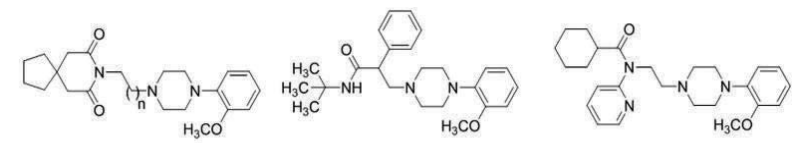

Figure 9. Chemical structures of **9**–**12**.

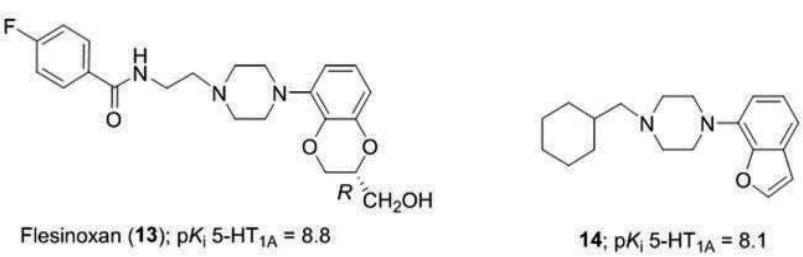

Figure 10. Chemical structures of **13**–**14**.

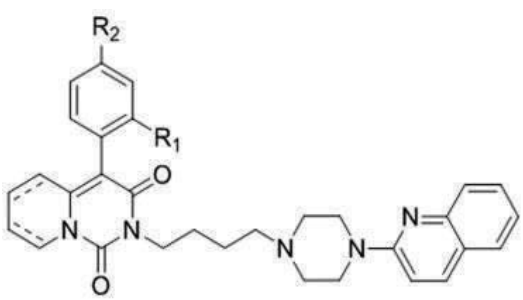

Figure 11. General structure of quinoline derivatives.

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5.2.2. Modification of the piperazine ring

*N*1-Arylpiperazine moiety plays an important role in the affinity for 5-HT$_{1A}$Rs. This template has been duplicated to successfully obtain selective homo- and heterobivalent ligands [18]. Indeed, compound **15** shows high affinity for 5-HT$_{1A}$Rs and selectivity over 5-HT$_7$Rs, whereas compound **16** selectively targets 5-HT$_7$Rs (pK_i = 7.4) (**Figure 12**).

The piperazine ring can be replaced by a piperidine one. The most representative example is befiradol (**17**), a very potent and highly selective 5-HT$_{1A}$R full agonist (**Figure 13**), that also shows efficacy in a rodent model of neuropathic, inflammatory, and surgical pain. It is endowed with potent analgesic and antiallodynic effects that are comparable to those of high doses of opioids. However, lower and fewer side effects are triggered, and little or no development of tolerance is manifested by **17**. In 2013, **17** was marketed by Neurolixis with indication for the treatment of L-DOPA-induced dyskinesia in Parkinson's disease [19]. The 3-chloro-4-fluorophenyl moiety of **17** can be bioisosterically replaced by both unsaturated and saturated lipophilic moieties [20]. Among the investigated compounds, the highly selective 5-HT$_{1A}$R superagonist benzothiophene-3-carboxamide **18** almost exclusively recognizes 5-HT$_{1A}$Rs (**Figure 13**).

A series of 2*H*-pyrido[1,2-c]pyrimidine derivatives, bearing a piperidinyl-indole residue in their pharmacophore (**Figure 13**), shows very high-affinity values for both 5-HT$_{1A}$Rs and SERTs. Compound **19** is a representative example [21]. The presence of a tetrahydropyridinyl-indole moiety reduces binding to 5-HT$_{1A}$Rs, while a Cl substituent in R$_3$ reduces binding to both 5-HT$_{1A}$Rs and SERTs.

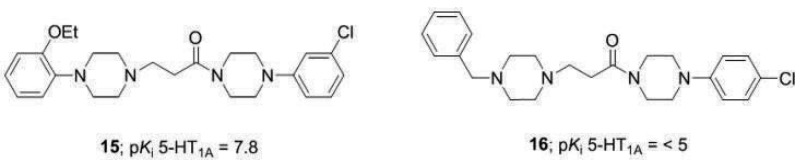


Figure 12. Chemical structures of **15** and **16**.

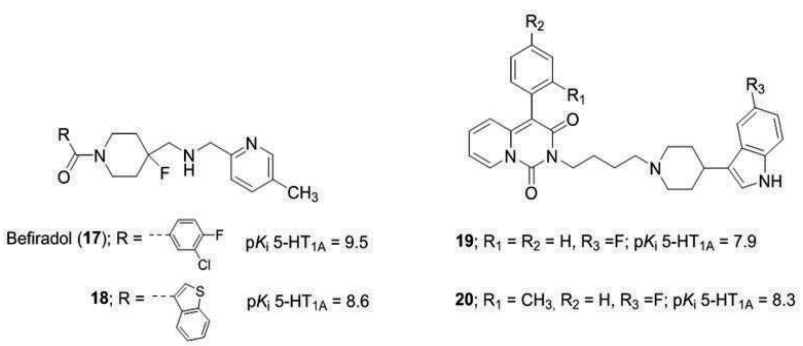


Figure 13. Chemical structures of **17–20**.

Finally, the presence of a 3β-aminotropane moiety instead of the piperazine or piperidine ring is unfavorable for the development of High affinity 5-HT_{1A}R ligands (**Figure 14**) [22].

5.2.3. Modification of the spacer

In LCPAs, the four-carbon alkyl chain seems to be the most favorable for high 5-HT_{1A}R affinity. Indeed, its shortening reduces affinity, according to the rank order of potency C-4 > C-2 > C-3 [4].

However, the butyl chain can be substituted by a propylthio bridge, as confirmed by the high 5-HT_{1A}R affinity of compound **21** (**Figure 15**). The NH_2 function is responsible for its selectivity over α_1-ARs (5-HT_{1A}R/α_1-AR = 55) [15].

The oxybutynin chain of aripiprazole (**7**) is also favorable for high 5-HT_{1A}R affinity. Besides its main use in the treatment of schizophrenia and bipolar disorder, **7** is also employed as an add-on treatment in major depressive disorder, tic disorders, and irritability associated with autism. In addition, its systemic or local administration induces antinociceptive effects. Unlike other atypical antipsychotics approved by FDA (e.g., clozapine, olanzapine, quetiapine, ziprasidone, and risperidone), which are D_2R antagonists, **7** behaves as a D_2R and D_3R partial agonist. Moreover, it shows partial agonism at 5-HT_{1A}Rs and, similarly to the other atypical antipsychotics, is an antagonist at 5-HT_{2A}Rs and 5-HT_7Rs as well as a partial agonist at 5-HT_{2C}Rs [23].

The presence of a hydroxyl group in the butyl chain is well tolerated. BMY 14802 (**22**) (**Figure 16**), for example, is a 5-HT_{1A}R agonist that also attenuates dyskinesia produced by L-DOPA.

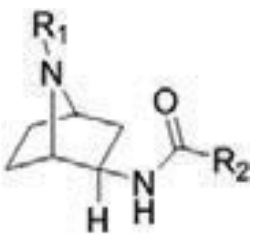
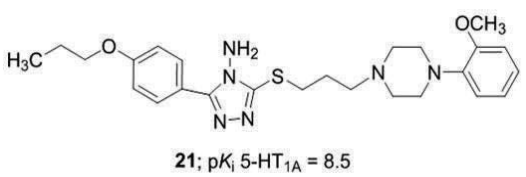


Figure 14. General structure of 3β-aminotropane derivatives.

A hydroxyalkyl chain also characterizes a series of molecules (**23**–**26**) (**Figure 17**), in which the combination of structural elements favoring the affinity for 5-HT_{1A}Rs (heterocyclic nucleus, hydroxyalkyl chain, and 4-substituted piperazine) was used to obtain ligands with high



21; pK_i 5-HT_{1A} = 8.5

Figure 15. Chemical structure of **21**.

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5-HT$_{1A}$ affinity and selectivity over other 5-HT subtypes [24]. In particular, while compounds **23–25** show an outstanding 5-HT$_{1A}$R affinity, compound **26** is selective for 5-HT$_{2C}$Rs (pK_i = 8.3).

In a series of compounds prepared to discover mixed 5-HT/dopamine receptor agents as novel antipsychotics, amide **27** (**Figure 18**) emerges for its high affinity for D$_3$Rs, 5-HT$_{1A}$Rs, and 5-HT$_{2A}$Rs. Its low affinity for D$_2$Rs, 5-HT$_{2C}$Rs, and hERG channels reduces extrapyramidal side effects, risk of obesity under chronic treatment, and incidence of torsade des pointes, respectively [25]. The replacement of the ether/amide bridge with a sulfonamide function affords a series of quinoline or isoquinoline derivatives endowed with multireceptor 5-HT$_{1A}$R/5-HT$_{2A}$R/5-HT$_7$R/D$_2$R/D$_3$R profile and behaving as 5-HT$_{1A}$R agonists, D$_2$R partial agonists, and 5-HT$_{2A}$R/5-HT$_7$R antagonists (**Figure 18**). They produce significant antidepressant activity in mice [26]. In particular, **28** also displays remarkable antipsychotic effects in MK-801-induced hyperlocomotor activity in mice.

The inclusion of the alkyl chain of LCAPs in a cyclohexyl ring leads to more conformationally constrained analogues (e.g., **29**) (**Figure 19**) [15]. Trans derivatives show 5-HT$_{1A}$R affinity significantly higher than that of their corresponding cis isomers (e.g., trans **29** and cis **29**). The insertion of a hydroxyl substituent in the cyclohexyl moiety is also well tolerated (**30**). Interestingly, compared to flexible 4-carbon alkyl chain analogues, 1e,4e-disubstituted cyclohexane derivatives maintain very high 5-HT$_{1A}$R affinity, but in some cases, the functional profile is modulated from partial agonism to antagonism [27].

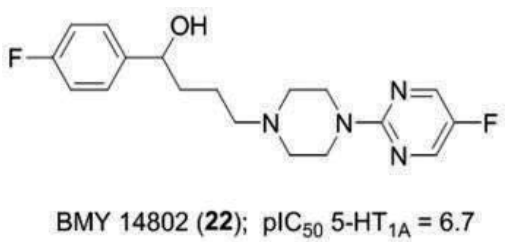


Figure 16. Chemical structure of BMY 14802 (**22**).

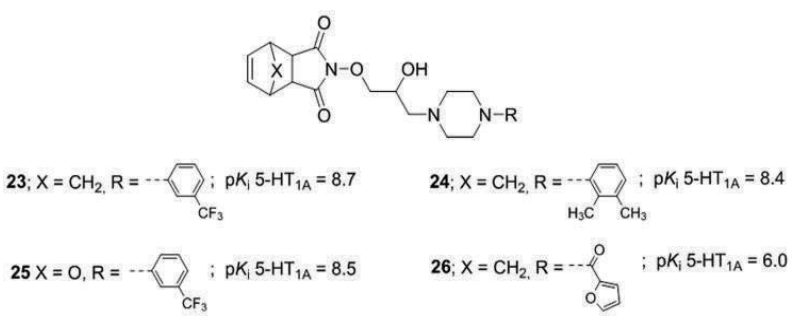


Figure 17. Chemical structures of **23–26**.

The alkyl chain can be partially included in aromatic functions, including pyrrole (RWJ 25730, **31**), phenyl (mazapertine, **32**), or benzimidazole (**33**) (**Figure 20**) [15]. The multireceptor affinity of **32** can be ascribed to its ability to adopt a variety of low-energy conformations. Indeed, constraining its 2-isopropoxyphenyl and piperazine moieties, affording compound **34**, significantly reduces affinities for α_1-ARs and D_2Rs, but not that for 5-HT_{1A}Rs.

The insertion of the 1,3-dioxolane nucleus in the chain is also well tolerated. Compound **35**, for example, is a potent partial agonist and shows moderate selectivity over α_1-ARs (**Figure 21**) [28]. Substitutions at C-8 position of the 1,4-dioxaspiro[4, 5]decane moiety reduce 5-HT_{1A}R/α_1-AR selectivity ratio because of the significant decrease of binding affinity and intrinsic activity for 5-HT_{1A}Rs with respect to α_1-ARs. The isosteric replacement of one (oxathiolane derivative **36**) and especially of two (dithiolane derivative **37**) oxygen atoms with sulfur atoms proves to be tolerated (**Figure 21**). The replacement of the piperazine ring with a more flexible basic chain affords compound **38**, which behaves as a potent and selective 5-HT_{1A}R partial agonist endowed with neuroprotective activity in vitro and potent antinociceptive activity in an in vivo model [28]. A similar profile is shown by the unsubstituted analogue **39** characterized by good 5-HT_{1A}/α_1-AR selectivity (**Figure 21**).

Similar structure-activity relationships (SARs) can be observed when the spiro-cyclohexyl terminal fragment in both piperazine and open-chain series is replaced by a 2,2-diphenyl moiety.

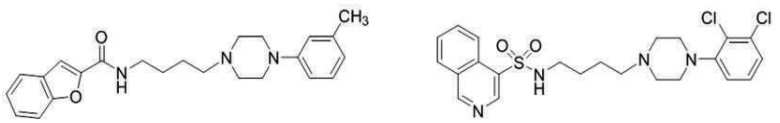


Figure 18. Chemical structures of **27** and **28**.

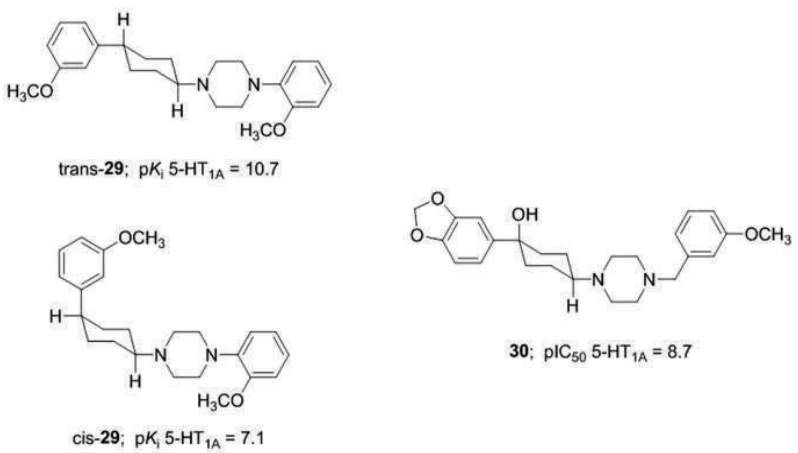


Figure 19. Chemical structures of **29** and **30**.

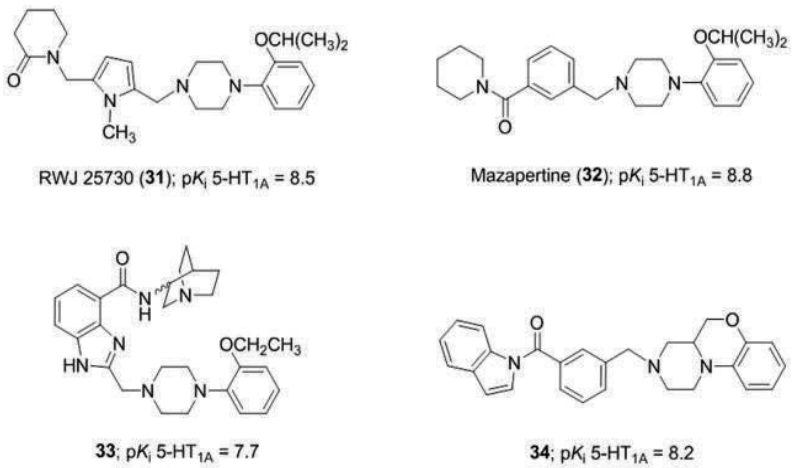


Figure 20. Chemical structures of **31–34**.

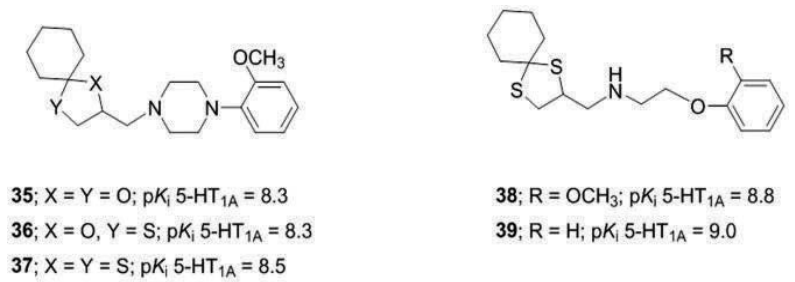


Figure 21. Chemical structures of **35–39**.

The replacement of the 1,3-dioxolane nucleus with other pentatomic rings bearing H-bond acceptor groups (tetrahydrofuran or cyclopentanone) or an H-bond acceptor and donor group (cyclopentanol) (**Figure 22**) causes an overall reduction of affinity at α_1-ARs, while both potency and efficacy are increased at 5-HT$_{1A}$Rs.

5.2.4. Modification of the terminal fragment

The numerous structurally different terminal fragments, as already seen for ligands reported above, demonstrate that this moiety is less critical for 5-HT$_{1A}$R interaction [8]. The dual SSRI and 5-HT$_{1A}$R agonist vortioxetine (**40**), approved by FDA for the treatment of major depressive disorders in adult in 2013, even lacks this function (**Figure 23**).

The replacement of the azaspirodecanedione moiety of **9** with an *N*-phthalimido group affords the nonselective ligand **41** (**Figure 24**) [15]. Shortening the length of its butyl chain to three or two units significantly decreases the affinity. The presence of an isosteric sulfonyl function instead of a carbonyl group of the phthalimide moiety, as in ipsapirone (**42**), is compatible with

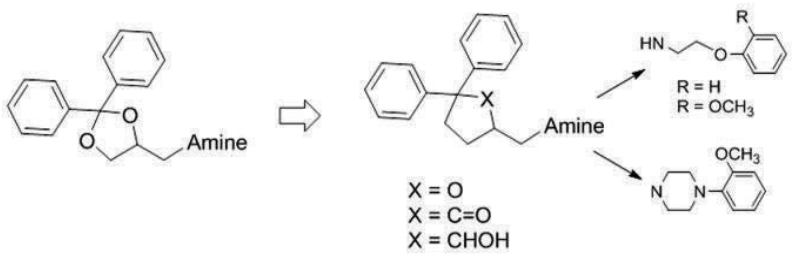
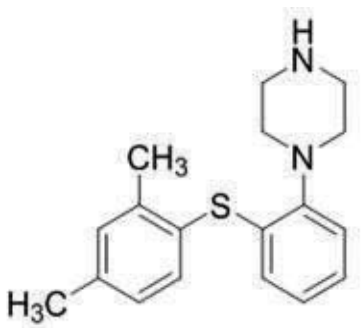


Figure 22. Bioisosteric replacement of oxygen atoms of 5-HT_{1A}R 1,3-dioxolane ligands.

the maintenance of similar 5-HT_{1A}R affinity and improved selectivity over α_1-ARs (**Figure 24**) [15]. The replacement of the phthalimide moiety of **41** with an adamantyl amide group, leading to **43**, also increases the selectivity for 5-HT_{1A}Rs over α_1-ARs (**Figure 24**) [15]. As in the case of the prototypical 5-HT_{1A}R antagonist **12**, substituents can be present at amidic NH [15]. The replacement of the pyridine ring of **12** with a pyrimidine substituent leads to the similarly potent 5-HT_{1A}R antagonist **44**. The isosteric inversion of the amide function and the presence of a phenyl group in the bridge, affording **45**, are tolerated (**Figure 24**). Considering both affinity and selectivity for 5-HT_{1A}Rs, among some 5-methoxy-1,2,3,4-tetrahydronaphthalen-1-yl derivatives obtained by inserting an alkyl chain of variable length (preferably a three-membered alkyl chain) in the α, β, or ω position, the best derivatives are **46** and **47** (**Figure 24**) [15].

Several molecules, bearing an isonicotinic moiety as the terminal fragment of LCAPs, show nanomolar and subnanomolar affinities for 5-HT_{1A}Rs, 5-HT_{2A}Rs, and 5-HT_{2C}Rs and moderate or no affinity for other relevant receptors (D_1Rs, D_2Rs, α_1- and α_2-ARs) [29]. In particular, derivative **48**, bearing a propyl chain as a spacer, shows the highest affinity for 5-HT_{1A}Rs and selectivity over dopaminergic, adrenergic, and other serotoninergic receptors (**Figure 25**). LCAPs bearing a 1,2,3,4-tetrahydroisoquinoline-3-carboxamide in the terminal fragment can show affinity for 5-HT_{1A}Rs and/or 5-HT_7Rs [30]. Indeed, while compounds **49** and **50**, with a methylthio substituent in the ortho-position show high



Vortioxetine (**40**); pK_i 5-HT_{1A} = 6.6

Figure 23. Chemical structure of vortioxetine (**40**).

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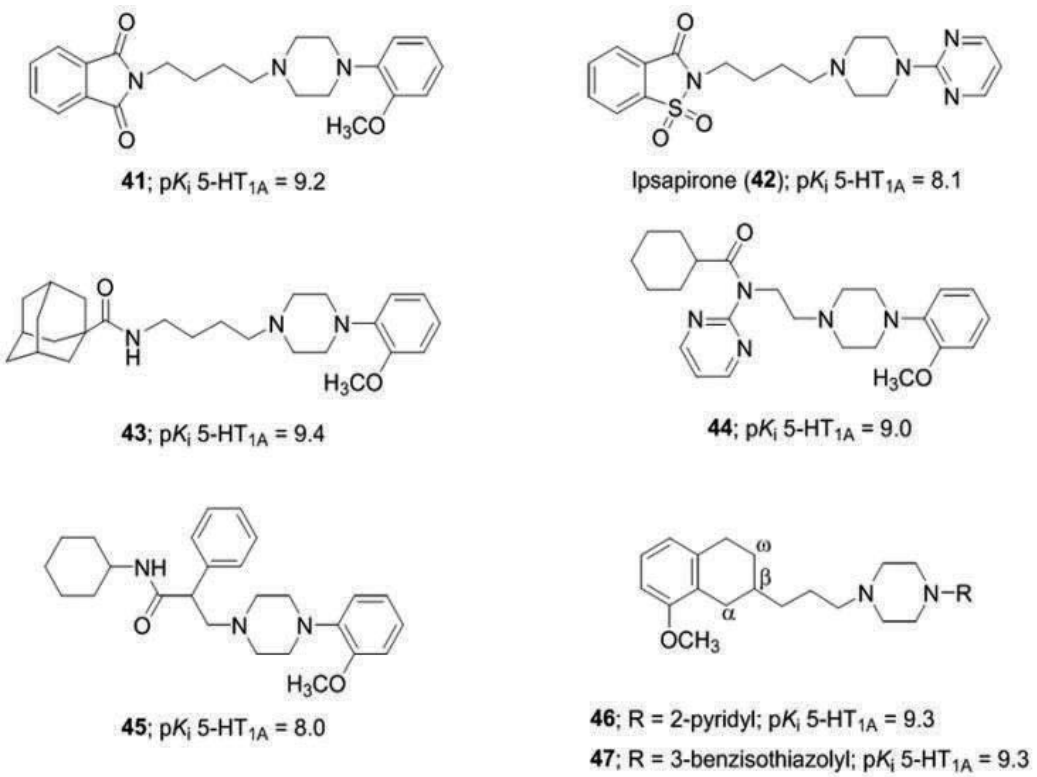

Figure 24. Chemical structures of **41**–**47**.

5-HT$_{1A}$R affinity, the replacement of the phenyl ring in the arylpiperazine moiety with a benzisoxazole system, affording, for example, **51** and **52**, significantly increases the affinity for 5-HT$_7$R (pK_i = 7.7 and 7.6, respectively) (**Figure 25**). The insertion of a spiro-cyclopentane or cyclohexane in position 3 of pyrrolidin-2,5-dione leads to a series of arylpiperazines, among which derivatives **53** and **54** with an ethylene spacer and a CF_3 substituent in meta position of the phenyl ring show both anticonvulsant activity and high 5-HT$_{1A}$R and 5-HT$_{2A}$R affinity (**Figure 25**) [31].

A β-tetralonohydantoin as terminal fragment characterizes a series of compounds, which show high 5-HT$_{1A}$R affinity (pK_i = 7.3–8.2) combined with moderate to high 5-HT$_{2A}$R affinity (pK_i = 6.7–7.3). Among them, compound **55** (**Figure 26**) is a postsynaptic 5-HT$_{1A}$R antagonist and produces the characteristic effect of presynaptic 5-HT$_{1A}$R agonists [32]. Moreover, it behaves as a 5-HT$_{2A}$R antagonist. Due to its interesting 5-HT$_{1A}$/5-HT$_{2A}$ functional profile, **55**, tested for its potential psychotropic activity, shows diazepam-like anxiolytic activity and behaves as a weak antidepressant.

Among new LNCPs with structural modifications in the terminal fragment, in the alkyl chain length and in the substituents of the piperazine fragment, the 2-ethoxy quinazolinone derivatives **56** and **57** are the most interesting ligands, showing high affinity for 5-HT$_{1A}$Rs and 5-HT$_7$Rs (**Figure 26**) [33].

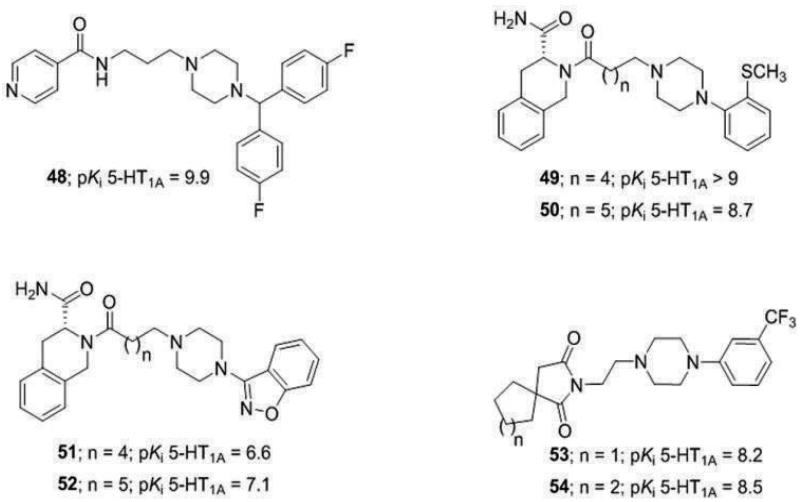

Figure 25. Chemical structures of **48**–**54**.

In a more recent work, the quinazolinone system has been replaced by 6-phenyl-4(3*H*)-pyrimidinone as a result of splitting bicyclic quinazolinone system [34]. The benzo-cracking strategy (compounds **58**–**62**) causes a decrease in affinity for both receptors. In functional assays, these derivatives behave as weak 5-HT_{1A}R and 5-HT_7R antagonists (**Figure 26**).

1,2,4-Triazine-6(1*H*)-one derivatives also display dual affinity for 5-HT_{1A}Rs and 5-HT_7Rs. SAR studies have revealed that receptor affinity and selectivity depend on the nature of the substituent in position 3 of the triazinone fragment as well as on the substitution pattern of the phenylpiperazine moiety [35]. The best 5-HT_{1A}R affinity values and selectivity over 5-HT_7Rs are displayed by compounds **63** and **64** (**Figure 26**).

The 3,5-dioxo-(2*H*,4*H*)-1,2,4-triazine-tethered arylpiperazines have been identified as agonists with high affinity for 5-HT_{1A}Rs. Several members of this series such as **65** show nanomolar affinity for 5-HT_{1A}Rs, high selectivity over α_1-AR, and potent agonist activity (**Figure 26**) [36]. The 1,2,3-benzotriazin-4-one terminal fragment characterizes some 5-HT_{1A}R antagonists prepared as potential antiproliferative agents in cancer cell lines [37]. These compounds are endowed with high 5-HT_{1A}R affinity and moderate or no affinity for other receptors (5-HT_{2A}Rs, 5-HT_{2C}Rs, D_1Rs, D_2Rs, α_1- and α_2-ARs). In particular, derivative **66** shows picomolar affinity for 5-HT_{1A}Rs (**Figure 26**).

MP 3022 (**67**), the lead compound of a large series of 4-alkyl-1-(*o*-methoxyphenyl)-piperazines containing a benzotriazole terminal fragment, behaves as a potent pre- and postsynaptic 5-HT_{1A}R antagonist, but it is not selective for 5-HT_{1A}Rs over α_1-ARs (**Figure 26**) [15]. 4-Benzoyl-1,2,3-triazole derivatives (e.g., **68**), open-chain analogues of their benzotriazole bioisosteres, bind to 5-HT_{1A}Rs in a nanomolar range and are highly selective over 5-HT_{2A}Rs and 5-HT_{2C}Rs (**Figure 26**) [15].

Purine 2,6-dione core has also been used as a terminal fragment to combine the 5-HT_{1A}R activity with the phosphodiesterase (PDE) inhibition [38]. Both effects might be advantageous in

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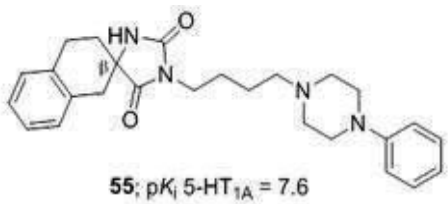
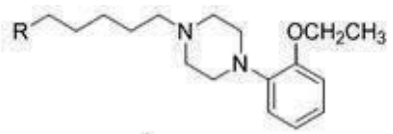
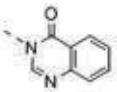
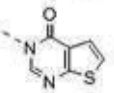
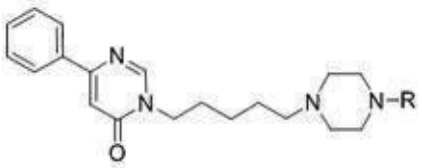


55; pK_i 5-HT$_{1A}$ = 7.6

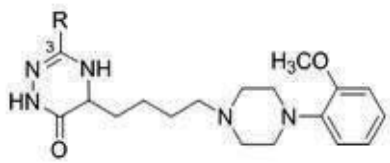


56; R =  ; pK_i 5-HT$_{1A}$ = 9.0

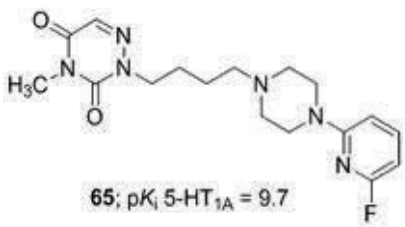
57; R =  ; pK_i 5-HT$_{1A}$ = 9.0



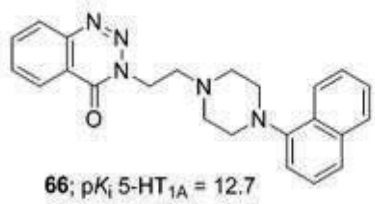
58; R = C_6H_5; pK_i 5-HT$_{1A}$ = 7.0
59; R = $CH_2C_6H_5$; pK_i 5-HT$_{1A}$ = 6.1
60; R = 3-Cl$CH_2C_6H_4$; pK_i 5-HT$_{1A}$ = 7.7
61; R = 4-Cl$CH_2C_6H_4$; pK_i 5-HT$_{1A}$ = 6.8
62; R = 2-$CH_3CH_2OC_6H_4$; pK_i 5-HT$_{1A}$ = 7.6



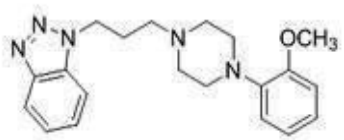
63; R = C_6H_{11}; pK_i 5-HT$_{1A}$ = 8.3
64; R = C_6H_5; pK_i 5-HT$_{1A}$ = 8.0



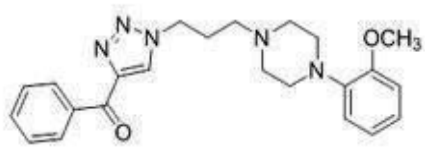
65; pK_i 5-HT$_{1A}$ = 9.7



66; pK_i 5-HT$_{1A}$ = 12.7



MP 3022 (**67**); pK_i 5-HT$_{1A}$ = 9.7



68; pIC_{50} 5-HT$_{1A}$ = 8.6

Figure 26. Chemical structures of **55–68**.

the treatment of neuropsychiatric disorders. Among the compounds bearing this core, **69–72** show high affinity for 5-HT$_{1A}$Rs and, in the case of **69** and **70**, also for 5-HT$_7$R. At the same time, compounds **69–72** show a moderate to very low D_2R affinity. From functional assays, **69–71** behave as 5-HT$_{1A}$R antagonists, whereas **72** is an agonist (**Figure 27**) [38, 39]. The antidepressant activity of **69** and **70** at a dose of 1.25 mg/kg is similar to that of citalopram given at the same dose [38]. The annulation of the purine system at 7,8-positions with an imidazole moiety affords ligands with a wide spectrum of activities (high 5-HT$_{1A}$R or 5-HT$_7$R affinity,

mixed 5-HT_{1A}R/5-HT_7R affinity, and additional affinity for D_2R) [40]. The tested compounds are in the ranges defined by the "rule of five" (logP < 5), which indicates good intestinal permeability and metabolic stability. In preliminary pharmacological in vivo studies, the selected compound **73** behaves as a potential antidepressant in mice and, at the dose of 2.5 mg/kg, shows anxiolytic effect (**Figure 27**). Finally, purine 2,4,8-trione derivatives show affinity values lower than those of the corresponding purine 2,4-dione analogues (**Figure 27**) [41].

5.2.5. Main interactions of arylpiperazines with 5-HT_{1A}Rs

Two main interactions prove to be important for the affinity of arylpiperazines for 5-HT_{1A}Rs: (a) an ionic bond between the protonated nitrogen atom of the piperazine ring and the carboxyl oxygen of the side chain of Asp3.32 and (b) an edge-to-face CH-π interaction between the aromatic ring and the Phe6.52 residue, which stabilizes the ligand binding. The basic pharmacophore of the 5-HT_{1A}R is the same for agonists and antagonists and consists of an aromatic nucleus and a basic nitrogen atom, whose optimal distance is 5.2 Å, while the nitrogen lies at 0.2 Å above the plane defined by the reference ring (**Figure 28**) [4].

Due to the highly flexible linker (usually 2-4 methylene units), using different experimental and modeling techniques, various attempts have been conducted to determine the bioactive conformation of LCAPs [42]. Assuming that active conformations of LCAPs are closely related to those in solutions or in solid state, two-dimensional (2D) NMR and crystallographic methods were often applied. The 2D NMR studies indicated that compounds with tetramethylene spacer can adopt extended, bent, or folded conformations. On the other hand, analysis of Cambridge Structural Database showed that linear geometries predominated. Molecular

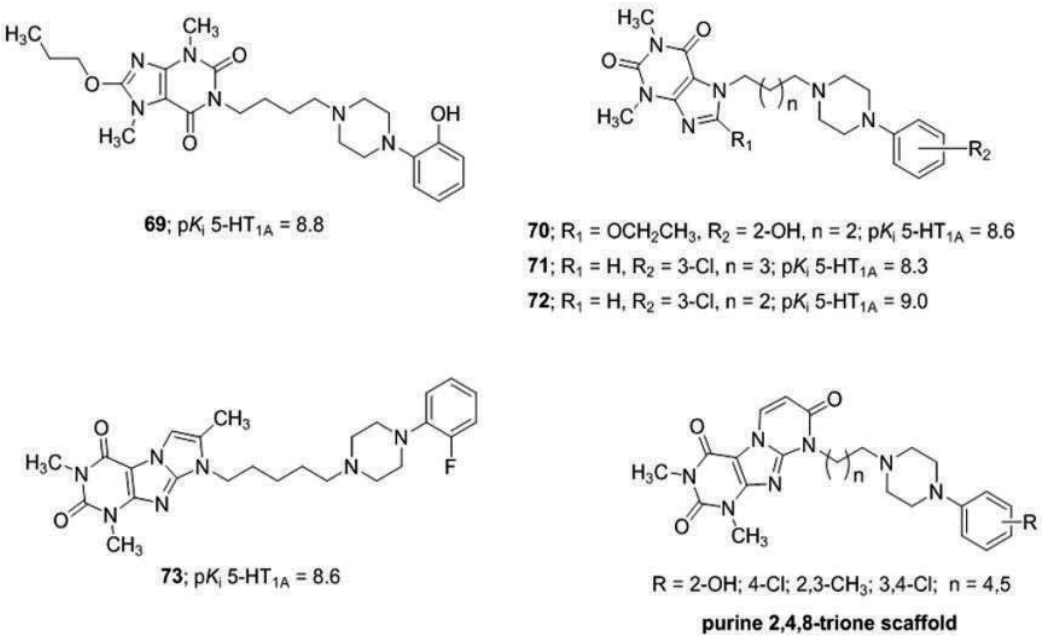


Figure 27. Chemical structures of **69–73** and general structure of purine 2,4,8-trione derivatives.

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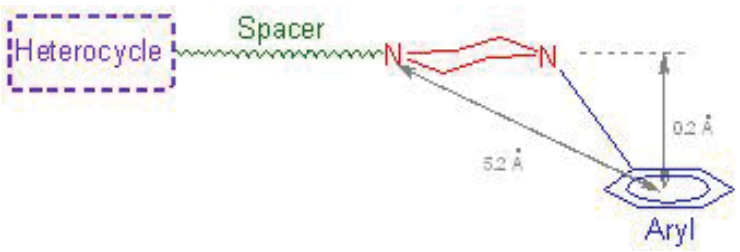


Figure 28. General structure of LCAPs and pharmacophoric model of 5-HT$_{1A}$R (Adapted with permission from Ref. [4]. Copyright (2014) American Chemical Society).

modeling studies (conformational analysis, docking, dynamics), provided with structural investigations or conducted separately, also gave equivocal results suggesting the possibility of different bioactive conformations of LCAPs.

5.3. Aminotetralins

For a long time, 2-aminotetralin structure has been known to be pharmacologically important. Initially, aminotetralins were characterized by their sympathomimetic action, i.e., the induction of mydriasis, contraction of the uterus, changes in blood pressure, and respiration, as well as increased intestinal motility in in vivo experiments. During the late 1960s, the discovery of their activity at central dopamine receptor led to active synthesis programs all over the world. The 2-aminotetralin structure has proven to be a valuable scaffold not only for the development of 5-HTR ligands, but it also characterizes dopamine and adrenergic receptor ligands, as well as compounds interacting with melatonin receptors [15]. The main SARs of aminotetralins are summarized in **Figure 29**.

The position of the hydroxyl group in the aromatic ring of the tetralin scaffold is crucial to address ligands toward 5-HT or dopamine receptors. Indeed, 8-hydroxy-2-(*N*,*N*-di-*n*-propylamino)tetralin (8-OH-DPAT, **74**) (**Figure 30**) is a very potent and selective 5-HT receptor ligand, while its 5- and 7-hydroxy regioisomers (5- and 7-OH-DPAT) are potent dopamine receptor ligands. [^{3}H]8-OH-DPAT is frequently used to label 5-HT$_{1A}$Rs. Both its enantiomers show high affinity for 5-HT$_{1A}$Rs. However, in functional experiments, the (*R*) enantiomer behaves as a full agonist while its antipode as a partial agonist.

Compounds obtained by replacing the 8-hydroxy substituent with 8-methoxy (8-MeO-DPAT, **75**), 8-acetyl (**76**), and 8-methoxycarbonyl (**77**) or 8-carboxamide (**78**) groups are about as potent as the parent compound, indicating that the proton of the 8-hydroxy group is not essential for drug-receptor interaction (**Figure 30**). A carboxylic group in the same position (**79**) is not favorable. Aryl and heteroaryl groups, such as phenyl, fluorophenyl, methoxyphenyl, acetylphenyl, 2-furyl, and benzylthio, are well tolerated. For most derivatives, the (*R*)-enantiomers are more potent than the (*S*)-enantiomers. The introduction of a fluorine atom at position C-5 of **74**, affording **80**, slightly decreases 5-HT$_{1A}$R affinity. In functional studies, the (*R*)-enantiomer behaves as a partial agonist, while the (*S*)-enantiomer is a pure antagonist at both pre- and postsynaptic receptors. An antagonist is also obtained by introducing a methyl group in 5-position of **74** (compound **81**) (**Figure 30**). The replacement of the *N*,*N*-di-*n*-propyl groups of **74** or **75** with smaller or larger di-*n*-alkyl substituents results in a significant

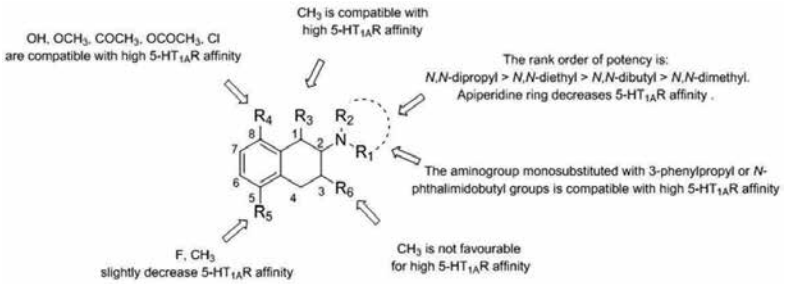


Figure 29. Main SARs of aminotetralins.

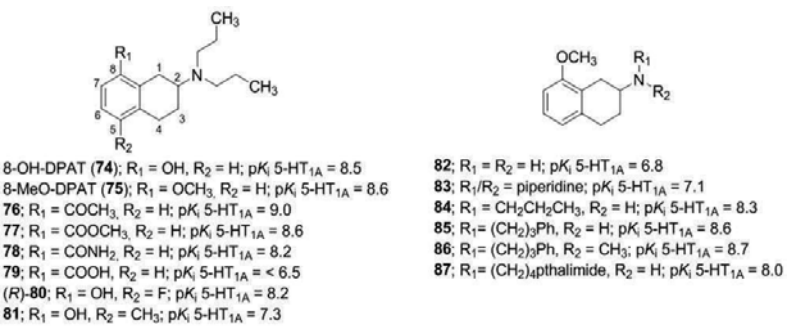


Figure 30. Chemical structures of **74**–**87**.

decrease in affinity. The rank order of potency is *N*,*N*-dipropyl > *N*,*N*-diethyl > *N*,*N*-dibutyl > *N*,*N*-dimethyl group.

Compared to the *N*,*N*-dialkylated 8-MeO-DPAT (**75**), the monoalkylated N-propyl derivative **84** shows slightly lower affinity, whereas the non-substituted 8-methoxy-2-aminotetralin (**82**) is almost inactive (**Figure 30**). The piperidine analogue **83** (**Figure 30**) is 16–29-fold less active than the *N*-mono (**84**) or *N*,*N*-dipropyl derivative (**75**). Compounds with high-affinity values are obtained if the amino group is monosubstituted with relatively large substituents as a phenylalkyl moiety, with the 3-phenylpropyl-8-methoxy group being optimal (**85**). Even an extra *N*-methyl group (**86**) or bulky substituents such as an *N*-(phthalimidobutyl) group are also well tolerated (**87**).

The incorporation of the nitrogen atom in the tetralin nucleus furnishes the series of 1,2,3,4-tetrahydroisoquinoline (THIQ) derivatives, which bind to 5-HT_{1A}Rs and 5-HT_{2A}Rs. SAR studies performed on the THIQ class lead to the synthesis of 1-adamantoyloaminoalkyl derivatives endowed with high affinity for 5-HT_{1A}Rs (pK_i = 7.3–8.3) and behaving as postsynaptic 5-HT_{1A}R partial agonists (**Figure 31**).

Ring contraction (indamines) or ring expansion (benzocycloheptamines) of the cycloexyl ring of 2-aminotetralins decreases 5-HT_{1A}R affinity. The replacement of the tetralin scaffold with the chroman nucleus does not influence affinity and selectivity.

Among the four enantiomers obtained by the introduction of a methyl group in position 1 of **75**, only (*S*,*R*)-**88** displays high affinity for 5-HT$_{1A}$Rs (**Figure 32**). In functional tests, it behaves as a mixed partial 5-HT$_{1A}$R agonist/D$_2$R antagonist.

The restriction of the conformation of **88** by the incorporation of the C-1 methyl and the C-2 nitrogen into an azetidine (**89**) or pyrrolidine (**90**) ring significantly enhances 5-HT$_{1A}$R affinity (**Figure 32**). These more rigid four/six and five/six fused angular tricyclic 2-aminotetralins are *N*-substituted with either *n*-propyl or its bioequivalent 2-propenyl group. The cis racemates of both series are more potent than cis-**88**. The hydroxy derivatives display selective 5-HT$_{1A}$R agonist activity, whereas the methoxy analogues show mixed 5-HT$_{1A}$R agonist and dopamine antagonist activities. In general, the cis analogues are more potent than the corresponding trans analogues, and in the cis series, the (*S*,*R*)-enantiomers display higher potency (**Figure 32**). Nitrogen substitution with either an *n*-propyl or an allyl group leads to ligands with similar activities, whereas their replacement with a bulky *α*-methylbenzyl group produces a decrease in activity. The incorporation of the C-1 methyl and the C-2 nitrogen into a more flexible six-membered piperidine ring (**91**) is less favorable for 5-HT$_{1A}$R affinity. In contrast to the pyrrolidine series, in these six/six fused angular tricyclic 2-aminotetralins, the trans enantiomers are more potent than the cis antipodes (**Figure 32**).

The introduction of a methyl group in position 3 of **75** is not favorable for high 5-HT$_{1A}$R affinity. Consequently, the incorporation of the C-2 nitrogen and C-3 methyl into a five-membered

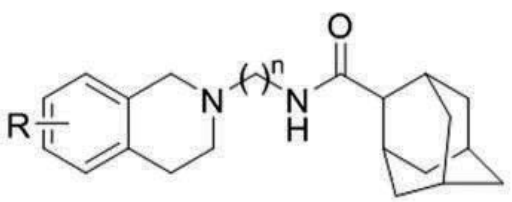


Figure 31. General structure of THIQ derivatives.

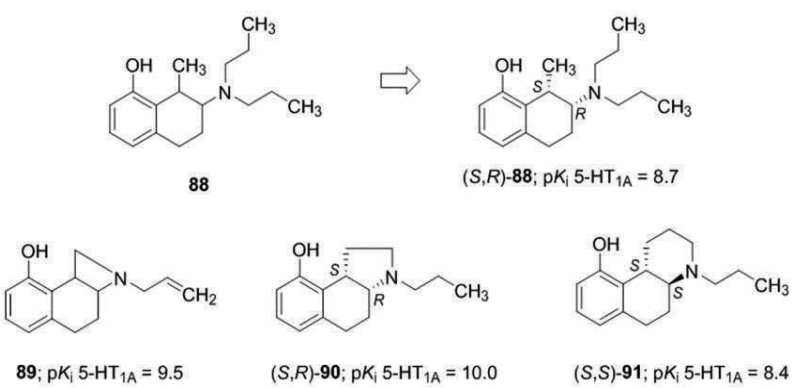


Figure 32. Chemical structures of **88**–**91**.

pyrrolidine ring also leads to five/six fused linear tricyclic 2-aminotetralins, which are only moderately active.

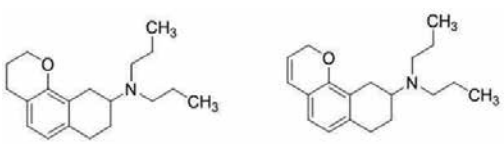
A different six/six fused angular tricyclic of 2-aminotetralin is obtained by incorporating the 8-oxygen atom and C-7 into a six-membered ring, obtaining **92** and **93**, respectively. However, these modifications reduce affinity. The (*R*) configuration is more favorable than the (*S*) one (**Figure 33**).

A further decrease in affinity is shown by compounds bearing an annulated pyrrole ring in which the NH moiety is in the same position as the hydroxy group of **74**. On the contrary, the annulation in which the indole NH is in C-7 of the tetralin nucleus affords potent 5-HT_{1A}R ligands (**94**) (**Figure 34**).

The introduction of a formyl group at C-1 of **94**, affording **95** (**Figure 34**), modulates the pharmacological profile from a mixed D_2/5-HT_{1A}R agonist to a selective 5-HT_{1A}R agonist. The enantiomers of **95** are full agonists with affinities comparable to that of **74**. Both affinity and selectivity for 5-HT_{1A}Rs are improved by the substitution at C-1 of the pyrrole ring with a cyano group. In fact, the enantiomers of the 1-cyano derivative **96** are almost equipotent to the corresponding formyl derivative **95**, while 1-chloro (**97**) and 1-(1,1,1-trifluoroethyl) (**98**) substituents lead to less potent derivatives. The substitution at the C-2 of the pyrrole with a carboxamide (**99**) or cyano function (**100**) is also well tolerated, compound **100** being a potent 5-HT_{1A}R agonist. In the C-1 and C-2 substituted series, the (*R*)-enantiomers display high and moderate affinity for 5-HT_{1A}Rs and D_2Rs, respectively. The (*S*)-enantiomers are somewhat less potent but even more selective 5-HT_{1A}R ligands. An unsubstituted indole-NH moiety is crucial for the interaction with 5-HT_{1A}Rs. Indeed, the *N*-methyl compounds are significantly less potent. Without loss in 5-HT_{1A}R affinity, one of the propyl groups can be replaced by a variety of large substituents such as the glutarimide-butyl one (**101–103**) (**Figure 34**). In functional tests, most of the (*R*)-enantiomers behave as full agonists, whereas the corresponding (*S*)-enantiomers are partial agonists.

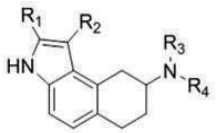
5.4. Indolylalkylamines

The prototype of this class of compounds is the endogenous ligand 5-HT (**Figure 1**), which behaves as a potent 5-HT_{1A}R agonist (pK_i = 8.4). The alkylation at α or β positions of tryptamine moiety, as well as the incorporation of its alkylamine side chain into a 4-substituted tetrahydropyridine ring, strongly decreases 5-HT_{1A}R affinity [15]. The removal of the hydroxyl group at position C-5 also reduces 5-HT_{1A}R affinity, the unsubstituted tryptamine analogue



(*R*)-**92**; pK_i 5-HT_{1A} = 7.7 (*R*)-**93**; pK_i 5-HT_{1A} = 7.6

Figure 33. Chemical structures of **92** and **93**.



94; $R_1 = R_2 = H$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 7.9
95; $R_1 = H$, $R_2 = CHO$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 8.9
96; $R_1 = H$, $R_2 = CN$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 8.7
97; $R_1 = H$, $R_2 = Cl$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 7.9
98; $R_1 = H$, $R_2 = CH_2CF_3$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 6.7
(*R*)-**99**; $R_1 = CONH_2$, $R_2 = H$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 8.8
(*R*)-**100**; $R_1 = CN$, $R_2 = H$, $R_3 = R_4 = CH_2CH_2CH_3$; pK_i 5-HT_{1A} = 10.0
(*R*)-**101**; $R_1 = CN$, $R_2 = H$, $R_3 = CH_2CH_2CH_3$, $R_4 = (CH_2)_2$-glutarimide; pK_i 5-HT_{1A} = 9.2
(*R*)-**102**; $R_1 = CONH_2$, $R_2 = H$, $R_3 = CH_2CH_2CH_3$, $R_4 = (CH_2)_2$-glutarimide; pK_i 5-HT_{1A} = 9.7
(*R*)-**103**; $R_1 = H$, $R_2 = CHO$, $R_3 = CH_2CH_2CH_3$, $R_4 = (CH_2)_2$-glutarimide; pK_i 5-HT_{1A} = 9.2

Figure 34. Chemical structures of **94–103**.

being 30-fold less potent than 5-HT. However, the 5-hydroxyl group can be replaced by a 5-methoxy or 5-carboxamide function, leading to 5-MeOT (**104**) and 5-CT (**105**), respectively, which show high 5-HT_{1A}R affinities (**Figure 35**).

The 4-substituted tetrahydropyridine analogue of **104** (RU 24969, **106**) and the *N*,*N*-di-*n*-propyl analogue of **105** (DP-5-CT, **107**) also show high 5-HT_{1A}R affinities and behave as potent and selective 5-HT_{1A}R agonists (**Figure 35**). The incorporation of the side chain of **105** into a 3-substituted tetrahydropyridine, affording **108**, slightly decreases 5-HT_{1A}R affinity, which is further reduced by the removal of the 5-carboxyamido function or its replacement with substituents such as a methoxy or cyano group. Linking the indolyl moiety to an *N*-substituted piperazine ring through a proper alkyl spacer (LCAPs) also proves to be compatible with high 5-HT_{1A}R affinity and selectivity [43]. In particular, hydroxy, methoxy, or carboxamide groups in position 5 of the indole moiety yield ligands with high 5-HT_{1A}R affinity. Such ligands tolerate several substituents in the piperazine ring. Though the optimal linker to connect the indolyl moiety to the *N*-substituted piperazine is the *n*-butyl chain, an *n*-propyl spacer is also suitable, as demonstrated by the good 5-HT_{1A}R affinity showed by compounds **109** and **110** (**Figure 36**) [44].

A compound with an *n*-butyl chain is the potent and selective 5-HT_{1A}R ligand **111** (**Figure 36**). Within this series of derivatives, the introduction of a residue in the para position of the phenyl ring reduces dopaminergic activity and, consequently, improves 5-HT_{1A}R selectivity [45].

The indolylalkylamine moiety is also present in multitarget compounds simultaneously acting as SSRIs and 5-HT_{1A}R antagonists and potentially useful for the treatment of depression. Among these, the benzoxazine derivative **112** shows high affinity for both 5-HT_{1A}Rs and SERTs (pK_i SERT = 8.5), but no selectivity over α_1-ARs. It behaves as a 5-HT_{1A}R partial agonist [46]. On the contrary, the aryloxyalkylamine derivative **113** (pK_i SERT = 9.3) behaves as a full 5-HT_{1A}R antagonist (**Figure 37**) [47].

The hybridation between the chromane-based structure, present in 5-HT_{1A}R antagonists, and the 3-indolyl-alkylamine moiety, embedded in numerous SSRIs, leads to compounds with mixed profiles. 5-Carboxamide-8-fluoro derivatives as well as 5-carboxamide-8-des-fluoro analogues with proper *N*-alkyl chains display good affinities for both 5-HT_{1A}Rs and 5-HT reuptake site [48]. In particular, **114** (**Figure 37**) behaves as a very potent 5-HT_{1A}R antagonist and SSRI. The constrained amide conformation inherent in the lactam group results in less potent 5-HT_{1A}R antagonist activity [49]. Another LCAP, obtained by combining 3-indolyl-alkylamine and arylpiperazine through a butyl chain (vilazodone, **115**), proves to be suitable for the interaction with both SERTs and 5-HT_{1A}Rs. Indeed **115**, showing subnanomolar 5-HT reuptake inhibitor activity and subnanomolar 5-HT_{1A}R affinity, behaves as a 5-HT_{1A}R agonist high selective over other GPCRs [43]. 5-Substituted *bis*-3-propylindole derivatives connected to *N*1 and *N*4 atoms of the piperazine ring also bind both SERTs and 5-HT_{1A}Rs, as suggested by compounds **116** and **117** (**Figure 37**), which show good affinities for both targets [50].

5.5. Ergolines

The tetracyclic ergoline skeleton is a common structural element contained in all ergot alkaloids. Such compounds are used in the treatment of several pathophysiological conditions, because of their wide spectrum of central and peripheral pharmacological activities. They can be considered as rigid analogues of both indolylalkylamines and catecholamines. Therefore, it is not surprising that they are able to nonselectively bind to adrenergic, dopaminergic, and serotoninergic receptors. Potent and selective 5-HT_{1A}R ligands have been developed by combining the structural elements of the indolylethylamines and the 2-aminotetralins into a

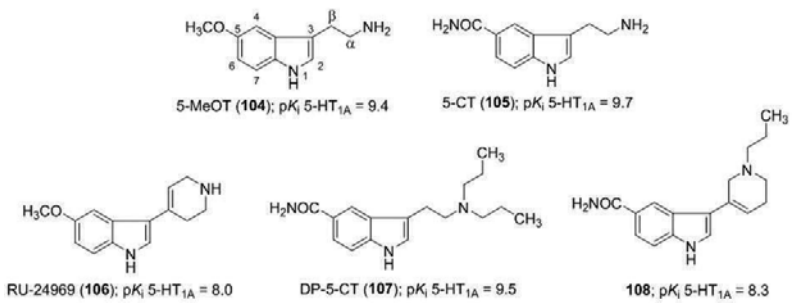


Figure 35. Chemical structures of **104–108**.

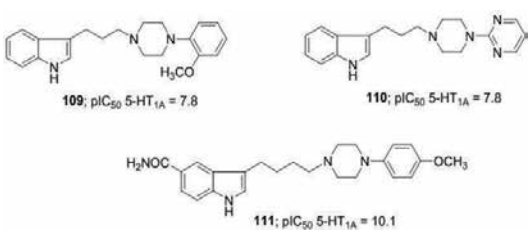


Figure 36. Chemical structures of **109–111**.

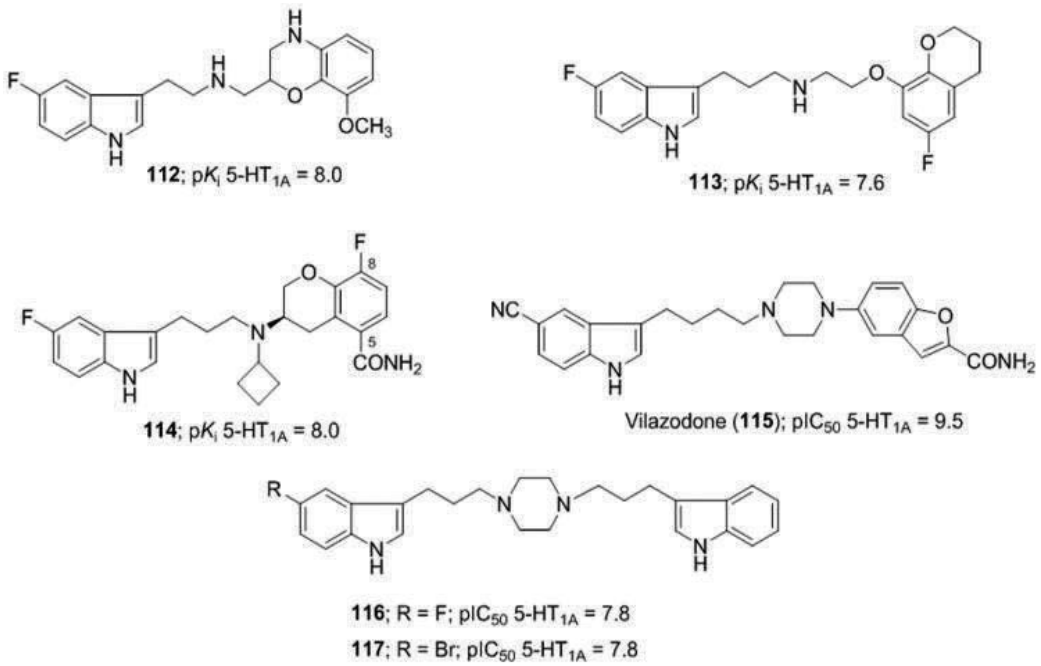


Figure 37. Chemical structures of **112–117**.

partial ergoline skeleton [15]. Among the compounds belonging to this series, LY228729 (**118**; **Figure 38**) displays the highest affinity for 5-HT$_{1A}$Rs and good selectivity over a lot of other monoaminergic receptors. In functional assays, **118** behaves as a both pre- and postsynaptic 5-HT$_{1A}$R agonists.

Though several tetracyclic ergolines, such as LSD (**119**), lisuride (**120**), or pergolide (**121**), show high affinities for 5-HT$_{1A}$Rs, they lack of selectivity over the other monoaminergic receptors. The improvement of the selectivity for 5-HT$_{1A}$Rs over 5-HT$_2$Rs as well as D$_1$Rs, D$_2$Rs, and α-ARs can be obtained by introducing the bulky and metabolically stable *tert*-butyl group in the phenyl ring at C-13 of the ergoline skeleton. Some derivatives (**122–124**; **Figure 38**), bearing a heteroaryl substituent at C-9, display nM affinity for 5-HT$_{1A}$Rs and at least 100-fold selectivity over the other tested receptors. In contrast, the presence of a *tert*-butyl group at C-14 favors the selectivity for 5-HT$_2$R.

Among the 5(10→9)*abeo*-ergoline derivatives, compound **125** displays good 5-HT$_{1A}$R affinity and selectivity over 5-HT$_2$Rs, D$_1$Rs, D$_2$Rs, and α-ARs. In this class of compounds, 5-HT$_{1A}$R affinity is enhanced by the conversion of the 8β-hydroxymethyl group into a methyl group. Indeed, the transformation of **125** into the deoxy derivative **126** leads to appreciable increase of 5-HT$_{1A}$R affinity. An improvement of 5-HT$_{1A}$R selectivity can be obtained by the reduction of the 2,3-double bond of **126**, leading to the indolines **127** and **128** (**Figure 39**).

The stereochemistry at C-3 is very important for the 5-HT$_{1A}$R profile. In particular, compound **128** displays an outstanding selectivity for 5-HT$_{1A}$Rs over 5-HT$_2$Rs, D$_1$Rs, D$_2$Rs, and α_1- and α_2-ARs.

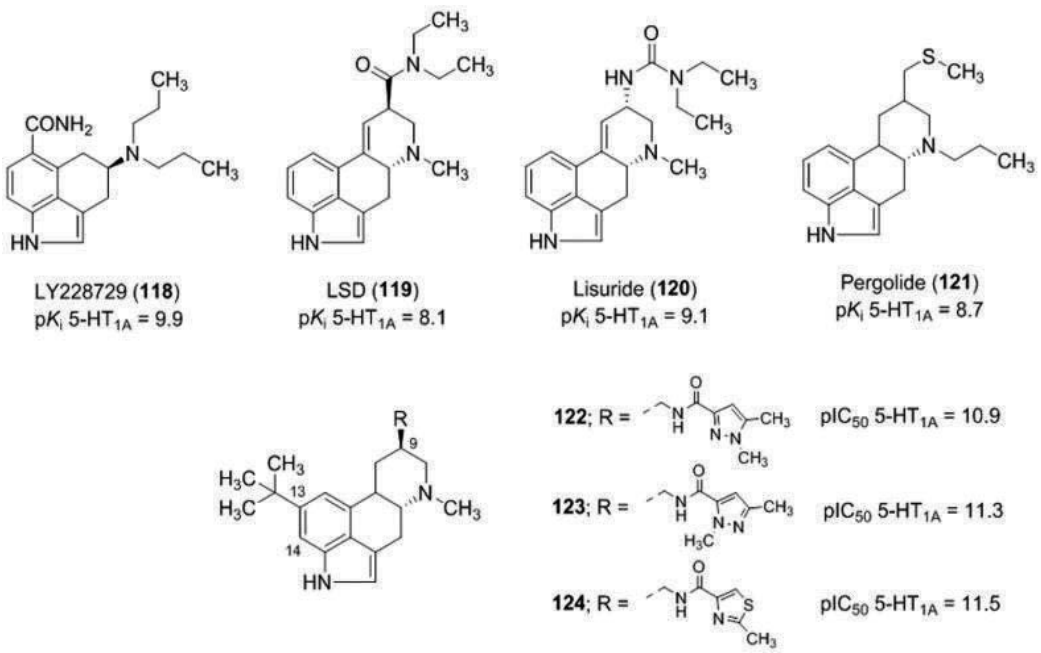


Figure 38. Chemical structures of **118–124**.

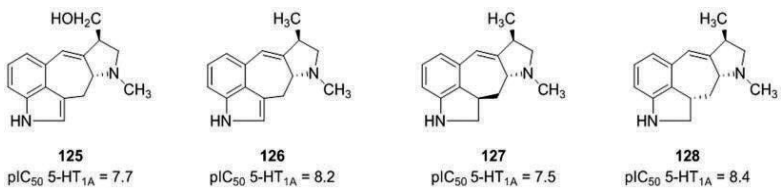


Figure 39. Chemical structures of **125–128**.

5.6. Aporphines

These compounds, whose prototype is (*R*)-apomorphine (**129**), have extensively been studied for their interaction with dopamine receptors in the CNS. In the effort to extend SAR studies of (*R*)-aporphines at dopamine receptors, (*R*)-(−)-10-methyl-11-hydroxyaporphine **130** (**Figure 40**), the 10-methyl substituted derivative of **129**, was surprisingly discovered [15] as a potent and selective 5-HT_{1A}R agonist devoid of dopaminergic activity. The corresponding (*S*)-enantiomer behaves as an antagonist at postsynaptic 5-HT_{1A}Rs and is tenfold less potent than its antipode. Changes in steric bulk and/or electronic properties of the C10-substituent as compared to a C10-methyl group produce a decrease in 5-HT_{1A}R affinity. For example, the substitution of the methyl at C-10 with an ethyl group (**131**) reduces the 5-HT_{1A}R affinity of about 20-fold. Compound **132**, the *N*-desmethyl derivative of **130**, shows about 7-fold lower than 5-HT_{1A}R affinity (**Figure 40**). However, such a modification mostly reduces the affinities

for D_1Rs (62-fold) and D_2Rs (>9.3-fold) and, consequently, improves 5-HT_{1A}R selectivity. The removal of the substituent at position C-10 is compatible with 5-HT_{1A}R interaction. In particular, among the C-11-monosubstituted aporphines, ethyl (**133**) and phenyl (**134**) derivatives show the highest affinities for 5-HT_{1A}Rs and good selectivity over both D_1Rs and D_2Rs (**Figure 40**).

Rigidifying (*R*)-aporphines derivatives by linking C-1 and C-11 into a fused pentacyclic or hexacyclic ring strongly reduces 5-HT_{1A}R affinity. However, among the compounds within this series, the imino derivative **135** displays poor selectivity for 5-HT_{1A}Rs over both 5-HT_7Rs and D_2Rs, whereas the regioisomer **136** is selective for 5-HT_7Rs.

5.7. Imidazolines

The observation that the beneficial properties of the α_{2C}-AR agonists and α_{2A}-AR antagonists allyphenyline (**137**) and cyclomethyline (**138**) on morphine dependence proved to be associated to a significant antidepressant effect led to the hypothesis that ligands bearing the 2-substituted imidazoline nucleus as a structural motif can also be suitable to interact with 5-HT_{1A}Rs (**Figure 41**).

Experiments carried out in the presence of the 5-HT_{1A}R antagonist WAY100135 confirmed that 5-HT_{1A}R activation is involved in the observed antidepressant-like activity [51]. The investigation of a wide series of 2-substituted imidazolines linked to an aromatic moiety by

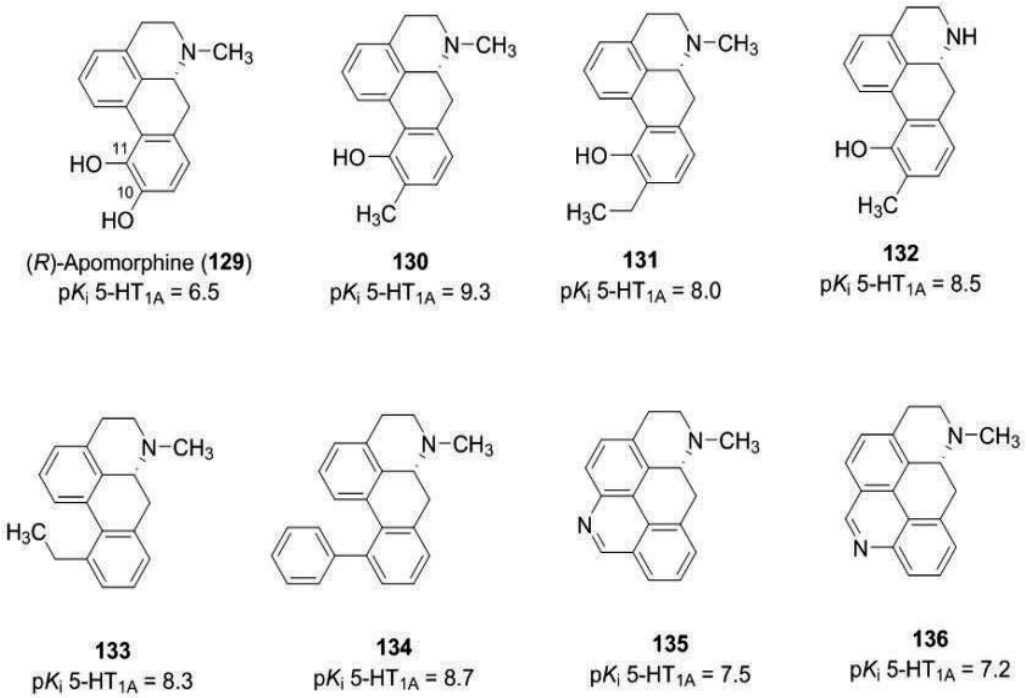


Figure 40. Chemical structures of **129–136**.

a biatomic bridge highlighted that a polar function (-O- or –NH- group) and a methyl group in the bridge as well as the suitable chirality and a proper steric hindrance in the aromatic area favor 5-HT_{1A}R recognition and activation. In particular, (*S*)-naphthaline (**139**) shows the highest 5-HT_{1A}R affinity within the series (**Figure 41**). In mice it displays antidepressant-like effect at a very low dose (0.01 mg/Kg) and proves to be more efficacious and potent than amitriptyline (15 mg/kg), a tricyclic antidepressant commonly used in human therapy [52].

5.8. 1,4-Dioxanes

The design and synthesis of 5-HT_{1A}R ligands bearing the 1,4-dioxane nucleus were inspired by the observation that the potent α_1-AR antagonist WB4101 (**140**) also shows high 5-HT_{1A}R affinity [53]. In the effort to discriminate between 5-HT_{1A}R and α_1-ARs, the quite planar 1,4-benzodioxane structure of **140** was replaced by the less conformationally constrained 6-aryl-1,4-dioxane ring, maintaining the 2,6-dimethoxy substitution or removing one or both methoxy groups of the phenoxy terminal. The most interesting results are shown by the 6,6-diphenyl substituted compounds **141–143**, which display nanomolar 5-HT_{1A}R affinities (**Figure 42**).

In particular, **143** behaves as a potent full 5-HT_{1A}R agonist with a pD_2 value significantly higher than those of the reference compounds 5-HT and 8-OH-DPAT. This derivative also shows a good selectivity for 5-HT_{1A}Rs over α_{1A}–, α_{1B}–, and α_{1D}–AR subtypes [54]. The stereogenic center in position 2 of the 1,4-dioxane nucleus appears to play a critical role in the

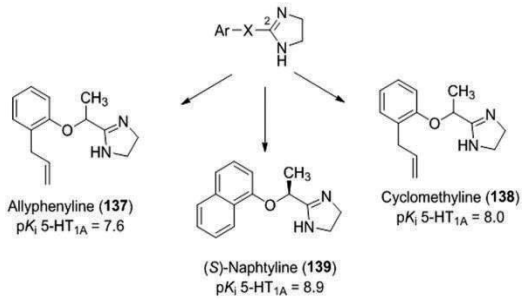


Figure 41. Chemical structures of **137–139**.

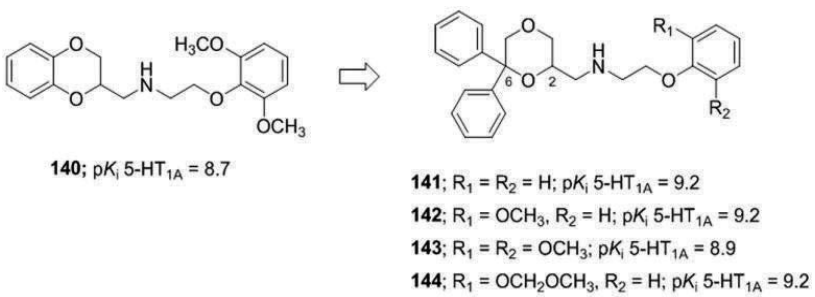


Figure 42. Chemical structures of **140–144**.

interaction with α_1-AR and 5-HT_{1A} R systems, a reversal enantioselectivity governing the 5-HT_{1A}R or α_1-AR recognition. Indeed, concerning 5-HT_{1A}Rs, the optimal affinity resides in the 2-(*S*) configuration, which, on the contrary, is less favorable for the interaction with α_1-AR subtypes. This result is particularly interesting because, as the eutomers for 5-HT_{1A}Rs behave as distomers for α_1-AR, the 5-HT_{1A}R/α_1-AR selectivity ratio significantly increases compared to the corresponding racemate [55].

A good selectivity for 5-HT_{1A}Rs over α_1-ARs and dopamine D_2-like receptors is also obtained by inserting a –OCH_2OCH_3 group in 2-position of the phenoxy terminal (compound **144**; **Figure 42**). The pharmacological profile of **144** and docking studies suggest that 5-HT_{1A}Rs also accommodate substituents bulkier than the methoxy group. Instead, both α_1-ARs and D_2-like receptors have more stringent steric requirements being intolerant to the increase of steric bulk itself. Due to its 5-HT_{1A}R activation, **144** significantly reduces anxiety-linked behaviors in mice [56].

6. Conclusion

In summary, the currently main knowledges of the four-wheel drive (4WD: who, why, where, what, and drugs) vehicle by which to travel inside the 5-HT_{1A}R world, have been presented. Such a travel, begun 30 years ago with the identification of 5-HT_{1A}R coding gene, is far from the conclusion. Indeed, despite no X-ray structure is deposited to date, it is possible to answer quite exhaustively the question "who" this receptor is. However, the most intriguing question is "why" it continues to be a so attractive target several years after its identification. Several evidences are available about "where" 5-HT_{1A}R is expressed throughout the body, at both central and peripheral levels. Between presynaptic (auto- and heteroreceptors) and postsynaptic receptors, are there differences which could allow us to target them selectively? Wider and wider is the field of "what" effects this receptor can elicit under physiological and pathological conditions directly or through the modulation of several other receptor systems or the stimulation of the secretion of various hormones. Well known is its involvement in anxiety, depression, epilepsy, mood disorders, learning, and memory. Consequently, growing is its importance in the treatment of such pathologies. Moreover, the interest for 5-HT_{1A}R as an attractive target of drugs is increased by further physiologically governed functions, including feeding/satiety, temperature regulation, sleep, pain perception, and sexual activity. The stimulation of 5-HT_{1A}Rs has been demonstrated to activate several different biochemical pathways and signals through both G-protein-dependent and G-protein-independent pathways. However, it cannot be ruled out that underlying mechanisms are far from being completely understood, making more and more complex the net of pathways through which the primary impulses unwind themselves. Finally, the discovery of "drugs" able to selectively activate or inhibit 5-HT_{1A}R might help to better characterize such a receptor and the physiological functions in which it is involved. Despite the numerous published papers and synthesized and tested molecules, the results are not completely satisfactory yet. The reasons can be ascribed partly to the great similarity of the ligand recognition transmembrane region of 5-HT_{1A}Rs with other members of the family or other GPCRs, partly to bimodal effect of 5-HT_{1A}R activation dependent on the neuroanatomical location of the receptors and the concentration of the ligand.

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Chapter 5

Sculpting Cerebral Cortex with Serotonin in Rodent and Primate

Tania Vitalis and Catherine Verney

Additional information is available at the end of the chapter

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Abstract

The mammalian cerebral cortex is critical for sensory and motor integrations and, for higher-order cognitive functions. The construction of mammalian cortical circuits involves the coordinated interplay between cellular processes such as proliferation, migration and differentiation of neural and glial cell subtypes followed by accurate connectivity evolving in complexity in primates. Alteration in cortical development may induce the emergence of various pathological traits and behaviours. Among the large array of factors that regulate the assembly of cortical circuits, serotonin (5-HT) plays important role as a developmental signal that impacts on a broad diversity of cellular processes. 5-HT plays distinct roles during specific sensitive periods and is produced from various sources depending on the perinatal stage. Its roles are mediated by more than fourteen 5-HT receptors that are all G-protein coupled receptors except the ionotropic 5-HT type 3A receptor (5-HT_{3A}) mediating rapid neuronal activation. Importantly, 5-HT metabolism and signalling are influenced by numerous epigenetic and genetic factors, including nutrition and gut microbiota, perinatal stress, infection and inflammation. In this review, we will recapitulate some evidences showing that dysregulation of 5-HT homeostasis and 5-HT_{3A} signalling impairs distinct steps of cortical circuit formation leading to the predisposition of the onset of various psychiatric diseases.

Keywords: development, human, monoamine, plasticity, 5-HT3 receptor

1. Introduction

The functions of the mammalian cerebral cortex are processed through the activation of multipartite neural networks composed of excitatory glutamatergic pyramidal neurons, local modulatory interneurons that release γ-aminobutyric acid (GABA), neuropeptides and vasoactive substances [1–5] and by 'glial cells' that do far more than just feeding neurones and scavenging debris [6, 7]. Developmental perturbations impacting the maturation of cortical circuits can trigger neuropsychiatric disorders [8–10]. Sensitive periods or windows of vulnerability have been demonstrated in various processes in particular for the rodent sensory systems as well as in the modulation of complex behaviours.

Mammalian cortical circuit formation is the result of a series of sequential events that take place mainly during embryonic and early post-natal development [11–14]. These events include the proliferation, migration and differentiation of neurons and 'glial cells' that are largely governed by genetic programs but are also sensitive to environmental factors. Such extrinsic signals are extremely diverse (including guidance cues, growth factors, cell adhesion molecules) and among them the monoamine serotonin (5-HT) has emerged as an important regulator of neural circuit formation [15, 16].

In mammals, cortical 5-HT arises from multiples sources depending on the developmental stage. At the onset of cortical development, 5-HT is of maternal and placental origin [17–19]. Later, by embryonic day 16 (E16 in mice) [15, 16, 20] and by gestational week 16 (GW16 in human) [13, 14], serotoninergic afferents invade the cerebral cortex and contribute to provide 5-HT locally. Not surprisingly, like in non-mammalian species, serotonin modulates neuronal proliferation, migration and differentiation. In addition, 5-HT is implicated in the emergence of many neuropsychiatric disorders, including mental retardation, autism, depression and anxiety [10, 15, 21–26]. Importantly, 5-HT signalling is influenced by numerous epigenetic and genetic factors, including nutrition and gut microbiota [27, 28], perinatal stress [29–31], infection and inflammation [32–35], 5-HT metabolism and storage [15, 36–38], pharmacological compounds such as selective serotonin reuptake inhibitors [38–40] and genetic alterations [41–44].

Our aim is to give a comprehensive overview on the possible roles of 5-HT receptor signalling and 5-HT homeostasis on the development of the cerebral cortex in rodent and primate with a specific emphasis on human. In this framework, we will highlight more particularly recent studies that have revealed new molecular targets of early-life 5-HT in the construction of cortical circuits; in particular, the ionotropic 5-HT type 3A receptor (5-HT_{3A}). We will also review recent clinical studies suggesting that altered 5-HT homeostasis or signalling could participate in the emergence of human psychiatric disease, in particular of mood and anxiety disorders.

In the following section, we will describe the general structure of the mammalian cerebral cortex focusing on rodent and then presenting the specificities observed in primate/human. Then we will describe the major steps of the development of the mammalian cerebral cortex that is governed by a series of sequential events including proliferation, migration and differentiation of neurons and glial cells. When numerous developmental similarities are observed very precociously in rodent versus primate, significant specificities arose later in development in primate especially in human.

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2. Structure and development of the mammalian cerebral cortex

2.1. Neuronal components and glial components

The mammalian cerebral cortex comprises of six lamina (layers), each containing specific combination of neurons and 'glial cells'. Cortical excitability is coordinated by the interplay of excitatory pyramidal neurons and inhibitory interneurons. Pyramidal cells, which make up the majority of all neurons in the adult cortex (80% in rodent cortex), are projection neurons that send axons to other areas inside or outside the cortex providing output excitatory drive by releasing glutamate [2]. Inhibitory neurons project locally, release the neurotransmitter GABA and refine cortical excitability. Although GABAergic interneurons are less abundant, they have crucial roles in the development and organization of cortical networks that underlie a wide range of cortical and mental functions [8, 45, 46]. They are extremely diverse, differing in shape, electrophysiological properties and in the combination of neuropeptides and calcium-binding proteins that they express in addition to GABA [1, 47]. To facilitate the description of GABAergic neurons, a consortium of experts has suggested using a unified nomenclature [4, 5]. Thus, one can distinguish four major and highly distinct classes of GABAergic neurons in the mammalian cerebral cortex (**Figure 1A**). First, fast-spiking interneurons expressing parvalbumin (PV) that gate incoming sensory information [48, 49]. Second, adapting Martinotti cells expressing somatostatin (SOM) that control dendritic information through local feedback inhibition [50]. Third, adapting bipolar interneurons expressing mainly the vasoactive intestinal peptide (VIP) and calretinin (CR) that preferentially target other interneurons and receive direct input from the thalamus [20, 51, 52]. Fourth, adapting neurogliaform interneurons expressing vasoactive substances, notably the neuropeptide Y (NPY) and/or nitric oxide (NO) that are responsible for the slow GABAergic inhibition of pyramidal cells and interneurons and vasomotion [53–56].

Although these different types of interneurons have been identified in the primate or human cerebral cortex, their diversity largely surpasses what is observed in rodent [12]. Interestingly, unique to human cerebral cortex, bipolar/von Economo neurons are present in layer V of the anterior cingulate and fronto-insular cortices expressing VMAT2 [57, 58]. Their possible involvement suggested in neuropsychiatric disorders needs to be further investigated [59]. In human and primate, the neuronal composition of the cerebral cortex is less homogeneous between areas with a higher level of arealisation than in rodent. Interestingly, the density of small interneurons appears very high in associative areas [60].

Besides neurons, mature 'glial cells' have been shown to exert roles that are extremely more complex than previously thought. Astrocytes are the largest glial population in the mammalian brain and are well-known to 'feed neurons' by transforming glucose into lactate that neurons can directly use as 'carburant', to scavenge debris and to regulate neural transmission and ionic homeostasis of the brain [61, 62]. Microglial cells play a role of sentinels of inflammatory state of the brain. In addition to these roles, astrocytes and microglial cells participate in regulating cell proliferation, neuronal migration and plasticity (for review, see Refs. [6, 61, 63]). Oligodendrocytes myelinate axons and increase their conduction velocity (they will not be further described in this chapter).

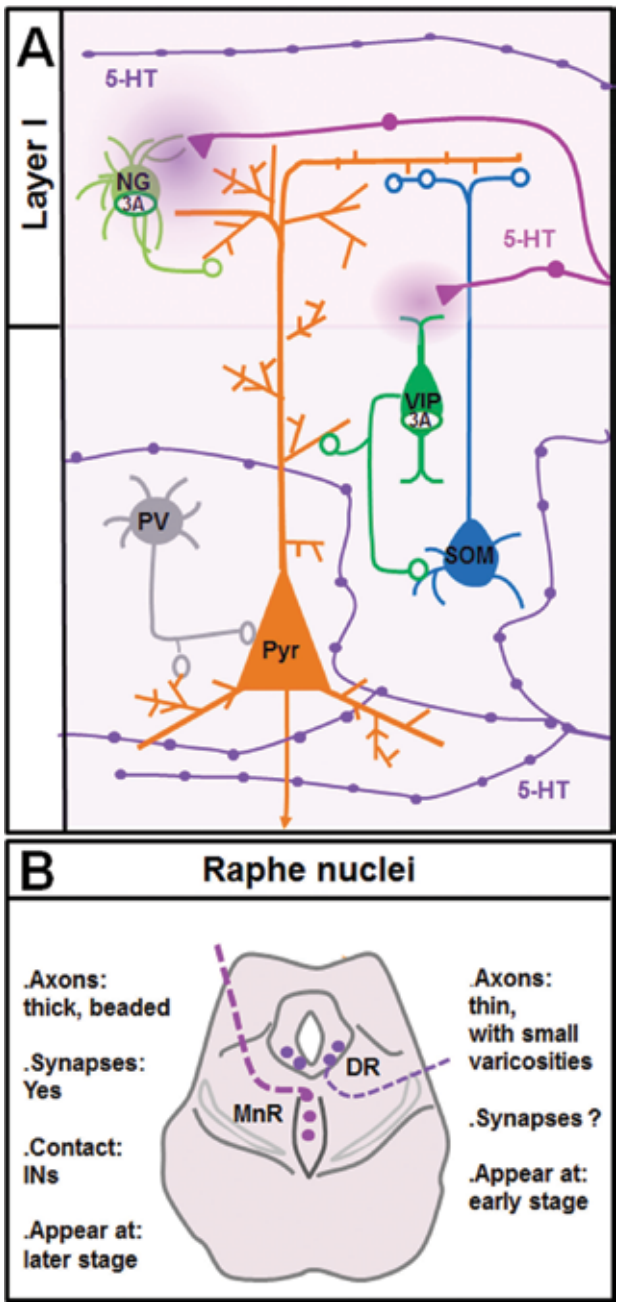

Figure 1. Structure of the rodent cerebral cortex and relation with serotoninergic afferents. A, The four main classes of interneurons (NG: neurogliaform, PV: parvalbumin+, VIP: vasoactive intestine peptide+, SOM: somatostatin+) and their relationship with a typical pyramidal glutamatergic neuron (adapted from [64]). B, Serotoninergic afferents arising from the median raphe (MnR) are thin, diffuse and display small varicosities. Serotoninergic afferents arising from the dorsal raphe (DR) are thick, beaded, preferentially located in superficial layer and make true synaptic contacts with small interneurons expressing VIP and with NG interneurons expressing the 5-HT receptor type 3A (3A). Adapted from [65]. 5-HT: serotonin.

2.2. Development of the rodent cerebral cortex

The cerebral cortex develops from neuroepithelial germinal cells of the telencephalic pallium and subpallium that massively proliferate by E11-E12 in mice and GW5-6 in human, to form the cerebral vesicles [66]. At this stage, microglial cells—of extracerebral origin—have already started to invade the telencephalon (from E9.5 in rodent [67] and GW5 in human [63]) before blood vessels start to penetrate and ramify in the telencephalon [68]. They will both participate in regulating neurogenesis [69]. The first generated neurons, Cajal-Retzius (C-R) cells and subplate cells (SP; from E10 in mice, GW5-7 in human), constitute transient and heterogeneous populations of cells that originate from both pallial and subpallial territories and form the preplate (PP; Boulder Committee; [66, 70, 71]). SP and reelin-secreting C-R cells provide positioning cues and instructions to developing cortical neurons and afferents [71–74]. The cortical plate, is formed from E13-E17 in mice and GW7-20 in human by post-mitotic excitatory pyramidal neurons migrated along radial glial (RG) fibres in an inside out gradient of development from layer VIa to layer II [13]. At the beginning of cortical plate formation (E13-E14 in mice), pyramidal cells are generated from radial glial cells (RGC), whereas later (E15-E17 in mice), they mainly originate from intermediate progenitor cells (IPC) or basal progenitors deriving from RGC cells [75, 76] (**Figure 2**).

The primate/human cortical neurogenesis is far more complex than that of rodent involving more germinal zones and a larger number of cell types [77, 78]. In particular, beside the early RGC in the VZ, a novel class of radial cells, the outer RG (oRG), located in the outer subventricular zone (SVZ) could be responsible for the increasing number of excitatory neurons and the formation of gyration in primate. The second stage of human cortical development (GW18-20) corresponds to the genesis of the supragranular layers that likely expand from the oRG [14] (**Figure 2A**).

In rodent, the cortical GABAergic interneurons are generated outside the cortical VZ, in the subpallium: mainly in the medial ganglionic eminence (MGE) (E11-E14 in mice) and the caudal ganglionic eminence (CGE) (E14-E17 in mice) [11, 20, 52]. These regions are specified through a combination of distinct transcription factors and morphogenes that produce different classes of interneurons [80]. The ventral and the dorsal parts of the MGE expressing the homeobox transcription factor Lhx6 generate fast-spiking/PV+ and adapting/SOM+ interneurons [81–85]. The CGE, a region that expresses the transcription factor Gsh2, COUP-TFII but lacks the transcription factors Nkx2.1, Nkx6.2 and Lhx6 [80, 86, 87], generates VIP+, CR+, NPY+ and nNOS+ interneurons [20, 52, 85, 88]. Once produced, interneurons are targeted towards specific brain regions, including cortex, depending on the transcription factors and guidance cues they express [87, 89]. They initially follow parallel migratory streams, first in the IZ and MZ and later on along the SVZ, before they switch their migratory mode and incorporate into the developing CP through radial migration (see **Figure 2B**). In mice, cortical migration is almost completed by P4, and is followed by cortical expansion. However, during the first two post-natal weeks and decreasing with age the SVZ retains the capacity to produce CR+ interneurons contributing to the pools of GABAergic neurons mainly populating lower cortical layers and cingulate cortex [90–92]. These events are recapitulated in **Figure 3A** and **B**.

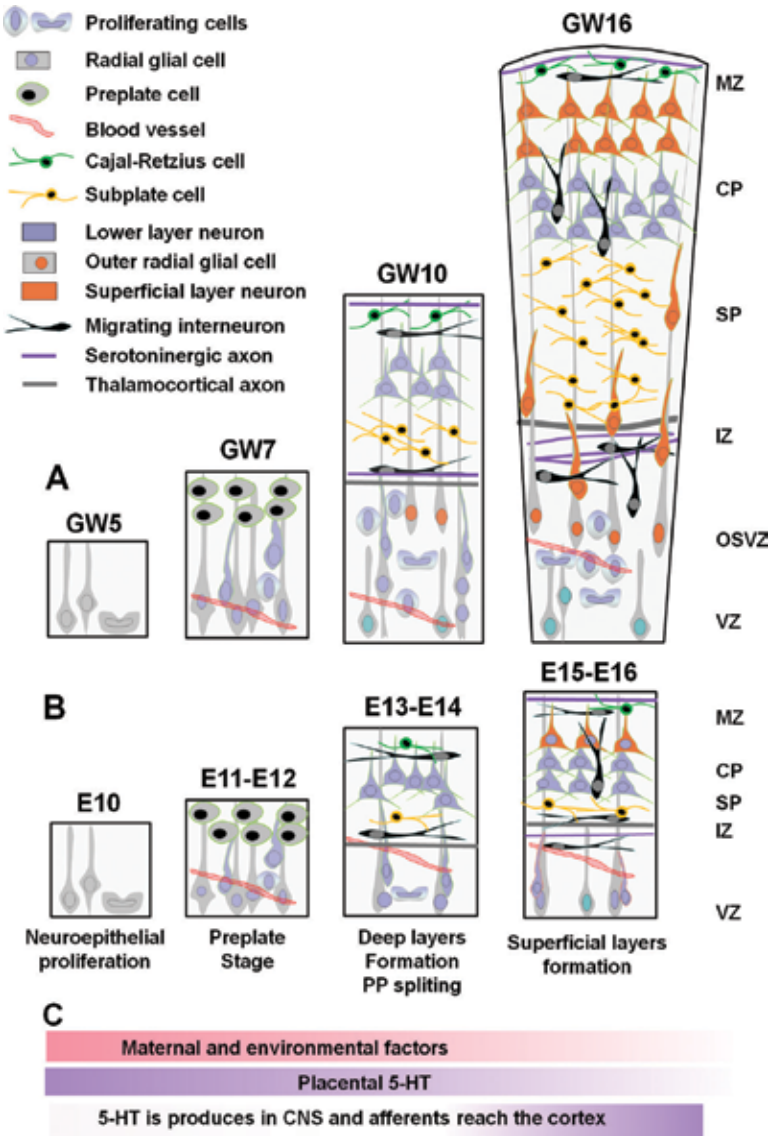

Figure 2. Early stages of development of the human (A) and mouse (B) cerebral cortex in relation with 5-HT afferents. A-B, Both in human and rodent intense proliferation of neuroepithelium and the formation of the preplate (PP) take place around (E10; GW5) and (E11-E12; GW6-7) respectively. By E13-E14 in mice and GW8-10 in human, PP is split by the migration of the first pyramidal neurons. Cajal-Retzius cells (C-R) will remain in the marginal zone (MZ) while subplate neurons (SP) will be positioned below the cortical plate (CP). In addition, in human around GW10, another source of progenitors arises: the outer radial glial (oRG) cells that do not maintain contacts with the apical surface. Monoaminergic axons and thalamocortical axons (TC) are already found in the MZ and in the intermediate zone (IZ) and, in the IZ respectively. By E15-E16 in mice most glutamatergic neurons are generated, 5-HT axons and TC run in the MZ and IZ and in the IZ respectively. By GW16 in human, SP occupy a large proportion of the cortical anlage and oRG are still producing a high amount of neurons. Interneurons migrating first tangentially to the pial surface and later radially to it, incorporating CP. C, Bars indicate the time at which different factors (maternal and environmental; 5-HT of placental origin, 5-HT produced by the embryo itself) could affect the development of the mouse embryo. A, is adapted from [20] and B is adapted from [13, 14, 79].

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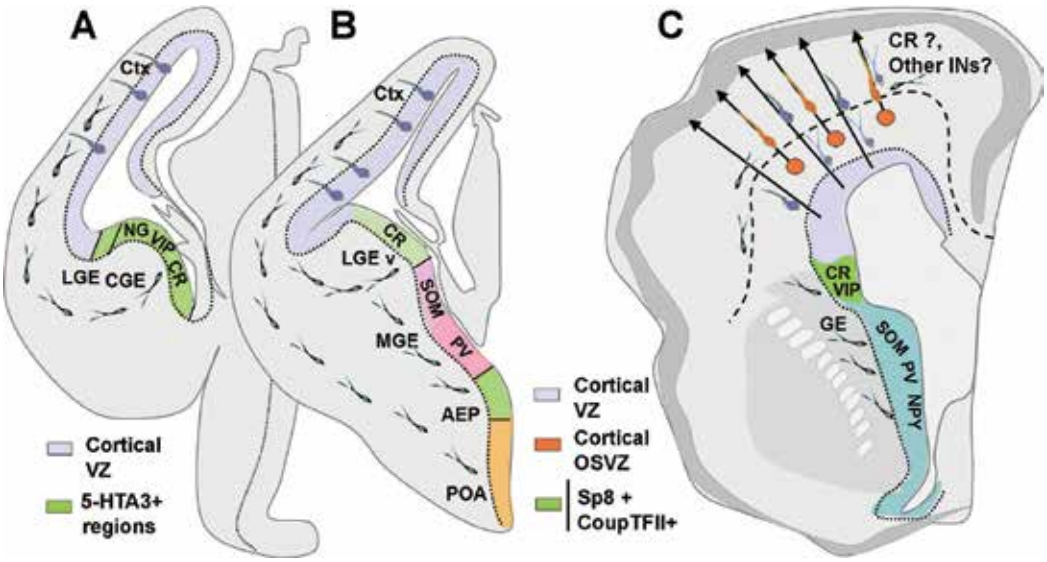

Figure 3. Presumptive genesis of cortical GABAergic neurons in the rodent and human/primate embryos and fetuses. (A and B) In rodent, PV+ and SOM+ interneurons (INs) are generated first from the medial ganglionic eminence (MGE) located in the anterior telencephalon. CR+, VIP+ and neurogliaform INs are generated mainly in the caudal GE (CGE) and in the lateral GE (LGE) located in the basal ganglia and to a lesser extent in the anterior entopeduncular area (AEP) and in the pre-optic area (POA). (C) In non-human primate and in human, the picture is less clear. However transcription factors expression suggest that the GE produce a large part of GABAergic neurons. By contrast to rodent brain numerous, INs may be generated in the cortical anlage. Panel C is adapted from Ref. [12]. CR: calretinin, NG: neurogliaform, PV: parvalbumin, SOM: somatostatin and VIP: vasoactive intestine peptide.

In non-human and human primate, the origin of the very heterogenous GABAergic interneurons is not so clear. Recently, studies have shown that in non-human primate, interneurons use a similar coding of transcription factors as in rodents and largely originate from the ganglionic eminences [93] (**Figure 3C**). However, a substantial proportion of them is likely to be generated in the pallium from the VZ and the SVZ [12, 94–96] (**Figure 3C**). Recently, migration of subclasses of human cortical interneurons has been reported to continue after birth [97].

2.3. Specificities of the human and primate cerebral cortex

As already mentioned, the first generated neurons, C-R and SP cells are located respectively in the presumptive Layer 1 and the SP zone of the human cortical anlage [66, 98, 99]. Specific to human, the SP zone is the largest transient compartment of the fetal neocortical anlage, about four times thicker than the cortical plate around midgestation [66, 100]. In humans and non-human primate, most SP neurons generated in the ventricular zone initially migrate radially, together with prospective layer VI neurons and secondarily get widespread into the expanding SP zone around midgestation [101]. Interestingly, at this stage, dispersion of SP cells in the extended SP zone is concomitant with the invasion of monoaminergic [102], thalamocortical and corticocortical axons in the cortical anlage [103]. SP zone begins slowly to disappear towards the end of gestation and during the early post-natal period. Finally, many

subplate neurons survive postnatally and transform into interstitial neurons of the subcortical white matter of the adolescent and adult brain [104]. GABA+ interstitial neurons express CB and CR [105]. Subcortical interstitial neurons in the white matter, which have been associated with a variety of neurological and psychiatric disorders of infant and adults, need to be further investigated [105, 106]. Comparison of the rodent/human cortical development could be obtained by comparing **Figure 2A** with **B** and **Figure 3A** and **B** with **C**.

Microglial cells take part in normal establishment and maturation of neuronal circuitry during development [107]. In human, amoeboid microglial cells infiltrate the brain via the choroid plexus, the meninges and the ventricles around GW4,5, progressively colonize the cerebral wall from GW7 and became ramified [108, 109]. Passing through walls from GW10 on. Interestingly, amoeboid microglial cells cluster in a band at the limit of the CP/IZ-SP zone at GW9-13 where early synaptogenesis takes place in the cerebral anlage [110]. They also clustered in major axonal crossroads in the corpus callosum at GW16 and in the coronal radiata at GW19-24 [63]. Interestingly, this last fibres tract area is the target of white matter injury observed in inflammatory process of premature infant in cerebral palsy [111]. Similarly, a cluster of microglia/macrophages is detected in the cingulum bundle in the perinatal rat models of hypoxia and growth restriction developed by Verney and collaborators [112–114].

In mammals, the numerous cortical astrocytes are reported to be mainly generated not only from radial glial cells but also from other cell types that are not clearly elucidated such as progenitors in the SVZ [62]. Human astrocytes are far more complex in diversity and size, and the ratio of glia to neuron is higher when compared to rodent [115]. The protoplasmic and fibrous astrocytes appeared in waves in the cortical anlage [115], begin to differentiate around midgestation and co-expression between vimentin and GFAP is observed [116]. Functional

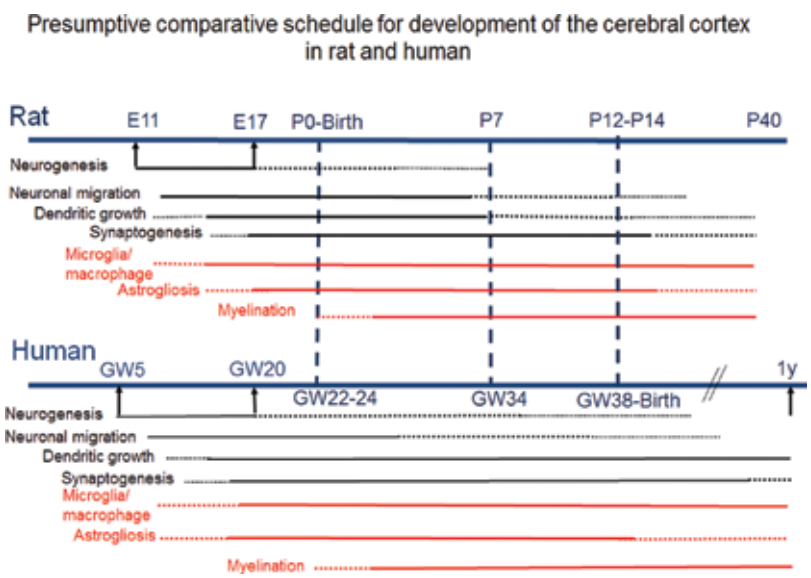

Figure 4. Presumptive comparative schedule for development of the cerebral cortex in rat and human.

astrocytes evolve in parallel with the maturation of the vascular endothelial cells involved in blood-brain barrier (BBB) formation [68, 117]. During development, monocarboxylates including lactate represent a major source of energy for the developing neurons [118]. The expression of monocarboxylate transporters such as MCT1 confirms the functionality of astrocytes in the energy trafficking occurring in the human visual cortex from GW19 [119].

Here, we provide a schematic drawing (**Figure 4**) comparing the schedule for the different key events occurring during the cortical development in human and in rat.

Serotonin is provided to the developing mammalian cerebral cortex via many sources. Numerous studies, cited in the section below, have described this in rodent but only sparse data are available in primate especially in human.

3. Sources of serotonin to the mammalian cortex

3.1. Serotonin synthesis and degradation

Serotonin is synthesized from the essential amino acid tryptophan. In the blood stream, tryptophan is linked to serum albumin but a proportion that decreases with age is free to cross the BBB (10% at post-natal day 12 when BBB is thought fully functional [120]). Tryptophan is then transported, accumulated in 5-HT-producing cells and hydroxylated by the tryptophan hydroxylase enzymes (Tph). Tryptophan hydroxylase type 2 (Tph2) is expressed in serotoninergic neurons of the raphe nuclei and myenteric neurons [121, 122], while Tph1 is expressed in the pineal gland, in the placenta and in various peripheral tissues [18, 19, 122, 123]. 5-hydroxytryptophan is then further decarboxylated into 5-HT by the aromatic amino acid decarboxylase (AADC). The availability of tryptophan to synthesise 5-HT depends on the inflammatory status of the organism. In case of inflammation, indoleamine 2,3-dioxygenase (IDO) is generated, which can lead to 5-HT depletion in the organism [35].

5-HT is catabolized by monoamine oxidases A or B (MAOA or MAOB [124, 125]). MAOA has higher affinity for 5-HT than MAOB and is strongly co-expressed with MAOB between E12 to P7 in rodent serotoninergic neurons [126]. After P7, the expression of MAOB is largely predominant in 5-HT+ neurons [126]. MAOs are also expressed by many non-aminergic structures, in particular the placenta and in a subpopulation of VZ-SVZ cells ([126, 127] and our unpublished results) where they may regulate the amount of 5-HT locally. Interestingly, MAOs expression and protein synthesis are tightly regulated and have been shown to be sensitive to environmental factors such as inflammation and ischaemia-like conditions [34].

During embryonic development, the telencephalon receives 5-HT arising from multiple sources that are mainly of extra-embryonic or maternal origin at the beginning of gestation. Later, they progressively arise from different embryonic regions. Below, we will briefly recapitulate the sources of serotonin provided to the embryonic telencephalon in relation with cortical development.

3.2. Development of the serotoninergic neurons and projections

In mammals, brainstem serotoninergic neurons are subdivided into 9 groups (B1–B9) forming a caudal and a rostral division. The rostral division (B5–B9; including the dorsal (B6, B7) and median raphe nuclei (B5, B8)) projects to the forebrain [65, 128, 129] (**Figure 1B**). Since these initial descriptions, recent mapping of 5-HT projections have been performed in mice revealing a higher level of refinement in the projections of raphe clusters towards specific targets [130]. Such level of analysis is lacking in primate and human.

In mice, the rostral division differentiates by E10-E11 (E12-E15 in rats); dorsal and median raphe send axons that reach the cortico-striatal junction by E14 in mice before entering the cortical anlage as two tangential streams, one above and the other below the CP [131, 132]. In the MZ, C-R cells and serotoninergic axons are in close apposition and make transient synaptic contacts [133, 134]. Below the CP, 5-HT afferents are mainly restricted to the IZ and the SP [131]. By E16-E17 in mice, thalamocortical axons (TCAs) invade the cortical anlage and are in close apposition with 5-HT axons running in the IZ. At the end of corticogenesis, 5-HT axons gradually arborize, sending numerous branches into the CP [131].

By P21, serotoninergic axons become evenly distributed in the different cortical territories showing their mature pattern of innervation [128]. Dorsal raphe axons are generally thin with pleiotropic varicosities that preferentially arborize in cortical layers IV and V. By contrast, median raphe axons show large spherical varicosities, form true chemical synapses, preferentially arborize in layer I and lower white matter, and contact interneurons containing VIP and cholecystokinin (CCK) [64, 65, 135] (**Figure 1**). Thus, 5-HT could be released along the entire axonal network through volume transmission or in synaptic clefts.

Anatomical studies have described the primate raphe nuclei and the serotonergic cortical innervation at mature stages [136–138], but only a few studies have reported their development. In Rhesus monkey, the genesis of raphe neurons was detected in the first quarter of gestation (E28-E45, birth: E165) [139] and 5HT+ fibres were reported in the entorhinal cortex at E70, similarly to tyrosine-hydroxylase+ catecholaminergic axons [140]. In human cortical anlage, one can suggest that the early afferents of serotoninergic axons as described for the catecholaminergic afferents may penetrate the cortical anlage around GW8 and invade the fetal cortex at midgestation in a mature-like pattern [102, 141]. In parallel, SERT expression in developing TCAs have been detected at GW10 in human cortical anlage [142]. Comparable expression has been described for the visual sensory system in the marmoset [143].

3.3. Other sources of serotonin

The first demonstrations showing that 5-HT was influencing very early embryonic development were provided by pioneer groups showing that *ex vivo* application of 5-HT or alteration of 5-HT levels altered normal development of various embryonic structures before serotoninergic neurons have innervated these structures [144–149]. Several studies suggest that 5-HT derives from maternal or placental sources (see **Figure 5** that recapitulates those studies).

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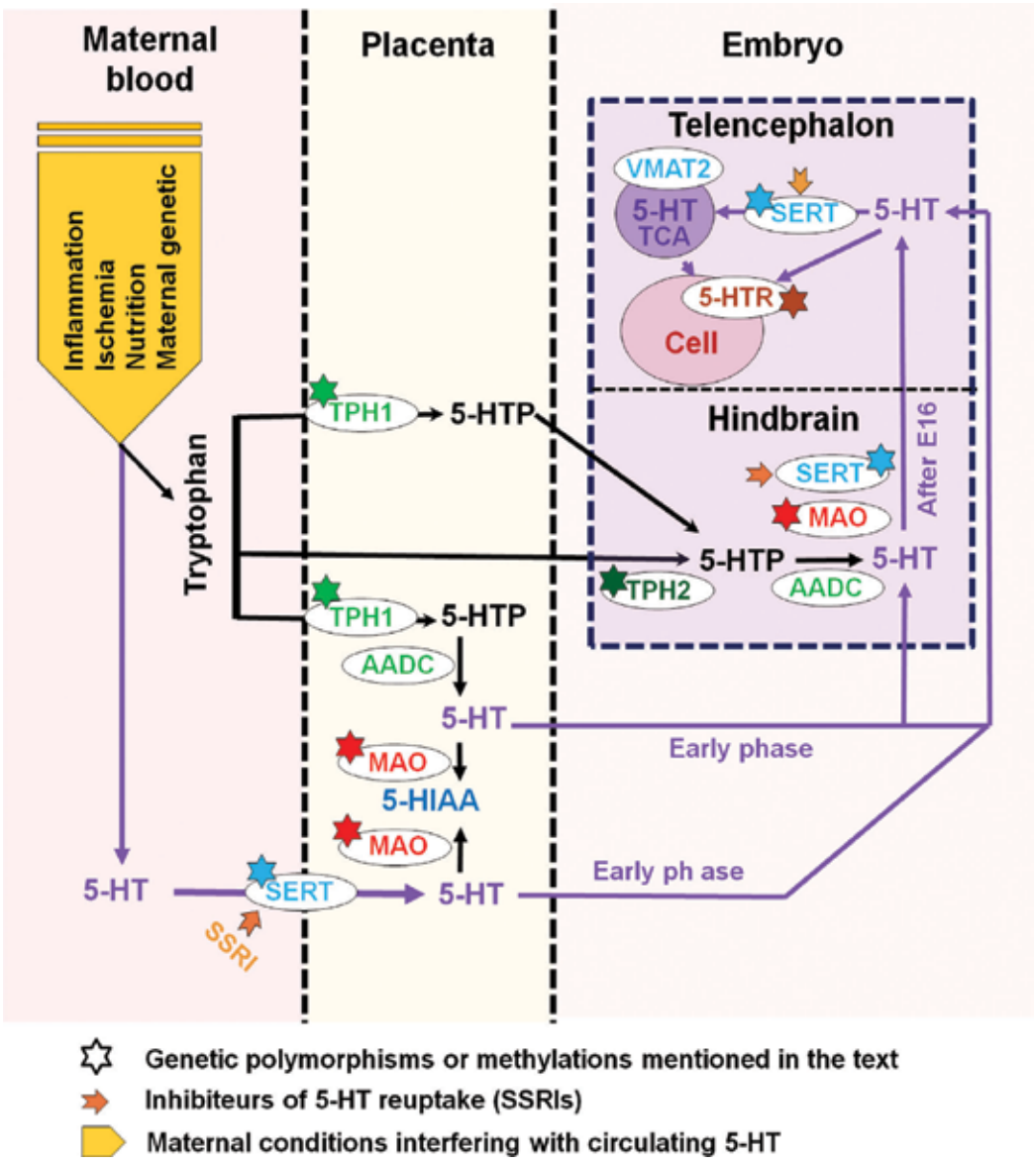

Figure 5. Maternal, placental, genetic and pharmacological conditions determining the amount of serotonin supply to the developing telencephalon. Tryptophan is provided to the embryo but could also be converted into 5-HTP (5-hydroxytryptamine) or further into serotonin (5-HT) in the placenta via the expression of various metabolic enzymes expressed in the placenta. In addition, 5-HT from maternal sources could be taken up by the placenta that also expressed serotonin transporter (SERT). During early embryonic stages 5-HT could be delivered directly to the developing embryo. After E15-E16, when 5-HT axons of the hindbrain reach the cortex, 5-HT could act on various target cells (Cell) expressing selected arrays of 5-HT receptors. At this stage 5-HT could also be taken up and stored by thalamocortical afferents (TC) and released after specific stimulation. In addition 5-HTP is provided to the (tryptophan hydroxylase type 2) Tph2 and the (aromatic amino acid decarboxylase) AADC containing neurons that synthetize 5-HT. In this drawing adapted from [19], we have pointed in the large left arrow the maternal conditions that are best known to interfere with 5-HT availability to the embryo. We have also indicated that inhibitors of 5-HT uptake (SSRIs) that cross all barriers affect SERT function at all levels. Genetic polymorphisms or methylations mentioned in the text are indicated by a star. The major catabolic enzymes of 5-HT, monoamine oxidases are indicated (MAO). Tryptophan hydroxylase type 1; Tph1.

Several groups have suggested that, at early stages, 5-HT arises from maternal sources. Indeed, this was suggested when analysing the phenotype of embryos generated from $Tph1^{+/-}$ or $Tph1^{+/+}$ mothers. $Tph1^{-/-}$ and $Tph1^{+/-}$ embryos obtained from crosses between heterozygous parents were indistinguishable from their wild-type littermates (the crown-rump length (CRL) was of 7.4–7.5 mm). By contrast, 80–88.9% of $Tph1^{-/-}$ and $Tph1^{+/-}$ embryos born $Tph1^{-/-}$ mothers displayed low CRL values (5.8–7.4 mm). This suggests that the partial lack of maternal 5-HT provided to the embryo may be sufficient to explain some of the littermates phenotypes [18, 123].

Recently, the placenta (that is of embryonic origin) has been identified as an important source of 5-HT for the developing embryo. The placenta (syncytiothrophoblastic cells and sinusoidal throphoblastic giant cells) of the placenta contain Tph1, AADC and MAO [124, 125, 127], and convert tryptophan of maternal origin into 5-HT as soon as E10-E11 [150]. Homozygote knock-out embryos in which 5-HT neurons fail to fully differentiate or to produce normal amounts of 5-HT levels do not display severe cortical defects when gestating in heterozygous dams. Examples include mice lacking the transcription factors Lmx1b [151] or Pet-1 [152], in which all or 70–80% of 5-HT raphe neurons fail to develop, and mice lacking Tph2 [153, 154]. Further analysis revealed that Pet-1 knock-out embryos developing in heterozygous dams have normal 5-HT levels before the closure of the BBB (before E15 [68]). These studies suggest that 5-HT produced by the placenta may buffer maternal deficiency. However, the compensatory mechanisms remain to be clarified.

Outside the CNS, 5-HT is also produced in the periphery of the developing embryo: from the myenteric plexus (from E15-E16), from enterochromaffin cells of the lining lumen of the digestive tract (from E18), from neuroepithelial cells of the respiratory tracts, from the parafollicular cells of the thyroid and from pinealocytes (belonging to the CNS; from E12). 5-HT could also be taken up by SERT expressing cells and further delivered to a distant region. SERT is expressed in platelets and mast cells [155, 156] that become numerous around E12 in mice. These cells could cross the BBB, transit across blood vessels that start to invade the developing cortex by E10-E11 in mice [68]. Whether these structures and mechanisms provide substantial amount of 5-HT to the developing telencephalon remains to be clarified.

Transiently, sensory thalamic neurons express SERT (E15-P15 in mice) and the vesicular monoamine transporter type 2 (VMAT2) that are respectively responsible for the uptake and packaging of 5-HT into synaptic vesicles [37, 157, 158]. Sensory thalamic neurons do not contain MAOs [159] but are equipped to release 5-HT, possibly with other transmitters (e.g. glutamate), after specific stimulation (review in Ref. [15]). Interestingly, it has been suggested that thalamocortical axons (TCAs) could be implicated in the proliferation and migration of glutamatergic neurons [160, 161] in addition to their well-known role on axonal refinement (see below).

Tryptophan is provided to the embryo but could also be converted into 5-hydroxytryptamine (5-HTP) or further into serotonin (5-HT; violet) in the placenta via the expression of various metabolic enzymes expressed in the placenta. In addition, 5-HT from maternal sources could be taken up by the placenta that also expressed serotonin transporter (SERT). During early embryonic stages, 5-HT could be delivered directly to the developing embryo. After E15-E16, when 5-HT axons of the hindbrain reach the cortex, 5-HT could act on various target cells (Cell; maroon) expressing selected arrays of 5-HT receptors. At this stage, 5-HT could also be

taken up and stored by thalamocortical afferents (TC) and released after specific stimulation. In addition, 5-HTP is provided to the tryptophan hydroxylase type 2 (Tph2) and the aromatic L-amino acid decarboxylase (AADC) containing neurons that synthesize 5-HT. In this drawing adapted from Ref. [19], we have pointed in orange the maternal conditions that are best known to interfere with 5-HT availability to the embryo. We have also indicated that inhibitors of 5-HT uptake (SSRIs) that cross all barriers affect SERT function at all levels. Genetic polymorphisms or methylations mentioned in the text are indicated by a star. The major catabolic enzymes of 5-HT, monoamine oxidases (MAO) are indicated.

Serotonin receptor signalling has been shown to regulate various cellular events. However, the large spectrum of serotonin receptors still need to be investigated in cortical development in rodent and even more in primate.

4. Serotonin receptors with specific attention to the 5-HT_{3A}

4.1. Transducing pathways

At least fourteen genes encoding for 5-HT receptors have been identified and cloned in the mammalian brain [162–165]. In addition, isoform diversity, alternative splicing of some subtypes and RNA editing add to the diversity of serotoninergic receptors. With the exception of the 5-HT_3 receptors, all 5-HT receptors are coupled to G-proteins. According to their second messenger coupling pathways, 5-HT receptors have been categorized into four groups. The 5-HT_1 and 5-HT_5 receptors are coupled to Gi/Go proteins and exert their inhibitory effects on adenylate cyclase, inhibiting cAMP formation. The 5-HT_2 receptors are coupled to Gq proteins and stimulate phospholipase C to increase the hydrolysis of inositol phosphates and elevate intracellular Ca^{2+}. The 5-$HT_{4,6,7}$ receptors are coupled to Gs proteins and are positively linked to adenylate cyclase and increase cAMP formation. 5-HT_3 receptors belong to a family of ligand-gated ion channel receptors that include nicotinic acetylcholine receptors, $GABA_A$ receptors and glycine receptors and are modulated by intracellular cyclic AMP [162]. The 5-HT_3 receptors respond to neurotransmitter release via direct (through the 5-HT_3 receptor itself) or indirect activation of the voltage-gated Ca^{2+} channels and lead to Ca^{2+} entry into the cell [166]. 5-HT_3 receptors are composed of five subunits, with the majority being homomers of 5-HT_{3A} receptors. Heteromeric 5-HT_{3AB} receptors have been observed in specific brain regions and display lower Ca^{2+} permeability than the homomeric 5-HT_{3A} receptors [167–169].

4.2. Expression patterns

Despite the efforts of many laboratories and open databases, a complete description of the developmental expression pattern of 5-HT receptors in the cerebral cortex is still lacking in rodent and very few studies have been performed in primate. However, pictures are emerging in the rodent brain. For example, 5-$HT_{1A,F}$ are expressed in neocortical proliferative zones in E14.5 rodent brain [17] and the 5-HT_{2B} are expressed in the proliferative zones of the human occipital cortex [129] and in all microglial cells [170, 171]. The 5-$HT_{1A,B,D}$, 5-HT_{2A}, 5-HT_{2C} and 5-HT_{3A}, are expressed in specific subpopulations of post-mitotic neurons [17, 88, 91, 167, 168, 172, 173], whereas the 5-HT_6 is expressed in both migrating interneurons and pyramidal neurons [174, 175].

The dynamic expression pattern of the 5-HT_{3A} receptor has been described in details recently in mice. In the developing cortex, 5-HT_{3A} is expressed as early as E11-E12 in neurons expressing reelin (Cajal-Retzius cells) and/or GABA cells located in the PP [88, 173]. The 5-HT_{3A} is expressed by newly post-mitotic GABAergic neurons located in the CGE and AEP/PO, where about 30% of cortical GABAergic neurons are generated ([52, 88]; see **Figure 3A** and **B**). Using homochronic in utero grafting in combination with a transgenic mouse line expressing GFP under the control of the 5-HT_{3A} promoter (5-HT_{3A}:GFP animals), we have shown that this expression was protracted in two large subpopulations of cortical GABAergic neurons: the multipolar interneurons expressing NPY displaying late spiking and accommodating properties and in VIP+ interneurons displaying adapting and bursting properties [52, 88, 176]. In addition, subpopulations of NO+ and reelin+ interneurons also express 5-HT_{3A} ([52, 55]; **Figure 1A**). By post-natal stages and decreasing with age, 5-HT_{3A} is also expressed by young neurons expressing doublecortin and/or calretinin generated in the SVZ and migrating towards the olfactory bulb (rostral medial stream) and various cortical and subcortical regions [90, 91]. In addition, we have reported that transient-amplifying precursors located in the white matter ventrally to the anterior cingulate cortex produced neurons destined to populate the anterior cingulate cortex and its vicinity [91].

Serotonin homeostasis and signalling act as a sculptor of cortical circuitry. In this section, we will review the different steps of cortical assembly that have been shown to be modulated by serotonin.

5. Impact of serotonin imbalance on cortical circuit assembly

5.1. Serotonin and cell proliferation

It has been postulated for some time that 5-HT regulates the proliferation of a wide variety of cell types including cortical neurons. Pharmacological studies inducing depletion of several monoamines triggered drawbacks due to the non-selectivity of the drugs used and they will not be discussed here.

Recently, transgenic models selectively targeting specific serotonin-related genes in different neuronal populations have started to provide more insights. For instance, mice deficient in Tph1 or Tph2 showed body weight reduction and delayed maturation of cortical layers [18, 153, 177]. Heterozygous embryos growing in null mutant $Tph1^{-/-}$ mice showed an average of 30% reduction in proliferating cells (BrdU+) in the VZ after a 2 h pulse of BrdU administration, an analog of thymidine that is incorporated during the S phase of the cell cycle [18]. Although these studies suggest that 5-HT from Tph1+ sources may regulate the proliferation of neuronal precursors, additional studies are needed to refine these observations.

Hyposerotonin-induced microcephaly could also be due to increased death of post-mitotic neurons or neuronal progenitors. Indeed, 5-HT_2 stimulation promotes the survival of glutamatergic neurons *in vitro* with a maximal effect observed for stages E16 and E18 in rats [178], and 5-HT_{1A} stimulation increases neuroprotection in models of ischaemia and protects neuronal cultures against serum withdrawal [179, 180]. Furthermore, activation of 5-HT_2 reverts

increased apoptosis observed in VMAT2:KO mice, in which dopamine, norepinephrine and 5-HT are depleted [181].

The analysis of mice lacking MAOA and B, which displays high 5-HT levels but normal dopamine and norepinephrine levels during development, revealed a specific reduction of symmetric divisions of intermediate precursors cells [76] in SVZ during late corticogenesis (E17.5) [182]. This unexpected alteration was reverted after pharmacological inhibition of 5-HT synthesis (with p-chlorophenylalanine; PCPA) between E14.5-E19.5. In addition, neurosphere formation was modulated by 5-HT in a dose-dependent manner *in vitro*, with proliferative effects observed for concentration ranging from 10 to 100 ng/ml and inhibitory effects observed for higher concentration (1000 ng/ml). In this study, these inhibitory effects were associated with decreased 5-HT_{1A} labelling of neuronal precursors [182] previously known to trigger neurogenesis in adult dentate gyrus. Hence, 5-HT might modulate cortical density through its proliferation-inducing action on progenitors.

During early development, 5-HT could also promote gap junction coupling through 5-HT_2 stimulation [183] that coordinates cell-cell assembly during cell cycle [184].

5.2. Serotonin and neuronal migration

In most phyla, 5-HT triggers motility of various cell types including vertebrate lymphocytes (chick, fish, rodent [185, 186]) and microglia towards the CNS [170]. In the mammalian cortex, a role for 5-HT in regulating the migration of cortical neurons has recently emerged. In this context, 5-HT produces opposite consequences depending on its concentration.

One of the first experiments to address this question was made *ex vivo* on cortical explants maintained in a serum-free medium and supplemented with low 5-HT concentration. The migration of glutamatergic neurons was examined and was found be faster in explants supplied with 5-HT suggesting that low 5-HT dosage may enhance the radial migration. Furthermore, decreasing 5-HT levels during development delayed or disrupted cortical migration, suggesting that 5-HT produces a positive drive on cortical migration [181]. In rats depleted in 5-HT by PCPA during the peak of migration (E12/E13 to E17 in rats), abnormal accumulation of GABAergic neurons below the subplate at E17 and a marked reduction of calretinin+ and CCK/VIP+ GABAergic neurons at adult stage were reported [187]. Interestingly, mice lacking Tph2 also display reductions of selective GABAergic populations in limbic structures [188]. 5-HT_{3A} is protractedly expressed by 30% of GABAergic neurons leading to calcium entry into the cell (see above). Using electrophysiological recording and calcium imaging, it was recently shown that CGE-derived interneurons that expressed 5-HT_{3A} increase their response to 5-HT_3 activation while they migrate radially and integrate the cortical plate (late phase of migration; see **Figure 6A**). This activation leads to an increased growth cone activity and to a decrease resting-state of 5-HT_{3A}+ interneurons. Further, using *in vivo* graft of 5-HT_{3A} deficient interneurons into wild-type host, it was shown that this role was cell-autonomous. Interestingly, long-lasting alteration in the positioning of reelin+ cortical interneurons was reported. This suggests that 5-HT_{3A} activation acts as a migratory signal for CGE-derived interneurons and alters definitively the positioning of their subpopulation [189]. A similar conclusion was suggested using SERT:KO animals that showed a specific increase in the migratory speed and positioning of VIP+ interneurons [92].

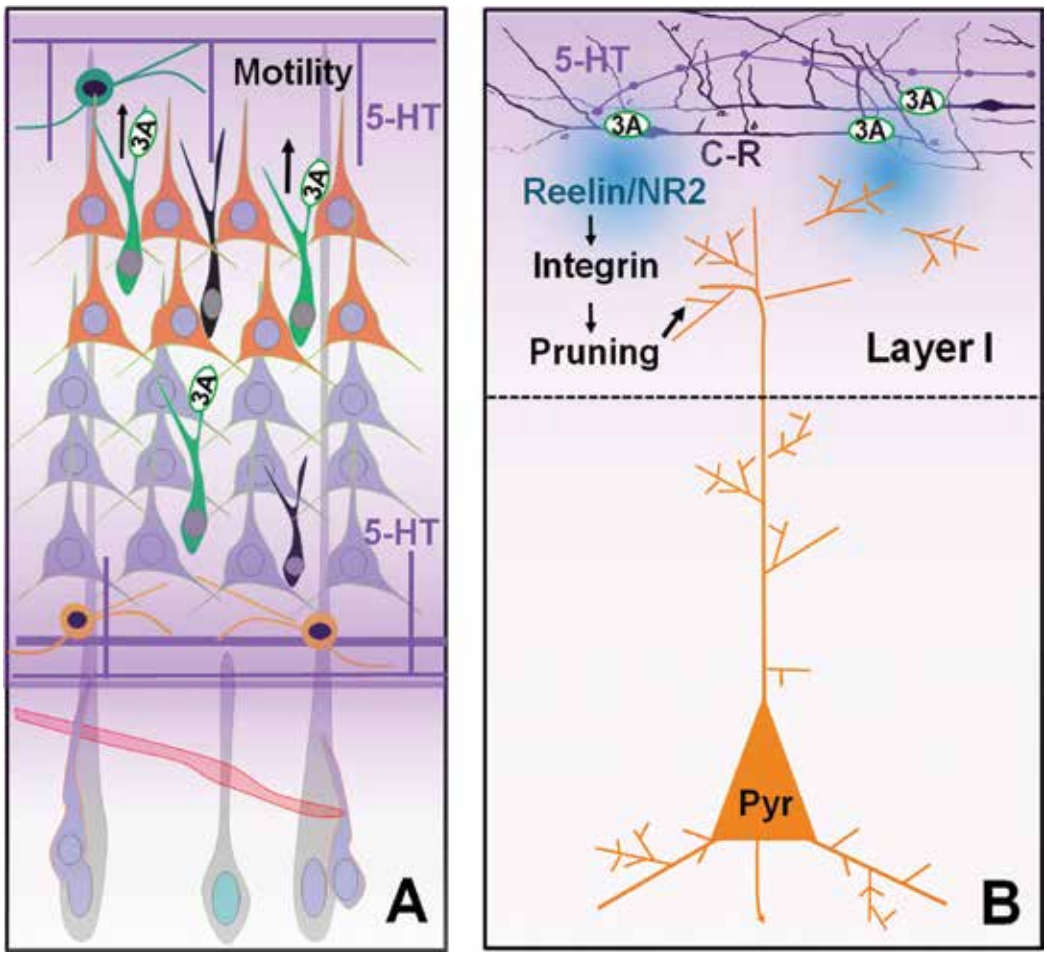

Figure 6. Modulation of cerebral circuit formation by 5-HT_{3A} A, 5-HT_{3A} (3A) is expressed by migrating interneurons generated in the caudal ganglionic eminence (CGE). Physiological concentration of serotonin (5-HT), induce an acceleration of the radial migration of 5-HT3A+ interneurons at E17. B, At early postnatal stage, Cajal-Retzius cells (C-R) that express 5-HT_{3A}, respond to 5-HT application by releasing reelin that through the activation of the integrin signaling pathway induce pruning of apical dendrites of pyramidal neurons (Pyr). This figure is adapted from [200].

Although dynamic expression pattern of 5-HT receptors is lacking in developing primate and human cortex, a very recent study by the group of Alvarez-Bulla showed that in human, late-born interneurons continue to migrate in the cingulate cortex even after birth. These interneurons expressed a combination of transcription factors and a substantial fraction of them expressed COUP-TFII or SP8 (22 or 28% respectively) that are mainly specific of 5-HT_{3A}+ interneurons suggesting that 5-HT could also modulate the migration and positioning of these neurons in human [97]. Interestingly, in the primate cortex, it was shown that 5-HT_{3A} is expressed by a subset of small GABA+, substance P+ or calbindin+ neurons and by medium-size CR+ neurons [190].

By contrast, 5-HT excess appears to have opposite role on migrating neurons. Using high dosage of 5-HT *ex vivo* on cortical slices, it has been shown that 5-HT induces a decrease in the

migratory speed of non-GABAergic and GABAergic neurons [174]. High 5-HT levels induced a retraction of the leading processes of GABAergic neurons migrating into the intermediate zone and cortical plate. This effect was shown to be mediated, at least in part, by the 5-HT_6 receptor activating the cAMP-signalling pathway [191]. Such role was also reported for glutamatergic neurons (for review, see Ref. [175]).

5.3. Serotonin and differentiation of cortical neurons and afferents

Lauder and Krebs were the first to report that depletion of 5-HT delayed the cessation of cell division, a marker of cell differentiation [144, 192]. After these pioneering studies, numerous groups have shown that 5-HT can influence dendritic and axonal morphogenesis during cortical development.

5.3.1. Serotonin and dendritic maturation of cortical neurons

5-HT was shown to regulate the physiology of C-R cells known to be key regulators of various aspects of cortical development including dendritic arborization. This role is largely mediated by the secretion of the glycoprotein, reelin [72, 74]. C-R cells receive serotoninergic projections with which they make transient synaptic contacts [134] and reelin secretion was shown to be regulated in part by the amount of brain 5-HT. Pharmacological perturbation of the serotoninergic system by 5-methoxytryptamine (a non-selective 5-HT receptor agonist) reduces reelin levels circulating in the blood flow at P0 [134], leading to the formation of abnormal microcolumns in the mice P7 presubicular cortex, a feature that is observed in autistic syndromes (ASDs). The activation of C-R cells was proposed to be modulated by 5-HT_{1A} or by the 5-HT_{3A} receptors, as they were both suspected to be expressed in the marginal zone during development [167, 193]. Interestingly, the 5-HT_{3A} has been shown to be expressed by C-R cells (averaging 80% at P0) and the synaptic activation of 5-HT_{3A} was shown to be sufficient to induce action-potential firing on C-R cells suggesting that 5-HT_{3A} could play a role in dendritic development [173]. The contribution of the 5-HT_{3A} was further analysed. The deletion or blockade of 5-HT_{3A} receptors was shown to induce excessive arborization of layers II-III apical dendrites of pyramidal neurons. Application of the N-terminal region of reelin, that induces the activation of a signalling pathway that is independent from the classic ApoER2/VLDL-pathway, rescued the dendritic phenotype of cortical pyramidal neurons in 5-HT_{3A}:KO cortical slices, whereas reelin blockade leads to an increased growth of apical dendrites ([173]; see **Figure 6B**). This study suggested that increased reelin secretion due to over-activation of the 5-HT_{3A} receptor could induce a decreased growth of apical dendrites. Interestingly, fluoxetine (an inhibitor of 5-HT uptake, SSRI) administration from E8 to E18 decreased the dendritic basal and apical arbor complexity of layer II/III pyramidal neurons in the somatosensory cortex. Such a role is specific to a selective developmental period and SSRIs have opposite functions at mature stages [194]. Furthermore, the effects of SSRIs on developing dendrites were abolished when administered in the 5-HT_{3A}:KO mice or after pharmacological blockade of the 5-HT_{3A} receptor [173, 195]. Moreover, the fine tuning of 5-HT_{3A} signalling has been shown to be responsible for the anxiety-like behaviours that are induced by prenatal fluoxetine treatment in wild type mice [196]. These results suggest that developmental excess of serotonin increases reelin secretion by over-

activating 5-HT_{3A} receptors expressed on C-R cells, consequently inhibiting dendritic growth of pyramidal neurons. Whether 5-HT_{3A}+ interneurons participate in this process remains unclear.

Animals fed with low tryptophan diet [197, 198] display cortical pyramidal neurons with decreased dendritic complexity and spine density. Thus, 5-HT may regulate dendritic maturation and spine density through different types of 5-HT receptors that remain to be identified. In this respect, the 5-HT_{1A} is strongly expressed in the developing cortical plate [17] and is known to be necessary for the dendritic maturation of CA1 pyramidal neurons [199]. The 5-HT_6 receptor also appears as a good candidate for controlling neuritic and dendritic development due to its ability to engage signalling pathways (e.g. Fyn, mTOR and Cdk5) playing roles in these processes. *In vitro* studies strongly suggest a role of 5-HT_6 on neuritic extension (for review see [175]). However, a clear view on the implication of the variety of 5-HT receptors expressed in the developing cortex remains to be elucidated.

5.3.2. Serotonin and axonal development within the cerebral cortex

The first clear demonstration that serotonin acts on cellular processes involved in the formation of cortical circuits comes from works performed on the rodent barrel field in the somatosensory cortex (S1). The serendipitous generation of a mouse displaying deficiency in the gene encoding for MAOA was at the starting point of these discoveries. These studies showed that excessive 5-HT amounts (ninefold increase at P0) in the developing cortex induced an abnormal organization of thalamocortical afferents (TCAs) growing in the layer IV of the primary somatosensory cortex [36, 37]. These alterations were later interpreted as an abnormal refining of TC axons due to a specific rise of 5-HT occurring during a sensitive period (P0-P4: [201]). In addition, pharmacological normalization of 5-HT levels in MAOA:KO mice by P0-P4 PCPA-treatment was sufficient to revert to normal the organization of S1 in MAOA:KO mice [37]. Later, it was shown that genetic SERT deficiency affected S1 organization similarly in rodent. These alterations are not only structural but also impair whisker-mediated perception [10]. Hyper-activation of the 5-HT_{1B} receptor, transiently expressed on TCAs during development, plays a key role in this process. Indeed, SERT:KO and MAOA:KO mice that are deficient in 5-HT_{1B} receptors are rescued [202–205]. Interestingly, serotonin excess does not only impairs S1 organization, but also such a role could probably be generalized in other regions displaying transient 5-HT uptake [158] as this was shown for the visual system [202, 205, 206]. Moreover, such a role could also occur in primate cortex since SERT is transiently expressed in the visual sensory thalamic neurons, at least in the marmoset [143]. So far due to the difficulty to obtain human embryonic samples of late stages, clear sets of data are still lacking but numerous non-serotoninergic fibres, presumably TCAs, labelled by SERT have been detected at GW10 [142].

Surprisingly, perinatal 5-HT deficiency only induces a reduction of barrel field organization without altering its general organization [177, 207, 208]. Nevertheless, further studies need to be carried since early reduction of 5-HT during embryonic development induces the emergence of altered behaviour [153].

Other studies suggest a prenatal role for 5-HT in regulating initial TCAs pathfinding. TCAs express SERT, 5-HT_{1B} and 5-HT_{1D} receptors at a time when TCAs are navigating towards the pallium. Embryonic down-regulation of 5-$HT_{1B/C}$ receptors in TCAs using *in utero* electropora-

tion leads to abnormal TCAs pathfinding [209]. Furthermore, it has been shown that 5-HT modifies the attractive versus repulsive responsiveness of TCAs to netrin-1 [209], an important guidance molecule for TCAs. Given these findings, it is thus likely that abnormal 5-HT levels could also affect these earlier stages of TCAs pathfinding and lead to abnormal long range of TCAs wiring [19, 150].

5.4. Serotonin and the regulation of astrocytes and microglial cell functions

Astrocytes and microglial cells have been shown to be implicated in key processes—from neurogenesis to synaptogenesis—involved in cortical development (for review, see Ref. [61]). These cells bear several 5-HT receptors depending on their stage and state (resting or activated) making 5-HT an indirect actor of cortical development via the modulation of their functions [170]. Pioneer studies have shown that 5-HT_{1A} and 5-HT_{2} are expressed by both immature and mature astrocytes in human and rodent cortex, and that 5-HT stimulates the release of several trophic factor produced by glial cells that promote neuritic extension and synaptogenesis of cortical and serotoninergic neurons such as S100β or BDNF. Conversely, lesions of the serotoninergic system were shown to increase GFAP and to decrease the release of several trophic factors [210, 211].

More recently, several groups have focused their attention on the implications of microglial cells that colonize the embryonic telencephalon at the very beginning of its formation in rodent and human (see above; [63, 212]). Through local phagocytic activities and the release of various molecules (such as interleukin-1beta or tumor necrosis factor-alpha), microglial cells have been shown to regulate neurogenesis, to participate in axonal and dendritic organizations and pruning [212–216]. From early stage of colonization, microglial cells have been shown to express, at least, the 5-HT_{2B} receptor and at later stages or upon stimulation (such as inflammation), several other 5-HT receptors have been detected in rodent (5-$HT_{1F,2A,2B,3B,5A}$ and 5-$HT_{7;}$ [170]). The activation of these receptors has been shown to regulate their motility, their phagocytic properties and selective reshaping of axonal and dendritic arborizations. For instance, 5-HT_{2B} has recently been shown to induce synaptic refinement of retinal projections to the thalamus since this process is impaired in mice lacking 5-HT_{2B} selectively in microglial cells [171].

During early development, the serotoninergic system is challenged by various genetic and epigenetic factors such as medications altering 5-HT transporter function, by nutrition and stress including ischaemia/hypoxia. In this section, we review how these factors may induce the emergence of various pathological disorders in primate and human.

6. Serotonin imbalance and consequences in human pathology

6.1. Serotonin imbalance and 5-HT_3 receptor modulation in human pathology

Developmental imbalance of 5-HT homeostasis or serotonin receptor signalling impacts various processes involved in the formation of cortical circuits and has consequences on the emergence of abnormal behaviour in rodent. Some similarities have been detected in primate and

human but many aspects remain to be tested, in particular, the cellular processes implicated (conditioned by SERT or 5-HT receptors expressions) and the time windows of vulnerability.

In human, three major causes of 5-HT imbalance leading to psychiatric diseases have been clearly identified: abnormal metabolism of 5-HT, exposure of fetuses to SSRIs and genetic inheritance of SERT variants (these points of vulnerability have been indicated in **Figure 5**). Following the discovery of the lack of MAOA in Norrie disease [217], abnormal regulation of the enzymes implicated in 5-HT metabolism has been known for long to be associated with neuropsychiatric diseases (recently reviewed by Naoi et al. [218]). However, it is not known whether the alteration in prenatal or post-natal human life induces such illness. Pharmacological SSRIs treatment gave clearer answers. Indeed, SSRIs during pregnancy are still largely used among women ((2–13%) [219]); despite the high incidence of mood disorders in pregnant women (around 20% of pregnant women are affected) and the deleterious effect of maternal stress on fetal development. However, SSRIs crossing the placenta, are detectable in breast milk, reach the developing brain. Both, short-term (e.g. fetal cardiovascular malformations) and long-term drawbacks of the treatments have been revealed (see below). During gestation, SSRIs induced a reduction of blood flow in the middle cerebral artery at GW36 [220] and reduced fetal head growth [221]. SSRIs induce reduced motor movements and altered speech perception at 6–10 months of age, increased irritability, and persistent blunted pain reactivity [222, 223]. Children exposed to SSRIs during pregnancy have poor scores on psychomotor developmental scales [224] and higher risks to develop autism spectrum disorders [225]. The risk appeared higher when exposure to SSRIs occurred during the second trimester and with higher dosage of SSRIs, suggesting deleterious effects on early neural circuit formation. The third well-known cause of excessive 5-HT-signalling in human is of genetic origin. There are two variants of SERT alleles leading to different levels of SERT expression: the short form that induces decreased levels of SERT expression and SERT hypofunction [41] and the long form. Hypofunctional s-allele has been shown to increase the risk for a wide range of psychopathological traits. When combined with maternal anxiety during pregnancy, infants and children carrying the s-allele showed higher levels of negative emotionality compared to l-allele carriers [42] and increased scores of anxiety and depression [43, 226]. Interestingly, platelets that bear SERT (generally accepted to be identical to neuronal SERT), VMAT2 and 5-HT_2 receptors have been suspected to play a role in the emergence of autistic disease in human. Dysregulation in platelets function has been largely used as a marker of autism, however clarifications need to emerge from further studies (for review, see Refs. [227, 228].

Although the consequences are subtle, they reveal that both genetic and environmental SERT deficiency impact human development and increase the risks of future psychiatric diseases [229, 230]. Overall, these findings point to the general conclusion that various clinical pathological traits, including autism, depression and anxiety-related phenotypes are associated to conditions of SERT deficiency during development. One should also consider that alteration of other genes may have synergistic effect on the emergence of those diseases or by contrast that bearing allelic variants of other genes could dampen the negative effects of SSRIs [231].

Rodent studies have revealed that the 5-HT_{3A} regulates cellular events involved in cortical circuit formation (see above). Human genetic studies have recently explored more deeply the involvement of 5-HT_{3A} polymorphisms and methylation in the emergence of various pathological

traits and they now provide compelling evidence for such a role. In human genetic studies, it has been shown that a single-nucleotide polymorphism in 5-HT_{3A} (SNP; rs1062613) was associated with bipolar disease [232]. Moreover, allelic variants or specific levels of methylation of the 5-HT_{3A} have been shown to be tightly linked with alcohol-dependence, modulation of emotional networks and increase of depressive-related symptoms [233]. The emergence of depressive-like diseases was associated at the structural level with a decreased grey matter in the fronto-limbic region. Interestingly, 5-HT_{3A} has been shown to interact with the brain-derived neurotrophic factor, a key factor for circuit formation and consolidation [234, 235]. Thus, genetic polymorphism or methylation of 5-HT_{3A} appears as a marker of susceptibility to develop a large panel of diseases.

Together, this further confirms complex connections between early-life stress and the serotoninergic systems.

6.2. Linking serotoninergic system and neonatal inflammation/ischaemia with the emergence of neuropsychiatric diseases in children and adults

Early-life inflammation modulates adulthood-inflammatory response [236]. In early brain injuries, activation of the immune system during fetal and neonatal life affects critical phases of brain development, with long-lasting consequences for neurological and mental health [237]. Neonatal stroke, systemic infection, or excitotoxicity/hypoxia-ischaemia (see **Figure 5**) induce perinatal insults activating the immune system and trigger peripheral and central responses that involve immune mediators (cytokines and chemokines), reactive oxygen species (ROS), reactive nitrosative species, excitotoxicity, mitochondrial impairment, and vascular integrity. In general, neonatal encephalopathy is of complex aetiology, encompassing several causal events, with strong evidence of fetal exposure to infection. The complex and multifactorial process of perinatal brain injury involves sensitization, whereby factors not severe enough by themselves to induce significant brain damage make the developing brain more susceptible to a second insult [238]. Substantial numbers of preclinical studies have demonstrated the sensitizing effects of gestational or neonatal systemic inflammation, gestational chronic mild maternal stress, and gestational hypoxia on perinatal excitotoxic or hypoxic-ischaemic lesions. Genetic factors have also been shown to influence the developing brain's response to sensitizing factors. Efforts to design therapies aimed to reduce the sensitizing effects of inflammation have been undertaken as neuroprotective agents, such as therapeutic hypothermia which have been performed mainly in models of pure hypoxia-ischaemia [238]. One of the main alterations following perinatal infection/inflammation is a persistent low-grade inflammation characterized by higher expression of inflammatory mediators and also microglial reactivity during adulthood [236]. Adult rodent exposed during early-life to LPS-enhanced expression of CD11b, IL-1β and IL-6 and also more activated microglia in the hippocampus, the striatum and substantia nigra/ventral tegmental area [239, 240]. This persistent low-grade inflammation sensitizes the brain to secondary injuries, which can lead to neurological disorders such as cerebral palsy, mood disorder, schizophrenia, or Parkinson disease [241].

Serotoninergic central system is vulnerable following a neonatal hypoxic-ischemic insult induced in a rat model [242] with a significant reduction in 5-HT levels, 5-HT transporter expression and 5-HT+ neurons is the dorsal raphe, 6 weeks after insult compared to control

animals. Inhibition of neuroinflammation by Minocycline within the first week after injury is sufficient to prevent long-term neuroinflammation as well as serotonergic system damage still. The loss of dorsal raphe 5-HT+ neurons has been suspected to be induced by an alteration of one of their major target tissues: the prefrontal cortex [243].

7. Conclusion and perspectives

Both genetic and environmental factors that influence serotonin signalling during specific sensitive periods of development impact specific cellular events involved in the development of cortical circuits. Such alterations depending on the cellular target and the time of occurrence could result in a predisposition to a large spectrum of cognitive or psychiatric illnesses including autism and depression.

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Chapter 6

Association of 5-HT_{1A} Receptors with Affective Disorders

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Abstract

Serotonin or 5-hydroxytryptamine (5-HT) is synthesized in both the brain and peripheral system, which exert their actions at a wide family of receptors classified as 5-HT_1 to 5-HT_7. Pharmacological, behavioral, and clinical studies involve particularly to the 5-HT_{1A} receptors (5-HT_{1A}-R) - auto-receptors (presynaptic) and heteroreceptors (postsynaptic) - in the control of motivated behavior, and consequently in the physiopathology of affective disorders and in the action mechanism of antidepressant drugs. In this way, some research support that 5-HT_{1A}-R participates in the delayed effect of different types of antidepressants, including selective serotonin reuptake inhibitors (SSRIs), and tricyclic drugs, principally. The therapeutic effect of serotonergic drugs as the SSRIs, starting with the binding to auto-receptors, which produces increases of 5-HT in the synaptic cleft as consequence of blockade of serotonin reuptake. While these molecular events occur initially, in the long-term are produced plastic changes at neuronal level, as well as down-regulation of the 5-HT_{1A}-R, which is associated with the therapeutic effects of antidepressant drugs. The purpose of this chapter is to analyze and discuss the current information about of 5-HT_{1A}-R-mediated signaling cascades, the intracellular signaling of 5-HT_{1A}-R, in addition to their expression and pharmacology that are important to treatment of affective disorders symptoms.

Keywords: 5-HT_{1A} receptors, affective disorders serotonin

1. Introduction

5-Hydroxytryptamine (5-HT) regulates many important physiological processes, including body temperature, sleep, appetite, pain, motor activity, and affective disorders. One type of 5-HTergic functions is performed by the release of 5-HT into targeted areas and its action via at least 16 different pre-and postsynaptic 5-HT receptor (5-HTR) [1]. 5-HTRs are subdivided into seven groups—from 5-HT_1-R to 5-HT_7-R—according to their distribution, molecular structure, cell response, and function. Except for the 5-HT_3-Rs, which are ligand-gated ion channels, all other 5-HTR are G-protein-coupled receptors that influence different transduction pathways (**Table 1**). 5-HT_{1A}-R auto-receptors located on the soma of 5-HTergic neurons are key components of the negative feedback loop that inhibits neuronal signaling and 5-HT release [2], while 5-HT_{1A}-R heteroreceptors located on postsynaptic 5-HTergic and non-5-HTergic neurons [3, 4], particularly those in the limbic system, are involved in emotional states.

2. Distribution and ontogeny of the 5-HT_{1A}-R

The 5-HT can interact with different types of receptors, whose effect depends on the activation of different subtypes and location of these [1, 3] (**Table 1**). In this sense, the use of such techniques as ligand binding, immunohistochemistry, and hybridization *in situ* in the brains of the rat, mouse, cat, and human has reported significant levels of 5-HT_{1A}-R in almost all regions of the brain [5–9]. A study performed in cats used positron emission tomography (PET) and 2′-methoxyphenyl-(N-2′-pyridinyl)-p-fluoro-benzamidoethyipiperazin marked with fluorine (MPPF [^{18}F]) in combination with *in vitro* autoradiography with [^{3}H] MPPF, 8-hydroxy-2-(di-n-propylamino)tetralin ([3H] 8-OH-DPAT) and [^{3}H] paroxetine, to visualize the distribution of the 5-HT_{1A}-R. These showed high levels of expression in the hippocampus, cingulate, septum, infralimbic cortex, and raphe nuclei, with low levels being detected in the cerebellum [9]. However, studies with PET using [^{11}C] WAY-100635 reported some regional heterogeneity of the 5-HT_{1A}-R in the human cerebellum [10]. The absence of 5-HT_{1A}-R expression was observed in

Receptor family	Subtype	Mechanism	Cellular response
5-HT_1	1A, 1B, 1D, 1E, 1F	Adenylate cyclase	Inhibitory
5-HT_2	2A, 2B, 2C	Phospholipase C	Excitatory
5-HT_3	3A, 3B, 3C	Ligand-gated ion channel	Excitatory
5-HT_4	54	Adenylate cyclase	Excitatory
5-HT_5	5A, 5B	Adenylate cyclase	Inhibitory
5-HT_6	56	Adenylate cyclase	Excitatory
5-HT_7		Adenylate cyclase	Excitatory

Table 1. Classification and mechanism of the 5-HT receptor.

the cerebellar white matter, while the other regions displayed detectable levels of this receptor. On the other hand, studies of the cellular distribution of this receptor and its messenger ribonucleic acid (mRNA) have reported that approximately 60% of all glutamatergic cells express the transcript 5-HT$_{1A}$-R, and about 25% of cells that express the enzyme glutamate decarboxylase (GAD) contain mRNA for 5-HT$_{1A}$-R [5]. In addition, studies using immunohistochemistry, *in vitro* autoradiography with [^{3}H] 8-OH-DPAT, and *in situ* hybridization have reported mRNA and protein expression for the 5-HT$_{1A}$-R in the pyramidal neurons of layer 2 of the prefrontal, insular, and occipital cortex [9], but labeling with [^{3}H] 8-OH-DPAT is only detected the layers 1 and 2 of the prefrontal and occipital cortex and in the pyramidal neurons of the cloister and the anterior olfactory nucleus. Neurons of the hippocampal CA1 region expressed the mRNA of the 5-HT$_{1A}$-R, and [^{3}H] 8-OH-DPAT labeling was observed in the stratum oriens and stratum radiatum. Low receptor expression was observed in CA3 pyramidal neurons, but the granule neurons in the dentate gyrus contained moderate concentrations of this receptor.

Turning now to the ontogeny of the 5-HT$_{1A}$-R, immunohistochemistry has shown that almost all neurons of the hippocampus begin to express the 5-HT$_{1A}$-R at the end of mitosis [11]. It is well known that at day 5 of postnatal age (P5), this receptor is expressed mainly in the cell bodies, while at day P10 it appears in the cell bodies and proximal apical dendrites. At the end of neuronal maturation (P21), a relatively scarce distribution is seen in the dendrites of the stratum radiatum and oriens of the hippocampus. During the early postnatal development of the hippocampus, glial cells that are positive to S100 (protein saturated ammonium sulfate soluble) and glial fibrillary acidic protein (GFAP) temporarily express the 5-HT$_{1A}$-R and more than 90% of astrocytes that are positive to S100 in CA1, CA3, and the dentate gyrus also show moderate immunoreactivity to the 5-HT$_{1A}$-R in P7, though this decreases sharply in P16. Although the specific distribution of the 5-HT$_{1A}$-R has been studied in different brain regions, this does not ensure that receptor signaling activity will always be proportional to the levels of receptor expression. 5-HT$_{1A}$-R signaling in neurons is important for functionality, and this intracellular effect is regulated by the coupling of second messengers.

3. Presynaptic and postsynaptic 5-HT$_{1A}$-R and their signaling effects

The main electrophysiological response to the activation of the 5-HT$_{1A}$-R in neurons is mediated by the hyperpolarization of K^+ channels [12, 13], which attenuates the propagation of action potentials, causing a consequent decrease in the release of the neurotransmitter. The hyperpolarizing effect is observed in both pre- and postsynaptic terminals; however, the desensitization profiles of those receptors and molecules activated in the pre- and postsynaptic terminals seem to differ. One of the mechanisms that cause desensitization of G-protein-coupled receptors is internalization, and studies have demonstrated the internalization (i.e., transfer of the plasmatic membrane in the cytoplasm) of the 5-HT$_{1A}$ auto-receptors in the dorsal raphe nucleus (DRN) of rats after acute treatment with the specific 8-OH-DPAT agonist to the 5-HT$_{1A}$-R, or with recapture inhibitors of the 5-HT (selective inhibitors of serotonin reuptake, SSRIs). Although this phenomenon has not been observed in the hippocampus, we know that in this structure the 5-HT$_{1A}$-Rs are located in the soma and dendrites of neurons (heteroreceptors) [14]. The

SSRIs in the presynaptic terminals, in turn, increase the release of 5-HT, which binds to the 5-HT_{1A}-R auto-receptors present in the soma of the raphe neurons, thus inhibiting neuronal firing. Subsequently, these auto-receptors are internalized, causing the end of 5-HT_{1A}-R signaling in the presynaptic neurons, and again at onset of the 5-HT release of the rape neurons in the synapse with the dendritic terminals of the postsynaptic neurons. In the absence of the 5-HT_{1A} auto-receptors, the 5HT released binds only to the postsynaptic 5-HT_{1A}-R, thereby eliciting the anxiolytic effect of the SSRIs [15]. On the other hand, agonists to 5-HT_{1A}-R, such as buspirone or flesinoxan, show an antidepressant effect, probably due to the desensitization of the 5-HT_{1A} auto-receptors [16, 17]. Thus, the acute agonist treatment has its effect due to interaction with the auto-receptors present in the soma of the raphe neurons. The hyperpolarizing effect of the activation of this auto-receptor inhibits the release of 5-HT in the presynaptic terminal. It has been reported that under this treatment, the free or excess agonist can activate the postsynaptic (dendritic) 5-HT_{1A}-R, resulting in the inhibition of postsynaptic neurons. Thus, an overstimulation of the receptor by an agonist causes desensitization and internalization of the 5-HT_{1A}-R in raphe neurons, but not in postsynaptic neurons. The absence of 5-HT_{1A} auto-receptors in the presynaptic raphe terminal facilitates neural firing by blocking inhibition by 5-HT, which is attached to the 5-HT_{1A}-R in the postsynaptic neurons and causes the anxiolytic effect.

The activation of both pre- and postsynaptic 5-HT_{1A}-R and their subsequent signaling seems to differ in at least one biochemical pathway. It has been shown that HN2-5 cells derived from neurons in the hippocampus, as well as in organotypic cultures of slices of the hippocampus, which are agonists to the 5-HT_{1A}-R, stimulate the protein kinase pathway activated by mitogen (MAPK) [18]. However, in the raphe-derived cell line RN46A, activation of this receptor by agonists inhibits the basal activity of the MAPK pathway [19]. Nonetheless, it has been reported that activation of the 5-HT_{1A}-R located both pre- and postsynaptically with the agonist inhibits intracellular cyclic adenosine monophosphate (cAMP) [20]. There are also reports that activation of 5-HT_{1A}-R in a postsynaptic neuron-derived cell line and in non-neuronal cells promotes synthesis of phospholipase C (PLC), but this response has not been reported in presynaptic (serotonergic) or raphe-derived neurons [20, 21].

4. Aberrant 5-HT_{1A}-R expression, anxiety and depression disorders

In recent decades, such psychiatric disorders as anxiety (mainly generalized anxiety) and depression (mainly severe) have increased in prevalence and are now responsible for 3.12 and 6.86%, respectively, of years lived with disability (YLDs), according to estimates by the Global Burden of Diseases in 2015. Anxiety is a normal human emotion that allows us to respond to everyday stress situations, where the stressor—work, for example—can be identified. However, anxiety becomes a disorder when it no longer allows the individual to remain functional in her/his daily activities and when no trigger can be identified [22]. Both anxiety and depression have been attributed to a varied etiology that includes the person's social, economic, family, employment and academic condition, combined with the persistence of an inherent biological factor. In this sense, the findings of clinical and preclinical studies have identified a dysfunctionality of the serotonergic system associated with low availability

of L-tryptophan (a precursor of 5-HT), low concentrations of 5-hydroxyindoleacetic acid (5-HIIA)—the main metabolite of 5-HT in the cerebrospinal fluid—a reduction in the synthesis, release, recapture, and metabolism of 5-HT, a decrease in the density of 5-HT$_{1A}$-R pre- and postsynaptic, low neural activity in brain areas involved in regulating the emotions (such as the septum and prefrontal cortex), factors that increase the propensity (serotoninergic vulnerability) to suffer mood disorders like anxiety and depression. This is reinforced by the fact that serotonergic antidepressant treatments are prescribed to reverse these types of alterations [23–25]. In addition, functional brain imaging and postmortem studies of the limbic structures of depressed patients—which are responsible for integrating the emotions, and include the striatum, amygdala, and frontal cortex—have reported a low capacity for recapture 5-HT coupled with a decrease in the expression of 5-HT (5-HTT) transporters, which are responsible for recapturing the unused 5-HT in the 5-HT synapse and so regulate the magnitude and duration of serotoninergic neurotransmission [26]. Alterations of this kind in the 5-HTT have also been detected in patients with major depression using PET, which reveals a low capacity for 5-HT recapture in the thalamus, an area involved in controlling cortical excitability that contributes to establishing anxiety in patients so affected [27].

In addition, the involvement of deregulation of pre- and postsynaptic 5-HT$_{1A}$-R in anxiety and depression is widely known, since it has been observed in patients with panic disorder by PET studies. There, reports indicate a reduction in the availability of both pre- and postsynaptic 5-HT$_{1A}$-R in brain areas that regulate cognitive and emotional responses, such as the raphe, the orbitofrontal cortex, the temporal cortex, and the amygdala [28]. In support of this, preclinical studies have reported that knockout mice for 5-HT$_{1A}$-R present an anxious phenotype that includes observations of such behaviors as a decrease of thigmotaxis (i.e., exploratory activity in central areas of an open field), increased fear in aversive environments, increased reactivity to stress, autonomic activation, and neuroendocrine alterations in models of experimental anxiety using the open-field, elevated-zero maze, and novel-object tests. However, an antidepressant-like effect has been observed in the tail suspension model of experimental depression, more markedly in females than in males. This is not associated with morphological abnormalities in brain tissues or changes in cell bodies or 5-HTergic fibers, nor is there evidence of changes in brain levels of 5-HT and 5-HIIA in the striatum, dorsal raphe, or frontal cortex [29, 30], though there is an increase in the turnover of 5-HT [31] and the firing of 5-HTergic neurons [32] in knockout mice to 5-HT$_{1A}$-R. However, the possibility of such long-term changes cannot be discarded [33]. This situation can be interpreted as a disinhibition of 5-HTergic neuronal activity that increases the release of 5-HT in limbic areas, causing the establishment of anxiety through its interaction with other receptor subtypes, but without modifying levels of 5-HT or its metabolite, since the amount of stored 5-HT greatly exceeds the extracellular 5-HT content.

In support of this, differences in the function of the pre- and postsynaptic 5-HT$_{1A}$-R in different brain areas seem to be decisive in establishing anxiety and depression, given that stimulation of the postsynaptic 5-HT$_{1A}$-R in the dorsal hippocampus and amygdala produces anxiogenic effects, while anxiolytic effects are seen in areas such as the middle and dorsal raphe (where the 5-HT$_{1A}$ auto-receptors are located) [33–35]. In contrast, stimulation of the presynaptic receptors produces anxiolytic effects by suppressing 5-HTergic neuronal

activity with the resulting decrease of 5-HT in axonal terminals of limbic areas [36]. These findings suggest that there are differences in the role played by pre- and postsynaptic 5-HT_{1A}-R receptors in regulating emotions. This may be reflected in the fact that acute administration of antidepressants causes a reduction in neural activity due to the immediate stimulation of the 5-HT_{1A} auto-receptors, while chronic antidepressant treatments cause desensitization and, consequently, the downregulation of the 5-HT_{1A} auto-receptors, though with no changes in postsynaptic 5-HT_{1A}-R. This leads to the recovery of 5-HTergic neuronal activity, which matches the long latency to the onset of the therapeutic effects of SSRIs antidepressants.

It is important to note that mice require proper 5-HTergic signaling through 5-HT_{1A}-R stimulation of the prosencephalon during the early postnatal period as this produces lasting chemical and structural changes in the brain that are essential for effective response behaviors in the face of normal anxiety during adulthood [37]. Thus, clinically effective antidepressant or anti-anxiety treatments must stimulate the 5-HT_{1A} auto-receptors with direct agonists (such as buspirone) or indirect agonists like fluoxetine to obtain therapeutic efficacy. This suggests that in both the developmental and adult stage efficient activation of the 5-HT_{1A} auto-receptors can produce changes that decrease expressions of pathological anxiety.

Donaldson et al. [38] reported that a decrease in the 5-HT_{1A} auto-receptors in the 21st postnatal leads to increased long-term anxiety levels but does not modify depressive behaviors. In this regard, lifelong abolition of the 5-HT_{1A} auto-receptors suffices to increase anxiety behaviors in adult mice [39], though without necessarily affecting depressive-like behaviors in the forced swimming test [40]. Based on these results, it has been suggested that 5-HT_{1A} auto-receptors are involved in establishing anxious and depressive phenotypes, while the heteroreceptor is implicated in the depressive phenotype observed in experimental tests of depression [40]. Moreover, Albert and François [41] suggest that a reduction in the activity of postsynaptic receptors is involved in anxiety and that an increase in the transcription of 5-HT_{1A} auto-receptors is associated with both depression and resistance to chronic treatment with SSIR drugs [41]. Hence, the reduced expression of the auto-receptors with no modification of postsynaptic 5-HT_{1A}-R expression is enough to produce depression-like behaviors in mice [42].

5. Therapeutic agents that function by regulating 5-HT_{1A}-R signaling

5-HT_{1A}-R is involved in the pathology and treatment of mental disorders, such as anxiety and depression [23, 43, 44]. Several studies have suggested that the 5-HT_{1A}-Rs are potential targets for these psychiatric disorders [45–49]. In this regard, agonists (total and partial) to the 5-HT_{1A}-R have shown antidepressant and anxiolytic properties and have been employed as adjunct treatments to improve the therapeutic action of several antidepressant and anxiolytic drugs in several preclinical and clinical studies [50–53]. They offer a different pharmacological mechanism from that of the monoamine oxidase inhibitors (IMAO), tricyclic drugs, SSIRs, and other antidepressants.

Buspirone is perhaps the most widely studied partial 5-HT$_{1A}$-R agonist. It belongs to the chemical class of the azapirones [54, 55] and has been used primarily due to its anxiolytic effects and absence of side effects such as sedation and dependence that are often associated with benzodiazepines [56]. It is also utilized to treat patients who are resistant to the SSRI$_S$, due to its capacity to stimulate the release of catecholamines [57]. In this regard, a clinical trial carried out with ambulatory patients diagnosed with generalized anxiety disorder (GAD) found that after weeks 3 and 4, buspirone showed efficacy in relieving patients' symptoms with a therapeutic effect comparable to that of lorazepam. Also, after discontinuing this therapy, the individuals treated with buspirone showed no withdrawal symptoms, while those medicated with lorazepam saw their symptoms worsen in week 9 after ceasing treatment [58]. Similarly, buspirone (15 mg/day) prescribed for 4 weeks to ambulatory patients with GAD produced a significant reduction of anxiety symptoms compared to alprazolam. Moreover, the patients treated with buspirone experienced fewer adverse effects and symptoms of abstinence than those who received alprazolam [59]. The anxiolytic properties of buspirone have been confirmed in animal models. For example, in a study conducted with Swiss Albino mice that received buspirone at 2.5 and 5 mg/kg, i.p., the drug significantly increased the number of step-through by 46 and 61%, respectively [60]. This demonstrates that buspirone is effective in treating anxiety disorders without causing adverse effects or signs of benzodiazepine dependence.

Gepirone is another component of the class of the azapirones that has shown antidepressant properties [61] due to its partial 5-HT$_{1A}$-R antagonism, which improves 5-HTergic activity [62]. The structure of this azapirone is similar to that of buspirone, and it has similar anxiolytic properties that have been identified in clinical studies [63, 64]. But it also has antidepressant action. In a study of patients with major depressive disorder (DDM), prolonged-release gepirone (60–80 mg/day) administered for 3 weeks produced a significant reduction in total HAM-D17 scores (Hamilton Depression Scale) compared to a placebo group, thus improving the symptomatology of patients [65]. Similarly, gepirone (40–80 mg/day) prescribed for 8 weeks improved the sexual function of male patients diagnosed with DDM, in addition to its antidepressant action [66].

Tandospirone is a partial 5-HT$_{1A}$-R agonist that has been shown to have antidepressant effects. In a study with male Sprague-Dawley rats, chronic treatment (28 days) with tandospirone at 10 mg/kg inhibited changes induced by psychosocial stress in the neurogenesis of the dorsal and ventral hippocampus, thus producing a type of antidepressant effect. It has been suggested that chronic administration of tandospirone desensitizes the 5-HT$_{1A}$-R in the raphe. This decreases self-inhibition mediated by the somatodendritic receptor and, consequently, increases the firing rate and release of 5-HT [67].

Brexpiprazole is a second-generation antipsychotic that exerts partial antagonism to the 5-HT$_{1A}$-R and D2. A study in adults diagnosed with DDM, but inadequate responses to antidepressants, showed that brexpiprazole as an adjunct therapy improved patients' symptoms. In that research, a series of drugs—escitalopram, fluoxetine, paroxetine, sertraline, duloxetine, and venlafaxine—all significantly improved scores on the Clinical Global Impressions Scale (CGI-I scale), Zung Self-Rating Depression Scale (SDS), and HAM-D17

scale when administered jointly with brexpiprazole (2 mg) for 6 weeks. Improvement was remarkable from the first week of treatment [68]. Finally, flesinoxan is a phenylpiperazine derivative initially developed as an antihypertensive [69]. This drug has total antagonism to 5-HT_{1A}-R with high affinity [70]. Various studies have demonstrated its antidepressant properties, particularly in treatment-resistant DDM patients [71]. For example, in a double-blind, placebo-controlled and fixed-dose study of treatment-resistant DDM patients, flesinoxan (1.2 mg/day) administered for 6 weeks improved scores on the HAM-D17, Montgomery-Asberg Depression Rating Scale (MADRS) and CGI scales with improvement in subjects' mood. Nausea and dizziness were the most common side effects reported [72]. The therapeutic effects of flesinoxan have also been reported in animal models. In research with male Sprague-Dawley rats after olfactory bulbectomy, subjects were given flesinoxan (1 and 3 mg/kg, s.c.) for 17 days. They presented reduced total immobility time on the forced swimming test [73]. This therapeutic action may be associated with the desensitization effect of the 5-HT_{1A}-R in the nucleus of the dorsal raphe as an action mechanism [71].

The antidepressant activity of agonists to the 5-HT_{1A}-R in presynaptic and postsynaptic neurons has been widely reported. Studies using the model of experimental learned helplessness in relation to depression have reported that stimulation of the 5-HT_{1A}-R with 8-OH-DPAT at dosages of 0.03, 0.06, 0.125, 0.25, and 1 mg/kg i.p. for 5 days shows an antidepressant effect. To explore the role of the pre- and postsynaptic 5-HT_{1A}-R, in that study, 8-OH-DPAT (0.1 and 1 μg/0.5 μl) was microinjected into the raphe and septum. While this showed an antidepressant effect when microinjected into the septum, no such effect was seen in the raphe of male rats [74]. This indicates that stimulation of the postsynaptic 5-HT_{1A} receptors is responsible for establishing the antidepressant effect caused by 5-HT_{1A}-R agonists when managed through a systemic pathway, since stimulation of the 5-HT_{1A} somatodendritic auto-receptors in the raphe inhibits the release of 5-HT and the electrical activity of the raphe [75].

In recent years, administration of vilazodone has shown antidepressant [75, 76] and anxiolytic effects by eliminating physical and somatic symptoms in women with generalized anxiety disorder, after 8 weeks of treatment at daily doses of 20–40 mg [77, 78]. This effect is due to the action mechanism of this SSRI, which is a partial agonist of postsynaptic 5-HT_{1A} receptors. In addition, it desensitizes 5-HT_{1A} auto-receptors in the raphe more quickly than fluoxetine or paroxetine [79], is 30 times more powerful than serotonin transporter (SERT), and causes a larger increase of extracellular 5-HT in the ventral hippocampus and frontal cortex [80]. These facts justify the short latency to the appearance of therapeutic effects. Similar data have been reported in models of experimental anxiety using ultrasonic vocalizations. Observations suggest that vidazolam produces an anxiolytic effect that can be reversed by coadministration with an antagonist of the presynaptic 5-HT_{1A} receptors such as WAY-100635.

This substance also produced an anxiolytic effect in the model of predator-induced stress at doses of 20–40 mg/kg and in the defensive burial model at doses of 10–40 mg/kg. However, no anxiolytic effect was seen in the elevated arms maze model [81]. Antidepressant effects at doses of 1 mg/kg were found in models of experimental depression based on the forced swimming and tail suspension tests [82].

6. Recent advances in the use of serotonin-norepinephrine reuptake inhibitors (SNRIs) for treating affective disorders

Affective disorders are characterized by vigorousness in neurotransmission pathways at the cerebral level with reductions in serotonergic, noradrenergic, and dopaminergic concentrations, among other neurochemical and neuroanatomical changes. Consequently, therapeutic strategies designed to treat affective disorders include combinations of drugs and, in other cases, chemical compounds that act on one or more neurotransmission systems [83]. In this way, serotonin-norepinephrine reuptake inhibitors (SNRIs) have the capacity to block serotonin and noradrenalin reuptake in the brain, and so have been used successfully to treat such affective disorders as depression, emotional disorders like anxiety, and other illnesses related to the control of overweightness, fibromyalgia, peripheral diabetic neuropathic pain, and attention deficit-hyperactivity disorders, among others (**Table 2**).

The SNRIs were introduced into therapeutic use in the USA in 1993 under the name venlafaxine, a chemical compound included in a group of molecules named phenylethylamines, whose action mechanism principally involves the reuptake inhibition of serotonin and noradrenaline, though a lower degree of dopamine reuptake inhibition has also been reported. Through their dual action, these substances quickly increase concentrations of both neurotransmitters, apparently producing better therapeutic actions in major depression disorders than conventional antidepressant drugs that act upon only a single neurotransmission system. But SNRIs can produce side effects that include loss of appetite, reduced body weight and sleep, fatigue, headaches, nausea/vomiting, sexual dysfunction, and urinary retention, among others. To a lesser degree, they can also produce anxiety and high blood pressure. It is important to point out that some patients treated with SNRIs have increased suicidal thoughts, though this is still subject to controversy [84]. Despite their side effects, SNRIs are used frequently to control several depressive disorders due to their therapeutic efficacy.

Active compound	Therapeutic use	Reference
Venlafaxine	MDD, AD, syndrome of chronic pain, BDD	[85, 86]
Desvenlafaxine	MDD in adult patients	[89]
Duloxetine	MDD, DPNP, fibromyalgia	[96, 104, 107]
Atomoxetine	ADHD in adults and pediatric patients under 6 years old	[103, 108]
Sibutramine	Treatment of obesity	[106]
Milnacipran	MDD, fibromyalgia	[85, 105]
Levomilnacipran	MDD, AD in adult patients	[90]

Abbreviations: MDD, major depression disorder; BDD, bipolar depression disorder; AD, anxiety disorder; DPNP, diabetic peripheral neuropathy pain; ADHD, attention deficit hyperactivity disorder.

Table 2. Principal serotonin-norepinephrine reuptake inhibitors and their therapeutic uses.

Indeed, in some cases they work better than classic antidepressant drugs (e.g., SSRIs and tricyclic drugs) in certain groups of patients. For example, a clinical study of patients diagnosed with major depression disorder (aged 18–65) found remission of symptoms after 24 weeks of treatment with venlafaxine (initial dose of 75 mg/day, maximum dose of 225 mg/day) and milnacipran (50 mg twice a day), with a greater effect than that produced by 20 mg/day of the SSRI paroxetine [85]. However, in patients diagnosed with Alzheimer's and major depression disorders, the SSRIs sertraline and venlafaxine had a greater effect than the tricyclic antidepressant desipramine, all at doses of 150 mg/day during 12 weeks of treatment [86]. In a randomized, double-blind, parallel group study that evaluated the effect of long-term treatment (12 weeks) with venlafaxine in adult patients, there was a significant reduction of depressive symptoms compared to patients under the same conditions but treated with a lithium monotherapy [87]. Another SNRI used to treat major depression disorder is desvenlafaxine [88]. An integrated analysis of the efficacy of this drug found that treatment with 50 and 100 g/day reduced depression symptoms in patients diagnosed with major depression disorder compared to a placebo group [89].

Similarly, treatment with levomilnacipran (40–120 mg) in patients aged 18–80 diagnosed with some depression disorder, significantly reduced symptoms after 8–10 weeks of treatment [90]. These data show that the effect of SNRIs in treating major depression disorders depends on the characteristics of patients and the dosage schedule. One double-blind, controlled, randomized study compared two treatment schedules with venlafaxine: one fixed (75 mg/day) the other flexible (75–225 mg/day). It found that the fixed program gave a better response to this antidepressant treatment than the flexible approach [91]. Similarly, the use of SNRIs in young depressed patients (7–18) did not produce better therapeutic effects than a placebo treatment, though duloxetine has shown therapeutic potential in such patients [92]. A meta-analysis of the efficacy of venlafaxine, duloxetine, fluoxetine, and imipramine in children and adolescents found that SNRIs and tricyclic antidepressants do not seem to offer a significant advantage in treating major depression disorder in this population, as only fluoxetine produced an adequate therapeutic effect in those patients [93].

SNRIs are also often used to treat depressive symptoms associated with menopause. It is well known that in this biological phase, women are more susceptible and vulnerable to socio-environmental factors that predispose them to develop emotional and affective disorders [94]. Menopausal women diagnosed with major depression disorders and vasomotor symptoms treated with duloxetine for 8 weeks experienced a reduction in their depressive and vasomotor symptoms, positive anxiolytic effects, and improved sleep quality, so it is believed that SNRIs may be an effective therapeutic option for treating mood and emotional disorders, as well as the more general symptoms associated with menopause [95]. In addition to its role as an effective treatment for major depression disorders associated with menopause, duloxetine is used to control other symptoms, such as hot flashes and anxiety [96]. Meanwhile, menopausal women treated with venlafaxine (75–300 mg/day) or fluoxetine (20–60 mg/day) felt a reduction in their depressive symptoms after 6 weeks of treatment, with no significant differences between these two antidepressants [97]. Administration of desvenlafaxine (50, 100, or 200 mg/day) to peri- and postmenopausal women also reduced depressive symptom compared to a placebo [98].

7. Mechanism of action of selective serotonin reuptake inhibitors (SSRIs) and affective disorders

The action mechanism of SSRIs consists in inhibiting the 5-HT transporters (SERT) in the soma of raphe dorsal neurons (**Figure 1**). It has been shown that SSRIs, such as fluoxetine, that have an antidepressant effect possess a mechanism that inhibits SERT, thus increasing the availability of 5-HT in the synaptic cleft. This is accompanied by an increase in 5-HTergic neurotransmission associated with the establishment of the antidepressant effect [99]. This pharmacological effect is not immediate, suggesting that the 5-HT$_{1A}$ transporter blockade, *per se*, does not produce therapeutic effects during acute treatment, since in the first week of antidepressant therapy with SSRIs increases 5-HTergic neurotransmission due to the availability of 5-HT, which causes an overstimulation of the 5-HT$_{1A}$ auto-receptors, located in the cell body and dendrites of neurons in the raphe. Therefore, its neuronal activity, which is in charge of releasing 5-HT, is reduced in limbic areas, though we know that treatment with SSRI antidepressants requires 2–3 weeks to establish its therapeutic effect, because regulation of 5-HTergic neurotransmission in depressed patients requires the desensitization and subsequent internalization of the 5-HT$_{1A}$ auto-receptors of presynaptic neurons that eliminate the negative feedback on the raphe, thus increasing its neuronal activity and normalizing the release of 5-HT to the synaptic cleft that, finally, translates into an antidepressant effect.

The postsynaptic mechanism and cellular signaling of the 5-HT$_{1A}$-R in relation to mood control are very complex. In this regard, it has been reported that some accompany the establishment of the therapeutic effect of SSRI antidepressants. One of the most important effects is the desensitization of the 5-HT$_{1A}$ auto-receptors. Normally in 5-HTergic neurotransmission, once the 5-HT is released into the synaptic cleft, it mainly has a three-point coupling. The

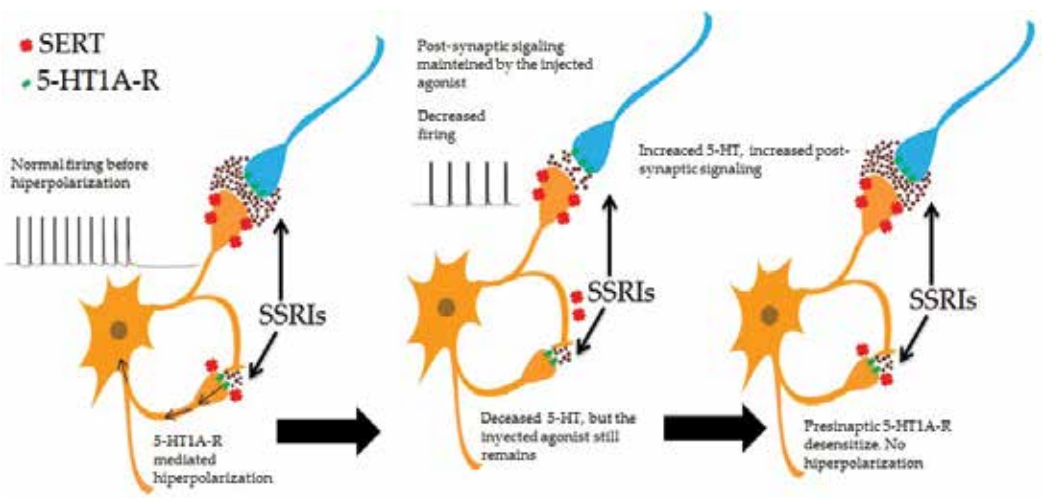

Figure 1. Mechanism of SSRIs: the 5-HT transporters (SERT) in the soma of raphe dorsal neurons; modified according to Garcia-Garcia et al. [40].

first is to the postsynaptic serotonergic receptors, mainly 5-HT_{1A}. These receptors are coupled to the inhibition of protein G (Gi/o) and the consequent decrease in AMPc synthesis due to the inhibition of adenylate cyclase which, in conjunction with other second messengers, are responsible for activating the opening of ion channels, including Na^+ and K^+, for its input and output, respectively (**Figure 2**). This contributes to the hyperpolarization of the postsynaptic neurons so that they can go with the flow of neural inhibition. The second coupling is with the SERT, which are responsible for the reuptake of unused synapse 5-HT, which is returned to the presynaptic neuron through recycling, where it is stored for later release or to be metabolized to reset the synthesis of 5-HT. The energetic cost of its production is very high. The third coupling is with the 5-HT_{1A} auto-receptors and, to a lesser extent, 5-HT_{1B} and 5-HT_{1D}. This causes inhibition of the opening of Ca^{2+} channels from the presynaptic neuron, which then inhibits the release of 5-HT into the synaptic cleft, thus regulating the intensity and duration of the nerve impulse from the presynaptic neuron (i.e., negative feedback or self-inhibition), mainly in neurons of the raphe, exerting the end the signaling of the presynaptic neurons and the resumption of the release of 5-HT neurons from the raphe to the postsynaptic neurons through the limbic areas [100]. In this context, chronic administration of SSRIs induces internalization of the 5-HT_{1A} auto-receptors and the neurons of the raphe [101], since the increase in the availability of 5-HT in the cleft overstimulates those auto-receptors while also desensitizing and internalizing them. This process is associated with the phosphorylation of the carboxylic chain

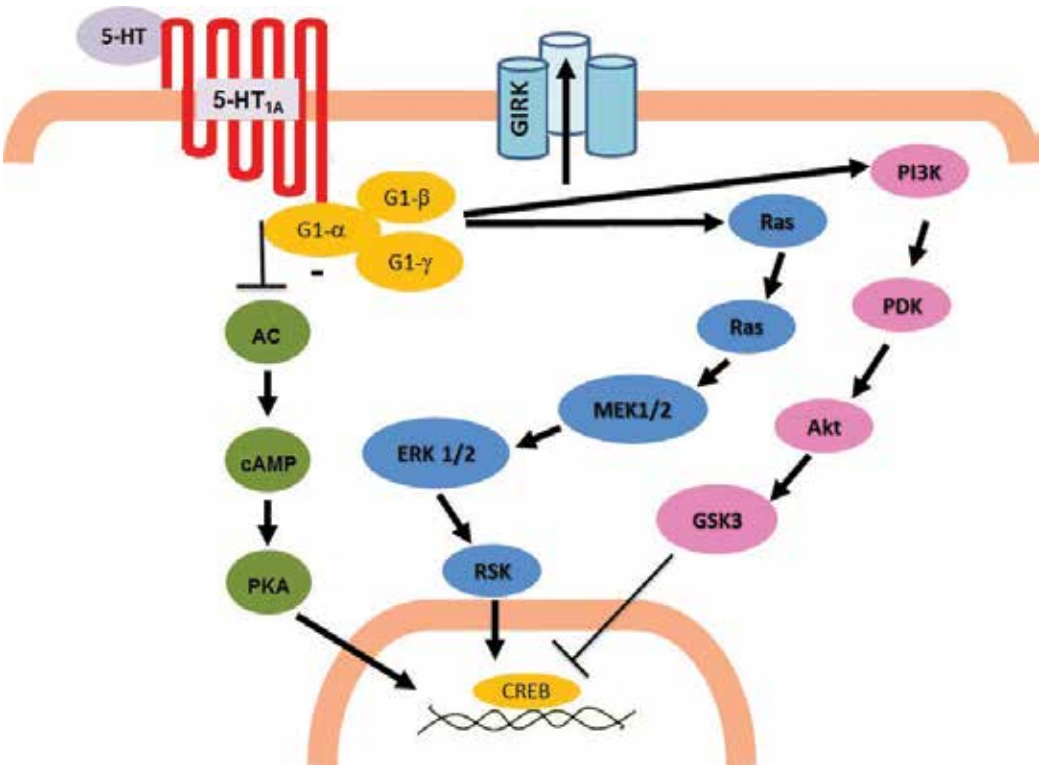

Figure 2. Model of the transduction pathways that may be activated by the 5-HT_{1A}-R; modified according to Polter and Li [100].

and the third intracellular loop of the receptor. The absence of 5-HT$_{1A}$ auto-receptors induces the binding of 5-HT only to postsynaptic 5-HT 1A receptors, which in turn triggers the antidepressant effect of SSRIs, though only after 2–3 weeks of treatment. However, this desensitization effect on the auto-receptors depends on the type of SSRIs administered, as it has not been observed when sertraline is administered chronically in humans [102].

8. Conclusion

Multiple antidepressant drugs are known to function through the 5-HT$_{1A}$-R. New findings related to dysfunctions in the serotoninergic system, specifically in both pre- and postsynaptic 5-HT$_{1A}$-R in the signaling pathways that modulate the 5-HT$_{1A}$-R, demonstrate that 5HTergic alterations—whether in the expression or functionality associated with such disorders as anxiety and depression, and their subsequent association with alterations in signaling pathways that indirectly modulate and involve survival and neuronal development—can interfere with responses to antidepressant treatment. However, we require additional studies that accurately identify signaling mechanisms in different brain areas and differentiate their functions between the pre- and postsynaptic 5-HT$_{1A}$-R present in intact animals and animals subjected to clinically effective antidepressant and anti-anxiety treatments. Since we know that differences in the distribution of receptors in the brain determine the physiological and behavioral functions, a better understanding of the underlying mechanisms associated with abnormal activity of the 5-HT$_{1A}$-R will contribute to the search for novel therapeutic strategies that explore new ways of enhancing treatment of the most common psychiatric disorders around the world, including those of anxiety and depression, which severely impair the quality of life of individuals.

In general, the participation of the 5-HT$_{1A}$-R in psychiatric disorders such as anxiety and depression has been widely explored in numerous clinical studies and animal models. All findings seem to indicate that including agonist components to the 5-HT$_{1A}$-R in drug treatment of individuals with anxiety and depression is a promising option for improving the efficiency and implementation of the therapeutic effect of conventional drugs. It is important to emphasize that stimulation of the 5-HT$_{1A}$-R activates indirect signaling mechanisms that have not yet been studied, so further research is necessary to explore possible alternative signaling mechanisms that accompany the establishment of the antidepressant effects mediated by 5-HT$_{1A}$-R. Finally, in order to better understand the etiology of many disorders of brain development and advance in the elaboration of drugs that target 5-HT$_{1A}$-R, it is important to study the profile of this receptor's activity in brain signaling during development.

In summary, there is ample clinical evidence to support the idea that SNRIs may be used to treat major depression disorder and other psychiatric disorders in certain groups of patients. However, the scarcity of controlled clinical studies and the wide age range of patients included in existing work, in addition to the scarce comparisons of the effects of SNRIs and classic antidepressant drugs (e.g., SSRIs and tricyclic antidepressants), raise the challenge of determining whether SNRIs produce greater, similar, or lower therapeutic effects than traditional therapeutic schedules. Nonetheless, the data currently available open doors for future research designed to explore new therapeutic options that will benefit patients with major depression disorders or other affective or emotional alterations.

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Section 3

Metabolism

Chapter 7

Application of 5-HT-SO_4 in Biomarker Research

Raimond Lozda

Additional information is available at the end of the chapter

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Abstract

A serotonin catabolite, serotonin O-sulphate (5-HT-SO_4), is hypothesised to accentuate the intensity of serotonin metabolism in the central nervous system (CNS). We hypothesised that serotonin O-sulphate could be quantified in human plasma using modern liquid chromatography-mass spectrometry. To test our hypothesis, we performed a critical literature review and a three-stage trial. First, a suitable liquid chromatography-mass spectrometry (LC-MS/MS) method for detection of 5-HT-SO_4 in human plasma samples was developed. Second, a pilot phase involving four healthy volunteers was executed. Finally, nine healthy volunteers were selected for the main study, where a basal plasma level of 5-HT-SO_4 was measured before and after serotonergic stimulation of the central nervous system. One h after stimulation, six study subjects showed a decrease in 5-HT-SO_4 levels, while three subjects showed an increase. This was the first study in which naturally occurring 5-HT-SO_4 was detected by liquid chromatography–mass spectrometry (LC-MS/MS) in the samples of human plasma obtained from healthy volunteers. The method developed was specific to the measurement of 5-HT-SO_4 and opens up new possibilities to evaluate minor pathways or serotonin metabolism by minimally invasive methods.

Keywords: serotonin, serotonin O-sulphate, biomarkers, depression

1. Introduction

For several decades, it is noted that biomarkers are playing an increasingly important role in drug discovery and development from target identification and validation to clinical application, thereby making the overall process a more rational approach.

Indisputably, serotonin (5-HT) plays a significant role in the course of depressive disorders, and majority of the drugs developed interferes with this pathway. Therefore, we decided to ascertain the metabolic processes to find perspective areas of research based on experience gathered so far. The most exploited laboratory biomarker methods investigating central nervous system

(CNS) processes use the cerebrospinal fluid (CSF) as far as it is site specific and reveals local processes. Thus, measurement of indoleamine metabolites in the cerebrospinal fluid (CSF) remains an analytical evaluation method of drug efficacy during clinical trials. Nevertheless, due to patient's safety concerns and clinical convenience, a method employing less invasive approach is highly appreciated by health care professionals.

Thus, our objective was based on critical literature review approach and practical liquid chromatography-mass spectrometry (LC-MS/MS) method implementation to investigate a possibility to use 5-HT-SO_4 as a potential CNS-specific serotonin metabolism biomarker based on a less invasive laboratory method suitable for clinical and pharmacological studies.

2. Literature evidence related to 5-HT-SO_4 appearance in animal and humans

Sulphonate conjugation was first described in 1876 and has since been shown to be a significant pathway in the biotransformation of many neurotransmitters.

During a critical literature review, our special attention was drawn to the final phase of 5-HT degradation by sulphotransferases (SULT) and the end product of biotransformation, 5-HT-SO_4. We identified 66 papers and excluded 51 papers that did not contain data related to 5-HT-SO_4. In total, 15 papers were included in the final review with 10 analysed in terms of outcomes.

We found that during the last century, a sulphation of serotonin was described by Kishimoto and a final product of such biotransformation, serotonin-O-sulphate, was found [1]. Furthermore, during later years in animal experiments, it was approved that 5-HT-SO_4 is the final product of serotonin metabolism which is rapidly excreted from the organism [2, 3]. Later, a similar compound was found in human urine, cerebrospinal liquor and platelets [4–6]. During recent years, some research was done with marine molluscs determining 5-HT-SO_4 in their nervous system [7, 8]. Findings showed that the serotonin metabolite 5-HT-SO_4 forms from 5-HT uptake and metabolism in central ganglia and other structures of nervous system but not in haemolymph itself.

Table 1 summarises the evidence related to 5-HT-SO_4 appearance in animals and humans.

As shown in **Table 1**, 5-HT-O-SO_4 was intensively investigated by G.M. Tyce during the 1980s and 1990s. Initially, a considerable amount of acid-hydrolysable conjugates of dopamine, norepinephrine (NE) and 5-HT were detected in lumbar CSF of normal individuals. The amounts of conjugated amines were small in comparison to the amounts of homovanillic acid and 5-hydroxyindoleaceticacid [9]. In a further study performed with CSF from humans and ventriculocisternal perfusion of African green monkeys found that the sulphates of NE, dopamine and 5-HT are present in the CSF of laboratory animals and humans. All amines and metabolites were quantitated by using high-performance liquid chromatography (HPLC) with electrochemical detection. The amounts of sulphated amines in human CSF always greatly exceed the amounts of the free amines [6]. This gave us a preliminary impression of 5-HT-SO_4 site

Date	Research name	Results	Analytical methods applied
2009	Analysis of intact glucuronides and sulphates of serotonin, dopamine, and their phase I metabolites in rat brain microdialysates by liquid chromatography-tandem mass spectrometry	The LC-MS/MS method was validated by determining the limits of detection and quantitation, linearity and repeatability for the quantitative analysis of 5-HT and DA and their glucuronides, as well as of 5-HIAA, DOPAC and HVA and their sulphateconjugates.	LC-MS/MS
2008	5-HT and 5-HT-SO_4 but not tryptophan or 5-HIAA levels in single feeding neurons track animal hunger state	Changes in levels of 5-HT-SO_4 in the metacerebral giant neurons of Pleurobranchaea californica related to feeding were observed	Capillary electrophoresis with laser-induced wavelength-resolved fluorescence detection (CE-LIF)
2007	Serotonin catabolism in the central and enteric nervous systems of rats upon induction of serotonin syndrome	Serotonin sulphate showed surprisingly large increases in rat intestinal tissues after induction of serotonin syndrome	*Capillary electrophoresis with laser-induced native fluorescence detection (CE-LINF)*
2004	Systemic serotonin sulphate in opisthobranch molluscs	5-HT-SO_4 forms in neural cells. Not detected in haemolymph	Capillary electrophoresis (CE) system
2003	Serotonin catabolism depends upon location of release: characterisation of sulphated and gamma-glutamylated serotonin metabolites in Aplysia californica	The pathway of serotonin inactivation with further formation of 5-HT-SO_4 depends upon the type of neuronal tissue subjected to neurotransmitter incubation	CE-LIF LC-MS
1988	Presence of phenolsulphotransferase activity in microvascular endothelial cells: formation of 5-HT-O-sulphate in intact cells	Existence of phenolsulphotransferase in the endothelial cells and formation of 5-HT-SO_4verified	Not available
1986	Amine sulphate formation in the central nervous system	Origin of the central nervous system of amine sulphates (also 5-HT-SO_4) in monkeys and humans observed. 5-HT-SO_4 was detected in CSF of monkeys and humans but not in the plasma	High performance liquid chromatography (HPLC) with electrochemical detection
1985	Free and conjugated amines in human lumbar cerebrospinal fluid	5-HT-SO_4 was detected in the CSF of healthy humans	HPLC with electrochemical detection
1983	Exploration of the role of phenolsulphotransferase in the disposition of serotonin in human platelets: implications for a novel therapeutic strategy against depression	Existence of phenolsulphotransferase in the platelets and formation of 5-HT-SO_4 verified	The assay technique-purified alveolysin toxin
1966	Isolation of serotonin-O-sulphate from human urine	5-HT-SO_4 isolated from human urine	Ion exchange resins

Table 1. Summary of the evidence found related to 5-HT-SO_4 appearance in animals and humans.

specificity. At the time of above mentioned studies, 5-HT-O-SO_4 could not be detected in the plasma of untreated monkeys and the concentration of 5-HT-O-SO_4 in brain perfusates versus plasma increased after injection of 5-HT sulphate. The ratio of amine sulphate in the brain versus amine sulphate in plasma was greater for 5-HT-O-SO_4 than for DA-O-sulphate at 60 and 100 min after injection. Finally, it was concluded that although 5-HT-O-SO_4 could not be detected in the plasma of monkeys or humans under normal conditions, the 5-HT-O-SO_4 in ventriculocisternal perfusates undoubtedly originates in the CNS [6].

This obstacle inspired us to analyse more recent studies selected during review.

Some research was conducted with marine molluscs determining 5-HT-O-SO_4 in their nervous system [7, 8].

In one such research done in 2003, incubation of neuronal tissue of Aplysia revealed three novel 5-HT catabolites. **Figure 1** summarises the metabolism of 5-HT found in Aplysia central ganglia compared to human.

As seen from **Figure 1**, there is no difference of 5-HT-O-SO_4 formation between humans and molluscs.

As shown in **Table 2**, the 5-HT-O-SO_4 can be detected in CNS and its formation though is site specific, and later animal studies confirm detection in periphery. Moreover, as far similar sulphotransfares exist in sea molluscs and mammals, an equal process should be theorised for humans [10]. Also, literature evidence exists that 5-HT-SO_4 was proposed to be measured in the animal urine or plasma which makes it to be relative simply detected by HPLC with various detectors [11].

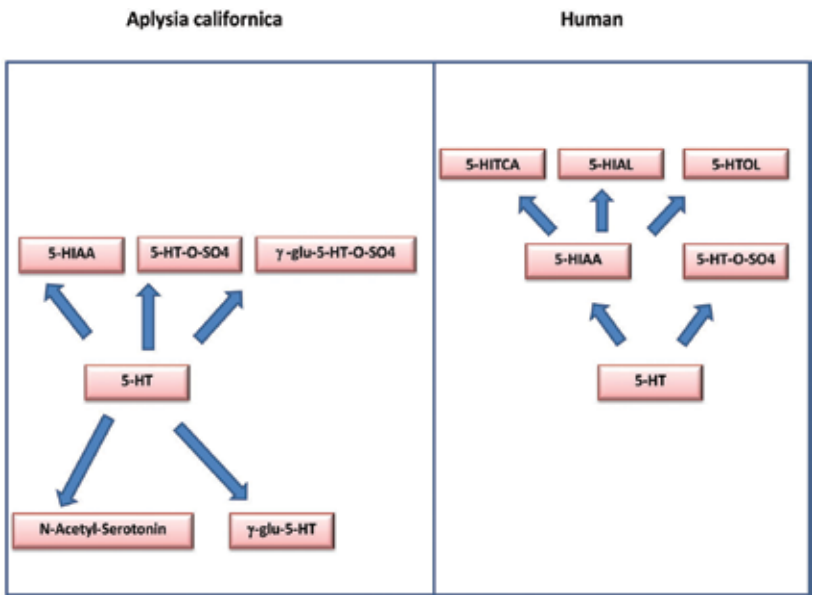

Figure 1. Metabolites of 5-HT in the marine mollusc *Aplysia californica* versus humans. The novel metabolites are shown for *Aplysia californica*. Only detailed 5-HIAA metabolism is shown for humans.

Trial	Quantity of trial subjects	Species	5-HT-O-SO_4 qualified	5-HT-O-SO_4 quantified	5-HT-O-SO_4 found in nervous system	5-HT-O-SO_4 found outside nervous system	Conclusions
Tyce et al. [9]	22	Humans	No. defined as conjugate of 5-HT	No. defined as conjugate of 5-HT	No. defined as conjugate of 5-HT	No	CSF contains 5-HT conjugate
Tyce et al. [6]	12/4	Humans/ animals	Yes	Yes	Yes	No	5-HT-O-SO_4 originates from CNS
Stuart et al. [7]	N/A	Animals	Yes	No	Yes	No [c]	5-HT-O-SO_4 formation is nervous-system specific
Stuart et al. [8]	11	Animals	Yes	Yes	Yes	Yes	5-HT-O-SO_4 forms in the nervous system but not in haemolymph itself
Uutela et al. [12]	N/A	Animals	Yes	Yes	Yes	No	5-HT-O-SO_4 in rat brain microdialysates was analysed using a direct LC-MS/MS method

Table 2. Summary of trials involving 5-HT-O-SO_4.

Summarising the literature review, there is evidence of similarities between human and animal metabolic pathways, and as far as there is literature evidence of site-specific 5-HT-SO_4 formation in animals, we can extrapolate the same to humans.

We noted that historically used methods assumed highly invasive approach of CSF sample collection from human subjects. Therefore, search for a minimally invasive method has significant clinical benefit. Moreover, the latest 5-HT-SO_4 research revealed experience with LC-MS application for such a purpose.

3. The latest findings related to 5-HT-SO_4 appearance in humans

The evidence in the scientific literature justifies the decision to employ detection of 5-HT-SO_4 in clinical practice. As far as there was no literature data particularly on 5-HT-SO_4 detection by LC-MS/MS in the human plasma, we initiated a development of the chromatography method based on literature evidence related to detection of similar compounds such as indoleamines in the human plasma. The objective concerning analytical procedure was to demonstrate that it is suitable for its intended purpose—qualitative detection of 5-HT-SO_4 in the human plasma.

Tandem mass spectrometric analysis (MS/MS) was made in a positive-ion mode (ESI+). The electrospray ionisation of 5-HT-SO_4 was weak. Thus, for the further quantitative analysis, a following ion transition was used: (257>>160) + (240>>160).

Specificity of the method was assessed visually by comparing multiple reaction monitoring (MRM) chromatograms of plasma sample spiked with serotonin O-sulphate, samples of plasma and purified water. As seen in **Figure 2**, in the plasma-based calibration standard (A) and plasma (B), some 1.79–1.80 min retention time peaks can be observed [13]. In the plasma-based calibration standard and "pure" plasma, some peaks with a retention time of 1.79–1.80 min were observed. The purified water samples treated similarly do not show such signals (C).This signal might be induced by native content of serotonin sulphate found in plasma samples. Conclusion was reached because in the analytical solution made of 5% serum albumin, such a signal was not seen [13]. For the MRM chromatograms, the test solution of 5% serum albumin (buffered to pH = 7 in a phosphate buffer) was prepared.

The results obtained lead to conclusion that the method developed is specific to the compound of interest, 5-HT-SO_4.

The results obtained lead to the conclusion that the method developed is specific to the compound of interest, 5-HT-SO_4, in samples of human plasma. The linearity of detection was evaluated three times in different days by analysing calibration standard solutions of 5-HT-SO_4 [13].

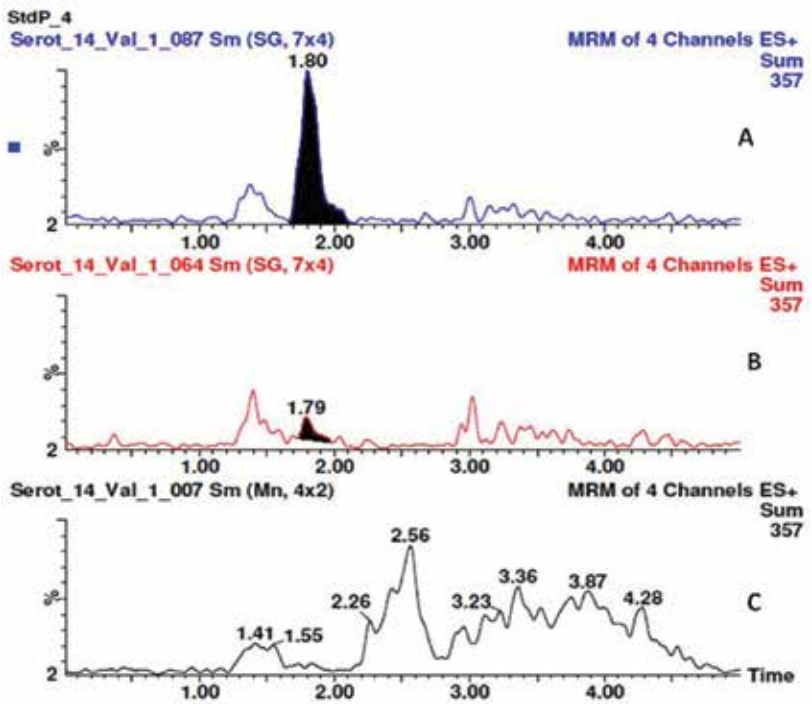

Figure 2. Chromatograms of 5-HT-SO_4 samples. (A) Plasma standard solution (containing 96 ng/mL of 5-HT-SO_4); (B) "pure" plasma sample and (C) water.

This method resulted in a linear relationship between concentration of the analyte (from 10 to 225 ng/mL) and a mass spectral signal of 5-HT-SO_4 with a calibration curve correlation coefficient of >0.98.

The optimal detection limit of 5-HT-SO_4 in the plasma sample was determined to be 26.5 ng/mL. Four different concentrations of 5-HT-SO_4 were used for a recovery testing and the method gave correct 5-HT-SO_4 detection results, which were justified by the average level of recovery of the analyte at 116 ± 8%. The relatively high interval of recovery can be explained due to the matrix effect [13].

The intra-laboratory accuracy of the method over a 3-day period was characterised by a standard deviation of ± 11.95% [13]. Taking into account above mentioned results, the method was concluded to be a suitable technique for measuring 5-HT-SO_4 in human blood samples.

The findings of method development phase led to the decision to perform the first-in-humans study in order to assess the clinical applicability of the LC-MS/MS method developed, and the studies were designed to quantify intra-individual results using a cohort of healthy subjects. The clinical study had a two-stage design: a plot study and main Study.

The pilot study confirmed that the peaks with retention time 1.79–1.80 min are detected in the samples of plasma of healthy volunteers. These peaks corresponded to the signal of 5-HT-SO_4. One study subject was exposed to oral intake of L-5-hydroxytryptophan (5-HTP) containing food supplement to observe the influence of serotonergic stimulation to 5-HT-SO_4 level. The pilot study proved that the 5-HT-SO_4 could be qualified in plasma samples obtained from healthy volunteers. The increase in 5-HT-SO_4 level after serotonergic stimulation was observed. Unfortunately, all results were below the detection limit of the method and probably due to several matrix-, method-, or analyte-specific reasons.

Our primary interest was to ascertain quantitative differences of basal 5-HT-SO_4 levels, the intra-individual sensitivity of the quantitation as well as detection limit issues obtained in the pilot study on a larger number of subjects.

Thus, after measurement of the basal 5-HT-SO_4 levels in nine subjects, all of them were exposed to serotonergic stimulation with a food supplement containing 100 mg of 5-HTP and a second blood sample was analysed. In six study subjects, a decrease in 5-HT-SO_4 levels was observed 1 h after 5-HTP ingestion. Three subjects, however, showed an increase in 5-HT-SO_4 1 h after 5-HTP ingestion. Out of nine study subjects and one pilot subject, an increase in 5-HT-SO_4 1 h after 5-HTP ingestion was observed in three women and one man, respectively. The others—five men and one woman—showed decrease. A graphical chart of study results is shown in **Figure 3**.

The outcome of our studies is that we developed a liquid chromatography method, which is specific to the measurement of 5-HT-SO_4 in the samples of human plasma. It is the first time when 5-HT-SO_4 was detected in the plasma obtained from healthy volunteers [14]. The sensibility of the LC-MS/MS method to detect intra-individual changes of the compound in the healthy volunteers undergoing supplementation with 5-HTP was observed, but the majority of results were below detection limit [13].

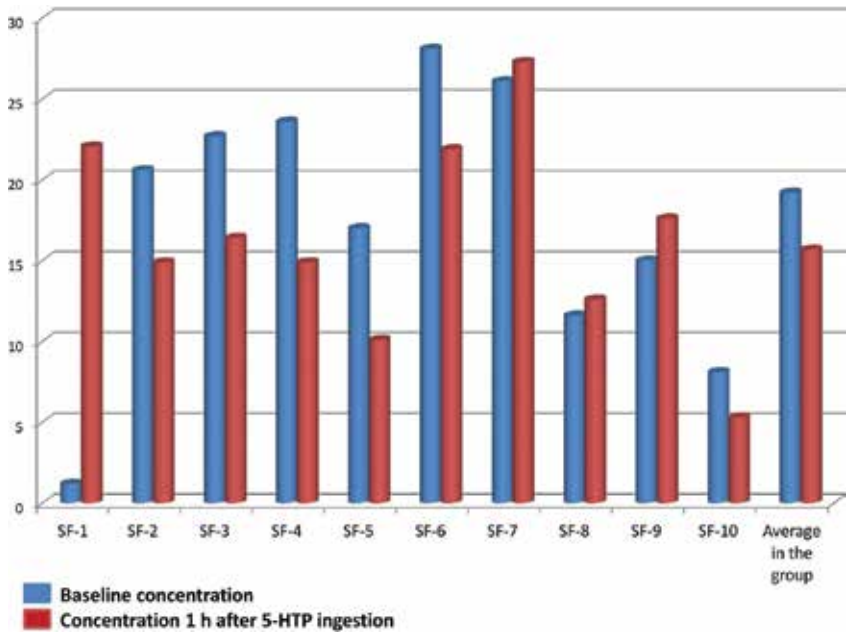

Figure 3. A graphical view of studies results.

4. Conclusions and discussion

This chapter ascertains a possibility for **5-HT-SO$_4$** to be employed as a potential serotonin metabolism biomarker based on a less invasive laboratory method. Based on critical literature review, **5-HT-SO$_4$** is identified as a potential 5-HT metabolism biomarker to be detected by a minimally invasive approach in human plasma. The novel LC-MS/MS method, which is specific to the measurement of **5-HT-SO$_4$** in the human plasma, has been developed [13]. It was the first time when **5-HT-SO$_4$** was detected in the plasma obtained from healthy volunteers [13]. However, the clinical applicability of the method was not justified as the majority of results were below detection limit of 26.5 ng/mL [13].

Concerns regarding the issue of whether 5-HT-SO$_4$ we found in the plasma has CNS origin or not should be evaluated. It is known that L-amino acid decarboxylase acts both in the periphery and in the CNS which can result with the ingested 5-HTP being converted into serotonin in the periphery of the body too [15], but plasmatic serotonin mostly derived from peripheral tissues is primarily metabolised in the liver to 5-hydroxyindole acetate and then excreted in the urine [16, 17]. Regarding 5-HT locating in the gastrointestinal tract, it is known that once serotonin reuptake transporter (SERT) has brought serotonin into the epithelial cells, it is metabolised to 5-HIAA by monoamine oxidase which is localised to all intestinal epithelial cells [18]. Alternatively, 5-HT released into the lamina propria may enter the portal vein circulation and be detected either as free serotonin or within platelets (via the actions of SERT). As the liver processes the portal circulation, enzymes rapidly degrade the

free 5-HT. The monoamine oxidase degrades about one-third to urine detectable 5-HIAAn. The remaining two-thirds of serotonin is degraded to the metabolite 5-HT-O-glucuronide. It should be noted that 5-HT taken up by platelets is protected from degradation in the liver and enters the general blood circulation [18]. Coincidentally, the only sites of 5-HT sulphation identified in humans are the CNS and enteric nervous systems [6, 19] while enteric nervous system 5-HT-SO_4 was found only after induction of serotonin syndrome [19]. Some isoforms of SULT have been shown to have sulphate serotonin [20]. Also, findings in sea molluscs indicate that metabolism of serotonin with further formation of 5-HT-SO_4 depends upon the location of release. Thus, haemolymph 5-HT- SO_4 most probably originates from the nervous system [7, 8]. There is also no doubt about the entrance of ingested 5-HTP into CNS [21]. Also, earlier studies concluded that under normal conditions, the 5-HT-O-SO_4 originates from CNS [6]. The most significant finding was that 5-HT-O-SO_4 freely crosses blood-CSF barrier, so physiological circumstances are not preventing the appearance of CNS-originated 5-HT-O-SO_4 in the venous blood circulation. Therefore, taking into account all aforementioned facts, we are more concerned that 5-HT- SO_4 detected in the study [13] mimics serotonin metabolism in CNS. Future investigations are needed to justify this assumption.

The most disputable outcome of our research is the elevation or reduction of 5-HT-SO_4. The majority of volunteers from the study phase, six out of nine, had a drop of plasma sulphate concentration [12]. Although there is no data available regarding diurnal rhythmicity of serotonin sulphate levels in the human plasma, we are not able to confirm whether these changes are due to the direct influence of serotonergic stimulation. To investigate the possible link to the health status of study subjects with 5-HT-SO_4 level changes, we performed questioning. Hamilton depression rating scale results revealed mild depression in four subjects. Unfortunately, we cannot make any clinical conclusion related to the correlation between symptoms and laboratory findings due to results below the detection limit, but the trend seems to be very intriguing.

Nevertheless, in the light of literature data, we tend to explain phenomena observed by substrate inhibition of SULT 1A3 [22, 23]. It could be concluded that under normal circumstances, quantity of serotonin synthesised and metabolised is kept under certain limits [20]. Data favouring this is evidence of paradoxical actions of the 5-HTP on the activity of identified serotonergic neurons in a simple motor circuit. It was found that more serotonin did not lead to more potent swim motoraction, implying that serotonin synthesis must be kept withincertain limits for the circuit to function properly. Also, alteration of neurotransmitter synthesis can lead to grave consequencesfor the output of neuralnetworks [24]. Described mechanisms could be taken into account explaining our results. Thus, we hypothesise that a drop of 5-HT-SO_4 in plasma would be related to the overproduction of serotonin, leading to inhibition of SULT 1A3. Elevation of 5-HT-SO_4 was probably a sign of serotonin deficiency, but such an opinion also requires further investigation. The latter would correlate with the experiment made in sea molluscs when hungry animals had significantly higher levels of serotonin and 5-HT-SO_4 than their satiated partners [25]. It remains for future investigations to determine whether serotonin sulphate found in plasma has central nervous system origin and the reason for elevated or lowered 5-HT-SO_4 levels after serotonergic stimulation [13].

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Chapter 8

Energy Homeostasis by the Peripheral Serotonergic System

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Additional information is available at the end of the chapter

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Abstract

Energy homeostasis is maintained by balancing energy intake and energy expenditure. In addition to the central nervous system, several hormones play a key role in energy homeostasis in the whole body. In particular, serotonin is regarded as one of the key regulators of energy homeostasis. Serotonin is unique in that it is able to act in both the brain as a neurotransmitter and the peripheral tissue as a gastrointestinal hormone. In the brain, serotonin is thought of as a pharmacological target for anti-obesity treatments because it greatly inhibits meal size and body weight gain. In contrast, serotonin in the periphery has not been targeted as a strategy for anti-obesity treatment, even though almost all of the serotonin produced in the body is produced in the peripheral tissue. Recently, the peripheral serotonergic signal has been shown to regulate glucose and lipid metabolism through autocrine and paracrine signals in energy homeostasis-related tissues, including the pancreatic β cell, liver, white adipose tissue, brown adipose tissue, and skeletal muscle. Thus, it is possible that the serotonergic system in the peripheral tissue is a new therapeutic target for metabolic disease, including obesity and diabetes. Here, we summarize the role of peripheral serotonin in the regulation of energy homeostasis.

Keywords: peripheral serotonin, energy homeostasis, obesity, pancreatic β cell, adipose tissue, skeletal muscle

1. Introduction

Serotonin is a monoaminergic neurotransmitter that modulates central and peripheral functions. Serotonin has an association with food intake, sleep, anxiety, sexual behavior, and mood in the central nervous system, and about 2% of the body's serotonin is stored here. On the other hand, around 98% of the body's serotonin is found peripherally, where it functions

as a peripheral hormone. It affects vasoconstriction, intestinal motility, primary hemostasis, liver repair, and the control of the T-cell-mediated immune system [1–4].

The synthesis of serotonin from tryptophan begins with the enzyme tryptophan hydroxylase (TPH), which is also the rate-limiting enzyme in its biosynthesis. It is reported that TPH has two isoforms, TPH1 and TPH2 [5]. TPH1 mainly exists in the pineal gland, thymus, spleen, and enterochromaffin cells of the gastrointestinal tract. TPH2 is found only in neuronal cells, such as in the raphe nuclei of the brain stem. Moreover, serotonin is thought not to be able to pass the blood-brain barrier. Therefore, there are thought to be two independent serotonin systems in the body: one in the central nervous system and the other in the periphery.

Since serotonin has been shown to affect fat metabolism and feeding behavior, through independent molecular mechanisms in *Caenorhabditis elegans* [6], serotonin has therefore been suggested to contribute to energy homeostasis with independent modulation from the central nervous system. There are several peripheral tissue serotonin receptors (Htr's), and TPH1 has been shown to be expressed in peripheral tissues, which are related to energy metabolism of not only the gut but also pancreatic β cells and adipose tissue [7, 8]. The roles of serotonin in energy metabolism in these tissues have been further exposed after these discoveries. In the following section, the function of serotonin in peripheral tissues is summarized (**Figure 1**).

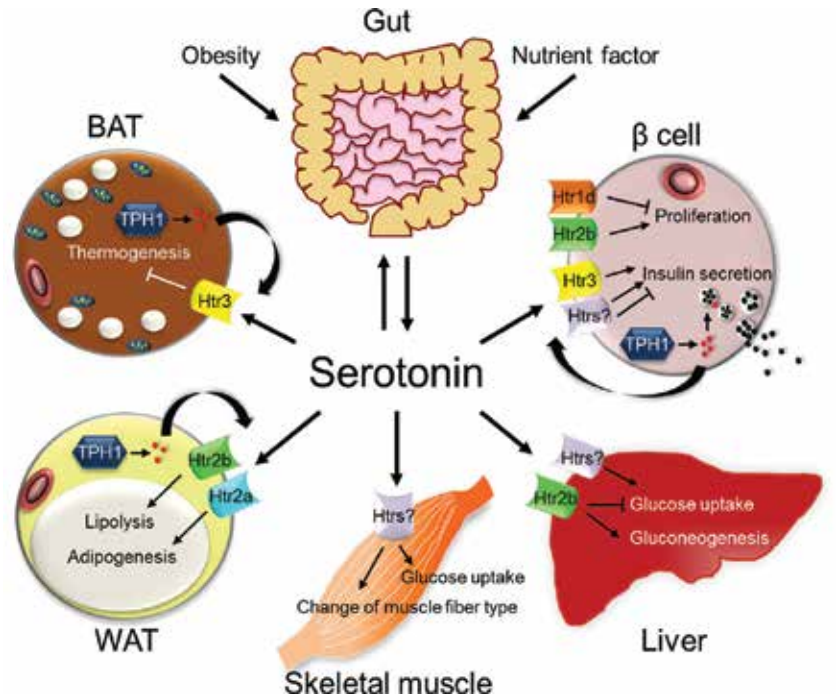

Figure 1. Role of serotonin in peripheral tissues related to energy homeostasis. Peripheral serotonin is mainly provided from the gut and regulates glucose and lipid metabolism through endocrine, autocrine, and paracrine matter. Gut-derived serotonin suppresses glucose uptake and promotes gluconeogenesis through Htr2b. Serotonin regulates proliferation and insulin secretion in pancreatic β cell through several Htr's. Serotonin is also provided from adipocyte and may increase energy storage and adipogenesis in WAT and inhibit adaptive thermogenesis in BAT. BAT, brown adipose tissue; Htr, serotonin receptor; TPH, tryptophan hydroxylase; and WAT, white adipose tissue.

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2. Role of serotonin in insulin secretion

2.1. Insulin and glucose metabolism

Insulin is secreted from pancreatic β cell and plays a key role in glucose homeostasis. Generally, insulin regulates plasma glucose level by suppression of gluconeogenesis in the liver and induces glucose uptake in the skeletal muscle and adipose tissue. Obesity induces insulin resistance in these tissues and glucose intolerance. To compensate for insulin dysfunction, the β cells increase their mass and secretion of insulin. The failure of compensation for this insulin resistance eventually results in type 2 diabetes.

Recently, serotonin has been implicated insulin secretion in the β cell. Pancreatic β cells express both Tph1 and Tph2 and synthesize serotonin [7, 9]. Indeed, mice lacking Tph1 ($Tph1^{-/-}$) have impaired insulin secretion and are characterized as mildly diabetic [9]. Additionally, it has been reported that serotonin injection elevates plasma insulin levels [10, 11]. Therefore, serotonin might regulate insulin secretion from β cells by both local and systemic actions.

2.2. Insulin secretion by intracellular serotonin function

Serotonin is synthesized and localized within β cell and co-released with insulin following stimulation by glucose [12, 13]. $Tph1^{-/-}$ mice have a normal pancreas mass and islet size. On the other hand, the level of serotonin in the pancreas is decreased by 90% compared with that of wild-type mice [9]. Insulin secretion in the $Tph1^{-/-}$ mice is suppressed, and high blood glucose and insulin resistance are observed. The adjustment of insulin secretion of this animal model has connection with GTPases (Rab3a and Rab27a) and serotonylation. The concentration of intracellular Ca^{2+} is raised by glucose stimulation that activates transglutaminase (TGase). The serotonylation of GTPases is promoted by TGase, and exocytosis of insulin granule is induced. $TGase2^{-/-}$ mice deteriorate β cell function as well as that of $Tph1^{-/-}$ mice [14]. Consequently, these data suggest that intracellular serotonin controls glucose-stimulated insulin secretion (GSIS) through modification of GTPase.

2.3. Insulin secretion by extracellular serotonin function

2.3.1. Insulin secretion by Htr3

It has been reported that extracellular serotonin regulates insulin secretion through several Htr's. First of all, Htr3 is a ligand-gated cation channel [15]. Htr3 deletion mice have normal β-cell mass and amount of insulin. There is no change when Htr3 deletion mice are fed normal-fat diet, but impaired insulin secretion and glucose intolerance are shown when a high-fat diet (HFD) is fed. The islets derived from β-cell-specific Tph1 deletion mice show the same impaired insulin secretion as seen in Htr3 deletion mice, and these recover following serotonin treatment. However, the islets derived from Htr3 deletion mice do not recover. Thus, Htr3 is thought to be necessary in order to maintain normal GSIS from the β cell by serotonin.

2.3.2. Insulin secretion by Htr2b

In addition, other research has determined that Htr2b, G-protein-coupled receptor, also has an impact on GSIS [16]. In human and mouse islets, Htr2b is expressed in β cells but not in α cells. Htr2b knockdown depresses GSIS. Alpha-methylserotonin maleate salt (an Htr2 agonist) increases GSIS in wild-type INS-1 cells, but the effect of this drug does not show itself in Htr2b knockdown INS-1 cells.

2.3.3. Insulin secretion by Htr2c

In contrast, Htr2c was reported to inhibit insulin secretion from pancreatic β cells in a mouse model of diabetes [17]. Htr2c expression is increased in pancreatic islets of db/db mice compared with that of lean mice. Treatment with an Htr2c antagonist increases insulin secretion from pancreatic islets isolated from db/db mice in a dose-dependent manner. This implies that Htr2c controls insulin secretion in diabetic subjects.

2.3.4. Insulin secretion by serotonin transporter

Furthermore, GSIS is obstructed by selective serotonin reuptake inhibitors (SSRIs) [18]. Short-term treatment with SSRIs increases Ser/Thr phosphorylation of IRS-2 and inhibits IRS-2 functions and results in impaired GSIS from murine pancreatic islets. Long-term treatment with SSRIs induces ER stress and cellular apoptosis.

As a result, the former study shows that GSIS is adjusted through the Htr signal and the serotonin transporter, by both extracellular serotonin and intracellular serotonin.

2.3.5. Role of serotonin in insulin function during pregnancy

Pregnancy dramatically changes maternal metabolism. In order to maintain the flow of nutrition to fetus, insulin resistance in the mother increases, resulting in an increasing demand for insulin. In order to compensate for this, the mother enlarges the mass of the β cells and increases the secretion of insulin. This change in insulin secretion during gestation is intimately related to the synthesis of serotonin through lactogenic signaling [9]. Lactogenic signaling is increased during pregnancy (though prolactin and placental lactogen), which raises Tph1 expression in pancreatic β cells and enhances serotonin synthesis. Serotonin in islets regulates insulin function in a paracrine-autocrine fashion during pregnancy. The expression of Htr2b rises in β cells during the pregnancy period, and this returns to normal levels after the delivery of the young. Serotonin is increased during pregnancy, which raises β-cell proliferation and mass through the Htr2b signal. On the other hand, there has been shown to be an elevation in the expression of Htr1d in pancreatic β cells at the end of pregnancy and postpartum. After that, Htr1d restrains the proliferation of β cells.

In addition to the regulation of β-cell mass through Htr2b and Htr1d during pregnancy, insulin release is increased through Htr3 signaling as well in mice on a high-fat diet [19]. Because of the impaired insulin secretion, $Htr3^{-/-}$ mice demonstrate glucose intolerance during pregnancy.

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In conclusion, β-cell mass and function during pregnancy are controlled by serotonin through several Htr's.

3. Role of serotonin in the liver

3.1. The liver and glucose metabolism

The liver has an important role in postprandial nutrient metabolism and in response to food deprivation. In particular, the liver maintains blood glucose levels through degradation of glycogen and gluconeogenesis in the fasted state and through glucose uptake in the fed state. It is known that diabetes is caused by an increase of gluconeogenesis and decline of glucose uptake in the liver. Moreover, the liver also controls the concentration of blood cholesterol and triglycerides. It is suggested that hormones such as insulin and glucagon mainly signal these liver functions.

Serotonin is a gastrointestinal hormone and is directly able to regulate the liver, as it is known that serotonin mediates liver regeneration [20]. Although there is still room for debate, several studies report that serotonin has a connection with glucose and lipid metabolism in the liver.

3.2. Serotonin and gluconeogenesis

Sumara et al. revealed that gut-derived serotonin (GDS) increased gluconeogenesis in the liver through Htr2b [8]. Plasma glycerol, produced by adipose tissue and used for gluconeogenesis, is not increased in gut-specific Tph1 knockout mice during food deprivation, though that is increased in fasted wild-type mice. Additionally, the fat-specific Htr2b knockout mice do not also show an increase in plasma glycerol levels in these fasted mice. Mice lacking Tph1 in the gut demonstrate a reduction in hepatic glucose production during hyperinsulinemic-euglycemic clamps and a decrease in plasma glucose levels during pyruvate tolerance tests. Liver-specific Htr2b knockout mice also show similar phenotype as gut-specific Tph1 knockout mice in glucose metabolism. These data support the idea that serotonin signals play an important role in the control of gluconeogenesis in the liver through Htr2b signaling. Consequently, it is suggested that serotonin provides glycerol to the liver from the adipose tissue through Htr2b and thereby contributes to gluconeogenesis in the liver.

3.3. Serotonin and glucose uptake in the liver

Sumara et al. also report that hepatic glucose uptake decreases in liver-specific Htr2b deletion mice compared with wild-type mice. This is because serotonin is related to the degradation of glucose transporter 2 [8]. Nevertheless, not all studies agree. Injection of serotonin does not impact on the uptake of 2-deoxy-glucose in the liver of fasted mice [10]. Additionally, in an experiment using conscious dogs, portal vein injection of serotonin induced hepatic glucose uptake during a hyperinsulinemic-euglycemic clamp [21]. Agonists of Htr 1/2a reduce blood glucose and increase hepatic glycogen after oral glucose loading. The same study also reported that these agonists stimulate glycogen synthesis in freshly isolated hepatocytes.

Furthermore, serotonin inhibits glycogen synthesis at micromolar concentrations but stimulates it at nanomolar concentrations in hepatocytes [22]. Thus, there are several reports on the control of hepatic glucose uptake by serotonin. It could be argued that the results vary according to the method used: genetic study or in vivo treatment study. Further progress in this field is expected.

3.4. Serotonin and enterohepatic circulation of bile acids

Bile acids are produced from the gallbladder and are deposited into the duodenum following feeding. They are associated with the absorption of nutrients and especially lipids. Nowadays, there is discussion about the role of bile acids with respect to glucose, lipid, and energy metabolism. It is suggested that activation of the farnesoid X receptor (FXR), a bile acid receptor, stimulates the liver concentrations of glycogen [23, 24]. In addition, hepatic triglyceride accumulation, very low-density lipoprotein (VLDL) secretion, and the elevation of serum triglyceride in mouse models of hypertriglyceridemia are impaired by bile acid cholic acid. In brown adipose tissue, administration of bile acids to mice raises energy consumption, preventing obesity and insulin resistance by inducing cAMP-dependent thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (D2) [25, 26].

In the enterohepatic circulation, bile acids are mainly reabsorbed from the ileum and return to the liver through the portal vein. The hepatocytes take up about 80% of this, and the remainder enters the general circulation. Serotonin is known to signal the enterohepatic circulation of bile acids. By stimulating the contraction of the smooth muscle in the gallbladder, serotonin induces the excretion of bile acids in a direct manner from the gallbladder into the duodenum [27, 28]. In addition to the excretion of bile acids from the gallbladder, serotonin is reported to enhance reabsorption of bile acids from the ileum and raise the level of plasma bile acids (**Figure 2**) [10]. Serotonin injection has been shown to cause an elevation of the expression of the apical sodium-dependent bile acid transporter (ASBT), which actively causes the reabsorption of bile acids from the lumen of the intestine into the body and decreases the content of bile acids in the feces. However, ASBT expression is negatively regulated by bile acids and FGF15 through the FXR-FGF15 signaling pathway. These data suggest the possibility that serotonin may increase ASBT expression through the FXR-FGF15-independent pathway.

3.5. Serotonin and lipid in the liver and the circulation

It has been suggested that serotonin may affect the concentrations of lipid in the liver and blood [10]. In practical terms, plasma triglyceride, cholesterol, and nonesterified fatty acid concentrations are reduced following serotonin injection. The same report suggested that the level of the concentration of cholesterol in the liver was increased following the passage of 60 min after serotonin treatment. On the other hand, there was a reduction of the plasma concentration of cholesterol at the same time. These data show that the intake of the cholesterol by the liver from the blood through serotonin stimulation may cause a decrease in the plasma cholesterol concentration.

Besides this, Haub et al. have suggested that serotonin may raise the fat concentration of the liver [29]. Comparing lean control mice, there is an increase of duodenal Htr3a protein

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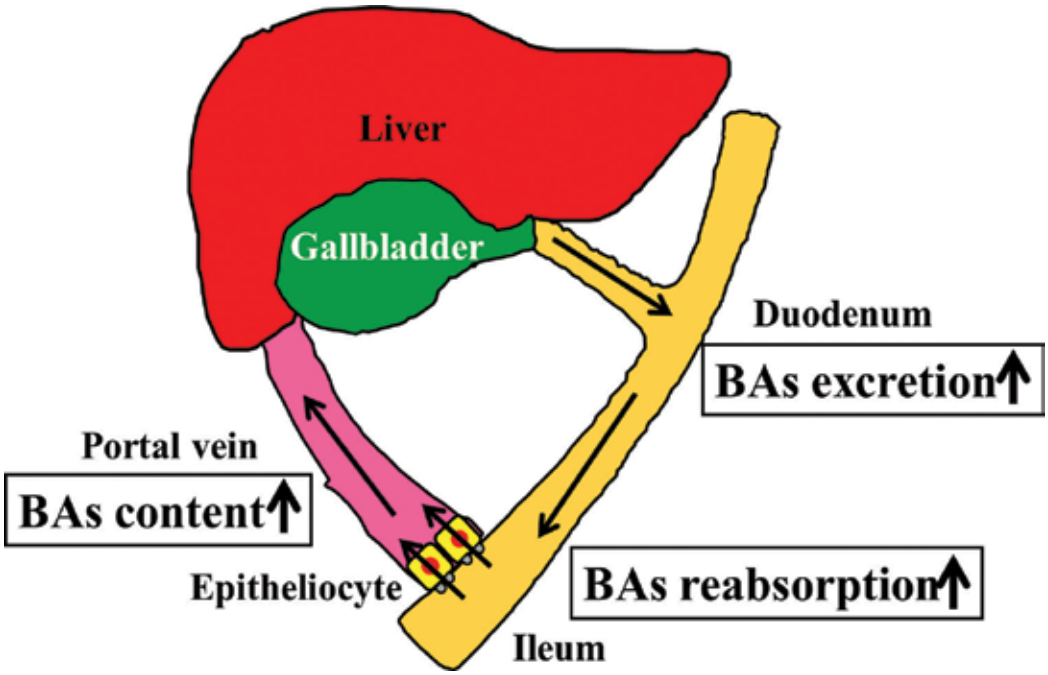

Figure 2. Upregulation of bile acid turnover by peripheral serotonin. Peripheral serotonin induces excretion of bile acids from the gallbladder to the duodenum, reabsorption of bile acids in the ileum, and an elevation of the concentration of bile acids in the plasma of the portal vein (Ref. [1]). BAs, bile acids.

expression and plasma serotonin levels in ob/ob mice. The fat concentration, inflammation, and cell necrosis in the liver of ob/ob mice are all decreased following treatment with an Htr3 antagonist, this by means of reducing the elevated serotonin levels in the intestine. As a result, serotonin is indicated to regulate lipid metabolism functions in the liver, both directly and indirectly.

4. Role of serotonin in the adipose tissue

4.1. Adipose tissue and energy metabolism

One of the features of the adipose tissue is to store a huge amount of energy. Adipose tissue can be roughly categorized into two types: white adipose tissue (WAT) and brown adipose tissue (BAT). In addition, more recently, the existence of beige adipose tissue has been noted. This is thought to be derived from WAT and has some of the features of BAT [30]. WAT functions as the main storage of energy in the body, in the form of triglycerides. WAT takes dietary absorbed glucose and lipids from the blood in the fed condition. On the other hand, in the fasted condition, WAT resolves stored triglyceride and releases free fatty acids and glycerol into the blood and supplies energy to the body [31]. The characteristic of BAT, on the other hand, is to have a small fat droplet and a large number of mitochondria, and it is considered

as an important internal organ, because it produces heat for temperature homeostasis [30]. Indeed, these two adipose tissues are crucial for energy homeostasis and are intimately associated with the development of metabolic diseases such as obesity and type 2 diabetes.

4.2. Role of serotonin in white adipose tissue

4.2.1. Serotonin and adipocyte differentiation

An expansion and growth in the number of individual adipocytes is a manifestation of obesity, and peripheral serotonin of adipose tissue origin is an autocrine element that is necessary for the adipocyte differentiation through the Htr2a and Htr2c receptors [32]. Serotonin production has been demonstrated in 3T3-L1 preadipocyte cells, and there was a gradual increase in the expression level of Tph1 protein and the concentration of serotonin after adipogenic induction in the same cells. Comparing wild-type 3T3-L1 preadipocyte cells, adipogenesis in 3T3-L1 preadipocyte cells was associated with a lack of Tph1 after treatment with differentiation-inducing agents. This phenotype in Tph1 mutant cells is recovered following treatment with serotonin. Furthermore, antagonists of Htr2a and Htr2c also inhibit the adipogenesis in 3T3-L1 preadipocyte cells. Additionally, it is suggested that serotonin metabolites operate as endogenous agonists for peroxisome proliferator-activated receptor gamma (PPARg) so that they control adipogenesis by means of directly binding to helix H12 of the PPARg binding site [33]. Consequently, these reports indicated that serotonin directly affects the differentiation from preadipocyte to adipocyte.

4.2.2. Regulation of energy homeostasis in white adipose tissue by serotonin

There is a report that adipocyte-derived serotonin has the important role in energy homeostasis in the whole body [34]. Tph1 expression and serotonin concentrations were increased in epididymal and subcutaneous WAT in a diet-induced obesity mouse model. Intraperitoneal injection of the Tph1 inhibitor PCPA led to a reduction of weight gain and lower adiposity after a high-fat diet (HFD). Surprisingly, treatment of PCPA promotes beige adipogenesis in inguinal WAT by elevating UCP1 and DIO2 expression. Adipocyte-specific Tph1 KO also causes a reduction of bodyweight, an improvement of insulin resistance and beige adipogenesis in inguinal WAT. Therefore, adipocyte-derived serotonin is suggested to play important role both to induce adipogenesis and to maintain the feature and function of WAT.

4.3. Regulation of energy homeostasis in brown adipose tissue by serotonin

Serotonin is involved in energy homeostasis not only of WAT but also of BAT [35]. In a diet-induced obesity mouse model, Tph1 expression and tissue serotonin concentrations were increased in BAT as well as in WAT. Tph1-deficient mice on a high-fat diet (HFD) are prevented from becoming obese, as well as succumbing to insulin resistance and nonalcoholic fatty liver disease (NAFLD) while expressing energy generation by BAT and exhibiting energy expenditure in whole body. This BAT function in Tph1 KO mice elevated UCP1 dependently. These data are supported by Oh et al. [34]. Tph inhibitor PCPA and LP-533401 promoted the expression of UCP1 and DIO2 in BAT. Furthermore, it is indicated that Htr3 is involved in

serotonergic signal to BAT. Tph1 KO mice demonstrate an increased expression of UCP1 and DIO2 of both WAT and BAT, whereas Htr3 KO mice show elevation of these thermogenic gene expressions solely of BAT. Thus, serotonin regulates BAT activation through Htr3. On the contrary, WAT of Htr3 KO mice did not show the beige adipogenesis in inguinal WAT which was observed in Tph1 KO mice. As a result, the serotonin function of WAT is connected to Htr's, for example, Htr2a which associated with adipogenesis, except for Htr3.

5. Role of serotonin in the skeletal muscle

5.1. The skeletal muscle and energy metabolism

The skeletal muscle is an essential tissue in energy metabolism and glucose utilization, especially during exercise. Slow- and fast-type myosin heavy chain isoforms exist in normal mature muscle fibers. There is a high concentration of mitochondria in slow-type muscle fibers, and it produces energy by oxidative metabolism. On the other hand, glycolysis is utilized by fast-type muscle fibers as the chief adenosine triphosphate (ATP) source [36, 37]. Peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 a (PGC-1a) is confirmed as a nuclear receptor coactivator of PPARγ, and it is a principal physiological controller for slow-type muscle fiber specification [37, 38]. There is a significant impaired glucose tolerance in skeletal muscle-specific PGC-1α knockout mice [39], whereas humans with lower adiposities have a significantly higher percentage of slow-type muscle fibers than obese humans.

5.2. Effect of serotonin on glucose uptake and glycolysis in the skeletal muscle

Some studies report that serotonin increases glucose uptake in the skeletal muscle. Serotonin promotes a fast stimulation in glucose uptake by 50% in both L6 myotubes and independent rat skeletal muscle mediated through the Htr2a receptor [40]. Apart from this, this serotonin function does not depend on the components that participate in the insulin signaling pathway. The other thesis insists that incubation with serotonin induced an increase in 2-deoxyglucose uptake in a concentration-dependent fashion by translocated GLUT4 to the cell membrane [41]. This GLUT4 translocation is thought to be caused by serotonylation of the small GTPase Rab 4.

In addition, serotonin signals 6-phosphofructo-1-kinase (PFK) through the Htr2a. This has been reported as the major rate-limiting enzyme of glycolysis and is related to the entire glycolytic pathway each other in the skeletal muscle [42]. Serotonin provokes PFK from the skeletal muscle via phospholipase C (PLC). The stimulation of PLC in the skeletal muscle promotes the recruitment of protein kinase C (PKC) and calmodulin and the activation of calmodulin kinase II, which connects with PFK upon serotonin action. Thus, serotonin may increase glucose uptake and glycolysis through Htr2a and intracellular serotonylation of Rab 4.

5.3. Effect of serotonin on skeletal muscle fiber type

Obesity induced by feeding a high-fat diet is improved in Tph1 KO mice by increasing beige adipogenesis in WAT and thermogenic gene expressions in BAT. In contrast, Watanabe et al. report

that long-term treatment of mice with peripheral serotonin interferes with weight gain, hyperglycemia, and insulin resistance and completely inhibited the enlargement of intra-abdominal adipocytes without having any impacts on food intake when on a high-fat diet, but not on a chow diet [43]. Amazingly, serotonin raises the percentage of slow muscle fibers and reduces the percentage of fast muscle fibers in serotonin-injected mice fed a high-fat diet (**Figure 3**). As a result, serotonin increases energy metabolism, O_2 consumption, CO_2 production, and the respiratory exchange ratio (RER). The function is caused by increase of PGC-1α expression in

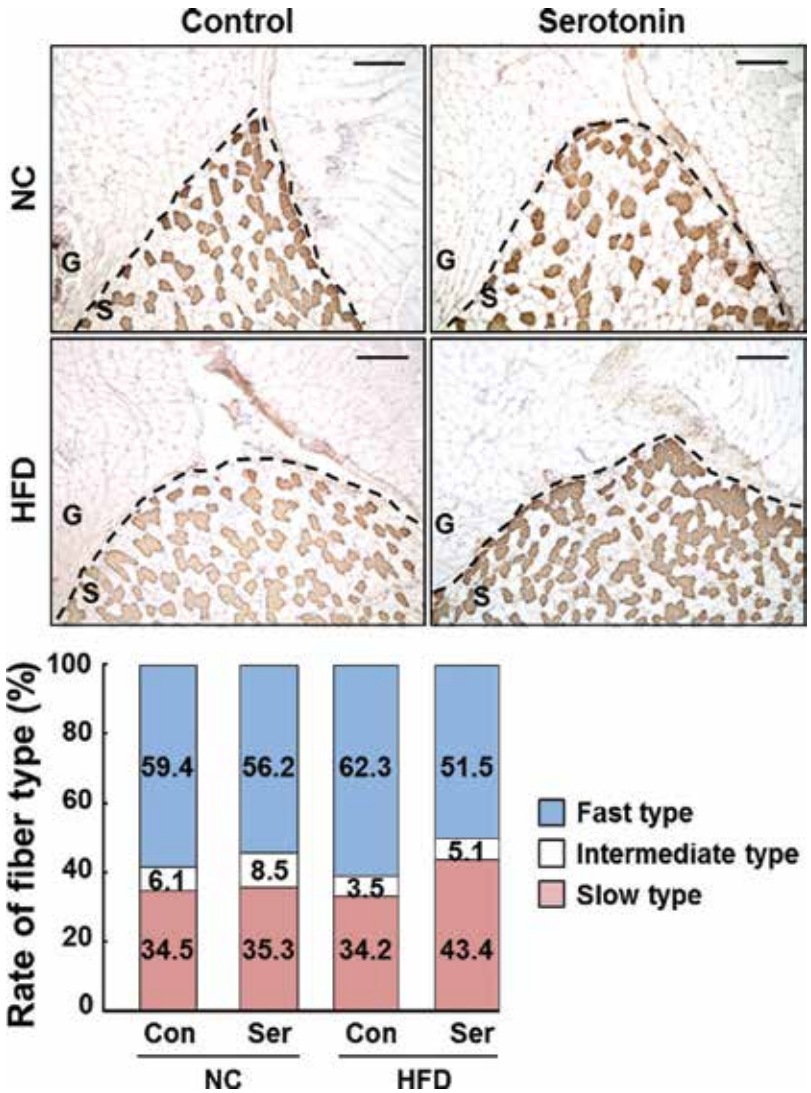

Figure 3. Induction of the transformation of skeletal muscle fiber type into slow muscle fiber by serotonin. Serotonin increases the proportion of slow muscle fibers, which have a high concentration of mitochondria and produce energy by oxidative metabolism, in the soleus muscle from mice fed a high-fat diet (Ref. [43]). Serotonin may play an important role in the relief of obesity by accelerating energy consumption in the skeletal muscle. Con, control; G, gastrocnemius muscle; HFD, high-fat diet; NC, normal chow; S, soleus muscle; and Ser, serotonin.

the skeletal muscle. PGC-1α is a major regulator that induces mitochondrial biogenesis and a fiber switch to decelerate muscle fiber type in the skeletal muscle [37, 38]. The fact that PGC-1α mRNA has three isoforms, PGC-1α-a, PGC-1α-b, and PGC-1α-c, has been revealed recently [44]. There was an elevation of the expression of total PGC-1α in serotonin in the soleus muscle of mice on a high-fat diet, although following a significant increase of PGC-1α-b and PGC-1α-c expressions through the Htr2a and Htr7 signaling pathways. Previous reports suggest that an Htr2 agonist led to an increase of PGC-1α promoter activity, and it supports serotonin's PGC-1α expression promoter activity in the skeletal muscle [45].

Serotonin in WAT and BAT has the possibility to reduce energy expenditure, whereas the skeletal muscle may transform skeletal muscle fiber type into slow muscle fiber and increase energy expenditure.

6. Will a tomato (with a high serotonin content) a day keep the doctor away?

A variety of vegetables and fruits contain serotonin [46]. We have also reported that good plant sources of serotonin are the cherry tomato, tomato, kiwi, banana, and potato, using the HPLC-fluorescence detection method (**Table 1**) [47]. We have confirmed that the serum concentrations of serotonin increased in a dose- and time-dependent manner after oral administration and that a serotonin metabolite, 5-hydroxyindole-3-acetic acid, was detected in urine at higher concentrations in treated than in untreated mice [48]. The foods with a high serotonin content may represent excellent dietary sources of serotonin, and serotonin action may well offer new drug strategies for developing therapeutic drugs for the treatment of metabolic diseases such as hyperlipidemia, hypercholesterolemia, diabetes, and obesity. In the future, we may say that a tomato with a high serotonin content a day keeps the doctor away.

	Names (botanical names)	**Serotonin (μg/g)**
Vegetables	Cherry tomato (*Solanum lycopersicum* var. *cerasiforme*)	12.44 ± 0.19
	Tomato (*Solanum lycopersicum*)	8.81 ± 0.08
	Asparagus (*Asparagus officinalis*)	0.55 ± 0.26
	Carrot (*Daucus carota*)	0.34 ± 0.01
	Potato (*Solanum tuberosum*)	0.26 ± 0.14
Fruits	Kiwi (*Actinidia deliciosa*)	9.52 ± 0.62
	Banana (*Musa acuminata*)	9.48 ± 0.09
	Pineapple (*Ananas comosus*)	9.11 ± 0.13
	Avocado(*Persea americana*)	5.37 ± 0.41
	Mikan (*Citrus unshiu*)	2.14 ± 0.08

Table 1. Serotonin levels in common vegetables and fruits in Japan [47].

7. Conclusions

Serotonin in the central nervous system has been studied as good strategy for dealing with obesity since the late twentieth century, because it affects behavior, especially food intake. On the other hand, despite the fact that peripheral tissue has almost all of the serotonin of the whole body, research into the function of serotonin in peripheral tissue has not significantly progressed. Since the beginning of the twenty-first century, the role of the peripheral serotonergic system in energy homeostasis has gradually been clarified and has been noticed as a new treatment target.

Peripheral serotonin is central to the control of energy homeostasis by means of stimulating several organs but especially pancreatic β cells, the liver, white adipose tissue, brown adipose tissue, and the skeletal muscle. These functions of peripheral serotonin are thought to operate through autocrine and paracrine means through at least the 14 Htr's or serotonin transporter.

It is considered that receptor-specific activation or inhibition is a better strategy for the development of drugs from this knowledge. Nevertheless, it has been reported that peripheral serotonin acts differently in different tissues, by functioning through different receptors in different cells. Thus, peripheral serotonin functions operate in a very complex manner when peripheral serotonin is considered as a therapeutic agent for the whole body. Indeed, there are still many points that need unraveling. For instance, serotonin in WAT and BAT regulates energy expenditure, while serotonin in the skeletal muscle increases glucose uptake and the proportion of slow muscle fibers and raises energy expenditure. This question may be resolved by using cell-specific deletion of Htr's and Tph1 mice. The solution of this question is expected to develop soon, because we anticipate that affecting energy homeostasis using the peripheral serotonergic system will eventually be a new treatment strategy for metabolic disease.

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Chapter 9

Serotonin Effects on Expression of the LDL Receptor Family Member LR11 and 7-Ketocholesterol–Induced Apoptosis in Human Vascular Smooth Muscle Cells

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Additional information is available at the end of the chapter

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Abstract

We previously confirmed the effect of sarpogrelate hydrochloride (sarpogrelate), 5-hydroxytryptamine (5-HT) 2A receptor antagonist on cardio-ankle vascular index (CAVI) as a marker of systemic arterial stiffness. After 6 months of treatment with sarpogrelate for 35 type 2 diabetic patients, decreased CAVI, indicating the ameliorated arterial stiffness, was observed. Therefore, via 5-HT2A receptor blockade, sarpogrelate might effect as a vasoactive agent, as well as an inhibitor of platelet aggregation. 5-HT is a known mitogen for vascular smooth muscle cells (VSMCs). In addition, the pathogenic change of VSMCs such as dedifferentiation and proliferation/apoptosis represents one of the atherosclerotic changes. On the other hand, LR11, a mosaic LDL receptor family member, may involve in the invasion of VSMCs into neointimal thickening. We therefore investigated an *in vitro* study to clarify whether 5-HT was concerned to LR11 expression and apoptosis of human VSMCs induced by 7-ketocholesterol (7KCHO), a major oxidation product of cholesterol involved in plaque destabilization. Resultantly, 5-HT accelerated the proliferation of VSMCs, and this effect was suppressed by simultaneous addition of sarpogrelate. Sarpogrelate also attenuated the 5-HT–induced LR11 mRNA expression in VSMCs. Additionally, 5-HT attenuated the 7KCHO-induced apoptosis of VSMCs through caspase-dependent pathway. These results suggest new knowledge on the modification of human VSMCs induced by 5-HT.

Keywords: arterial stiffness, vascular smooth muscle cells, LR11, apoptosis, 7-ketocholesterol

1. Introduction

Invasion of vascular smooth muscle cells (VSMCs) to intima takes a principle finding in the progression of atherosclerosis and the incidence of restenosis after vascular intervention [1–3]. Lately, LR11, a mosaic low-density lipoprotein (LDL) receptor family member, is known to exist largely in VSMCs of the hyperplastic intima, but not the media and induce the invasion and migration potential of intimal VSMCs, speculated to originate from medial VSMCs [4–6]. Meanwhile, reduced extracellular matrix, reduced number of VSMCs, thin fibrous cap, and extracellular oxysterol accumulation have been observed in unstable plaques [7–9]. We have previously shown that 7-ketocholesterol (7KCHO), a major oxidation product of cholesterol, revealed as an apoptosis inducer on VSMCs [10, 11], and attenuated the migration of VSMCs [12]. These reports propose that the presence of 7KCHO may reduce the number of VSMCs and contribute to unstable plaque. Generally, proliferation/apoptosis and dedifferentiation of VSMCs in the arterial intima identify one of the pathological findings observed in atherogenesis [2, 13, 14]. Meanwhile, the factors modulating proliferation/apoptosis of VSMCs and the potential cellular mechanisms are not fully elucidated.

Serotonin (5-hydroxytryptamine, 5-HT), secretion from activated platelets, is recognized to be a naturally occurring vasoactive mediator involved in vascular inflammation and atherogenesis [15]. 5-HT has multiple receptor subtypes [16] and induces platelet aggregation, vasoconstriction, and VSMC proliferation [17, 18]. In addition, the plasma level of 5-hydroxyindole-3-acetic acid (5-HIAA; a derivative end product of 5-HT) is relatively high in subjects with visceral adiposity, revealing that 5-HT is one of the potential mediators for atherogenesis in lifestyle diseases [19].

Sarpogrelate hydrochloride (sarpogrelate), a selective 5-HT2A receptor antagonist, is used for diabetic patients with chronic arterial occlusive diseases [20] and is known to suppress platelet aggregation, vascular endothelial dysfunction, and smooth muscle contraction mediated via 5-HT2A receptor [21, 22]. The restorative effects of sarpogrelate on cardiovascular disturbance in experimental diabetic rats were also reported [23]. Furthermore, we investigated prospectively the effect of sarpogrelate on systemic arterial stiffness assessed by cardio-ankle vascular index (CAVI) in type 2 diabetic patients [24]. After 6 months of treatment with sarpogrelate for 35 Japanese type 2 diabetic patients, decreased CAVI, indicating the ameliorated arterial stiffness, was observed (**Table 1**). Sarpogrelate is known to inhibit 5-HT–induced vascular smooth muscle contraction and/or cell proliferation [25, 26]. Moreover, Shirai et al. have reported that CAVI might be affected by change in contractility of vascular smooth muscle [27]. Therefore, these results suggest that sarpogrelate may ameliorate arterial stiffness through inhibiting vascular smooth muscle contractility. However, the effects of 5-HT on vascular composition are not fully understood. We hypothesized that 5-HT was concerned to the invasion and migration of VSMCs through the regulation of LR11, besides the apoptosis of VSMCs.

We confirmed the effect of 5-HT on LR11 expression in human VSMCs. Additionally, whether there was an interaction of 5-HT with 7KCHO in inducing VSMC apoptosis was investigated.

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	Baseline	After 6 months	*p* Value
N (male/female)	35 (21/14)	-	-
Age (y)	67.4 ± 9.1	-	-
Height (cm)	159.5 ± 8.1	-	-
Body weight (kg)	61.9 ± 10.1	61.9 ± 10.1	0.982
BMI (kg/m^2)	23.4 ± 2.9	23.4 ± 2.9	0.966
CAVI	10.11 ± 0.92	9.87 ± 0.97	0.013
sBP (mmHg)	128 ± 11	132 ± 12	0.076
dBP (mmHg)	75 ± 8	76 ± 8	0.231
TG (mg/dl)	161 ± 234	178 ± 243	0.068
HDL-C (mg/dl)	58 ± 14	55 ± 14	0.304
LDL-C (mg/dl)	98 ± 14	104 ± 34	0.054
FPG (mg/dl)	154 ± 53	153 ± 60	0.771
HbA1c (%)	7.2 ± 1.2	7.1 ± 1.2	0.463
Medication: n (%)			
Sulfonylurea	11 (31.4)	-	-
Biguanide	6 (17.1)	-	-
Alpha-glucosidase inhibitor	10 (28.6)	-	-
Thiazolidinedione	4 (11.4)	-	-
ARB/ACE-I	8 (22.9)	-	-
Calcium channel blocker	7 (20.0)	-	-
Statin	15 (42.9)	-	-
Fibrate	5 (14.3)	-	-

Data are presented as mean ± standard deviation. Paired *t*-test was used in comparing baseline and 6-month data. BMI, body mass index; CAVI, cardio-ankle vascular index; sBP, systolic blood pressure; dBP, diastolic blood pressure; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; ARB, angiotensin receptor blocker; ACE-I, angiotensin converting enzyme inhibitor.

Table 1. Participant characteristics before and after 6 months of sarpogrelate treatment.

2. Materials and methods

2.1. Cell cultures

VSMCs prepared from human femoral artery were cultured in growth medium of Dulbecco's modified Eagle's minimal essential medium supplemented with delipidated calf serum

mixture or 5–10% (v/v) heat inactivated fetal calf serum (FCS), 40-μg/mL gentamicin, and 2-mmol/L L-glutamine, maintained at 37°C in 5% carbon dioxide.

Sarpogrelate was a gifted reagent from Mitsubishi-Tanabe Pharma Co., Osaka, Japan. 5-HT, 7KCHO, and other reagents were generously provided by Sigma (St. Louis, Missouri).

2.2. Proliferation of VSMCs

VSMCs were plated into 12-well microtiter plates in triplicate (10^4 cells per well). After 72 h of growth, the medium was changed to Dulbecco's modified Eagle medium containing 5% FCS and sarpogrelate and/or 5-HT was administered. Thereafter, proliferation of VSMCs was evaluated by a hemocytometer during 0 and 8 days after the administration of sarpogrelate and/or 5-HT.

2.3. Reverse transcription polymerase chain reaction

Total RNA was isolated from VSMCs by RNeasy kit (Qiagen, Courtaboeuf, France), and complementary (c) DNA was prepared by reverse transcription (RT-) polymerase chain reaction (PCR) kit (TaKaRa, Tokyo, Japan) as already described in the manufacturer's instructions. RNA concentrations were evaluated by measuring absorbance at 260 nm. Thereafter, RT-PCR was carried out using 1 μg of reverse transcribed total RNA. Expression of β-actin internal standard was adopted as housekeeping gene for quantifying RNA levels. The specific primers were as follows:

LR11:

sense 5′-AGGAGGGCATCCTGCAGTATTGCCAAGAAG-3′

antisense 5′-TGGCGACGGTGTGCCAGTGA-3′

β-actin:

sense 5′-CTCTTCCAGCCTTCCTTCCT-3′

antisense 5′-AGCACTGTGTTGGCGTACAG-3′

PCR was run on a Gene Amp PCR System 9700 (Applied Biosystems, Foster city, CA) for 35 cycles both for β-actin and LR11. The terminal cycle for denaturation, annealing, and elongation was required at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for each. The amplified products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized by UV irradiation. Furthermore, the images were photographed using an Olympus digital camera (Tokyo, Japan).

2.4. Caspase activity of VSMCs

Two methods were used in order to measure caspase activity in VSMCs. First, a luminescent assay for measuring caspase-3 and -7 (caspase-3/7) activities was adopted. The other was flow

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cytometric analysis by the fluorescein-5–isothiocyanate (FITC) Active Caspase-3 Apoptosis Kit (BD Pharmingen, La Jolla, California). After two washes in ice-cold phosphate-buffered saline, VSMCs incubated in 96-well microplates were scraped off the tissue culture dish, and caspase activities were evaluated using the Caspase-Glo® 3/7 Assay (Promega, Wisconsin) as described in the manufacturer's protocol.

WST-8 cell counting kit (Dojindo Laboratories, Kumamoto, Japan) was adopted to evaluate bioavailability of VSMCs. When serial dilutions of VSMCs were plated into 96-well microtiter plates and analyzed, an absorbance response at 460 nm was observed linearly (data not shown). The number of VSMCs was measured using the regression equation. Caspase-3/7 activity was corrected for mean cell number measured for each.

2.5. Apoptosis of VSMCs

VSMCs were harvested by trypsinization and kept into 5-mL fluorescence-activated cell sorting (FACS) tubes in phosphate buffered saline (PBS) (pH 7.4) containing 5% FCS. Thereafter, samples were processed in a Becton Dickinson FACScalibur (Immunocytometry Systems, San Jose, California) equipped with a 15 mW, 488 nm argon laser, and filter configuration. BD™ Biosciences Propidium Iodide Staining Solution was adopted to quantify DNA content. FITC Active Caspase-3 Apoptosis Kit (BD Pharmingen) was used to evaluate active caspase-3. Cell samples (20,000 cells) were analyzed on a FACSort cytometer using Cell Quest Pro software (BD Biosciences). The percentage of apoptotic cells in each sample was quantified by manual counting of adherent VSMCs using fluorescence microscopy.

2.6. Statistical analysis

Statistical analyses were carried out using SPSS software (version 11.5, Chicago, IL, USA). The data are presented as mean ± standard deviation (SD). The effects of reagents were compared by one-way ANOVA followed by Bonferroni multiple comparison test and values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of 5-HT and/or sarpogrelate on VSMC proliferation

Figure 1A proposes VSMC proliferation in the absence or presence of 5-HT for 8 days. Administration of 5-HT (100 μM) to VSMCs significantly increased proliferation at days 5 and 8. The cell counts of VSMCs on day 8 after the administration of 5-HT (1, 10, or 100 μM) with or without sarpogrelate (10 μM) were shown in **Figure 1B**. The administration of 5-HT to VSMCs induced a dose-dependent increase in cell number and simultaneous addition of sarpogrelate attenuated the effect of 5-HT.

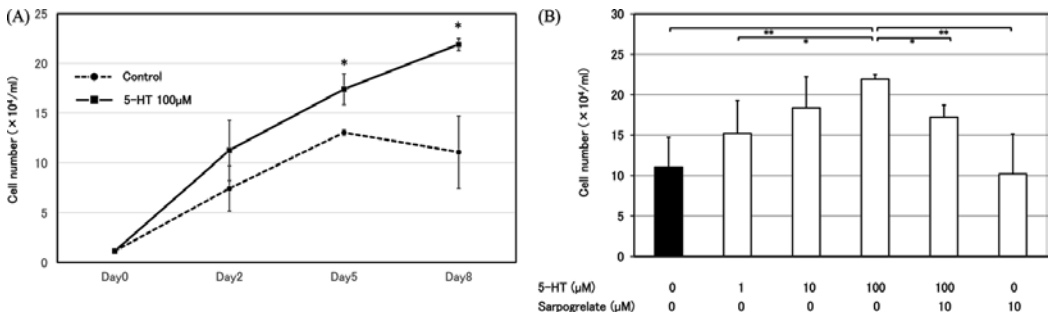

Figure 1. Effects of serotonin (5-HT) and sarpogrelate on proliferation of vascular smooth muscle cells (VSMCs). (A) Changes in cell number over time. After seeding VSMCs in 12-well microplates (1×10^4/well, in triplicate) and culturing for 72 h, 5-HT (100 μM) was added. Cell number was counted from day 0 to day 8 after the addition of 5-HT. Data are presented as mean ± SD of triplicate samples. *Significantly higher ($p < 0.05$, unpaired *t*-test) cell count compared with control. (B) Effects of 5-HT concentration and sarpogrelate on cell proliferation. After seeding VSMCs in 12-well microplates (1×10^4/well, in triplicate) and culturing for 72 h, 5-HT and sarpogrelate at indicated concentrations were added. Cell numbers were counted on day 8 after addition of 5-HT and/or sarpogrelate. Data are presented as mean ± SD of triplicate samples. $^*p < 0.05$, $^{**}p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

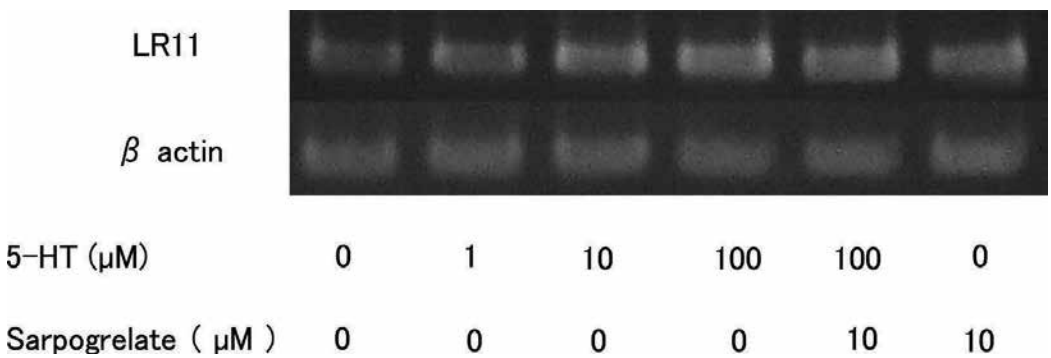

Figure 2. Effects of serotonin (5-HT) and sarpogrelate on LR11 mRNA expression in vascular smooth muscle cells (VSMCs). After seeding VSMCs in 6-well microplates (6×10^4/well, in triplicate) and culturing for 72 h, 5-HT and sarpogrelate at indicated concentrations were added and cultured for another 72 h. Upper panel shows LR11 mRNA expression determined by reverse transcription PCR. Beta-actin expression shown in the lower panel was used as internal standard.

3.2. Effects of 5-HT and/or sarpogrelate on LR11 mRNA expression in VSMCs

RT-PCR showed that LR11 mRNA expression was enhanced dose dependently by administration of 5-HT at 1–100 μM in VSMCs. Additionally, simultaneous addition of sarpogrelate at 10 μM attenuated the effect of 5-HT (**Figure 2**).

3.3. Effects of 5-HT and/or 7KCHO on caspase activity in VSMCs

The effects of 5-HT and/or 7KCHO on caspase activity in VSMCs were examined using two methods. Flow cytometric analysis was carried out by VSMCs stained with FITC-conjugated

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antiactive caspase-3 monoclonal antibody. The histograms in **Figure 3A** propose the distribution of VSMCs. Administration of 5-HT to VSMCs alone caused a slight leftward shift of the peak from control, revealing an attenuation in active caspase-3 expression [28]. Contrastingly, administration of 7KCHO alone caused an enhancement in active caspase-3 expression as revealed by a rightward shift of the histogram, and this effect of 7KCHO was attenuated by simultaneous addition of 5-HT (**Figure 3B**).

Next, a luminescent assay was carried out in order to evaluate caspase-3/7 activities using the same protocol shown in the previous experiment. Administration of 5-HT alone did not change the caspase-3/7 activities in VSMCs. On the other hand, administration of 7KCHO alone enhanced caspase-3/7 activity ninefold compared to the control, and this

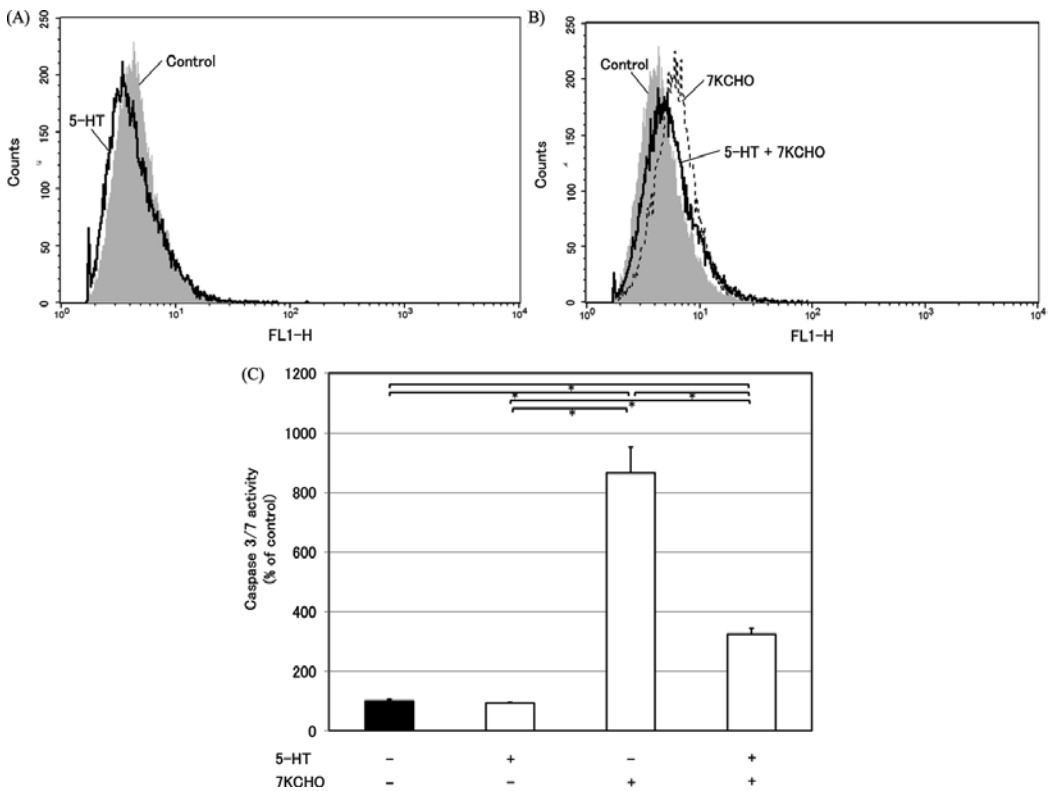

Figure 3. Effects of serotonin (5-HT) and/or 7-ketocholesterol (7KCHO) on caspase activity in vascular smooth muscle cells (VSMCs). (A and B) Caspase-3 activity assayed by antiactive caspase antibody and flow cytometry. After seeding VSMCs in 6-well microplates (8×10^4/well, in duplicate) and culturing for 48 h, VSMCs were incubated with no addition, addition of 5-HT (100 μM) alone, or addition of 5-HT (100 μM) and 7KCHO (50 μM) for another 48 h. The cells were maintained in Dulbecco's modified Eagle's minimal essential medium containing 10% FBS and 1% non essential amino acid, and incubated at 37°C, 5% CO_2. Caspase-3 expression was analyzed using FITC-conjugated monoclonal anti active caspase-3 antibody followed by flow cytometry. Changes in active caspase-3 activity are shown in FL1 histograms. (C) Caspase-3/7 activities assayed by luminescent assay. VSMCs were seeded into 96-well microplates (1×10^5/well, in triplicate), and incubated with or without the addition of 5-HT (100 μM) and/or 7KCHO (50 μM) for 48 h. Luciferase activity was measured according to the protocol from Promega. Data are presented as mean ± SD of triplicate samples. $^*p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

effect of 7KCHO was attenuated by simultaneous addition of 5-HT (**Figure 3C**). To sum up, almost same effects of 5-HT were shown in both methods for measuring caspase activity in VSMCs.

3.4. Effects of 5-HT and/or 7KCHO on quantitation of apoptosis in VSMCs

Apoptotic DNA fragmentation in VSMCs was evaluated by propidium iodide fluorescence to clarify the apoptosis-inducing effect of 7KCHO in the absence or presence of 5-HT (**Figure 4**). The apoptotic rate was elevated after administration of 7KCHO (50 μM) alone, but this effect of 7KCHO was suppressed by simultaneous addition of 5-HT at 100 μM.

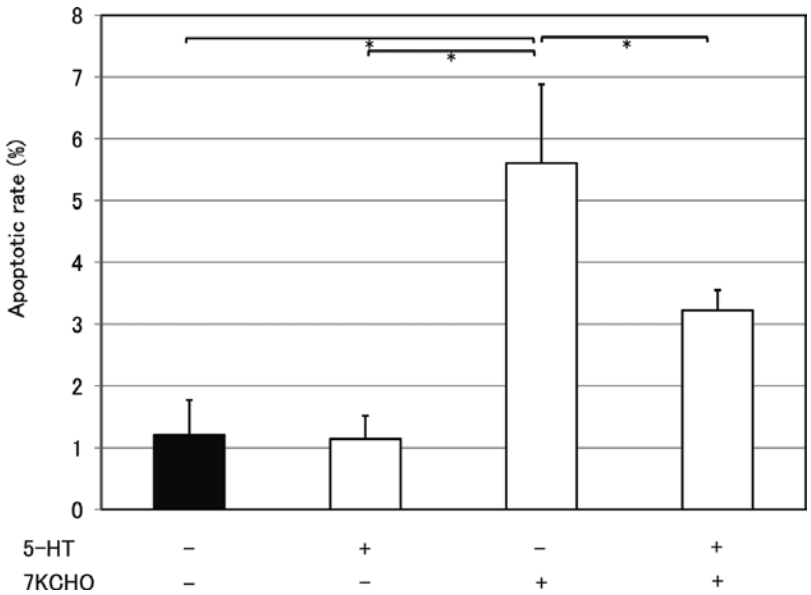

Figure 4. Effects of serotonin (5-HT) and/or 7-ketocholesterol (7KCHO) on quantitative analysis of apoptosis of vascular smooth muscle cells (VSMCs). After seeding VSMCs in 6-well microplates (8×10^4/well, in duplicate) and culturing for 48 h, VSMCs were incubated with or without the addition of 5-HT (100 μM) for another 96 h. 7KCHO (50 μM) was added to some wells 72 h after the addition of 5-HT. Cells were stained with 50 μg/ml of propidium iodide after cell lysis and analyzed by flow cytometry. Apoptotic rate is the percentage of nuclei in the sub-G1 population representing DNA fragmentation as shown in FL2 histograms. Data are presented as mean ± SD of three independent experiments. $^*p < 0.01$; one-way ANOVA followed by Bonferroni multiple comparison test.

4. Discussion

In the present study, 5-HT accelerated the proliferation of human VSMCs and this effect of 5-HT was attenuated by simultaneous addition of sarpogrelate. Moreover, sarpogrelate also suppressed the 5-HT–induced enhancement of LR11 mRNA expression in VSMCs. 5-HT attenuated the 7KCHO-induced VSMC apoptosis through caspase-3/7–dependent pathway

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besides. There is so far no evidence providing the effect of 5-HT on LR11 expression and apoptosis in VSMCs. The present report demonstrates the effects of 5-HT on such pathogenic changes in VSMCs.

The mechanism by which 5-HT regulates the number of intimal VSMCs has not been fully clarified, so that the modulators for migration of VSMCs from the arterial media to the intima should be examined. Previous reports have shown that LR11 expression was largely involved in the differentiation of VSMCs. Furthermore, the VSMCs with a contractile phenotype observed in the arterial media do not express LR11, whereas the VSMCs in an active synthetic phenotype located in the intima highly express LR11 [29, 30]. Additionally, circulating soluble form of LR11 concentrations in serum is known to correlate with the degree of coronary organic stenosis, carotid intima-media thickness, and pulmonary arterial hypertension [31–33]. These findings suggest that LR11 may play key role of modification in VSMCs during atherogenesis. Our data suggested that 5-HT enhanced the expression of LR11 mRNA in VSMCs, and simultaneous addition of sarpogrelate attenuated this effect of 5-HT. These results reveal that 5-HT may participate to neointimal thickening through stimulating not only proliferation, but also invasion of VSMCs accompanied by upregulation of LR11. Therefore, sarpogrelate may show the pleiotropic effect on vascular tissue, such as decreased systemic arterial stiffness, partially through down-regulation of LR11 in VSMCs. Additionally, sarpogrelate was recently reported to ameliorate the development of chronic hypoxic pulmonary hypertension through the occurrence of increased apoptosis and decreased proliferation of VSMCs [34]. However, we cannot definitely consider that the effect of 5-HT on VSMCs is absolutely malignant to human body.

Apoptosis of VSMCs located in atheroma is known to be associated with vulnerable plaque ruptures [35, 36]. In this study, luminescent assay and flow cytometric analysis revealed that 7KCHO enhanced the caspase-3/7–dependent apoptotic pathway. In a phase of progressive atherosclerotic plaque formation, 7KCHO is speculated to induce the absence of VSMCs, which make plaque unstable leading to rupture. Meanwhile, simultaneous addition of 5-HT suppressed 7KCHO-induced VSMC apoptosis. These findings reveal that 5-HT may reduce the occurrence of plaque rupture through the attenuation of 7KCHO-induced VSMC apoptosis.

Whether 5-HT is favorable or not for the vascular remodeling process is still controversial. In state of vascular injury, subsequent platelet activation accompanied by endothelial damage provides increasing local plasma level of 5-HT. Furthermore, 5-HT causes the proliferation, contraction, and migration of VSMCs through the 5-HT2A receptor amplified by various intracellular signaling pathways [37–39]. Thus, 5-HT plays the basic principle of vascular repair, which spreads to neointimal thickening and decrease of peripheral blood flow. Note that the administration of 5-HT to VSMCs resulted in the enhancement of potential for cell migration caused by up-regulation of LR11 in the present study. Moreover, the suppressive effect of 5-HT on VSMC apoptosis might concern to the suppression of vulnerability in atheromatous plaque caused by 7KCHO. These effects of 5-HT can be protective for vascular composition.

It is still controversial whether modification of VSMCs along with upregulation of LR11 directly concern to the attenuation in apoptosis-inducing effect of 7KCHO. Further elucidation about the causal relationship of LR11 expression with VSMC apoptosis should be examined.

Resultantly, 5-HT accelerated the proliferation of VSMCs, and this effect was suppressed by simultaneous addition of sarpogrelate. Sarpogrelate also attenuated the 5-HT–induced LR11 mRNA expression in VSMCs. Additionally, 5-HT attenuated the 7KCHO-induced apoptosis of VSMCs through caspase-dependent pathway. These results suggest new knowledge on the modification of human VSMCs induced by 5-HT.

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Systems

Chapter 10

Serotonin in Neurological Diseases

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Additional information is available at the end of the chapter

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Abstract

Serotonin (5-HT) is responsible for anxiety, aggression, and stress. Alterations in a serotonergic system play a significant role in pathogenesis of neurological diseases and neuropsychiatric disorders. A wide range of disturbances associated with serotonergic neurotransmission results from different functions of 5-HT in a nervous system. It is believed that 5-HT may be involved in the pathogenesis of migraine, epilepsy, Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), attention-deficit hyperactivity disorder (ADHD), and autism spectrum disorder (ASD). In these diseases, disturbances of 5-HT and its metabolites, such as 5-hydroxyindoleacetic acid (5-HIAA), were observed in the plasma, blood platelets, and cerebrospinal fluid (CSF). Changes in the level of this biogenic amine (5-HT) may be associated with malfunction of 5-HT receptors, reuptake transporter for 5-HT (5-HTT, SERT), the enzymes responsible for the synthesis and metabolism of 5-HT, and genetic variants for serotonergic system. It seems that 5-HT and its metabolites may be used as a diagnostic and prognostic marker for neurological diseases or a target for more efficient therapy in neurology in the future.

Keywords: serotonin, molecular factors, neurological diseases

1. Introduction

Serotonin (5-HT) is a neurotransmitter responsible for anxiety, aggressive behavior, stress, blood pressure regulation, peristaltic movements, heart rate, and the coagulation system. 5-hydroxytryptamine (5-HT) is produced in neurons and gut cells, as well as in the walls of blood vessels and the heart. On the periphery, 5-HT is located in platelets, which enters via 5-HT reuptake transporter (5-HTT, SERT) [1].

The level of 5-HT in whole blood is in the range of 65–250 ng/ml and in the plasma has a lower value, 5.6–23.9 ng/ml [2]. 5-HT enhances the response of the adrenal medulla, and other sympathetic ganglia using 5-HT2A/3. It has been shown that the impairment of serotonin transporter (SERT) function in addition to the increased 5-HT in the extracellular fluid and increased turnover of 5-HT and its decreased level in nerve cells causes an abnormal stress response in the form of anxiety, as well as an excessive response of the adrenal medulla, including triggered by the hypothalamic-pituitary axis (with no effects on the expression of tyrosine hydroxylase and AT_2 receptors) [3]. Furthermore, 5-HT released from the terminals of afferent vagal neurons enhances the activity of the catecholaminergic neurons of the solitary tract nucleus (pulsed through potentiation of glutamatergic) and the effect on food intake and cardiovascular reflexes [4]. 5-HT acting on 5-HT4 receptors in the human heart causes stimulation of the atrium, pro-arrhythmic effect, produces a positive inotropic effect. At the same time, stimulation of 5-HT1B/1D endings of the sympathetic cardiac causes decreased release of norepinephrine (NE) [2]. 5-HT binds competitively to the binding site of catechol-O-methyltransferase (COMT) in binding site S-adenosyl-S-methionine, inhibiting methylation substrates for this enzyme [5]. It also acts antiapoptotic by stimulating the expression of cystathionine-beta-synthase (CBS) and increases the level of hydrogen sulfide (H_2S) and antioxidant activity [6].

Currently, it is believed that disturbances in the level of 5-HT may be associated with the pathogenesis of few neurological diseases such as migraine [7], epilepsy [8], Parkinson's disease (PD) [1], multiple sclerosis (MS) [9], and amyotrophic lateral sclerosis (ALS) [10] and other disorders (attention-deficit hyperactivity disorder (ADHD) [11], autism spectrum disorder (ASD) [12]).

2. Biosynthesis and metabolism of serotonin

Biosynthesis of 5-HT is a process consisted of coupled reactions, with amino acid tryptophan (Trp) as a primary substrate. The first reaction is hydroxylation of Trp yielding 5-hydroxytryptophane (5-HTP). The next step is decarboxylation of 5-HTP to 5-hydroxytryptamine (5-HT). 5-HT is further metabolized in the body. The main metabolic pathways of 5-HT are shown in **Figure 1** [1, 13].

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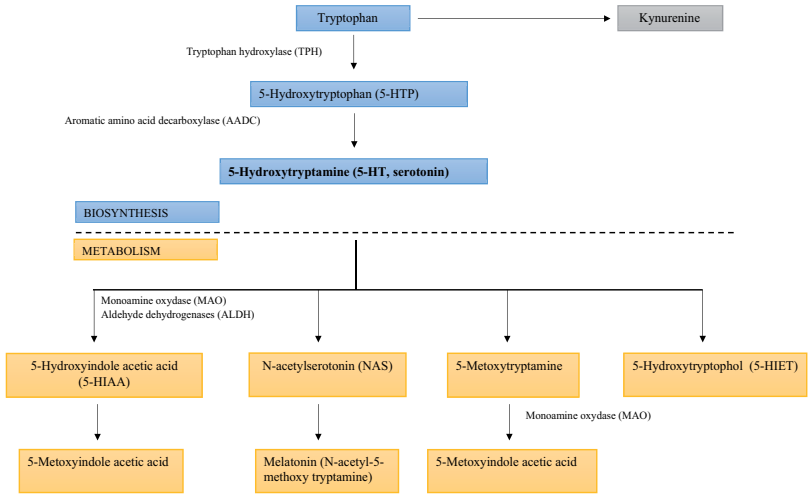

Figure 1. Biosynthesis and metabolic pathways of serotonin.

3. Serotonin and its metabolites in migraine

The disturbances in serotonergic system are a hallmark of migraine. Migraine is a common primary headache disorder that affects 11% of adult worldwide. It occurs three times more often in females (15–18%) than in males (6–8%) [14]. The disease is divided into two main clinical forms: migraine with aura (MA) and migraine without aura (MO). The exact pathomechanism of migraine is unknown, but it is postulated that disease has neurovascular origin in which cortical spreading depression (CSD) and trigeminovascular system (TGVS) play an important role [15]. The TGVS regulates vascular tone and transmission of pain signals [16]. It is believed that activation of TGVS during the head pain phase initiates a chemical cascade of vasoactive neuropeptides such as substance P, calcitonin gene-related peptide, neurokinin A, and nitric oxide. These molecules cause vasodilatation, which can contribute to headaches [17]. The TGVS transmitting migraine pain may be controlled by serotonergic neurons. 5-HT can modulate the trigeminal nerve function, as well as inhibit or promote the pain perception [18]. A decreased level of platelet 5-HT and its metabolite, N-acetylserotonin (NAS), during migraine activate TGVS by CSD [19].

It is known that migraine is a consequence of chronically low 5-HT disposition due to disturbances in its synthesis. The 5-HT metabolism has a cycling character in course of migraine. The plasma concentration of 5-HT is lower and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), is higher during attack-free period, with transient increase of 5-HT and decrease of 5-HIAA during attacks [20–22]. The changes of 5-HT and its metabolite in plasma reflect the situation in the brain as the elevated 5-HIAA level was also found in cerebrospinal fluid (CSF) of migraine suffers [23].

Although the role of 5-HT in migraine pathogenesis is known from ages, the reason of abnormalities of the central 5-HT synthesis remains unknown. The neuroimaging studies have found some answers for a serotonergic mechanism in migraine brain [24]. The electrophysiological studies of Sand et al. [25] indicated that reduced level of serotonergic neurotransmission caused the increase in visual evoked potentials (VEPs) amplitude (P100-N145) in MA patients compared with controls and individuals with MO. It may be associated with the presence of visual aura and increased sensitivity to light in patients with migraine. Authors suggested that disturbances in 5-HT metabolism may be more important in MA than in MO. Dysregulation of 5-HT in brainstem of migraine patients may be caused by a higher level of 5-HTT compared with controls. The higher the availability of 5-HTT, the lower the synaptic level of 5-HT, and in consequence, the lower the brain 5-HT level [26]. The reduction of brain 5-HT synthesis and serotonergic neurotransmission may lead to symptoms related to migraine, such as nausea, dizziness, photophobia, and pain sensitivity [27].

Numerous studies searched the polymorphisms and mutations in genes involved with 5-HT homeostasis in migraine patients. No association between migraine and polymorphisms in genes encoding tryptophan hydroxylase (TPH), aromatic l-amino acid decarboxylase (AADC), monoamine oxidase A (MAO-A), monoamine oxidase B (MAO-B), and most of 5-HT receptors (5-HT1A, 5-HT2A, 5-HT2C, 5-HT1B, and 5-HT1F) were found [13]. Genetic variants in 5-HTT gene, SLC6A4, have also been analyzed in migraine. There are two widely studied polymorphisms: the first is 5-HTTLPR insertion-deletion polymorphism located in the regulatory region of SLC6A4 and the second is STin2 VNTR (variable number of tandem repeats) with four different alleles that correspond to the number of tandem repeats (12, 10, 9, or 7). Both polymorphisms are associated with lower 5-HT reuptake. According to meta-analyses, the short allele of 5-HTTLPR is a risk factor for migraine among European women, while the non-STin2.12 alleles have the protective effect against migraine compared with STin2.12 genotype in the European population [28, 29]. According to the review of Margoob and Mushtaq [30], the S allele and S/S genotype are also associated with many neuropsychiatric diseases, such as major depressive disorder, unipolar or bipolar depression, and seasonal affective disorder. This may explain the fact that patients with migraine more often suffer from depression and anxiety disorders.

A control of the 5-HT level is a means of migraine treatment. Triptans—the 5-HT1B/1D receptor agonists—are successfully used in migraine therapy. The medications that inhibit the reuptake of 5-HT (e.g., selective 5-HT reuptake inhibitor, SSRI) are efficient in chronic pain conditions among which are chronic headaches [31].

High prevalence of migraine was noted in a population of fibromyalgia (FM) sufferers; therefore, it is suggested that both disorders share the same pathomechanism with disturbances in 5-HT metabolism [32]. FM is a chronic pain syndrome, characterized by widespread musculoskeletal pain with diffuse tenderness in specific areas. It affects 3–6% of the world population and 80% of suffers are women [33, 34]. The plasma and CSF levels of 5-HT are decreased in individuals with FM and correlate with clinical symptoms. The low level of Trp and 5-HT precursor, 5-HTTP, as well as high concentration of metabolites in the kynurenine pathway

suggest that the synthesis of 5-HT is decreased in FM. Additionally, 5-HTP supplements are recommended for people with FM. A combined therapy of 5-HTP and MAO inhibitors is more effective that each substance alone [35, 36]. The disturbances in 5-HT concentrations may be associated with changes in 5-HTT, as well. The binding capacity of 5-HTT was found to be lower in FM patients compared with controls. A negative correlation was noted between the binding capacity and rate of 5-HTT and severity of symptoms. The lower expression of 5-HTT in FM patients may be caused by genetic changes [37]. The genetic studies in FM have found that short allele of 5-HTTLPR polymorphism is associated with decline in 5-HTT expression and is a risk factor for developing the disease, similarly to migraine. The T102C polymorphism in *HTR2A* gene encoding 5-HT2A is also postulated to be a risk factor for FM [38]. As 5-HT2 and 5-HT3 are involved in pain perception, the treatment with 5-HT3 antagonist or inhibition of 5-HT reuptake is effective in FM patients [39]. The SSRI administration is necessary as depression is a common disorder among FM patients and it is present in up to 80% of individuals [34]. Participation of 5-HT in the pathogenesis of migraine attacks requires further study.

4. Serotonin levels in epileptic patients

Inhibitors of 5-HT reuptake are also used in another common neurological disorder, epilepsy. Since 1957 it is known that 5-HT can inhibit epileptic attacks [40]. Epilepsy is defined as a set of somatic, vegetative, and mental symptoms. The disease affects 1% of the world population [41]. The disease occurs with a comparable frequency in women and men. Two peaks of incidence are noted: one in childhood and the other over the age of 65 years [42]. In the pharmacotherapy of epilepsy old (e.g., carbamazepine, CBZ; valproate, VPA) and new generation (e.g., lamotrigine, LTG) of antiepileptic drugs (AEDs) are used. Their mechanisms of action among others involve also changes in serotonergic system: CBZ and VPA release the 5-HT, while LTG inhibits 5-HT uptake [8]. The increase in extracellular 5-HT level inhibits both limbic and generalized seizures [43]. Lower values of 5-HIAA concentration were observed in CSF of individuals with epilepsy; this in turns suggests hypofunctional serotonergic neurotransmission in the course of the disease [44].

Moreover, alterations in 5-HT1A, 5-HT2C, 5-HT3, and 5-HT7 receptor subtypes have been analyzed in epilepsy [8]. The binding capacity of 5-HT1A is lower in epilepsy. Reduction in 5-HT1A binding and changes in 5-HT2C and 5-HT7 are features of depression, thus it is unsurprising that 25% of epilepsy cases are accompanied by depression [45, 46]. There is also an age-related decline in 5-HT1A receptors and as it was mentioned before the onset of epilepsy increases in older people. SSRI has anticonvulsant effects because of nonspecific receptor activation, as the volume of 5-HT1A and 5-HT2C receptors is decreased in the temporal regions of brain in epileptic patients. Studies on a mouse model of epilepsy have found that disturbances in the serotonergic system may lead to postictal depression of breathing due to inadequate response to increased CO_2 blood level. Moreover, SSRI drugs are thought to be effective in prevention of hypoventilation after a seizure incident, and of sudden unexpected death in epilepsy in consequence [8, 47].

5. Serotonin and Parkinson's disease

PD was first described in 1817 by an English physician James Parkinson. PD is still an incurable neurological disease, and its pathological mechanism is not fully explained. It is known that in PD there is an imbalance of motor and nonmotor functions, including the autonomic system [1]. Biogenic amines: catecholamines and 5-HT are involved in the regulation of autonomic functions such as blood pressure. In PD degeneration of serotonergic system may also result in depression, psychiatric, and sleep disorders [48]. Moreover, factors regulating levels of biogenic amines such as COMT [49], MAO-A [50], and *5-HTT* gene encoding SERT [51], and bradykinin [52] are involved in the regulation of pain sensation involving neuropeptide Y (NPY). Neuropeptides, Y_1 and Y_2, are also involved in controlling the level of calcium ions regulating by calbindin-B and inflammatory conditions, underlying degenerative changes in the course of PD [1].

Moreover, so far the participation of MAO-B enzyme in the pathogenesis of PD is well known. While the role of MAO-A enzyme in this pathogenesis is not clear. The results of association studies between genetic variants of the *MAO-A* gene and the disclosure of PD are divergent. Hotamisligil and Breakefield [53] have shown that *Eco*RV and *Msp*I polymorphisms of the *MAO-A* gene occurred with threefold higher frequency in patients with PD compared with controls. In contrast, the study of Costa-Mallen et al. [50] did not confirm this association. It was also shown that *MAO-A* polymorphism in the intron 1 in both Japanese population [54] and Caucasians [55] was not associated with PD. On the other hand, a study of Parsian et al. [56] confirmed that *MAO-A* polymorphism was linked to the general population of patients with PD but it did not demonstrate significant differences between familiar PD (FPD) and sporadic PD (SPD).

Preliminary study of Dorszewska et al. [1] indicated that the use of selective MAO inhibitors for depression treatment (by increasing the levels of biogenic amines) in PD may be a particularly effective therapy for patients with genotype *MAO-A* TT (c.1460C>T) and lower levels of NA and 5-HT. Antidepressant MAO inhibitors lead to an inactivation of MAO-A and they promote an increase of 5-HT concentration [57].

It has been shown that SERT (or 5-HTT) is involved in regulating of 5-HT level. SERT is encoded by *5-HTT* gene (SLC6A4, SLC6 member 4) located on the long arm of chromosome 17 in the region 17q11.1-q12 [58]. The *5-HTT* gene may play an important role in revealing and development of mental illness, depression, and feeling of pain as well as SPD [51, 59–63]. In SPD, changes in the SERT level are observed within the raphe nuclei, cingulate, and hypothalamus, as well as increase of SERT activity and decrease of 5-HT level in the striatum, thus leading to depression in these patients [64, 65]. It has been shown that depressive symptoms occur in 50% of patients with PD [1].

Influence of genetic variants of the *5-HTT* gene on SERT concentrations in specific brain structures in PD is not clear. The literature data indicated that 5-HTTLPR polymorphisms and the *5-HTR2* gene lead to lower SERT expression in the dentate rim and caudate nucleus. There was no correlation between the *5-HTT* polymorphism and disclosure of SPD [66]. In contrast,

the study conducted on 393 Caucasian PD patients indicated the influence of 5-HTTLPR polymorphism on a risk of SPD disclosure [67]. Mutations in the *5-HTT* gene related to pathogenesis SPD are summarized in the work of Dorszewska et al. [1].

It seems that in patients with PD, there are many mechanisms involved in controlling levels of biogenic amines, including catecholamines and 5-HT, associated with the appearance of motor and nonmotor symptoms and impaired blood pressure regulation. Furthermore, changes in levels of biogenic amines may also be a consequence of genetic variants influencing their level and the activity of enzymes responsible for the metabolism.

6. Disturbances of serotonin levels in multiple sclerosis

MS is a complex and not fully recognized neurological disorder. Both the environmental and genetic factors are a probable cause of this disease. MS is mainly characterized by myelin destruction and a consequent dysfunction of the central nervous system (CNS). This disease is caused by inflammatory processes, linked with increased levels of Th17 and Th1 cells and decreased levels of regulatory T cells. All the MS patients are at risk of disease progression over time. This progression affects not only physical ability but also mental functions. The disease may have different forms, such as relapsing-remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), primary progressive multiple sclerosis (PPMS), and progressive relapsing multiple sclerosis (PRMS). Serotonergic system disturbances are one of the studied areas in MS patients [68].

In MS, the level of 5-HT precursor, Trp, is reduced in both plasma and CSF [68–70]. Monaco et al. [68] found that not only the Trp level in plasma was decreased, but also the level of leucine and valine was decreased. The neutral amino acids to Trp ratio were found to be significantly higher in MS than in other analyzed neurological diseases. The low concentration of Trp in CSF and plasma of MS patients stays in line with decreased of brain 5-HT synthesis and overactivation of kynurenine pathway of Trp metabolism. The kynurenine pathway competes with the melatonin pathway for Trp. Moreover, overactivation of the kynurenine pathway leads to severe imbalance between emerging neuroprotective and neurotoxic metabolites [6, 71, 72].

It is known, that in MS, the decreased of 5-HT synthesis in the brain may lead to the local 5-HT-deficit. A significant role in this deficit may play 5-HT metabolites, N-acetylserotonin (NAS) and melatonin. The levels of these metabolites are dependent on availability of 5-HT. NAS and melatonin exhibit antioxidant and anti-inflammatory properties. It also acts as immune signaling agents [73]. NAS exerts similar as a brain-derived neurotrophic factor (BDNF), activating the brain-derived neurotrophic factor (BDNF) receptor. However, melatonin decreases the number of Th1 and Th17 cell populations and the cytokines synthetized [74]. It also exerts a positive effect on mitochondrial function and reduces oxidative stress [74, 75]. It has been shown that NAS and melatonin in experimental autoimmune encephalomyelitis (EAE) in mice reduce clinical scores and the loss of mature oligodendrocytes, demyelination, and axon injury [74].

Literature data indicate that both synthesis and metabolism of 5-HT are disrupted in patients with MS. The low level of 5-HIAA was found in CSF of MS patients [9, 76]. Moreover, Markianos et al. [77] presented a negative correlation between 5-HIAA CSF level in RRMS patients and scores of disability scales: expanded disability status scale (EDSS) and multiple sclerosis severity scale (MSSS). What is interesting, the negative correlation was stronger between 5-HIAA level and MSSS than EDSS. MSSS scores not only disability status as EDSS, but also time of disease duration. Markianos et al. [77] also suggest that 5-HT turnover is more affected by the rate of accumulation of disability rather than disability itself. Reduced serotonnergic activity may lead to axonal loss. Therefore, it seems that 5-HIAA may be considered as a biomarker of severity and duration in RRMS.

It is believed that the serotonergic system also may be a target for therapy in MS. It has been shown that fluoxetine, a represent of SSRIs, reduces the formation of new enhancing lesions in magnetic resonance imaging (MRI) of nondepressed patients with RRMS. This explains the reason of elevated astrocyte-cAMP levels. The elevated levels of intracellular cAMP levels inhibit interferon-gamma induction of MHC class II in astrocytes. Normally, the MHC class II expressed on astrocytes in MS acts as antigen-presenting cells and take part in inflammation [78]. What is more, fluoxetine also promotes disease remission in acute EAE [79]. Moreover, escitalopram belonging to SSRI lowered the risk of stress-related relapse in women with MS [80]. Those studies implicated fluoxetine, and perhaps other SSRIs, may be analyzed as candidate drugs in MS.

The altered of 5-HT activity is linked not only to MS symptoms, but also to mental changes in these patients. For instance, the low concentration of platelet 5-HT may correlate with fatigue symptoms in MS [81]. Other studies have shown that SSRIs and duloxetine, which is 5-HT and NE reuptake inhibitor, are effective in depression treatment in MS [82]. Depression in MS is explained among others, as due to decreased 5-HT and melatonin synthesis.

Many studies suggested that platelet 5-HT may be used to estimate brain 5-HT level. Platelet 5-HT was found to strongly correlate with 5-HT level in CSF [83]. There are many similarities in serotonergic mechanisms in platelets and serotonergic neurons. The 5-HT uptake from plasma to platelets is similar to neuronal 5-HT uptake [84]. It is known that SERT transports 5-HT through the membrane. This transporter is encoded by the same single copy gene in platelets and neurons [85]. The 5-HT uptake in platelets and neurons is inhibited by the same drugs, tricyclic antidepressants and neuroleptics. Furthermore, 5-HT is stored in dense granules in both platelets and synaptic vesicles in neurons. Moreover, both types of cells contain MAO-B in a greater amount than MAO-A. This fact allows them to storage 5-HT which is not metabolized by MAO-B. These similarities justify treating platelets as models of serotonergic neurons [83]. Moreover, 5-HT in the blood is concentrated mainly in platelets what underlines their significant function in the serotonergic system. The 5-HT level in platelets is 24,000 times higher than in plasma [86] and the platelet 5-HT accounts for 98% of its total circulating amount [87].

As it has been mentioned that plasma 5-HT is transported to platelets by SERT. SERT is a member of the Na+/Cl-dependent solute carrier 6 (SLC6) family. In platelets, 5-HT may be deposited in dense vesicles by vesicular monoamine transporter (VMAT) or degraded by MAO [88]. Although the mechanisms of transport are recognized, the relations between 5-HT

plasma level, SERT, and platelets are still not fully understood. SERT is found to compete with dopamine transporter (DAT). Moreover, the SERT expression in relation to the 5-HT plasma level seems to be complicated and biphasic [86, 88]. These facts may play a significant role in regulation of SERT in platelets.

The similarities between neurons and platelets are mainly the complex transport regulation and lack of 5-HT synthesis in platelets. Despite that it can be used to estimate the brain 5-HT in many studies of neurological diseases, such as ALS and MS. These studies will be discussed further.

7. Amyotrophic lateral sclerosis and serotonin level

ALS is a neurodegenerative disease that affects upper and lower motor neurons. The etiology and pathogenesis of motor neuron degeneration are still not elucidated. Many of motor neuron functions are altered in ALS, especially motor neuron excitability and synaptic glutamate release. Due to disappointing results of treatment with riluzole, a glutamate action modulator, new mechanisms are under research. 5-HT system alterations may also be involved in ALS pathogenesis. The alterations of this system affect 5-HT synthesis and release. There are reports suggesting that some changes in serotonergic system may be used in clinical laboratory tests in ALS [89].

The role of 5-HT in ALS progression may be related to many mechanisms. 5-HT facilitates motor neuron activity by strengthening weak inputs—electrical impulses or excitatory neurotransmitters, such as glutamate. As in ALS 5-HT neurons are degenerated, the amount of glutamate needed to excitation of motor neuron increases. This leads to the pathological glutamate overexpression and neurotoxicity [10]. Moreover, in the brain 5-HT inhibits the glutamatergic system as a precursor of melatonin, which inhibits glutamate neurotoxicity. El Oussini et al. [89] have also indicated that 5-HT2B receptor limits degeneration of mononuclear phagocytes in CNS, which accompanied neurodegeneration in the disease.

Disturbances of serotonergic system in ALS may be found in studies of 5-HT precursor, Trp. Monaco et al. [68] shown that CSF and plasma level of Trp are reduced in ALS patients. Moreover, plasma levels of leucine and valine, which compete with Trp for uptake into the brain [90], were increased in ALS patients as a result of a larger uptake of neutral amino acids. However, its ratio was increased not only in patients with ALS, but also in patients with some other neurological diseases, such as MS. The authors of the study suggest that its different levels may be possibly used to differentiate these diseases.

The level of 5-HT itself may also have a prognostic value. Dupuis et al. [87] have shown that platelet 5-HT level is not only significantly decreased in ALS compared with controls, but it also predicts survival in ALS. In the study, the level of 5-HT was measured at one single time point in patients with diagnosed disease. The authors calculated the difference between platelet and plasma unconjugated 5-HT concentrations. The level of platelet 5-HT was more decreased in patients with bulbar onset, what corresponds with less 5-HT1A receptor binding in imaging studies [91]. Moreover, in all ALS patients, the platelet 5-HT level corresponded

with survival, from time of test to death. This can be related to some role of 5-HT alterations in the disease progression [87].

As it has been mentioned before, the serotonergic receptors can also play a significant neuroprotective role and its expression may be altered in ALS. The study of El Oussini et al. [89] has shown that the 5-HT2B receptor may limit progression in ALS by some mechanisms related to mononuclear phagocytes. On the other hand, the test of *5HT2B* gene, which encodes 5-HT2B receptors, may have some value as a survival predictor. Moreover, in the same study, patients carrying the C allele of single nucleotide polymorphism (SNP) rs10199752 in *5HT2B* gene, which encodes the 5-HT2B receptor, had a longer survival than patients carrying the more common A allele. This was also accompanied by decreased mononuclear phagocyte degeneration and increased concentrations of 5-HT2B mRNA in the spinal cord.

However, the imaging studies showed also decreased concentration of 5-HT1A receptors in the brain raphe and the cortex in ALS, even more decreased in patients with bulbar ALS onset [92]. The studies showed also alterations in concentration of 5-HIAA. This can be treated as evidence of 5-HT metabolism alterations in ALS. The *postmortem* studies of ALS patients showed decreased levels of 5-HIAA and 5-HT in the spinal cord and the brain tissue. The alterations were found particularly in the cervical and thoracic level of the spinal cord. One single study showed that concentrations of 5-HIAA were lower in the cervical spine of ALS patients with no difference in 5-HT level compared with controls [93]. However lower 5-HIAA concentration may be still linked to weak 5-HT metabolism.

8. Neuropsychiatric disorders and serotonin

ADHD, one of the most common childhood conditions, is categorized as a neurodevelopmental disorder. The group of behavioral symptoms of ADHD broadly encompasses inattentiveness, hyperactivity, and impulsiveness. The exact causes of ADHD remain unknown, but 5-HT plays a potential role in its pathomechanism. Studies provide evidence that altered availability and metabolism of 5-HT may lead to impulsivity [94]. Moreover, studies indicate that 5-HT deficiency leads to a failure of 5-HT-mediated inhibitory control of aggressive behavior and can occur also in adults [11]. Some of the studies have demonstrated decreased levels of 5-HT and 5-HIAA, in the blood, urine, and CSF in individuals with ADHD compared with in healthy controls, but other studies found no differences. However, the studies indicate that 5-HT levels in the platelets are much higher in impulsive children. There was no correlation between the platelet 5-HT concentration and other common ADHD symptoms, neither any significant difference between platelet 5-HT concentrations in ADHD children compared with controls [12].

Abnormalities in 5-HT receptors were observed in patients with ADHD: the aggression and impulsiveness are linked to increased 5-HT2A and decreased 5-HT1A receptor binding. Moreover, underexpression of 5-HT1B is a predictor of increased impulsive behavior, but not of impulsive choice [95]. Changes in 5-HTT activity in various brain regions are thought to be associated with ADHD [96, 97]. Alterations in the 5-HT level may also be caused by low activity of MAO-A and lead to impulsivity and aggressive tendencies in ADHD [98].

Disturbances in serotonergic system may be a result of many polymorphisms. Animal model studies have found that inactivation of the brain-specific Trp hydroxylase-2 (TPH2) gene leads to increased aggression due to impaired synthesis of neuronal 5-HT in the raphe neurons of the brain stem [99]. The several SNPs of the TPH2 gene are found to be strongly associated with altered functions of the prefrontal cortex during a response inhibition task in adults with ADHD [100].

Hyperserotonemia is one of the biomarkers of another neuropsychiatric condition, the ASD, and is presented in approximately 30% of patient. ASD is a group of neurodevelopmental disturbances, characterized by communication difficulties, social deficits, and repetitive behaviors, and associated by mental health issues, poor motor skills, gastrointestinal symptoms, and sleep problems [101]. The range of the symptoms varies from mild to severe. The pathomechanism of ASD is unknown, as well as the contribution of the 5-HT system to its pathophysiology.

One of the consequences of hyperserotonemia is increased catabolism of 5-HT. Blood 5-HT concentrations are regulated by the activity of peripheral 5-HT-associated proteins. It is suggested that an increased velocity of kinetics of MAO-B might be an answer to high 5-HT concentrations in the platelets [102, 103]. The hyperserotonemia in platelets in autism could be due to an increased uptake of 5-HT into the platelet. Children with autism carrying the short allele of 5-HTTLPR polymorphism associated with decreased 5-HTT expression showed better connectivity than youth with autism and long allele of this polymorphism [104].

Changes in 5-HT receptors were noted in patients with Asperger's syndrome. The abnormalities in 5-HT2A receptor density and reduction in 5-HT1A receptor binding density in several brain regions were demonstrated [105, 106].

Future studies are needed to understand the role of serotonergic system in ASD.

9. Summary

Neurological diseases, such as migraine, epilepsy, PD, MS, ALS, and neuropsychiatric disorders (ADHD, ASD) may be connected to abnormal 5-HT levels in a variety of mechanisms, as shown in **Figure 2**. Synthesis and metabolism efficiency of 5-HT is changed in neurodegeneration. Patients with ALS and MS present with reduced both plasma and CSF levels of Trp, what can be linked with a decreased 5-HT synthesis. Moreover, in MS 5-HT synthesis is decreased because of overactivation of kynurenine pathway, which drives Trp away from 5-HT synthesis. This pathway is overactivated by inflammatory molecules, such as tumor necrosis factor alpha (TNF-alpha) and interferon-gamma (IFN). In MS, 5-HT concentration is decreased due to production of its neuroprotective metabolites, such as NAS and melatonin.

In ALS, the platelet 5-HT level is decreased compared with controls and there is a positive relation between platelet 5-HT and survival. In MS, a lower platelet 5-HT level was found in patients with a more severe fatigue syndrome.

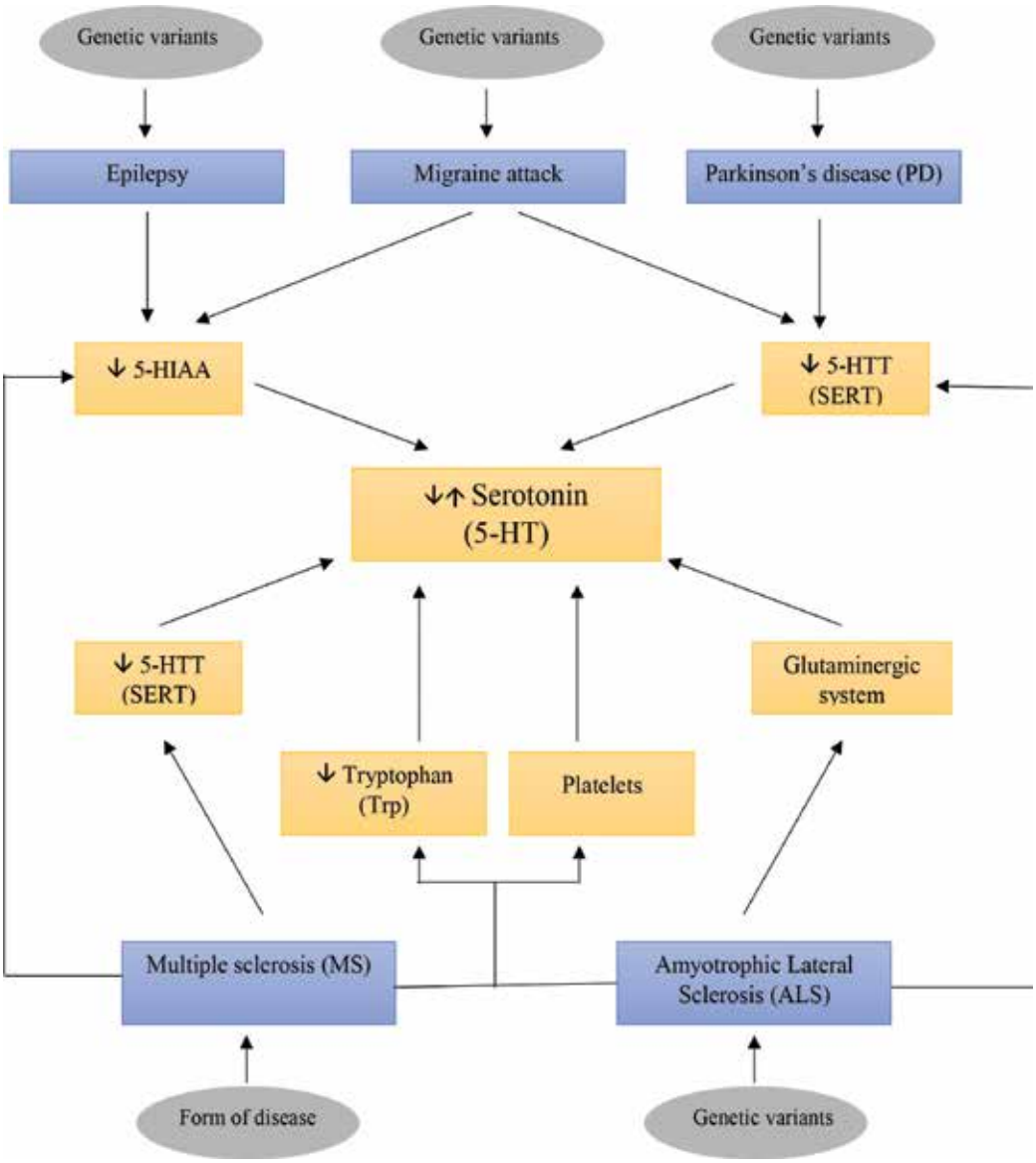

Figure 2. Disturbances of serotonin levels in neurological diseases.

Simultaneously, *postmortem* studies of ALS patients showed decreased levels of 5-HIAA and 5-HT in the spinal cord and brain tissue. However, in MS patients, lower levels of 5-HIAA were found in CSF. Moreover, in RRMS there was a negative correlation between 5-HIAA CSF level and scores of disability scales. A lower 5-HIAA level was also observed in CSF of epileptic patients as well as in migraine during attacks. However, a lower level of 5-HTT in FM and PD patients is associated with genetic variants.

Understanding the mechanisms of changes in the level of 5-HT and its precursors/metabolites in neurological diseases may contribute to finding new biomarkers relevant to the diagnosis and treatment of these diseases.

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Chapter 11

The Role of Serotonin in Aggression and Impulsiveness

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Additional information is available at the end of the chapter

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Abstract

Serotonin is a neuromodulator that has a critical role on the regulation of essential events in neuronal and glial development, such as cell proliferation, differentiation, migration, apoptosis, and synaptogenesis, and acts as a developmental signal. It has been known that a serotonergic system is associated with many psychiatric disorders. The serotonergic system also predominates on the etiopathogenesis of two important endophenotypes: impulsivity and aggression. Impulsiveness is defined as personality trait and an implusive temperament is associated with clinical conditions such as pathological gambling, eating disorders, and borderline personality disorder as well as being a risk factor for self-harm, suicide, and emotional liability. Aggression is not a personality trait like impulsivity, but it is the behavior of harm or injury to others. Besides being a natural human behavior toward survival, aggression can be harmful to the individual and the community when it is constant and excessive. In this chapter, we aimed to review the role of the serotonergic system on impulsivity and aggression, which are two important endophenotypes that identified in many psychiatric disorders.

Keywords: serotonin, aggression, impulsivity, impulsive aggression, psychiatric disorders

1. Introduction

Serotonin is a neuromodulator that acts as a developmental signal [1]. The serotonin is formed by decarboxylation of the 5-hydroxy-tripotafan that synthesized from tryptophan via the tryptophan hydroxylase enzyme [1]. Serotonin has a critical role on the regulation of essential events in neuronal and glial development, such as cell proliferation, differentiation, migration, apoptosis, and synaptogenesis [2]. Because of this broad spectrum of serotonin functions, pathologies in serotonergic system have been held to account on many psychiatric

disorders such as mood disorders, anxiety disorders, attention-deficit hyperactivity disorder (ADHD), and autism spectrum disorders (ASDs) [3]. Consider of heterogeneous clinic and different symptom clusters of psychiatric disorders, the serotonergic system predominates on the etiopathogenesis of two important endophenotypes: impulsivity and aggression [4, 5]. Impulsiveness is defined as personality trait, which is a multidimensionality [6]. An implusive temperament is associated with clinical conditions, such as pathological gambling, eating disorders, and borderline personality disorder as well as being a risk factor for self-harm, suicide, and emotional liability [3, 7]. Brain imaging and pharmacogenetic studies have demonstrated that serotonin dysfunction is associated with impulsive behaviors [8]. Aggression is not a personality trait like impulsivity, but it is the behavior of harm or injury to others [9]. It is harmful to the individual and the community when it is constant and excessive, besides being a natural human behavior toward survival [9]. Three types of aggression have been defined as psychotic, impulsive, and proactive [4]. The serotonergic system is associated with impulsive aggression which is manifested by provocation rather than proactive aggression which is goal-oriented and planned [4]. Nowadays, researchers are directed to endophenotypes in psychiatric diseases with heterogeneous clinic in order to develop new treatment methods and to elucidate etiopathogenesis. In this chapter, we aimed to review the role of the serotonergic system on impulsivity and aggression, which are two important endophenotypes that identified in many psychiatric disorders.

2. Impulsivity

Impulsivity is defined as the tendency to exhibit behavior without adequate mental assessment of possible outcomes [10, 11]. From this point of view, it can be said that impulsive dimension can be mentioned in the process of thought up to behavior [12]. In this dimension, there have been different definitions such as impulsive choice, impulsive reflection, and impulsive action that can be measured by different assessment tools that have subjective or objective qualities [10, 13, 14]. Impulsive choice described as prefer less valuable prize in soon afterwards rather than the more valuable prize in the distant future, the inability of the individual to gather adequate data on the risks describes as impulsive reflection and a lack of motor inhibition described as impulsive action [15]. The lowa gambling test provides data about impulsive choice known as delay-discounting [16]. Stop-signal reaction time and go/no go tasks are objective assessment methods that assess motor inhibition. In these tasks, individuals should wait until the appropriate signal arrives and stop the movement when no go or stop signal is received. “Waiting impulsivity” described as the failure to start the movement and “stopping impulsivity” described as the failure to stop or restrict the movement. The Barratt impulsivity scale and impulsive behavior scale are subjective self-report scales, each with different subscales and provide data on different dimensions of the impulsivity [10, 12, 13, 15, 17–19].

The main pathophysiological mechanism is the disruption of reciprocal equilibrium in corticostriatal cycles [10]. Impulsive behaviors come out as a result of impaired inhibitor function of the prefrontal cortex (PFC) to delay the award and stopping or restricting the behavior,

additionally increased striatal output to achieve a small and certain but definite near future reward rather than the far-future reward, with a high value but a low degree of uncertainty [10, 13, 15].

Recent studies showed that the basic region that rejects the award postponement when the award is quick earning despite small was nucleus accumbens; contrary the basic region that provides inhibition is the orbitofrontal cortex [20, 21]. Anterior cingulate cortex and right inferior frontal gyrus are two other important regions for inhibition [22, 23]. Nucleus accumbens is also associated with impulsive cycle inflicting from the striatum, also accompanied by amygdala and hippocampus [24]. This network includes dopaminergic, noradrenergic, and serotonergic neurotransmission.

Increased impulsiveness is associated with many psychiatric disorders, although healthy individuals have a personality trait and an advantage in situations where the organism needs to move quickly [10]. ADHD, substance abuse, eating disorders, bipolar disorder, behavioral addictions, and borderline/antisocial personality disorders are typical psychopathologies associated with impulsivity [25]. In these disorders, impulsive behavior patterns can be described in many expressions; but aggression is the most accentuated and evidence-based one.

3. Aggression

Aggression is the pattern of behavior that an individual exhibits in such a way as to damage himself or environment [4]. Natively, aggression is necessary to survive. For example, to protect ourselves and our beloved ones from danger, to supply the food and water for survive, and to react to possible risks of the organism on threat [26]. Investigating aggressive behaviors by subcategories is beneficial both in clarifying etiopathogenesis and in adjusting the treatment process. In previous papers, aggression had been categorized as offensive and defensive such as a dangerous or evasive response to a sense of fear, the most frequently preferred classification in the recent literature categorized into three groups: impulsive, proactive (also known as organized, instrumental, or predatory), and psychotic. Impulsive aggression (54%) is the most common category followed by proactive aggression (29%), and psychotic aggression (17%) [27, 28]. As predicted, psychotic aggression is a process related to positive symptoms of psychosis, such as hallucinations or delusional content. In proactive aggression, the individual exhibits this behavior in a planned manner to achieve a blazing benefit such as money or revenge. Impulsive aggression is a behavioral pattern which is accompanied by physical symptoms after stimulation of the sympathetic system, often associated with feelings of fear, inhibition, or anger, which are manifested by stress, threat, or provocation [28].

The main pathophysiological mechanism of impulsive aggression is the altered balance—to the detriment of prefrontal cortex—between the inhibitor stimulants from cortex to subcortex/limbic system and excitator stimulant as strong tendency to realizing behavior from cortex [4]. PFC dysfunction results in inadequate risk assessment and top-down inhibition is reduced [4, 27]. Bottom up outputs that have increased frequency and amplitude especially from the amygdala toward the orbitofrontal cortex contribute to impulsive aggression [27, 29]. In many human and

animal studies, ventral PFC has been shown to be associated with impulse and aggression [30]. Antisocial behaviors are also observed in specific lesions of ventral PFC [30, 31].

4. Serotonin on impulsivity

A significant part of serotonergic innervation in brain structures is derived from the dorsal raphe nucleus (DRN) [32]. It has been shown to increase premature response in the lesion of serotonergic areas at DRN by 5-choice serial reaction time task (5CSRT) although increased correct response [33]. These findings point to the role of serotonergic regulation in organizing behavior to optimize the performance of the cortex [34]. What a serotonin-stimulated neuron will ultimately do is related to the balance between the serotonergic receptors on it [35]. In addition, there are nonserotonergic neurons in the projection fields of raphe nucleus in PFC, where 5HT1A and 5HT2A receptors are postsynaptic located [36]. In PFC, 80% of the glutamatergic neurons and 25% of the GABAergic neurons have 5HT1A and 5HT2A receptors distributed in Ref. [36]. The 5HT2A receptor activates the neuron and increase glutamate release by the contrast with 5HT1A receptor that decreases glutamate release [34]. In molecular genetic studies, 5HT2A is the most prominent receptor in the role of serotonin on impulsivity.

5HTR2A is located in the genomic chromosome 13q14-q21 and contains three exons [37]. This gene codes for a receptor associated with the G protein, and this receptor stimulates phospholipase C, which reduces protein kinase C activity [37]. 5HT2A is most commonly expressed in the hippocampus, amygdala, and nucleus accumbens [38]. The 5HT2A receptor is associated with many common psychiatric disorders such as major depression, obsessive-compulsive disorder, anorexia nervosa, and schizophrenia. Dopamine and 5HT have also been shown to play an important role in the regulation of attention and response control in frontal cortex by animal models [39]. In the psychopathology mentioned above, impulsivity is one of the three core symptom clusters of the disease, ADHD is especially prominent at research. Therefore, in this section, ADHD/impulsivity will be discussed in the context of serotonin. Continuation of sedative effects of methylphenidate in knockout mice inhibited dopaminergic gene function supports the role of other systems. In this model, hyperactivity was also observed to be suppressed with fluoxetine. This effect is thought to be mediated by an increase in the concentration of extracellular serotonin through blockade of the serotonin transporter. In the direction of these findings, it has been suggested that the effect of methylphenidate on impulsivity also be demonstrated by increasing serotonin levels [40, 41]. The data obtained from pharmacological studies, which showed that stimulated striatal 5HT2A receptors increase dopamine release and regulate hyperactivity, confirm that the serotonergic and dopaminergic requirements are in interaction to mediate hyperactivity behavior [42]. Serotonin may affect ADHD and other impulsive behaviors indirectly by regulating dopaminergic functions. The nature of this regulatory effect is complex. It has been demonstrated that serotonergic neurons have inhibitory effects on dopaminergic neuron bodies in the midbrain region; both excitatory and inhibitory effects on dopamine projections in striatum, nucleus accumbens, and prefrontal

cortex by animal models [43, 44]. When serotonergic agonists supplied to striatum, it has been leading to inhibited striatal neuronal firing, decreased in synaptic dopamine, which may result in reduced synthesis or release of dopamine in neuronal projections. That effect has been thought to be mediated by the serotonergic receptor 5HT2A. By way of these data, it has been thought that 5HT2A receptors may contribute to the development of ADHD [45]. Interest in the 5HT2A receptor in ADHD began with the observation that decreased hyperactivity in mice given selective 5HT2A antagonists [42]. It has been shown that the 5HT neurotransmitter system, in parallel with the typical course of ADHD, develops an age-related developmental pattern, for example in developmental studies in monkeys, the 5HT receptor binding increased during infancy and childhood, peaked before puberty and slowly decreased during adolescence and early adulthood [46]. In humans, the 5HT2 receptor binding at 6 years was found to be higher than in neonates and 13–14 years of age [47]. The main result of activation of the 5HT2A receptor by serotonin is reduced noradrenalin and dopamine levels and increased glutamate levels [35]. In this context, 5HT2A antagonism contributes to attention functions by causing an increase in dopamine noradrenalin levels. In the light of those information, it has been aim to clarify the subtypes of impulsivity by referring to some important studies that have recently been made. The 5CSRT is a test for assessing impulsivity, as well as providing information on attention functions used in animal studies [48]. In that task, the individuals learn to get food by pressing the button after a certain goal. The pushing of the button by the animal without showing the target is regarded as a premature response and displays the waiting impulsivity [48]. In a study conducted by Fletcher et al., it was observed that 5HT2C and 5HT2A antagonists given to mice have different effects on 5CSRT [49]. While 5HT2A antagonists reduced prematurity responding, 5HT2C antagonists increased. As a result of that the researchers have also indicated that the impulsivity is not only related to the level of 5-HT, but it is also related to the balance between different serotonergic receptors [49]. In a study in which the effect of 5HT2A receptor gene polymorphism [1438G/A] on impulsivity was assessed by go/no go test, individuals with polymorphism were found to have significantly more commission errors [50]. It has been found that 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), a potent 5HT1A agonist, was ineffective on 5CSRT parameters by systemic administration; however, presynaptic 5HT1A autoreceptors at DRN had a markable effect on those parameters [34]. In a study conducted on women with bulimia nervosa in which impulsive behavior patterns were observed, it was determined that there was a decrease in 5HT2A binding in ventral PFC [51].

The decrease in central serotonergic activity was associated with negative emotional state, poor impulse control, aggressive behavior, increased alcohol and nicotine use, and increased food consumption [52]. The tryptophan depletion method reduces the amount of serotonin throughout the brain. In a study conducted with this method, the relationship between impulse serotonin in humans was examined and an increase in premature response was detected. By this means, it has been concluded that central serotonin levels are related to waiting impulsivity rather than stopping impulsivity. Interestingly, it has been determine that tryptophan-depleted individuals had an increase in motivation and accuracy in compliance with individuals who do not have this depletion [53, 54].

5. Serotonin on aggression

Serotonin is the main neurotransmitter in both top-down and bottom-up processes of neurobiological cycles associated with aggression [4]. Serotonergic hypofunction has been found to be associated with impulsive aggression in aggression subtypes [5]. It has been known that polymorphism of metabolic enzymes, carrier proteins, and receptors on the serotonergic system is associated with an increased aggressive behavior pattern [55]. The essential role of serotonin in the etiopathogenesis of impulsive aggression has been determined by brain imaging studies showing an increase in 5HT2A receptor concentration in orbital PFC in aggressive individuals, tryptophan depletion studies, molecular genetic studies that showed individuals who have monoamine oxidase a gene polymorphisms and have early stressful life events lean to aggression and violence at early adulthood period [38, 56–59].

Selective serotonin reuptake inhibitors (SSRIs) are generally recommended in the treatment of impulsive aggression. However, it should be kept in mind that special approaches are needed in special patient groups. For example, SSRIs have been found to be effective in the treatment of aggression in dementia patients and ineffective in patients with traumatic brain injury [27]. SSRIs are generally recommended in the treatment of impulsive aggression [27].

6. Conclusion

In this chapter, it has been argued the relationship between serotonin, one of the basic neurotransmitters, with the two endophenotypes—impulsivity and aggression—in the face of many psychiatric disorder. There is a consensus in the literature that the problems of the subunits of the serotonergic system result with impulsivity and aggression. Nowadays, researchers have elaborated this information and have identified impulsivity and aggression as subtypes. In the last decade, data from both animal and human studies have been suggested that serotonin has more associated with impulsive aggression than with aggression subtypes, with more "waiting impulsivity" in impulsivity subtypes. More clinical studies are needed on this issue in which genetic and neuroimaging techniques are combined in homogeneous samples that are well defined by subtypes.

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Chapter 12

Immuno-Thrombotic Effects of Platelet Serotonin

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Additional information is available at the end of the chapter

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Abstract

Platelets transport and store serotonin at a high concentration in dense granules and release it upon activation. Abnormal serotonin concentrations in the blood plasma or increased platelet serotonin release promote the development of thrombosis, sepsis, allergic asthma, myocardial infarction, and stroke. Consequently, experimental data suggest possible benefits of serotonin receptor blockade or inhibition of platelet serotonin uptake in the indicated human diseases. Here, we highlight the current state of basic biological research regarding the role of platelet serotonin in normal and pathophysiological conditions focusing on thrombotic and inflammatory diseases. We also describe the possible clinical applicability of targeting thrombo-immune-modulatory effects of platelet serotonin to treat common health problems.

Keywords: platelets, serotonin, inflammation, thrombosis, selective serotonin reuptake inhibitors

1. Introduction

Serotonin (5-HT) is a well-known neurotransmitter, which regulates neural activity and a variety of neuropsychological processes [1]. As it has been shown to be involved in the regulation of systemic and cellular functions, alterations in serotonin concentration in the body are associated with many different diseases, such as irritable bowel syndrome, restless legs syndrome, sudden infant death syndrome, autism, headache, insomnia, anxiety, depression, anorexia, schizophrenia, Parkinson's and Alzheimer's disease, pulmonary hypertension, and

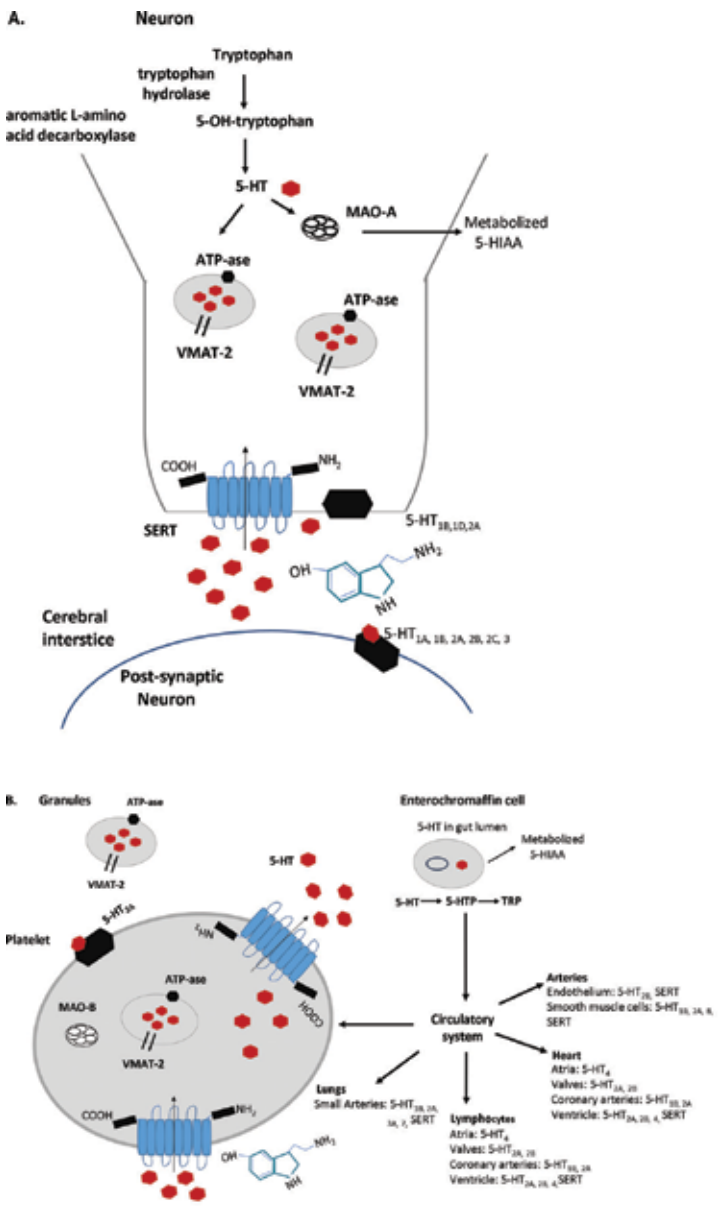

Figure 1. 5-HT biosynthesis and receptor distribution in brain (A) and periphery (B). Serotonin (5-HT), serotonin transporter (SERT), monoamine oxidase (MAO), 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptophan (5-HTP), tryptophan (TRP), and vesicular monoamine transporter (VMAT). For the details, see the text.

myocardial infarction. 5-HT was first described in 1930 by Vittrorio Erspamer who isolated it from enterochromafin cells of the gut [1]. Only a small amount of 5-HT is synthesized in brain (5%), whereas 95% is produced by the enterochromafin cells of the gastrointestinal (GI) tract. 5-HT is synthesized from the essential amino acid L-tryptophan (TRP) to 5-hydroxy-tryptophan (5-HTP) by the enzyme L-tryptophan hydroxylase (TPH)-1 in the brain and

TPH-2 in the periphery [2, 3]. The activity of these TPH enzymes is the rate-limiting step in the production of 5-HT in both organs. After its synthesis in the gastrointestinal tract, 5-HT is released into the bloodstream. 5-HT can bind and activate several isoforms of 5-HT receptors expressed throughout the body (**Figure 1**). 5-HT receptors were identified on different blood cells and in the vessel wall including lymphocytes, endothelial, and smooth muscle cells, respectively, which can respond to 5-HT under certain physiological conditions. These receptors constitute a family of seven different receptor sub-classes: 5-HT_1 (A-F, P, S), 5-HT_2 (A-D), 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 [3, 4]. All these receptors belong to the GPCR superfamily with the exception of 5-HT_3 [3], which is a member of nicotinic acetylcholine receptor superfamily and is a ligand-gated ion channel.

5-HT can also be taken up from plasma into several cells—such as platelets—via the 5-HT transporter (5-HTT, SERT). After uptake, 5-HT can be then stored in vesicles and granules through the action of vesicular monoamine transporter (VMAT)-1/2 which is expressed in neurons, neuroendocrine cells, and platelets. The largest quantity of serotonin is believed to be stored in platelets, from where it can be released upon platelet activation, for example, during thrombus formation or inflammatory reactions. Interestingly, chemical precursors of 5-HT can pass across the blood-brain barrier, but 5-HT cannot, thereby effectively isolating the brain 5-HT pool from the periphery and vice versa. In the brain, 5-HT regulates several complex networks, such as mood, perception, reward, anger, memory, appetite, attention, and sexuality. There are two major routes of 5-HT metabolism, which convert 5-HT to melatonin and 5-HIAA. 5-HT is metabolized by neurons and endothelial cells by monoamine oxidases (MAOs) and the products of this breakdown are then excreted by the kidney [3, 5–7].

Peripheral 5-HT regulates heart development and rate, valvulopathy, pain, nociception, embryonic development, vasoconstriction/vasodilatation, blood flow, hemostasis, and many other important processes. Platelets are not able to synthesize serotonin, but take it up from plasma via 5-HTT, store it in dense granules (via VMAT-1), and release it into the blood during their activation. Platelet serotonin has not only well-established autocrine functions during platelet activation and thrombus growth but also paracrine functions in the vasculature including modulation of endothelial, smooth muscle, and immune cell function.

2. Autocrine-regulatory mechanisms of platelet serotonin

Platelets store 5-HT in their dense granules at millimolar range and secrete it after activation [8]. Dense granule and 5-HT release support the recruitment of circulating platelets to preformed thrombi, thereby leading to thrombus growth. This process is mediated through the interaction between 5-HT and its receptor 5HT_{2A} expressed on circulating platelets. Activated 5-HT_{2A} receptor transduces the signal to G_q-phospholipase C (PLC) β-signaling cascade. Enhanced PLCβ activity results in intracellular Ca^{2+}mobilization from the store through inositol 3-phosphate (IP3) receptor and mediates 1,2-diacylglycerol (DAG)-dependent protein kinase C (PKC) activation, thereby amplifying platelet reactivity (**Figure 2**).

In addition to the mobilization of cytosolic Ca^{2+} [9, 10], receptor-ligand interactions are also known to regulate 5-HT uptake kinetics. In human platelets, the rise of cytoplasmic Ca^{2+} in

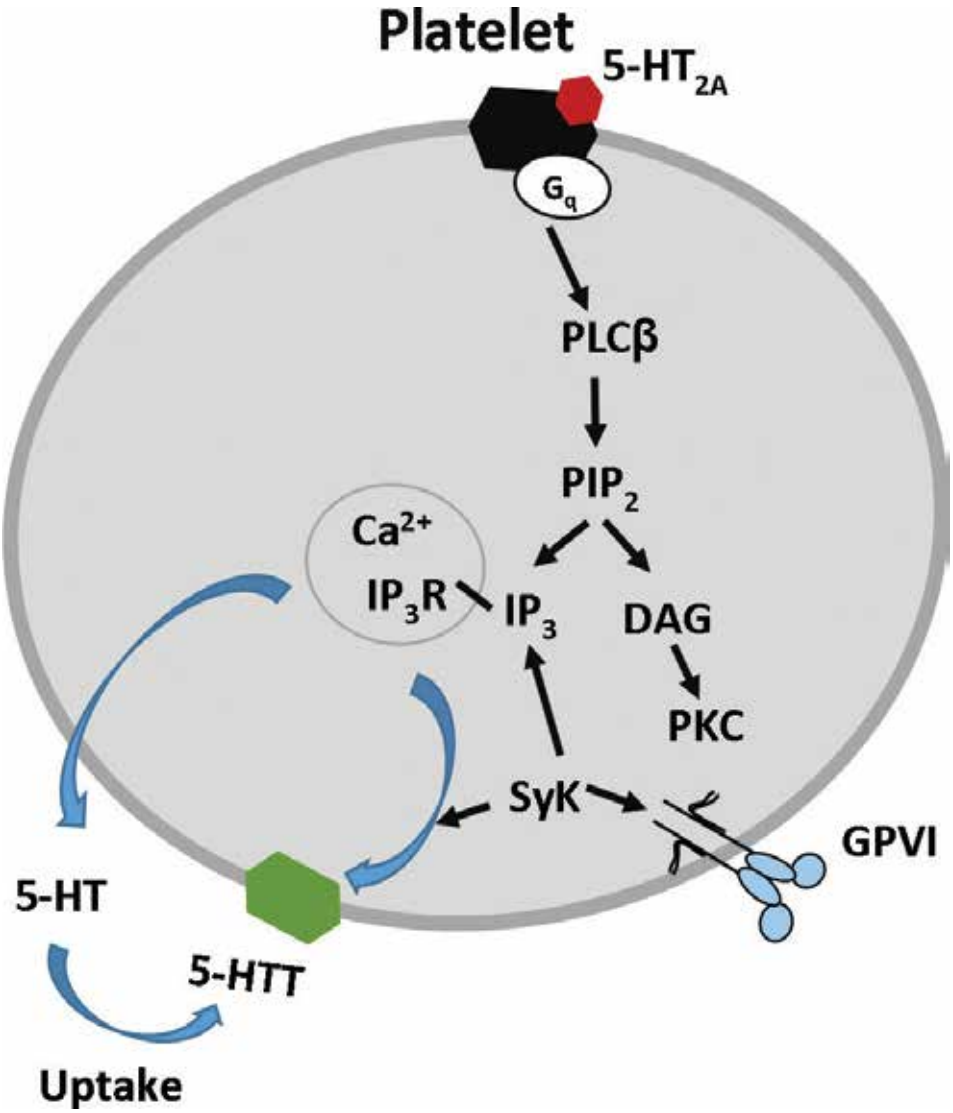

Figure 2. Autocrine effects of platelet 5-HT. Activated platelets release 5-HT, thereby amplifying platelet activation and the recruitment of circulating platelets. Binding of platelet 5-HT to the 5-HT_{2A} receptor induces activation of PLCβ-signaling cascade and upstream effectors which support platelet reactivity. Receptor-ligand interactions also regulate 5-HTT uptake kinetics by interconnecting several signaling pathways. For the details, see the text.

the absence of exocytosis reduces 5-HT transport into the cytoplasm, thereby decreasing the release of 5-HT [9]. Interestingly, rabbit platelets activated in the presence of the extracellular Ca^{2+} chelator ethylene tetraacetic acid also displayed a decrease in 5-HT transport activity [11, 12]. Consistently, human platelets treated with the membrane permeant Ca^{2+} chelator BAPTA-AM also had reduced 5-HT transport in the presence of extracellular Ca^{2+} [9]. Activation of the Orai1 Ca^{2+} channel induces a robust Ca^{2+} influx called store-operated Ca^{2+} entry (SOCE), which is triggered through the release of Ca^{2+} from intracellular stores.

This process is controlled by functional coupling of activated stromal interaction molecule 1 (STIM1) to Orai1 [13]. Interestingly, strongly reduced SOCE was found in *5Htt*$^{-/-}$ platelets [14]. This suggests that secreted platelet 5-HT contributes to the regulation of SOCE through binding to 5-HT$_{2A}$ which activates Gq-PLCβ-mediated Ca^{2+} store release, thereby further activating STIM1/Orai1 complex. Interestingly, SOCE-induced signal can strongly inhibit 5-HT uptake in human platelets via 5-HTT [9, 11]. This could be an important step to keep 5-HT outside of platelets, thereby increasing extracellular 5-HT concentration and permanently activating 5-HT$_{2A}$ on the platelet surface. Therefore, 5-HT cannot enter the platelet cytosol during SOCE. Interestingly, 5-HTT contains several consensus sites for PKC. It has been shown that PKC activity is required for the internalization of the transporter suggesting a link between 5-HT uptake and intracellular Ca^{2+} level [15–18]. Altogether, Ca^{2+} signaling, Ca^{2+} store release, and Ca^{2+} influx through SOCE play an important regulatory role for 5-HT cycling in human and mouse platelets.

After Ca^{2+} store release and PKC activation, integrins exposed and activated on the platelet surface support aggregation and thrombus formation. In β3 integrin-deficient platelets, 5-HT uptake was strongly reduced, indicating a functional crosstalk between 5-HTT and β3 integrin [19]. Integrin activation defect in response to glycoprotein VI (GPVI) or C-type lectin-like receptor 2 (CLEC-2) stimulation was found in *5Htt*$^{-/-}$ mouse platelets, which was fully rescued in the presence of extracellular 5-HT [14]. The physical interaction between 5-HTT and β3 seems to be dispensable for β3 integrin activation. The observed integrin activation defect is due to the lack of the secreted platelet 5-HT which further amplifies "inside-out" activation of integrins through Ca^{2+}-dependent and independent pathways mediated by Ca^{2+}- and DAG-regulated guanine exchange factor-1 (CalDAG-GEFI) and PKC, respectively.

Although 5-HT is mainly stored in dense granules, intracellular-free 5-HT in the cytoplasm has been proposed to activate diverse biological processes called serotonylation. It has been shown that small-guanosine triphosphate-binding protein (GTP)-ases covalently bind 5-HT, thereby changing the structure and activity of GTPase, leading to α-granule exocytosis from platelets. This process requires tissue transglutaminase and factor XIIIa, both activated by mobilized Ca^{2+}. Transglutaminase may mediate the transamidation of small GTPases, like cytoplasmic Ras homolog gene family member A (RhoA) and a small GTP-binding protein Rab4. Serotonylation in turn blocks the inactivation of both molecules. A complex composed of Ca^{2+} and calmodulin (CaM) may also activate guanine exchange factors (GEFs), which induce the exchange of guanosine di- (GDP) to triphosphate (GTP) on RhoA and Rab4 and thus stimulates activation of the respective protein. These two active molecules play an important role in cytoskeleton rearrangement, exocytosis of α-, and dense granule contents. Some bioactive molecules stored in platelet granules, such as fibrinogen and factor V, are also known to be serotonylated [8]. Upon platelet activation, these proteins are exposed at the platelet surface and are used to mark a subpopulation of highly activated, pro-coagulant platelets, the so-called collagen and thrombin-activated (COAT) platelets. Coated platelets express high levels of phosphatidylserine and strongly support prothrombinase activity [8, 20].

Besides the dopamine transporter (DAT), the noradrenaline transporter (NET), and the organic transporter (OCT), 5-HTT is an important 5-HT transporter to regulate 5-HT uptake from the blood plasma and reuptake of the released platelet 5-HT in certain physiological

conditions. 5-HTT is encoded by the SLC6A4 gene containing 14 exons. The protein structure of 5-HTT contains 12 transmembrane domains. In humans, the splice variants of 5-HTT and their mutations are associated with several pathologies, such as anxiety, suicide, depression, substance abuse, autism, and neurogenic disorders [21–24]. 5-HTT is abundantly expressed not only on neurons, endothelial cells, mast cells, immune cells, in intestine, and vasculature, but also in platelets [25, 26]. It is well established that in platelets 5-HTT plays an important role in the uptake of 5-HT from the circulation. Monoamine transporters are thought to be able to compensate for one another where they are co-expressed. For example, 5-HT may be taken up in venous vessels independently of 5-HTT expression [25, 27]. Interestingly, and in sharp contrast to venous vessels, genetic ablation of *5Htt* in mice completely abolished 5-HT uptake in platelets, since no detectable secreted 5-HT was observed upon platelet activation, indicating an essential role of 5-HTT for 5-HT uptake into platelets, which cannot be compensated by other transporters [14]. Altogether, these results highlight the cell-type-specific regulation of 5-HT uptake in mammalian cells.

5-HTT can be targeted by several antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) (cf., Section 5), which are widely used in the treatment of psychiatric diseases to increase 5-HTT concentrations in the synaptic space. The blockade of 5-HTT with the SSRI citalopram reduces the aggregation response to collagen in human platelets [28] due to reduced phosphorylation of a tyrosine-protein kinase Syk in the GPVI signalosome. Syk can also bind and phosphorylate 5-HTT suggesting an Syk-mediated functional crosstalk between 5-HTT and GPVI complex. Interestingly, *5Htt*$^{-/-}$ mouse platelets could not show any abnormalities in the tyrosine phosphorylation cascade of the GPVI signalosome, as Syk phosphorylation was normal after GPVI stimuli. Consequently, Syk and 5-HTT interaction seems to be dispensable for the initial activation of GPVI complex, but enhanced Syk activity may regulate the 5-HT uptake in platelets [29].

3. Paracrine-regulatory mechanisms of platelet serotonin

During degranulation, activated platelets secrete a significant amount of 5-HT from dense granules which is clinically relevant to induce acute thrombotic events [30, 31] by promoting vasoconstriction and cellular activation of neighboring platelets and lymphocytes through their 5-HT receptors.

5-HT receptors expressed on endothelial, smooth muscle, and immune cells respond to platelet-derived 5-HT (**Figure 3**). 5-HT has growth-promoting effects on endothelial cells, which may facilitate tissue healing after vascular damages [32]. However, 5-HT may also exert dual effects either stimulating constriction or dilatation of microvasculature. In the liver, 5-HT appears to mainly promote constriction of hepatic sinusoid vessels, since mice lacking peripheral 5-HT display elevated sinusoidal perfusion under physiological and pathological conditions [33]. By contrast, platelet-derived 5-HT coordinates the formation of gaps between endothelial cells in the joint microvasculature, which in arthritic conditions may contribute to inflammation [34]. How these processes are regulated is still not clear but presumably may involve differential signaling pathways through specific 5-HT receptors expressed on vascular endothelial and smooth muscle cells.

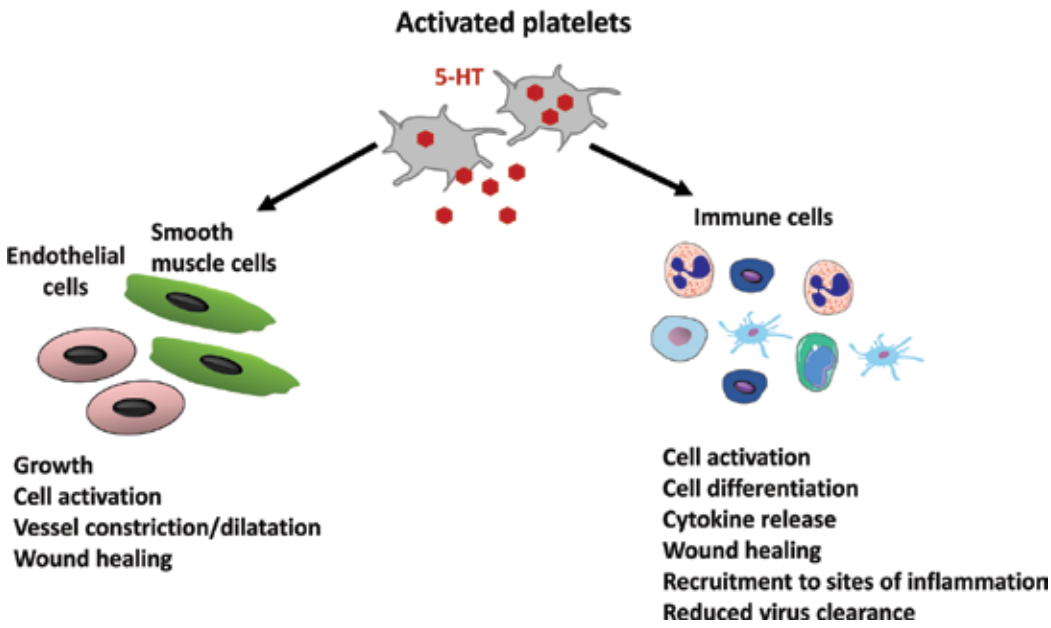

Figure 3. Paracrine effects of platelet 5-HT. Secretion of platelet 5-HT modulates the function of endothelial and smooth muscle cells either promoting vessel constriction or dilatation. Platelet 5-HT influences several functions of immune cells, indicating their importance in the regulation of immune cell response and activities under pathophysiological conditions. For the details, see the text.

Platelet-derived 5-HT can regulate the function of T- and B-cells, natural killer cells, monocytes, and neutrophils under certain conditions [35–38]. In the spleen, 5-HT increases monocyte differentiation into dendritic cells and early naive T-cell activation via the 5-HT_{2A} receptor [38, 39]. Furthermore, it also has been shown that lymphocytic cytokine levels in mice are reduced after treatment with SSRI [40]. In a mouse model of viral hepatitis, the release of 5-HT by platelets was responsible for tissues damage caused by CD8 (+) T-cells, microcirculatory events, and reduced clearance of infiltrated viruses [33]. Moreover, specific antagonism of 5-HT receptors in mice attenuated asthmatic attacks and sepsis [37, 41].

5-HT released from dense granules upon activation by the inflamed endothelium also contributes to the recruitment of immune cells to the vascular wall [37]. Indeed, platelet-derived 5-HT promotes leukocyte migration, possibly via activation of endothelial cells, thereby enhancing P-selectin exposure and IL-8 release [37], which trigger neutrophil rolling, adhesion, and extravasation. Moreover, locally increased levels of platelet-released 5-HT had paracrine effects on endothelial cells, thereby inducing microvasculature leakage through the activation of transglutaminase and the phosphorylation of vimentin [42]. By contrast, in solid tumors platelet-released 5-HT has been described as a major regulator of the tumor vascular homeostasis that continuously prevent bleeding. Interestingly, tumor-infiltrating leukocytes have been identified as the cause of tumor bleeding [43, 44]. Altogether, these studies suggest that under specific conditions, platelet-released 5-HT promotes clot formation and modulates immune cell functions.

In humans, 5-HT levels appear elevated in infection and autoimmune diseases, suggesting that SSRI could be applicable for vascular and immune system modulation. Since platelets are the major 5-HT store in the blood, pharmacological blockage of 5-HT uptake in platelets increases the level of 5-HT in the blood plasma transiently. Unexpectedly, *5Htt*$^{-/-}$ mice display reduced 5-HT levels in plasma [14]. In *5Htt*$^{-/-}$ mice, elevated urinary 5-HIAA levels were detected suggesting a faster 5-HT metabolism in the peripheral blood. Consequently, platelet 5-HT uptake and storage play an important regulatory role for controlling systemic 5-HT metabolic cycles. Future studies are needed to specify the exact mechanisms of platelet-derived 5-HT on vascular and immune system modulation in normal physiology and diseases.

4. Pathophysiological consequences of abnormal platelet serotonin release

5-HT plasma concentration was analyzed in several pathological contexts. It became widely recognized that 5-HT is an independent risk factor for platelet aggregation and for thrombus formation in animal models (cf., Section 6) and human patients [19, 45–49]. Plasma 5-HT can support platelet aggregation and thrombus growth through 5-HT$_{2A}$-dependent or independent signaling pathways. Pharmacological blockade of 5-HT$_{2A}$ receptor increases the 5-HT uptake rates in animal models of hypertension, as well as ex vivo platelet aggregation. Vikenes et al. detected a 10-fold increase of plasma 5-HT in patients undergoing angiography after admission for myocardial infarction [50]. In these patients, high plasma 5-HT was associated with cardiac events. In another study, more than 10-fold rise in 5-HT has been noticed in coronary vessels of patients following angioplasty. Importantly, in these patients the level of 5-HT in the systemic plasma was normal [51]. Together, these studies suggest that in vivo the interplay between circulating 5-HT and platelet function could be a predictive factor.

5-HT levels are drastically increased during myocardial ischemia, and blockade of the 5-HT$_2$ receptor improves the outcome after myocardial infarction in different mouse models [52, 53]. 5-HT also enhances the survival of cardiomyocytes via the 5-HT$_{2B}$ receptor. In hepatic ischemia models, platelets promote tissue repair [54], and proliferation of hepatocytes was shown to be partly mediated by platelet 5-HT after liver ischemia [55]. 5-HT also contributes to intratumoral homeostasis by dysbalancing permeability factors [44]. 5-HT-induced growth of human hepatocellular carcinoma cells and specific blockade of the 5-HT$_2$ receptor decreased recruitment of circulating tumor cells [56, 57]. It has been suggested that the inhibition of platelet granule contents might be effective to induce intratumoral bleeding, thereby decreasing tumor viability and growth. Additionally, plasma 5-HT levels are increased in patients with colorectal, liver, and intestinal cancers [58, 59].

Allergic airway inflammation provokes a local release of 5-HT in mouse models and human patients [41]. Interestingly, after challenge with an allergen, 5-HT increased 10-fold in broncho-alveolar lavage of predisposed patients, inducing asthmatic attacks. In line with these studies, 5-HT is known as a key regulator of pulmonary vascular resistance and vessel wall integrity [60, 61].

5. Clinical applications: effects of selective serotonin reuptake inhibitors on platelet functions

Selective serotonin reuptake inhibitors are commonly used drugs for the treatment of patients with severe depressive and anxiety disorders [62]. SSRIs were developed to selectively inhibit the uptake of 5-HT through the 5-HTT transporter in the brain, while having minimal side effects on DAT and NET proteins which can also transport 5-HT [63]. The action of SSRIs relies on the modulation of the allosteric region of the transporter, thereby leading to a conformational change and blocking of the uptake of 5-HT [63]. The uptake of 5-HT into neurons is very important for the clearance of the synaptic cleft, preventing firing rates and overstimulation of receptors [64]. This uptake and the later release are blocked upon treatment with SSRIs, such as fluvoxamine, fluoxetine, nortryptiline, citopram, and escitalopram [65]. The different SSRIs vary in kinetics being competitive and non-competitive inhibitors. Two distinct binding sites on 5-HTT have been identified, a low-affinity allosteric site, mediating the dissociation of SSRIs from their high-affinity site, which induces the blockade of 5-HT uptake [64].

There is evidence that targeting 5-HT receptors or using serotonin-like molecules is effective in the treatment of non-neuronal diseases. The use of tricyclic antidepressants, but not SSRIs, is associated with an increased risk of myocardial infarction. SSRIs have shown no cardiac toxicity, even in patients with heart disease. Several epidemiologic studies reported lower cardiovascular morbidity and mortality in patients treated with SSRI [66–68].

Depression is a significant risk factor for ischemic heart and cerebrovascular disease as well as mortality following myocardial infarction. The potential effects of SSRIs upon the cardiovascular system may therefore play an important role. These drugs had potential benefit in hypertensive patients after myocardial infarction and hypertensive responses to depression were reduced in patients who had been prescribed SSRIs [30]. In blood samples of depressive patients taking fluoxetine, the platelet aggregation response to submaximal collagen stimulation was decreased [69]. In this study, a significant decrease in 5-HT concentration was observed in platelet-rich plasma associated with the use of fluoxetine but not with the tricyclic antidepressant amitryptiline. It is intriguing whether lowered platelet 5-HT content translates into less 5-HT release during platelet activation in patients with thrombotic diseases. Enhanced platelet reactivity was observed in patients suffering from depression and chronic heart disease due to the upregulated β-thromboglobulin (β-TG) and platelet factor 4 (PF4) levels [70]. Lowered PF4 and β-TG levels have been observed upon treatment with SSRI paroxetine [71], suggesting that reduced platelet aggregation in vivo may impact coronary artery-related mortality. SSRI treatment also decreases platelet reactivity in patients with heart failure. Other SSRIs, sertraline, and N-desmethylsertraline were also shown to dampen platelet responses [72].

SSRIs have been shown to increase the risk of bleeding in patients with liver cirrhosis and liver failure. Importantly, SSRIs may also directly increase gastric acidity with ulcerogenic effect resulting in GI bleeding. The risk of SSRI-associated GI bleeding is increased with the concurrent use of nonsteroidal anti-inflammatory drugs, anticoagulants, and antiplatelet agents,

and is decreased by concurrent proton pump inhibitors [73, 74]. In conclusion, SSRIs appear to be protective against cardiovascular diseases and may enhance the risk for GI bleeding. However, to date this evidence is not yet conclusive.

6. Experimental studies on the role of platelet serotonin in arterial thrombosis and stroke

Over the past decades, the functions of peripheral 5-HT have received increasing attention. It has been shown that peripheral 5-HT plays a major role in a variety of important processes, including hemostasis and immune defense. This has been addressed by using *Tph1*$^{-/-}$ mice, which lack peripheral 5-HT in the circulation, due to the lack of the enzyme that converts hydroxylases tryptophan to 5-HT in the gut [75]. In humans, abolished or decreased level of TPH1 is associated with impulsive behavior, aggression, irritable bowel syndrome, anxiety, and other pathologies [76–79]. Genetic ablation of TPH1 function in mice not only leads to several disorders, such as mild anemia, cardiomyopathy, and diabetes, but also to other defects in hemostasis, erythropoiesis, pulmonary hypertension, and lung regeneration. The lack of 5-HT in this mouse model is associated with decreased neutrophil recruitment to inflammatory sites, diabetics, and mild anemia [37, 80].

Recent studies using wild-type mice infused with 5-HT or *Tph1*$^{-/-}$ mice have demonstrated that peripheral 5-HT is required for platelet aggregation [14]. Additionally, *in vivo* 5-HT infusion generates hyperreactive platelets with reduced bleeding time and shortened occlusion time of the carotid arteries in wild-type mice. *5-Htt*$^{-/-}$ mice have prolonged bleeding time, reflecting the increased bleeding risk described to occur using long-term SSRI treatment in human patients. In comparison to this relatively mild hemostatic defect, *5Htt*$^{-/-}$ mice were not able to form occlusive thrombi in response to mechanical injury of the abdominal aorta as compared to wild-type animals [14].

Platelets contribute to the progression of infarct growth after transient brain ischemia by thrombo-inflammation with platelet-immune cell interactions. SSRI treatment of stroke patients has been described to enhance brain function recovery, indicating a therapeutic benefit of the direct blockade of 5-HTT function. Neuroblast proliferation and cell migration have been shown to be enhanced and associated with increased microvessel density during SSRI treatment, explaining the possible role of 5-HTT in tissue repair after ischemic insults [81–83]. *5Htt*$^{-/-}$ mice have been studied in the tMCAO (transient intraluminal filament model of middle cerebral artery occlusion) model of ischemic stroke. Unexpectedly, these mice developed similar brain infarcts to wild-type controls and the 5-HTT neurological outcome was indistinguishable [14]. In line with this study, SSRI treatment could not reduce infarct size or cerebral edema in mice [82], suggesting that this treatment cannot protect neurons or other cells in the ischemic brain. Altogether, these results indicate that SSRI treatment may have a long-term effect in the ischemic brain tissue which positively influences post-stroke recovery. Further investigation is necessary to understand the specific role of peripheral and brain 5-HT in thrombo-inflammation during stroke and infarct progression.

7. Conclusions

5-HT is an ancient molecule that is better known for its functions in the brain than in the periphery. However, literature describing the contribution of peripheral 5-HT, including platelet 5-HT, is rapidly growing. It became evident that platelet 5-HT has a complex role involving many bidirectional interactions with tissue microenvironment to regulate platelet and immune cell functions. SSRI treatment in animal models appears to improve thrombotic and inflammatory diseases. Further fundamental and preclinical studies are needed for a better understanding of platelet 5-HT functions in humans. In conclusion, targeting thrombo-immune-modulatory functions of platelet serotonin may provide new important therapeutic approaches.

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Chapter 13

Production and Function of Serotonin in Cardiac Cells

Joachim Neumann, Britt Hofmann and Ulrich Gergs

Additional information is available at the end of the chapter

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Abstract

Serotonin [5-hydroxy-tryptamine (5-HT)] exerts a number of effects in the mammalian heart: increase in heart rate, increase in force of contraction, fibrosis of cardiac valves, coronary constriction, arrhythmias and thrombosis. These effects are, in part, mediated by 5-HT-receptors, in part, directly by 5-HT action on intracellular proteins. In the beginning, 5-HT was thought to be only produced in the gut and then transported into the heart via platelets, because platelets can take up 5-HT in the gut and enter the capillaries and thus the mammalian heart. 5-HT is to a large extent metabolized in the liver and excreted via the urine. Here, we will also overview data that argue for additional pathways, namely production and degradation of 5-HT in the cells of the heart itself.

Keywords: heart, human atrium, serotonin, 5-hydroxytryptophan, MAO

1. Introduction

Practically, all physiological systems of the mammalian body have been reported to be affected by 5-hydroxy-tryptamine (5-HT). Prominently affected systems are the central nerve system and the peripheral nerve system but 5-HT also plays a complex role in the gut, the liver and e.g. spleen. However, 5-HT also seems to have profound (patho)-physiological roles in the heart. Some drugs that are devised to treat non-cardiac diseases alter the level of 5-HT in the heart or act as agonists/antagonists on one or more of the 5-HT-receptors in the mammalian heart. Finally, there is evidence that in cardiovascular diseases 5-HT itself can affect the heart in a compensatory or detrimental way. Some newer aspects of the action and generation of 5-HT in the mammalian heart with special emphasis on the human heart will be addressed here. Finally, gaps in our knowledge, conflicting views, some challenging hypotheses and suggestions for further research will be put forward.

2. Historical aspects

The history of 5-HT goes back in time. Some write that the first hint of 5-HT's existence can be traced back to the work of Otto Weiss (1896, Göttingen, Germany) [1], who noted that serum in contrast to plasma exerted vasoconstrictory responses in dogs, a finding which one could now explained by high (serum) and low (plasma) levels of 5-HT in the samples of Weiss [2].

Enterochromaffin cells were first described in the lining of the gastrointestinal tract at the end of the nineteenth century (Charkow in ancient Imperial Russia) [3]. Using various staining reactions, these enterochromaffin cells were found in the gut of many species [4]. Early work described an increase in blood pressure in one rabbit with extracts from human carcinoid tumors (tumors derived from enterochromaffin cells [5]). Stimulated by these findings, Vittorio Erspamer in Italy (working in Pavia, Rome and later in Bari, Italy) studied acetone extracts of mucosal intestinal strips from animals (mainly rabbits) for decades. In an early paper, he presented an original recording for a positive inotropic effect (PIE) of "enteramine" (the putative active ingredient of his acetone extract) on the frog heart, conceivably the first inotropic effect reported for 5-HT [6]. Later he purified the extract further and reported the presence of a putative indolalkylamine that increased force in isolated hearts of various kinds of molluscs [7]. His efforts culminated in identifying his "enteramine" as 5-hydroxy-tryptamine [8]. 5-HT was, independently from Erspamer, studied by the group of Irvine H. Page in the USA (Cleveland, Ohio) and given its current name "serotonin" [9]. The American authors were investigating naturally occurring vasoconstrictory compounds (vasoconstriction being tested in rabbit ear arteries) in serum of humans in order to find a putative cause of peripheral arterial hypertension in humans. As a first step, they partially purified a vasoconstrictory compound from clotted serum of beef [10] and later this vasoconstrictor (their "serotonin") by chemical synthesis was identified as 5-hydroxy-tryptamine [11]. Synthetic 5-HT was shown in early work to act on blood pressure in animals (cats and rabbits [12]) and hypertensive patients [12]. These authors noted in patients an initial fall in blood pressure (measured in brachial arteries, absent in the presence of atropine) followed by a modest increase in blood pressure (up to 20 mm Hg increase). Patients injected with 5-HT complained of nausea, tightness of the breast, and dizziness, but the pulse rate was not reported [12]. A clear positive chronotropic effect (PCE) of 5-HT by venous injection in humans was published some years later [13, 14]. Initial attempts using biological tests failed [6] to detect 5-HT in the heart, but several decades later, the presence of 5-HT in the mammalian heart (hamster, human heart) has been proven (using more sensitive methods (fluorescence): e.g. [15]).

3. Sources of cardiac 5-HT

Ninety percent of 5-HT in the body is present in enterochromaffin cells in the gut, 5% in platelets, and about 2% in brain [16]. 5-HT in the blood is mainly assumed to be synthesized in enterochromaffin cells of the gastrointestinal tract [17, 18]. 5-HT is released from these enterochromaffin cells and is taken up rapidly by thrombocytes (review: [19, 20]). Alternatively,

5-HT enters the portal vein and passes into liver cells where it is rapidly degraded by monoamine oxidase-A (MAO-A) (see below) and its metabolites leave the body via the urine. Thus, platelets are commonly thought to be the main source of 5-HT that reaches the mammalian heart. Many cell types like mast cells also contain 5-HT (see below) and small numbers of mast cells are present in the mammalian heart [19]. Many immune cells contain 5-HT and are also found in small numbers in the heart. Newer data (using more sensitive analytical methods: HPLC, GC, MS) cast some doubt on this classical concept (see below). Thus, 5-HT was detected not only in hamster heart and human heart [15] but also in mouse heart [21], rat neonatal cardiomyocytes [22], and adult mouse cardiomyocytes [23]. The content of 5-HT in the hamster heart was reduced by the treatment of the living hamster with pargyline, an inhibitor of MAO-A [15] suggesting degradation of 5-HT in the heart via MAO-A. Moreover, the level of 5-HT in the hamster heart was not reduced by injecting in the living hamster a dose of 6-hydroxy-dopamine, sufficiently high to reduce cardiac norepinephrine levels, suggesting that cardiac 5-HT is not derived to a measurable extent from the neuronal cells in the hamster heart [15]. Compound 48/80, a substance that is known to release 5-HT from cells in other organ systems, increased force of contraction in isolated atrial preparation of 5-HT_4-receptor overexpressing mice, indicating that releasable 5-HT is present in these cardiac preparations, but leaving open the question of its cellular origin [24].

5-HT is formed from L-tryptophan by the enzyme tryptophan hydroxylase (TPH, see next paragraph). Peroral treatment of animals or humans with tryptophan (an essential amino acid) increases body concentrations of tryptophan. Uptake of tryptophan in the gut is brought about by the protein transporter enzymes SLC6A19 and SLC16A10 [25]. Protein-rich food is known to compete with these transporters and will lead to lower levels of tryptophan in the body [25]. Effects of 5-HT in the heart were usually thought to be due to 5-HT released from intact platelets. Indeed huge amounts of 5-HT can be released from activated thrombi in the heart. These thrombi are formed in atrial fibrillation and contain, of course, thrombocytes. 5-HT released from thrombi may then reach by diffusion endothelial cells. If these endothelial cells were lacking (for instance after local injury), 5-HT may act on 5-HT-receptors on the outer surface of smooth muscle cells or may reach cardiomyocytes. Conceivably, 5-HT in the plasma may reach the heart from the lungs (lung veins, left atrium, and left ventricle) and then enter the coronaries and thence affect the whole heart. In addition, 5-HT in the plasma may come from the periphery (via the right atrium and right ventricle) and exert right heart sided effects. Indeed, in tumors producing 5-HT (carcinoids, see below), such pathways are accepted to occur. Depending on the anatomic localization of the tumor, vasoconstriction would occur and should explain cases of left or right (or global) hypertrophy or contractile failure of the heart [26]. Furthermore, the 5-HT producing enzyme TPH1 (see below) was detected in pulmonary endothelial cells and could therefore generate 5-HT in the lung and it is conceivable that this 5-HT of pulmonary origin reaches the left heart via the pulmonary veins [27]:

There is precedence in the literature that a neurotransmitter like 5-HT can be formed in the heart. Here, we mention as an example noradrenaline which can act in an autocrine and paracrine fashion: there is evidence for the presence, synthesis (and degradation), and intracellular action of noradrenaline in heart muscle cells on β- [28] and α-adrenoceptors (most of the

latter being detected intracellularly in the nuclear membrane, for overview [29]). We speculate that the 5-HT-receptors (which are phylogenetically assumed to be much older than adrenoceptors) might also be detectable intracellularly in cardiomyocytes when more sensitive techniques will become available (**Figure 1**). It is notoriously difficult to detect endogenous G-protein-coupled receptors with specific, highly sensitive antibodies. Progress in this regard would be highly desired. Interestingly, 5-HT can exert intracellular effects via oxidation of 5-HT in mitochondria (e.g. in mouse heart) and formation of free radicals. In that way, 5-HT

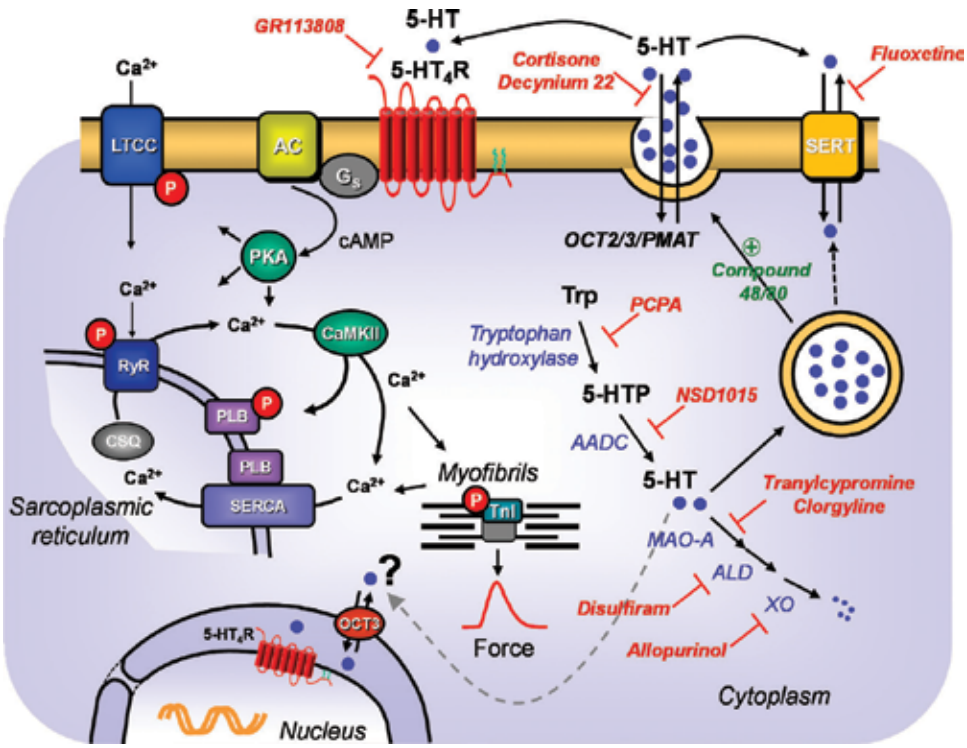

Figure 1. Hypothetical fates of 5-HT in the mammalian heart. Ca^{2+} enters the mammalian heart cell via the L-type Ca^{2+} channel (LTCC). This process can be enhanced by 5-HT via a cascade starting with the 5-HT_4 receptor (inhibitable by GR113808) occupation of which by 5-HT elevates activity of adenylyl cyclase (AC) in the sarcolemma via stimulatory G-proteins (Gs), elevates subsequent production of cAMP, and thereby activates cAMP-dependent protein kinase (PKA). PKA increases cardiac force generation and relaxation by increasing the phosphorylation state (P) of LTCC, phospholamban (PLB), and other regulatory proteins. Trigger Ca^{2+} initiates release of Ca^{2+} from the sarcoplasmic reticulum via ryanodine receptors (RYR) into the cytosol. There, Ca^{2+} activates myofilaments and this activation leads to increased inotropy. In diastole, Ca^{2+} is taken up into the sarcoplasmic reticulum via a sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), the activity of which is enhanced due to an increased phosphorylation state of PLB. We tentatively propose that 5-HT is stored in cardiomyocytes in a hypothetical locus from which it can be released by compound 48/80 and extruded from the cardiomyocytes, possibly via OCT2 and/or OCT3 and/or PMAT (inhibitable by cortisone, decynium 22). From the outside of the cardiomyocytes, 5-HT might be pumped back into the cardiomyocyte via SERT (inhibitable by fluoxetine). 5-HT might be formed in cardiomyocytes from tryptophan (Trp) via the enzyme tryptophan hydroxylase to generate 5-hydroxytryptophan (5-HTP) and thereafter decarboxylated by AADC (inhibitable by NSD1015) to 5-HT that is degraded via MAO-A (inhibitable by tranylcypromine) and its metabolites are substrates for aldehyde dehydrogenases (ALD; disulfiram-sensitive) or xanthine oxidases (XO; allopurinol-sensitive). In addition, one can speculate that 5-HT can pass through the outer nuclear membrane via OCT and then activate the inner nuclear membrane located 5-HT_4 receptor which activates than AC via Gs leading to phosphorylation of substrates in the nucleus and to altered gene transcription.

can also act receptor independently, at least in high concentrations to lead to apoptosis and necrosis, at least in the mouse heart [17, 30].

Furthermore, 5-HT can form covalent links to intracellular proteins and thence altering their functional role: transglutaminases can initiate covalent binding of 5-HT to fibrinogen, to small G-proteins, and to several other proteins present in platelets (review: [31]).

On the surface of platelets, a 5-HT_{2A}-receptor is known to be expressed. Its activation will activate thrombosis. 5-HT is thought to enter platelets via serotonin transporter (SERT) (for review, see Refs. [32, 33]). Within the platelet, 5-HT is either degraded via oxidation or transported via Vesicular monoamine transporter (VMAT) into vesicles in platelets which will store (VMAT1 and VMAT2 are also present in other non-neuronal cells, saliva cells [34], and renal tubular cells [35]; it is worthwhile to try to detect VMAT in cardiomyocytes, which has apparently not yet been reported) 5-HT and protect 5-HT from degradation. Upon an appropriate stimulus, 5-HT containing vesicles can reach the outer membrane of the platelets, fuse, and release 5-HT out of the platelets into the plasma. It has been shown that high blood platelet levels of 5-HT can serotonylate the protein rab4, which then inhibits the shift of SERT from the sublemmal space into the plasmalemma and hence quantitatively reduces its own uptake via SERT into platelets (which has been suggested to be of pathological relevance). There, 5-HT can act via the above-mentioned receptors in an autocrine or paracrine way [31, 36, 37]. Moreover, it stands to reason that 5-HT produced within cardiomyocytes might also exit the cardiomyocyte wherein it was formed (speculatively using uptake 1 or 2 and/or SERT, see below) to act in an autocrine or paracrine fashion at least under pathophysiological conditions.

5-HT can be compartmentalized in relevant cells: in peritoneal mast cells from rats, 5-HT was not only present in storage vesicles but also in the nucleus [38]. It is possible that mast cells produce relevant amounts of 5-HT because they contain their own TPH1 [19]. Compound 48/80 can release 5-HT from mast cells and PCPA (an inhibitor of TPH activity) can reduce the levels of 5-HT in the cytosol of mast cells but not the nucleus of mast cells [38]. Likewise, clorgyline (a MAO-A inhibitor) and fluoxetine (a SERT-inhibitor) could decrease the cytosolic but surprisingly also the nuclear amounts of 5-HT in mast cells [38]. These data argue for the existence of functionally distinct subcellular pools of 5-HT. Similar studies in cardiomyocytes are apparently lacking and are keenly awaited.

5-HT is not only produced in the mammalian body but also in the plant kingdom and is found in foodstuff such as nuts, bananas, oranges, coffee, and peaches [39]. This might be an additional source of 5-HT reaching the heart. Finally, there are data that in the lumen of the gut, bacteria form 5-HT, which may be absorbed and may also reach the heart [40]. Uptake via the intestine could be achieved by SERT which present and active in epithelial cell of the gut (in crypts of intestine, rat [41]).

4. Enzymes for synthesis of 5-HT in the heart

The isoform TPH1 is mainly expressed in the gut (but also in the pineal gland [21]), whereas TPH2 is mainly found in the CNS (but also in enteric nerve cells [21, 36]). Knockout of TPH1 reduced cardiac (adult mouse) 5-HT levels to about 10% of wild-type levels, indicating a relevant

production of 5-HT in the heart [21]. Some TPH1-knockout mice exhibited signs of left ventricular systolic failure without histologically detectable fibrosis [21] suggesting beneficial effects of the presence of 5-HT for cardiac function. In RNA from HL-1 cells and neonatal rat heart cells in Northern blots, TPH1 was detectable and TPH2 was missing [22] (similarly in adult hamster heart [42]). In RNA prepared from whole adult mouse hearts, TPH1 but not TPH2 was detected by PCR [43, 44]. Western blotting revealed low levels of TPH1 in mouse and rat adult heart homogenates. Fittingly, with the same antibody under similar conditions, no signal for TPH1 was noted in TPH1-knockout mouse hearts [44]. However, the localization of TPH is uncertain: in rat hearts in immunohistology, TPH1 was located only in cardiac mast cells, but in mouse heart no signal was noted in cardiac mast cells [44]. This might be explained by the antibody used, as others detected in immunohistology TPH in mouse cardiomyocytes as well as human atrial cardiomyocytes [45]. Amino acid decarboxylase (AADC, which is identical to dopamine decarboxylase [46]) on mRNA level was detected in heart (by PCR in neonatal rat cardiomyocytes but not in non-cardiomyocytes from neonatal rat hearts [22]). Subsequently, the activity of AADC will result in the generation of 5-HT. In apparent contrast to neonatal cardiomyocytes, AADC was detected via Western blotting in endothelial cells but not in cardiomyocytes of adult rat hearts and adult mouse hearts [44]. Whether this is due to age differences or lack of translation of RNA or too low protein levels of AADC or the features of the antibody used is still an open question. Others, however, noted in immunohistology the presence of AADC also in cardiomyocytes using slices from adult mouse heart and human right atrium [45]. Moreover, addition of 5-hydroxytryptophan (5-HTP, the direct precursor of 5-HT) enhanced 5-HT levels in these isolated cardiac mouse myocytes [23]. Interestingly, 5-HTP can exert functional effects in the heart. More specifically, in electrically driven left atrial preparations of transgenic mice (which overexpress the human 5-HT_4-receptor in the heart, see below), 5-HTP exerted time- and concentration-dependent positive inotropic effects (PIE) or increased the beating rate [positive chronotropic effect (PCE)] of right atrial preparations [45, 47]. Injection of 5-HTP into intact mice led to an increase of 5-HTP but allows of 5-HT in the cardiac tissue of mice [44]. Injection of benzerazide in intact mice, in contrast, reduced the cardiac levels of 5-HT [44]. Likewise, 5-HTP exerted a PIE in atrium from 5-HT_4 overexpressing mice or human right atrial preparations [47] (**Figure 2**). These contractile effects were blocked by NSD-1015 (**Figure 2**), suggesting they result from the enzymatic formation of 5-HT in these mouse or more importantly human cardiac preparations. Similarly, injection of 5-HPT in living whole mice (and in isolated buffer perfused hearts) led to a measurable increase in the cardiac content of 5-HT [44], and the effect was blocked by injection of benzerazide (an AADC inhibitor, used in treatment of Parkinson's disease). The authors posited that 5-HTP derived from platelets led to 5-HT synthesis in the heart [44]. In earlier work in the kidney, infusion of 5-HTP led to vasoconstriction which was reversed (or block by pretreatment) with carbidopa (a dopa decarboxylase inhibitor [48]). Infusion of 5-HTP led to increased levels of 5-HT in the renal venous effluent and in urine and these elevations of 5-HT returned to baseline values if carbidopa was additionally applied. These data are consistent with renal formation of 5-HT from 5-HTP via dopa decarboxylase activity [48].

A drawback of these pharmacological experiments is always that their interpretation is highly dependent upon the specificity of the inhibitory drugs used. It would be useful to refute or confirm these pharmacological experiments by studying cardiomyocytes from mice with

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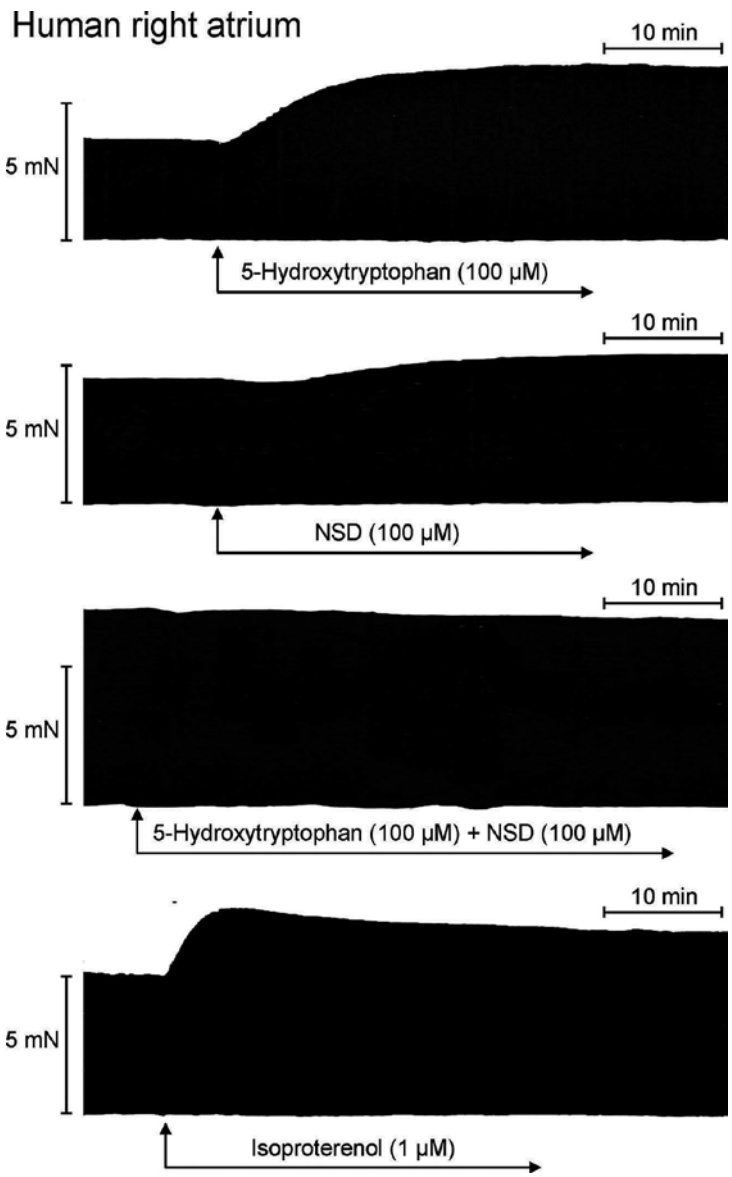

Figure 2. Typical original recording of isolated electrically stimulated trabeculae from a human atrium. The ordinate indicates force of contraction in milli Newton (mN), and the abscissae indicate time in minutes exemplified by scale bars. Of note, 5-hydroxytryptophan increases force of contraction (lane 1) and this effect was gone in the presence of NSD 1015, suggesting that 5-HT formation is necessary. NSD always exerted a small contractile effect of unknown origin. Isoproterenol, an unselective β-adrenoceptor agonist, was used as positive control.

cardiac-specific knockout of TPH1 and/or aromatic L-amino acid decarboxylase (AADC). Data for the local generation of 5-HT in peripheral arterial tissue (rat aorta, isolated human arterial coronary smooth muscle cells) are available and argue for local production and release independently of plasma or platelet levels of 5-HT [49, 50]. When one studied cardiac

tissue in adult human autopsies, in 72 or 80% of neurons within cardiac ganglia, tryptophan hydroxylase or dopa-decarboxylase immune reactivity was found, respectively, using commercial antibodies [51]. These levels were reduced in the presence of p-chlorophenylalanine (PCPA, an irreversible inhibitor of tryptophan hydroxylase activity) or 3-hydroxy-benzylhydrazine (NSD-1015), an inhibitor of aromatic L-amino acid decarboxylase (AADC [23]). Moreover, addition of 5-hydroxytryptophan (the direct precursor of 5-HT) enhanced 5-HT level in these isolated adult cardiac mouse myocytes [23].

5. Enzymes for degradation of 5-HT in the heart

As mentioned above in non-cardiac tissues, 5-HT is probably degraded by MAO-A. The same probably holds true for adult cardiac myocytes: levels of 5-HT were greatly elevated in the presence of tranylcypromine (clinically used as an antidepressant, inhibiting both MAO-A and MAO-B [23]) or in the presence of clorgylin (a MAO-A inhibitor [23]) but not by deprenyl (clinically used to treat Parkinson's disease, because it inhibits MAO-B [23]). MAO is especially active in gut, liver, and serotoninergic nerve cells. However, species differences exist. MAO-B is much less active in rat heart than MAO-A, and in human heart MAO-A and MAO-B are equally active [52]. The total activity of MAO is 100 times higher in the rat than in the wild-type mouse heart [53]. Likewise, MAO-B is mainly active in mouse heart, compared to MAO-A [54]. Hence, knockout of MAO-A in mice is probably not all that physiologically relevant for the human situation. At least in rat using ligand-binding experiments, even the regional cardiac distribution of MAO was found to be regionally different: there is a fivefold difference in MAO-A levels in parts of the ventricle of rat hearts [55]. The study of 5-HT levels in human cardiac tissue (preferably in cardiomyocytes, which is technically not highly reproducible, or stem cells, which have their own pitfalls) in the absence or presence of selective MAO inhibitors or genetic reduction of MAO levels in human cardiomyocytes are awaited with eagerness. Moreover, 5-HT can also be metabolized by the acrylalkylamine-N-acetyltransferase (present in the heart [56]). 5-HT can be degraded by MAO-A or MAO-B to 5-hydroxy-indole-acetaldehyde and by action of unspecific dehydrogenases and/or alcohol dehydrogenase 2 finally to 5-hydroxy-indole-acetic acid which leaves the body via the kidneys, and its concentration has been used in patients to monitor the presence of 5-HT-producing carcinoid tumors [26, 57]. Based on knockout experiments, 5-HT in the mouse is mainly degraded by MAO-A not MAO-B [58]. Inhibition of the activity of MAO by tranylcypromine potentiated the PIE of 5-HT in atrial preparations of 5-HT_4-receptor overexpressing mice [24]. 5-HT can also be metabolized by an indoleamine 2,3-dioxygenase (the rate limiting step in this pathway, with immunohistology detected in cardiomyocytes and active in mouse heart [59]) to kynurenine (present in mouse heart [60]). Indoleamine 2,3-dioxygenase (IDO) can be induced in infectious diseases like cardiac viral myocarditis [59]. Studying knockout mice for this enzyme supported an important role of IDO in acute viral myocarditis [59]. Furthermore, 5-HT can be metabolized even into melatonin (recent publication on levels of melatonin in rat heart: [61]) by hydroxyindole O-methyltransferase (enzyme present and active in mammalian heart: [56]) in the heart and this melatonin may play a role in protection against cardiac ischemia [62].

6. Uptake 1 of 5-HT in the heart

Classically, re-uptake of 5-HT (but also of neurotransmitters like histamine, noradrenaline, or dopamine) into nerve cells has been called uptake 1 and is assumed to be mediated for 5-HT by SERT (and by dopamine transporter (DAT) for dopamine, as well as by noradrenaline transporter (NAT = NET) for noradrenaline, however, their specificity of transport shows some overlap, which may explain compensations in knockout mouse models [63]). SERT is blocked by some antidepressant drugs like fluoxetine. Likewise, genetic deletion of SERT (total knockout) led to a decrease of 5-HT levels from 29 to 0.4 μM in whole blood, probably as a result of lack of reuptake via SERT into platelets, clearly indicating that SERT is not only active in the central nervous system but also in the periphery. Uptake 1 is energy dependent because it acts against a neurotransmitter gradient. Uptake 1 can also be blocked by cocaine (which is, however, unspecific because it blocks at least also NAT and DAT). Interestingly, the EC_{50} of 5-HT in the presence of cocaine for the PIE is much smaller in human isolated atrial preparations (39 nM) than in the absence of cocaine (230 nM: [64]): this could mean that cocaine inhibits the uptake 1 into nerve cells or that it inhibits reuptake of 5-HT into cardiomyocytes by inhibiting SERT in cardiomyocytes. At low concentrations of 5-HT (50 nM), about 70% of 5-HT is taken up via uptake 1 (the remainder via uptake 2 see below). SERT has been found in the lung (endothelial cells and smooth muscle cells: [65]; rat aorta: [49]) on cardiac valves (rat: [66], dog: [67], human valvular tissue: [43]), conduction system of the mouse, mouse cardiomyocytes, and mouse cardiac endothelial cells [68–70]. At least in fetal cardiomyocytes, SERT was seen in immunohistology [71]. Some detected SERT in the endocardium and endothelium of coronary arterial cells and capillaries, while they failed to detect SERT in cardiomyocytes from adult mice [43]. Others using different experimental conditions detected SERT in cardiomyocytes from adult mouse heart and human right atrium [45]. Functional evidence for the activity and therefore presence of SERT are also available: 5-HT, applied in cell culture of adult rat ventricular myocytes induced cellular hypertrophy and this hypertrophy was attenuated by imipramine [72, 73]. This is functional proof that cardiomyocytes can take up 5-HT and might argue for an involvement of SERT in this process. Knockout of SERT in mice was accompanied in whole blood by an about 10-fold reduction of 5-HT levels [43]. Interestingly, adult mice with global knockout of SERT showed left ventricular dilatation and systolic heart failure (decreased fractional shortening in echocardiography) which was accompanied and possibly caused, in part, by cardiac ventricular interstitial fibrosis as well as cardiac valve fibrosis effects present also on 5-HT_{1B}-receptor knockout mice and hence not 5-HT_{1B}-receptor mediated [43]. SERT is reversible in its transporter function: during ischemia, in the presence of tyramine of amphetamines, intracellular 5-HT can leave mouse cardiomyocytes [68]. The functional role of SERT in the heart is evident from the observation that fluoxetine can shift the concentration response curve for the positive inotropic effect of 5-HT to lower concentrations of 5-HT in the left atrium of mice overexpressing the 5-HT_4-receptor [24]. A prominent pathway is initiated by the enzyme indoleamine-2,3-dioxygenase (IDO, which opens and destroys the indole ring system of tryptophan), which feeds into the so-called kynurenine pathway (review: [19]).

7. Uptake 2 of 5-HT in the heart

Uptake of neurotransmitters (like 5-HT) into non-neuronal cells (such as smooth muscle cells, fibroblasts, endothelial cells, or cardiomyocytes—have been called "uptake 2") and is assumed to be mediated by proteins such as OCT1, OCT2, OCT3, and PMAT. Uptake 2 is not energy dependent because it follows a neurotransmitter gradient. Another difference between uptake 1 and 2 relies on the fact that uptake 2 is much less specific for 5-HT than uptake 1. Usually, proteins that comprise uptake 2 will also transport other neurotransmitters like dopamine and noradrenaline [63]. Uptake 2 is usually inhibited by cortisone (but also by synthetic dexamethasone, by aldosterone, and by budesonide) via unknown mechanisms and divergent specificity for OCT1-3 and PMAT [63, 74]) and more specifically by decynium 22 [57, 63]. At higher concentrations of 5-HT (10 μM), it is mainly transported via uptake 2 (in synaptosomes, regarding decynium 22 as uptake 2-specific [57]). In the CNS, proteins responsible for uptake 2 have been detected not only in nerve cells but also in non-nerve cells (glial cells [63]). Uptake 2 is functionally relevant in the heart because decynium 22 affects the concentration response curve of 5-HT on force of contraction in isolated atrium of 5-HT_4-receptor overexpressing mice [24]. OCT2, OCT3, and PMAT have been detected by immunohistology in mouse or human cardiomyocytes [45] and by immunofluorescence (OCT1, OCT3) in the human heart [75].

8. Inotropic effects of 5-HT in the heart, species differences

A positive inotropic effect (PIE) of 5-HT was described in the heart of many mammalian species. More specifically, a PIE was described in cardiac preparations from cats, guinea pigs, dogs, pigs, and rats [76–80]. The PIE in cats is indirectly mediated via release of endogenous noradrenaline [81]. The PIE in the same species can be region dependent: for instance, in rats, a PIE in left atrium but not in papillary muscle was reported [82]. Similarly, in human atrial but not ventricular preparations, 5-HT exerted a PIE [83, 84]. Later, it was noted that in ventricular preparations from patient in end-stage heart failure a noteworthy effect of 5-HT was detectable and this effect was more pronounced in the presence of the phosphodiesterase inhibitor 3-isobutyl-1methylxanthine (IBMX) [85]. Interestingly, similar findings were reported in pigs: only in the presence of IBMX in ventricular preparations of pigs, a PIE to 5-HT could be noticed [86] suggesting that the low number of 5-HT_4-receptors was unable to raise cyclic-3′,5′-adenosine monophosphate (cAMP) levels to inotropically relevant levels in the presence of substantial endogenous unopposed phosphodiesterase activity in ventricular preparations of humans and pigs. In isolated paced left atrial preparations of wild-type mice, no PIE in the absence [87] or presence (Käufler, Gergs, Neumann, unpublished observations, 2017) of 100 μM IBMX to 5-HT (1 nM–1 μM) was, however, observed, underscoring species differences. The EC_{50} value for the PIE of 5-HT in isolated preparations from human right atrium was between 309 and 230 nM [80]. In mouse adult cardiomyocytes, the 5-HT level was estimated to amount to 2.9 pmol/mg protein [23]. Concentrations of 5-HT in isolated samples from human hearts (freshly frozen, after autopsy, from the right atrium, from papillary muscles) were reported from 0.08 to 0.4 μg/g [15], recalculated as about 0.45 to 2.3 μM. Such

differences might be due to contamination with platelets (for very high values) or postmortal degradation (for low levels). Assuming a homogenous distribution of 5-HT in isolated mouse adult cardiomyocytes, intracellular concentrations of 200 nM for 5-HT have been calculated [23]. These concentrations are well in the range of EC_{50} values for the 5-HT-receptors like those responsible for inotropy in some mammalian species including humans [20, 88]. At high concentrations of 5-HT for prolonged times in the organ bath, a second negative inotropic effect of 5-HT was noted, which was alternatively explained as desensitization by activation of phosphodiesterases [89]. Homologous desensitization in the isolated atrium (also in left ventricle of the living animal) can be clearly shown for the PIE effect of 5-HT in 5-HT_4-receptor overexpressing mice [90, 91] like in isolated human cardiac preparations (review: [92]).

9. Chronotropic and proarrhythmic effects of 5-HT in the heart

Positive chronotropic effects of 5-HT were noted in isolated atrial preparations of rats [93], cats [94], pigs [95] as well as guinea pigs [96] and awake humans [14]. Even bradycardia can be elicited by 5-HT via the von Bezold-Jarisch reflex [97]. In whole pigs and isolated pig cardiac preparations, 5-HT increased the heart rate via 5-HT_4-receptors [86, 92, 98]. It is assumed that the effect is brought about by 5-HT_4-receptors initiating a cascade via Gs, AC, cAMP and then activating hyperpolarization-activated, cyclic nucleotide-gated cation channels (HCN) in the sinus node [86]. 5-HT increased the HCN-coded current called I_f in isolated human atrial cardiomyocytes and was mediated by 5-HT_4-receptors (using specific receptor antagonists [99–101]). In one of the first studies on humans, 5-HT induced cardiac arrhythmias *in vivo* (tachycardia and P wave inversions in two patients: [14]). Interestingly, 5-HT induced arrhythmias even in isolated electrically driven human atrial cardiomyocytes, proving that arrhythmia does not need indirect pathways but is sufficiently explained by direct activation of receptors (5-HT_4-receptors) [102]. Interestingly, the incidence of arrhythmias was enhanced in isolated atria from humans treated prior to surgery with β-adrenoceptor blockers [102, 103]. The arrhythmias could be explained on a single cell basis via late afterdepolarizations [104, 105]. In addition, arrhythmogenesis due to 5-HT might also involve stimulation L-type Ca^{2+}-channels and potassium channels [86, 106]. 5-HT may also be relevant to sustain an existing arrhythmia: during pre-existing atrial fibrillation, more 5-HT will be released from thrombocytes [107]. This can increase local concentrations of 5-HT, which can act on 5-HT_4-receptors to sustain fibrillation (for further hypothetical mechanisms: [92]). Mechanistically interesting is the observation that in some children autoantibodies against 5-HT_4-receptors exist which have been suggested to lead to AV blocks in neonates [108]. In 5-HT_4-receptor overexpressing mice, arrhythmias under basal conditions or after 5-HT stimulation have been observed [23, 87, 109]. The 5-HT_3-receptor might be antiarrhythmic: general deletion of the 5-HT_3-receptor in mice led to spontaneous ventricular tachycardia and increased sudden death in pregnant mice. It was speculated that 5-HT_3-receptor blockers should therefore be avoided in pregnant women [110]. Consistent with this, ondansetron, a blocker of 5-HT_3-receptors, has been reported to elicit arrhythmias in patients [111]. In addition, prolongation of P-waves and highly elevated T-waves (interpreted as a sign of repolarization abnormalities) were described in mice with knockout of 5-HT_{2B}-receptors [112].

10. Effects of 5-HT on cardiac vasculature, species differences

5-HT can induce vasoconstriction also in coronary arteries [20]. During reperfusion of coronary arteries, 5-HT can have detrimental effects like apoptosis and necrosis [72]. In man, a more subtle picture emerges: without endothelium or in defective endothelium (arteriosclerosis, coronaries injected *in vivo* with 5-HT in patients having received transplanted hearts), 5-HT induces vasoconstriction, but in the presence of functional endothelium, 5-HT induces vasodilation in human coronary arterial strips [113–115]. Others noted in human coronary vessel strips with intact endothelium (obtained from transplanted hearts) a 5-HT-mediated vasoconstriction that was in part ketanserin-sensitive [116]. In isolated strips from human pulmonary veins or arteries, 5-HT led to vasoconstriction (regardless of the presence or absence of endothelium) and was interpreted to be mediated by 5-HT_2- and 5-HT_1-receptors while ligand-binding studies presented evidence for the expression of 5-HT_4-, 5-HT_{2A}-, and 5-HT_{1D}-receptors in these tissues [117]. In summary, vasoconstriction can be mediated by 5-HT_{2A}- and 5-HT_{1D}-like receptors and the latter are more relevant for vasoconstriction [114, 118].

11. Use of genetically modified mice to study functional effects of 5-HT in the heart

Gain of function animal models like mice that overexpress in a cardiac specific way 5-HT_4-receptors [87], MAO-A [119], 5-HT_{2B}-receptors [120] or SERT [121] have been described and used to better understand the role(s) of 5-HT in the mammalian heart. However, animal models with loss of function are much more abundant (**Table 1**). The cardiac phenotypes of mice overexpressing SERT or 5-HT_4-receptors (in the heart) have been discussed in this text. 5-HT_{2B}-receptor overexpressing mice, however, had no defect in systolic function (unaltered ejection fraction). Interestingly, their heart weight to body weight ratio was increased (cardiac hypertrophy). This was explained by an increase in the number and size of cardiomyocytes. Further changes were an increase in the number and activity of mitochondria in hearts from transgenic mice but no cardiac fibrosis was noted. It is possible that the hypertrophy is due to constitutive activation of the PLC pathway by the overexpressed 5-HT_{2B}-receptor [120]. Interestingly, in 5-HT_{2B}-receptor knockout mice (made by the same group), a dilated cardiomyopathy (with decreased systolic function, small sized cardiomyocytes) was noted [112] (**Table 1**). Surprisingly, the knockout of the 5-HT_{2B}-receptor exhibited gender-specific differences in the phenotype. For instance, ECG alterations (prolongation of P-wave) were more pronounced in female than male 5-HT_{2B} knockout mice [112].

To the best of our knowledge, mice with cardiac-specific knockout of the genes listed in **Table 1** have not been described in the literature and might be meaningful new study systems. In addition to the mouse models listed in **Table 1**, there is also a SERT knockout rat in the literature [147]. This rat model should be useful in some regards, because historically most work on hypertension was done in rats. In this context, mice with cardiac-specific overexpression of 5-HT_{2B}-receptors could be generated by mating 5-HT_{2B} knockout mice and 5-HT_{2B}-receptor overexpressing mice, an interesting line of research for other 5-HT-receptors [148].

Protein	Function	References
5-HT1A	Serotonin-receptor	[122]
5-HT1B	Serotonin-receptor	[123]
5-HT1D	Serotonin-receptor	[124]
5-HT2A	Serotonin-receptor	[125]
5-HT2B	Serotonin-receptor	[126]
5-HT2C	Serotonin-receptor	[127]
5-HT3A	Serotonin-receptor	[110, 128]
5-HT4	Serotonin-receptor	[129]
5-HT5	Serotonin-receptor	[130]
5-HT6	Serotonin-receptor	[131]
5-HT6	Serotonin-receptor	[132]
5-HTT (SERT)	Serotonin-transporter	[133–135]
TPH1	Serotonin synthesis	[21, 37]
Dopa-decarboxylase	Serotonin synthesis	[136]
MAO-A	Serotonin degradation	[137]
MAO-B	Serotonin degradation	[138]
VMAT1	Serotonin uptake	[139]
VMAT2	Serotonin uptake	[140]
PMAT	Serotonin uptake	[141]
OCT1	Serotonin uptake	[142]
OCT2	Serotonin uptake	[143]
OCT3	Serotonin uptake	[144]
IDO	Serotonin degradation	[59]
Alcohol dehydrogenase	Serotonin degradation	[145]
Xanthine oxidase	Serotonin degradation	[146]

Table 1. Constitutive knockouts of genes relevant for serotonin handling.

12. 5-HT-receptors present in the heart: cell and species differences

The current thinking is that 5-HT can act via membrane bound receptors called 5-$HT_{1\text{-}7}$ ([20, 149], review: [150]). The 5-HT_3-receptor is a ligand-gated ion channel, whereas all other 5-HT-receptors are G-protein-coupled receptors. The 5-HT_1-receptors as well as 5-HT_7-receptors can inhibit the activity of adenylyl cyclase via Gi/q, whereas 5-HT_4-, 5-HT_5-, and 5-HT_6-receptors can increase the activity of adenylyl cyclase via Gs. 5-HT_2-receptors, via G_q/G_{11}, can activate PLC and thereby increase IP3 levels as well as generate diacylglycerol and

subsequently diacylglycerol can activate PKC. Moreover, 5-HT_{2A}- and 5-HT_{2C}-receptors can also activate phospholipase A_2. In the whole mouse heart, the following receptors have been described on mRNA level: 5-HT_{1A}-, 5-HT_{1B}-, 5-HT_{1D}-, 5-HT_{2A}-, 5-HT_{2B}-, 5-HT_{2C}-, 5-HT_3-, and 5-HT_4-receptors [43]. Surprisingly, others failed to detect the 5-HT_4-receptor in mouse heart and only reported on 5-HT_{2A}- and 5-HT_{2B}-receptors [151]. Others failed to detect 5-HT_{2C}-receptors in neonatal rat cardiomyocytes, which offers the possibility that the cardiac expression of 5-HT-receptors might be developmentally regulated or likewise be species dependent or in different cell types of the heart [22]. Apparently, the 5-HT_6-receptor was not found by PCR in adult mouse whole hearts [43]. Four isoforms of mouse 5-HT_4-receptors exist (on RNA level) in mouse atria [152]. On RNA level, 5-HT_{4a}- and 5-HT_{4b}-receptors are also present in human atrium [153, 154] and to a lesser extent in human cardiac ventricle [85, 155]. As mentioned before, 5-HT_{2A}-receptors mediate the effects of 5-HT in thrombocytes [156]. The PIE of 5-HT in rat atrium is probably mediated by 5-HT_{2A}-receptors [82]. 5-HT_{2A}- and 5-HT_4-receptors are, however, present on RNA in rats [82], but the 5-HT_4-receptors in rat hearts only become functional (mediating a PIE) in stress (myocardial infarction: overview in [157]). The 5-HT_2-receptors can activate phospholipase C and can elevate IP3 levels in the rat heart [82]. 5-HT_{2A}-receptors are found in human arterial smooth muscle cells and can lead to vasoconstriction [158, 159]. Initially, 5-HT_3-receptors seemed only to be present in nerve cells in the heart and might mediate the "von Bezold-Jarisch" reflex [97]. The 5-HT_3-receptors seem to be found in epicardial afferent sensory nerve ending of the vagus [160]. More recently, however, using a new knockout mouse, 5-HT_3-receptors were found at least in the ventricle of wild-type mice [110]. The 5-HT_4-receptor (but not, for instance, a 5-HT_2-receptor) mediates the PIE and PCE in the human heart [64, 92]. The study of the 5-HT_4-receptor structure is complicated because many splice variants are known which might have different physiological and/or pathophysiological roles [157]. No convincing antibodies to 5-HT_4-receptors let alone for splice variants have been published in the literature (and our own unpublished observations). Hence, protein levels of these receptors are difficult to assess. At least, some radioactive ligand-binding studies shed some light on the protein expression levels in the heart and found measurable but very low densities of 5-HT_4-receptors in the heart [161]. Really specific antibodies for 5-HT_4-receptors with high affinity are highly desirable. 5-HT_1-receptors are present in endothelial cells and smooth muscle cells in human coronary arteries and mediate vasoconstrictory effects of 5-HT [158] and can inhibit AC activity [162]. PIE of 5-HT in human atrium and ventricle are 5-HT_4-receptor mediated (trabeculae: [64]). 5-HT_{2B}-receptors are present in cardiac valves. Their simulation by 5-HT, fenfluramine (indirectly by inhibiting SERT or by releasing 5-HT from platelets), ergotamine derivatives, methysergide, and recreational drugs ("ecstacy") can lead to deadly valve ruptures [163–165]. Typically, these drugs are present in all parts of the blood circulation; hence, the valve dysfunction can take place in the right as well as in the left heart. An excellent review on 5-HT-receptors in the vascular system especially the heart of humans is to be found in the literature and will be helpful for in depth information [157].

13. Signal transduction mechanisms of 5-HT-receptors in the heart

Moreover, 5-HT in isolated atrial preparations from human hearts increased cAMP content, PKA activity [64, 80], and the phosphorylation state of phospholamban (PLB) and the

inhibitory subunit of troponin (TNI, [88]), and these effects were blocked by 5-HT_4-receptor antagonists [88]. Hence, these effects were probably 5-HT_4-receptor mediated [88]. In electrophysiological experiments, 5-HT elevated the L-type Ca^{2+}-current in human atrium [83, 166, 167] but not human ventricle [83, 168]. Mechanistically important, 5-HT increased the contractility in isolated atrial paced human cardiomyocytes [103]. Stimulation of 5-HT_{2A}-receptors led to increases of IP3 content [82]. Similarly, in transgenic mice that overexpress 5-HT_4-receptors in the mouse heart, 5-HT led to PIE and PCE in intact mice (using echocardiography), in isolated perfused hearts, in isolated left atria (electrically driven), or isolated spontaneously beating right atria. These effects were accompanied by cAMP increases, increased phosphorylation state of PLB (on amino acid serine 16 and threonine 17), increase in current through L-type Ca^{2+}-channels, and increase in the free Ca^{2+} content in the cytosol in mice ventricular preparations or whole hearts [87]. In addition, increased phosphorylation of PLB was also noted in atrial preparations from 5-HT_4-receptor overexpressing mice [169]. In these mice, the *in vivo* activity of agonists could be studied on contractility [90]. Here, one can recapitulate findings in cloned receptors, for instance, cisapride was less potent and effective to increase force of contraction than 5-HT. Moreover, cisapride induced concentration-dependent tachycardia (and arrhythmias) in spontaneously beating isolated right atrial preparations of 5-HT_4-receptor overexpressing mice [170], similar to tachycardias described in some patients treated with cisapride [171]. However, prucalopride was less potent but equieffective compared to 5-HT [109, 170, 172]. In addition, 5-HT is able to desensitize the 5-HT_4-receptor not only in 5-HT_4-receptor overexpressing mice in the atrium [90] but also in the ventricle [172, 173]. LSD and ergotamine *in vitro* displayed biased signaling for β-arrestin at 5-HT_{2B}- and 5-HT_{1B}-receptors [174].

14. Altered expression or function of cardiac 5-HT or its receptors under pathophysiological conditions

14.1. Carcinoid syndrome

In the carcinoid syndrome (typically due to tumors arising from enterochromaffin cells of the gut that in 10% of cases produce high levels of 5-HT: cf. [23] for a clinical example, large patient series: [26]), high circulating levels of 5-HT, which can stimulate 5-HT-receptors, and lesions of the *right* cardiac valves have been reported. Normally, the pulmonary circulation is assumed to remove free circulating 5-HT from the plasma and very little 5-HT in the plasma would reach the left ventricle (discussed in Ref. [43]). However, when a carcinoid tumor is located to the lung or an open foramen ovale is present or exceedingly high plasma 5-HT levels occur from a carcinoid in the gut or liver, which would spill over into the pulmonary veins, *left* cardiac lesions like valve failure have also been noted [43].

14.2. MAO-dependent oxidative stress

By altering cardiac levels of 5-HT, MAO might be of clinical relevance in patients. This can be tentatively concluded from the following animal studies. Cardiac-specific overexpression of MAO-A (in mice) led to decreased cardiac levels of 5-HT (and noradrenaline). This was

accompanied by increased levels of free radicals in the mouse heart, as well as oxidation of mitochondrial DNA, cardiac fibrosis, and ventricular heart failure [53]. Oppositely, a knockout of MAO-A in mice was functionally beneficial because it reduced left ventricular dilatation and left ventricular dysfunction after hypertension (via aortic banding). Accordingly, aortic banding (an experimental model to increase cardiac afterload) increased protein levels of MAO-A threefold [119]. High cardiac concentrations of 5-HT can lead to cardiac hypertrophy (and later on, possibly, to heart failure, see below) via receptor-independent mechanism(s) or 5-HT-receptor-dependent mechanisms. In more detail, high cardiac intracellular levels of 5-HT are oxidized in mitochondria via MAO activity: this leads to the generation of deleterious free radicals [119]. In aging rat hearts, MAO-A activity was increased, which may exacerbate deleterious effects of cardiac 5-HT [119]. In rat cardiac myocytes, high intracellular levels of 5-HT led to enhanced MAO-dependent oxidative stress followed by release of cytochrome c from cardiac mitochondria, upregulation of proapoptotic BAX protein, downregulation of antiapoptotic Bcl2 protein, and thus to detrimental apoptosis [72].

14.3. Hypertension

In peripheral arterial hypertension, more 5-HT can be found in the plasma, and via covalent modification of the protein rab4, the function of SERT in platelets is reduced and thus a circulus vitiosus might start [175]. Moreover, it has been suggested that 5-HT might act on small G-proteins (by inducing serotonylation of these proteins) in smooth muscle cells in pulmonary arteries, rendering the arteries more susceptible to 5-HT-induced vasoconstriction and thus leading to sustained pulmonary hypertension and death [36]. Serotonylation of several proteins in rat aorta has likewise been reported [176]. Pulmonary hypertension might be causally related to 5-HT: plasma levels of 5-HT are twofold to threefold higher in patients suffering from this disease and augmented 5-HT plasma levels may lead to constriction of pulmonary arteries and thus to pulmonary hypertension in humans [177]. This is in line with animal experiments: in TPH1 knockout mice, hypoxia (placing the animals into chambers with low partial pressure of oxygen) was less prone to rise right ventricular pressure compared to WT animals [178]. In a rat model of pulmonary hypertension (induced by monocrotaline), blocking 5-HT_{2B}-receptors was protective for right ventricular function [179].

14.4. Cardiac hypertrophy

Interestingly, a blocker of 5-HT_{2A}-receptors attenuated cardiac hypertrophy after aortic banding in mice, suggesting a role of this receptor in cardiac hypertrophy, and in this context, hypertrophy (transiently) increased the expression of the 5-HT_{2A}-receptors in mouse cardiomyocytes after aortic banding [180]. The classical isoproterenol-induced hypertrophy in a mouse model was reduced in mice treated with a 5-HT_{2B} blocker or in 5-HT_{2B}-receptor knockout mice, probably by inhibiting peroxide generation in mitochondria [2, 181]. Interestingly, a isoproterenol-induced cardiac hypertrophy (a classical animal model of hypertrophy) seemed to require 5-HT_{2B}-receptors on cardiac fibroblasts [148]. Fittingly, in patients with cardiac hypertrophy, the expression of 5-HT_{2B}-receptors was elevated (radioligand binding [148]). 5-HT_{2B}-receptors were detected with immunohistology in human cardiomyocytes and human

non-cardiomyocytes; however, it is an open question (and a mechanistically very important question) whether overexpression of these receptors in human heart failure occurs in cardiomyocytes and/or in non-cardiomyocytes [148].

14.5. Heart failure

In heart failure, 5-HT might be altered: increases in plasma 5-HT levels in patients with decompensated systolic heart failure [182] or diastolic heart failure [183] have been described. These studies concluded that 5-HT elevation may be a compensatory mechanism, trying to increase cardiac output by increasing heart rate and cardiac force [182]. In atrial samples from heart failure patients, the PIE of 5-HT was reduced. Moreover, biochemical correlates of receptor coupling like the extent to which 5-HT could increase AC activity [184] or increase L-type Ca^{2+}-currents, was attenuated in samples from heart failure patients [168]. Some of these effects were reversed after β-adrenergic blockade of patients prior to surgery [185]. In a rat model of heart failure (infarction), the mRNA of 5-HT_4-receptors increased and a robust PIE of 5-HT (which was lacking in normal rats without heart failure) became apparent [186]. Moreover, there are data that in human heart failure a PIE of 5-HT and upregulation of 5-HT_4-receptors become measurable [85, 155]. Interestingly, the PIE of 5-HT increased with NYHA class but the PIE of β-adrenergic stimulation decreased with NYHA class [85]. In a pilot study with heart failure patients, the EF increased after being treated with a 5-HT_4-receptor antagonist (piboserod [187]). This might mean that activation of 5-HT_4-receptors is deleterious in human heart failure. In apparent contrast to this conclusion, in lipopolysaccharide (LPS)-induced sepsis (another accepted model of heart failure), overexpression of 5-HT_4-receptors seemed to protect the heart by interference with the toll-like receptor 4 pathway [188].

14.6. Atrial fibrillation

In patients with chronic (more than 1 month persistent) atrial fibrillation, the expression of 5-HT_4-receptor mRNA levels was found to be decreased by about 36% (irrespective of β-adrenoceptor treatment) in comparison to controls in sinus rhythm and this change in receptors level was suggested to be a protective mechanism [189]. Others found the expression of 5-HT_{4b}-receptors to be downregulated (mRNA) in acute atrial fibrillation but upregulated with atrial fibrillation lasting more than 1 year [190]. Protein data for 5-HT_4-receptors in atrial fibrillation would clearly be desirable to resolve these somewhat contradictory findings. In aging, the uptake of 5-HT in platelets is augmented, concentrations of 5-HT in platelets are therefore higher, and thence 5-HT is more prone to induce aggregation of platelets and thus thrombosis (e.g. [191]).

14.7. Aging

At least in pigs, the PIE of 5-HT in atrium and ventricle *in vitro* is increased from neonates to adulthood [85, 86]. The opposite occurs in rats: fetal rat ventricles express highly the mRNA for 5-HT_4-receptors and are accompanied by (and probably causes) a large PIE to 5-HT in neonatal cardiac preparation. In contrast, in adult rats, as mentioned before, 5-HT is devoid of a

PIE in the rat ventricle [82, 192]. In human atria, 5-HT stimulates AC less in aging, which was explained by increased levels of Gi proteins [193]. Hence, age-dependent changes in cardiac response to 5-HT are known, but much more refined data are clearly needed from further work.

So sum up these findings, it is possible that 5-HT causes or at least contributes to cardiac hypertrophy, arterial or pulmonary hypertension, heart failure, cardiac aging, and cardiac arrhythmias.

15. Possible cardiac side effects of serotoninergic drugs

Drugs elevating 5-HT: SERT *inhibitors* (specific serotonin reuptake inhibitors, SSRIs), they are suggested to increase 5-HT not only in brain synapses but also in cardiac tissue and therefore they might be implicated in arrhythmias (citalopram: [194]). Warning notices have been sent out for citalopram in this regard by regulatory authorities (FDA: 2012). A recent study noted enhanced risks of valve disease in patients who take SSRI [195], presumably because high plasma membrane concentrations of 5-HT activate 5-HT_{2B}-receptors. *5-HTP* has been suggested to be used as add on to SSRI in order to treat depression, because it is metabolized to 5-HT. *5-HTP* is therefore predicted, indirectly, to lead to arrhythmias in patients.

5-HT_1-receptors: *Sumatriptan* and congeners are well known to have the ability to contract coronary arteries and can lead to myocardial infarctions [196]. Buspirone is a drug acting among others on 5-HT_{1A}-receptors and has been shown to lead to tachycardia.

5-HT_2-receptors: *Ergotamine* and *LSD* (but also *fenfluramine*) stimulate 5-HT_{1B}-receptors but also detrimental 5-HT_{2B}-receptors (leading to valve fibrosis) [174]. In addition, an important metabolite of fenfluramine called *norfenfluramine* could bind with high affinity to 5-HT_{2B}-receptors and could stimulate in the receptor-transfected HEK cells the IP3 levels, presumably initiating fibroplasia *in vivo* in humans [197]. In 2014, pimavanserin is an antagonist at 5-HT_{2A}-receptors entered the market in the USA as an antipsychotic drug and to treat Parkinson's disease [198]. Interestingly, the producing company lists as contraindications irregular heartbeat. In some countries, *ketanserin* (a classical 5-HT_{2A}-antagonist) has been used for many years to treat *hypertension*. However, the antihypertensive effect is probably due to an additional α_1-adrenoceptor antagonistic effect of ketanserin [2]. Moreover, its use has rapidly declined when it was suggested to lead to deadly arrhythmias (ketanserin can make QT prolongation and thereby lead to torsade de pointes). The reason for this is that the K^+ channel hERG (human ether-a-go-go-related gene) is blocked by ketanserin (reviewed in [2]).

5-HT_{2C}-receptors: A newer serotonergic drug is the 5-HT_{2C}-agonist *lorcaserin* that was approved by the FDA in June 2012 in order to bring about weight loss by action in the CNS [198]. Lorcaserin was developed because older drugs for weight loss were withdrawn, in the past, from the market (e.g. fenfluramine) because they led to fibrosis (reviewed in Ref. [70]). Clearly, one can speculate that lorcaserin might also have agonistic properties at 5-HT_{2B}-receptors and thus may have effects on valves. Initial studies did not detect an increased risk of lorcaserin for valvulopathies, however the incidence of headaches was increased versus placebo which may mean that lorcaserin can act agonistic on other 5-HT receptors [199] and

side effects should carefully monitored by physicians and communicated to the regulatory authorities.

5-HT_4-receptors: *Metoclopramide* acts on many receptors but in this context the activation of 5-HT_4-receptors is important [200]. One has speculated that 5-HT_4-receptor agonists might be useful to treat patients with sinus bradycardia, slowing of AV conduction, and autonomic dysfunction of the heart [201]. 5-HT_4-receptor agonists are used in some countries to treat irritable bowel disease (e.g. prucalopride [202]), bladder dysfunction [203], and Morbus Alzheimer (e.g. [204]). However, early on a clinical study detected arrhythmias in ECG of healthy volunteers [205], which used the 5-HT_4-receptor agonist *prucalopride*, and hence caution in its used is advised. The 5-HT_4-receptor agonist (RS 67333) *donecopride*, in addition, inhibits the activity of acetylcholine esterases [206] and might be useful for the treatment of Morbus Alzheimer. However, this compound is expected to lead to arrhythmias in sensitive patients via its agonist activity of cardiac 5-HT_4-receptors. 5-HT_4-receptor agonists have been developed to treat anxiety or chronic obstipation. *5-HT_4-receptor antagonists* have been suggested for the treatment of supraventricular arrhythmias [159, 189] but were not successful due to side effects [106].

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